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Inflammation and remodelling in experimental models of COPD

Mechanisms and therapeutic perspectives

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Table of contents

Chapter 1

General Introduction

COPD

Chronic obstructive pulmonary disease (COPD) is defined as “a disease state characterized by airflow limitation that is not fully reversible. The airflow limitation is usually both progressive and associated with an abnormal inflammatory response of the lungs to noxious particles or gases” (1). Tobacco smoke exposure is the major risk factor for the development of COPD, although other forms of insult to the lungs as well as genetic predisposition may also contribute (1, 2). COPD has been predicted by the Global Burden of Disease Study to become the third leading cause of death worldwide in 2020 (3).

Innate and adaptive immune system in COPD

The inflammatory response in COPD consists of infiltration of various innate immune cells, including macrophages and neutrophils, as well as adaptive immune cells, such as T and B lymphocytes (4-6). Macrophages clear the lung of inhaled particles, bacteria and apoptotic cells by phagocytocis. After phagocytocis, the macrophages are removed by mucociliary clearance, which constitutes an important mechanism of defense against infection. However, the phagocytic capacity of alveolar macrophages may be decreased in COPD (7), whereas the epithelial cilia function is impaired by cigarette smoke, resulting in increased inflammation in the lung (8-10). In addition to the removal of foreign material from the lung, macrophages are also considered important regulators of immune responses in the lung due to their ability to release various pro-inflammatory cytokines and chemokines. Macrophages release tumor necrosis factor-α (TNF- α) and interleukin-1β (IL-1β), cytokines which induce local and systemic inflammatory responses as well as increased mucus production. Furthermore, the release of chemokines such as interleukin-8 (IL-8) and leukotriene B_4 (LTB_4), which are potent neutrophil chemoattractants, may potentiate neutrophilic inflammation in the lung (4-6, 11, 12). In addition, macrophages are a source of proteolytic matrix metalloproteinases (MMPs) as well as reactive oxygen species which inhibit antiproteases, both of which may contribute to the development of emphysema (4, 6, 11, 12).

Neutrophils in the lung are also a source of proteolytic enzymes, including neutrophil elastase, an elastolytic enzyme which is considered to play a key role in the development of emphysema. In addition to its role in the breakdown of lung tissue, neutrophil elastase has also been implicated in increased mucus production and decreased mucociliary clearance, as well as stimulation of IL-8 and pro-fibrotic transforming growth factor-β (TGF-β) release. Neutrophils also release IL-8 and LTB_4, thereby perpetuating neutrophilic inflammation.

Azurophilic granules in neutrophils contain the enzyme myeloperoxidase (MPO), which participates in the formation of hypochlorous acid, a strong oxidant which may inactivate antiproteases in the lung, thus further augmenting tissue breakdown by elastolytic enzymes. Additionally, released MPO may result in lipid peroxidation and the formation of reactive nitrogen species, leading to tissue injury (4, 6, 13, 14).

Various studies have demonstrated increased numbers of T and B lymphocytes in the lungs of COPD patients, suggesting a role for the adaptive immune system in this disease (6, 13, 15-17). The numbers of T cells in the parenchyma and peripheral airways were shown to correlate with the degree of emphysema and airflow obstruction, respectively, suggesting an important role for these cells in the pathogenesis of COPD (15, 16, 18). Although $CD4^+$ and $CD8^+$ T cells are both increased in the lung, $CD8^+$ cells are the predominant type. $CD8^+$ T cells may contribute to tissue breakdown in COPD by releasing cytotoxic perforins and granzymes (6, 19). $CD8^+$ cells in sputum of COPD patients were shown to be highly activated and express high levels of perforins, associated with increased cytotoxic activity of these cells in vitro (20). In addition, the degree of $CD8^+$ cell activation in peripheral blood of COPD patients correlated with disease severity (21). Due to their strength in numbers, $CD8^+$ cells have received more attention in COPD research than the $CD4^+$ cells. The role of $CD4^+$ cells in COPD is still unclear, but it has been suggested that they contribute to the development of adaptive immune responses by priming and prolonging survival of $CD8^+$ cells as well as by taking part in the activation and differentiation of B cells (19).

B cell follicles have been observed in both the parenchyma and the airways of COPD patients (17, 18, 22, 23). Although the small airway area occupied by these follicles correlates with airflow obstruction in COPD (18), the contribution of B cells to the pathogenesis of this disease is unknown (24). There is evidence that B cells produce immunoglobulins, which are directed against antigens in the lung (25). The nature of these antigens is presently unclear, however. It has been suggested that the immunoglobulins may be directed against microbial antigens, since there is increased colonisation in the lungs of COPD patients (18). Alternatively, the protein content of cigarette smoke may be a source of antigens, as well as protein adducts formed in the lung as a result of cigarette smoke exposure (17). In addition, immunoglobulins directed against elastin peptides have been found in plasma of COPD patients, indicating that the breakdown of extracellular matrix proteins may result in the formation of antigens (26).

Structural changes in the lung

The persistent inflammatory response in the lung may lead to tissue damage and result in structural changes in the lung. The major structural abnormalities which develop in COPD include airway remodelling and emphysema. Emphysema is the destruction of alveolar structures, which leads to impaired gas exchange in the lung. In addition, loss of elastic recoil due to a decreased number of alveolar attachments results in collapse of the airways, contributing to airflow limitation (5, 27). As mentioned above, various inflammatory cells have been implicated in the development of emphysema and a particularly important role is attributed to elastolytic enzymes, such as neutrophil elastase.

Airway remodelling in COPD is characterized by peribronchial fibrosis, mucus gland hypertrophy, goblet cell hyperplasia and increased airway smooth muscle mass (5). These structural abnormalities result in thickening of the airway wall and mucus plugging of the lumen, thereby contributing to airflow obstruction. Although some features of airway remodelling may be observed in the large airways, they are most pronounced in small airways (<2 mm diameter), which are considered the major site of airflow obstruction in COPD (18, 27-29).

The pro-fibrotic growth factor TGF-β and its downstream Smad signalling pathway may be importantly involved in fibrotic processes in COPD (5). Increased TGF-β expression has been found in the airway epithelium of smokers and COPD patients (11, 30, 31). In addition, cigarette smoke exposure was shown to increase TGF-β expression and to induce Smad signalling in rat tracheal explants and in mouse lung (32-34). Furthermore, exposure of latent (recombinant) TGF-β to cigarette smoke in a cell-free system resulted in the release of active TGF-β (35).

Mucus gland hypertrophy and goblet cell hyperplasia in COPD result in mucus hypersecretion, which has been associated with morbidity and mortality in this disease (36-38). In the peripheral airways of COPD patients increased expression of the mucin MUC5B is observed in the lumen, whereas MUC5A/C expression is increased in the epithelium (39). The increased presence of mucus in the airways, combined with a cigarette smoke-induced deficiency of the ciliary apparatus, impairs the mucociliary clearance, leaving the lung susceptible to microbial colonisation (5, 6, 8, 36).

Airway smooth muscle remodelling is discussed separately below.

In addition to the airway remodelling, structural changes in the pulmonary vasculature have also been observed in COPD (5). Pulmonary vascular remodelling in COPD is characterized by thickening of the vessel wall, due to proliferation of the intima and the thickening of the media. In addition, muscularization of microvessels, which lack a smooth muscle layer under healthy condition, has also been observed (5). Remodelling of the pulmonary vasculature may contribute to pulmonary hypertension, a co-morbidity of COPD, which is present in a large proportion of the patients and may manifest itself at rest or during exercise. The mechanisms underlying pulmonary vascular remodelling in COPD are not known, but may involve hypoxia or pulmonary inflammation, leading to endothelial dysfunction and increased expression of growth factors in the vessel wall (5, 40-45). In addition, pulmonary hypertension results in remodelling of the right ventricle, which becomes hypertrophied as a result of the increased afterload (41).

Extrapulmonary manifestations of COPD

COPD may be associated with a wide range of extrapulmonary manifestations, including cardiovascular disease, loss and dysfunction of skeletal muscle, diabetes, osteoporosis, anemia, increased gastro-oesophageal reflux and clinical depression and anxiety (46, 47). These may be the result of a spill-over of lung inflammation into the circulation, causing low grade systemic inflammation or, alternatively, systemic inflammation which does not originate from the lung (46-49).

Pharmacological treatment of COPD

Currently, inhaled β-adrenergic receptor agonists and anticholinergics constitute the main bronchodilator therapy, whereas inhaled corticosteroids are the major anti-inflammatory therapy in COPD (50). Although glucocorticosteroids may be used to reduce exacerbations (51) and some recent studies have indicated that long-term therapy with high doses of inhaled corticosteroids, with or without long-acting bronchodilators, may decrease inflammation and the rate of lung function decline in (subgroups of) COPD patients (52, 53), the effectiveness of inhaled corticosteroids on progression of COPD is still a topic of discussion (54). Thus, it appears that the sensitivity to glucocorticosteroids is reduced in most patients with COPD, which may involve inhibition of histone deacetylase-2 by oxidative stress (54). PDE4 inhibitors, such as roflumilast, are a novel class of anti-inflammatory agents bearing perspectives in (severe) COPD (55).

The role of airway smooth muscle in the pathogenesis of COPD

Airway smooth muscle area may be increased in the small airways of COPD patients (16, 18, 56, 57). This contributes to the increase in airway wall area which leads to airflow obstruction. Indeed, both airway smooth muscle mass (16) and total airway wall area (18) were shown to correlate with a decrease of FEV_1 in COPD. Airway smooth muscle form COPD patients was shown to have an increased capacity to generate force in vitro (58, 59); the generated force negatively correlated with both FEV_1/FVC ratio and FEV_1 in these patients (59). These data suggest that increased area and altered function of airway smooth muscle may contribute to airflow obstruction and hyperresponsiveness in COPD (27).

In addition to their role as contractile cells regulating airway diameter, airway smooth muscle cells may also act as synthetic cells, producing chemokines, growth factors and extracellular matrix proteins in response to various G-protein-coupled receptor agonists, growth factors, pro-inflammatory cytokines or cigarette smoke (28). The increased release of chemokines, such as IL-8, may contribute to airway inflammation, whereas increased deposition of extracellular matrix proteins and the release of growth factors may contribute to fibrosis as well as airway smooth muscle cell proliferation (28, 60-67).

Cell proliferation is a mechanism underlying the increased airway smooth muscle area in obstructive airways diseases (68, 69) and may be induced by the release of growth factors from structural cells of the airway wall, such as epithelial cells, macrophages or even airway smooth muscle cells themselves (60, 70, 71). Growth factors and other mitogens, including various G-protein-coupled receptor agonists, cytokines and extracellular matrix proteins, can induce a proliferative phenotype of airway smooth muscle cells (66, 72-81). This phenotype is characterized by an increased rate of proliferation as well as a decreased expression of contractile proteins and decreased contractile function (74, 76-80). Phenotypic modulation is a reversible process; removal of the proliferative stimulus or altered extracellular matrix expression can induce maturation of the cells to a normo- or even hypercontractile phenotype, resulting in airway smooth muscle tissue with an increased force-generating capacity (74, 77, 82, 83).

Mitogen-activated protein (MAP) kinase pathways have been shown to play a major role in growth factor-induced signalling and the induction of a proliferative, hypocontractile phenotype of airway smooth muscle (72, 75, 77, 78, 84, 85). Even though airway smooth muscle mass may be increased in COPD, the potential contribution of disease-relevant stimuli, such as cigarette smoke or

lipopolysaccharide (LPS), to airway smooth muscle proliferation and phenotype modulation by direct action on the airway smooth muscle cells has not yet been addressed.

In vitro models of COPD

Cigarette smoke

Cigarette smoke extract (CSE) is the cornerstone of in vitro COPD modelling. CSE is made by passing CS through cell culture medium or a physiological buffer solution. There is no standard for preparing CSE. The number of cigarettes, volume of cell culture medium and type of cigarette used vary between different laboratories. Furthermore, cigarettes may be burned with or without filter and the CSE may be used as is or filtered. Therefore, a detailed description of the procedure used to prepare the CSE is required for comparing results obtained in different laboratories.

Various cell types have been exposed to CSE in order to mimic CS exposure in the lung. These include structural cells such as fibroblasts, epithelial and airway smooth muscle cells (62-65, 86-88) as well as inflammatory cell types such as macrophages and neutrophils (89-91). Upon CSE exposure, functional responses and gene expression changes, as well as the underlying intracellular signalling mechanisms, have been evaluated in these cells. Experiments in various cell types, including bronchial epithelial and airway smooth muscle cells as well as macrophages and neutrophils, have indicated that CSE induces increased release of the neutrophil chemokine interleukin-8 (IL-8) (63-65, 86, 89, 90). Some of these studies revealed that oxidative stress and activation of NF-κB and MAP kinase pathways play a role in this process (63, 64). In addition, experiments using CSE exposure of macrophages and neutrophils have indicated that TLR receptors may play a key role in CS-induced IL-8 release (89, 90). Various studies have also indicated that CSE may induce cell death (92-95). However, in the majority of these studies cells are exposed for prolonged periods of time (92, 93), whereas smokers' lungs are usually exposed for several minutes at a time. Effects of short, pulsatile CSE-stimulation of cells are still unknown.

CS instead of CSE has also been used to stimulate epithelial cells (96, 97), since these cells are directly exposed to inhaled air in the lung and this type of exposure may therefore be suitable for studying the effects of CS. Exposure of other airway wall cells, such as fibroblasts and airway smooth muscle cells, may possibly be more suitably modelled using CSE rather than CS, as in vivo components of CS may only reach these cells by diffusion through the airway wall.

Although most in vitro models have focused on CS or CSE exposure of cultured cells, airway or lung tissues have also been exposed to these stimuli (33, 98-100). A particularly elegant model using rat tracheal explants embedded in agar and exposed to CS on the epithelial side, thereby mimicking airway CS exposure in vivo, allows determination of the processes in the airway wall that are activated by CS without the involvement of immune cells (33, 101). Studies using this model have revealed that CS may induce pro-fibrotic changes in the airway wall in the absence of an inflammatory response. These findings were subsequently confirmed using an in vivo mouse model, showing that the pro-fibrotic gene expression in the airways is increased by CS before inflammatory cell numbers are increased (32).

Components of CS

Cigarette smoke is a complex mixture of over 5300 identified components (102). Exposure of cells to known components of CS, such as acrolein, formaldehyde or acetaldehyde, has been used to elucidate the contribution of these components to the release of pro-inflammatory cytokines and mucus production (103-106). This approach is indeed useful for identifying the contribution of individual CS components to the pathophysiology of COPD, but may be less suitable as a model as the contribution of a large number of other components is excluded.

Bacterial components

Stimulation of cells with bacteria (P. aeruginosa, H. infuenzae) or bacterial components, such as LPS, as well as viruses (RSV, rhinovirus) has been used to model exacerbations of COPD. Airway infections have been associated with exacerbations of COPD, which contribute to the progression of the disease (107, 108). Increased levels of LPS have been demonstrated in BAL fluid from COPD patients, indicating that it may be a relevant stimulus for disease progression (109). LPS has been shown to induce IL-8 release from lung fibroblasts as well as from epithelial and airway smooth muscle cells (110-112). LPS has also been shown to induce connective tissue growth factor release or mucin production from bronchial epithelial cells, indicating a possible contribution of LPS to fibrotic processes and mucus hypersecretion in the airways (113, 114).

Elastase

Elastase is a proteolytic enzyme, which is released by activated neutrophils in the lung and is considered to be a major contributor to the breakdown of alveolar tissue, resulting in emphysema (115). However, several studies have shown that elastase may induce pro-inflammatory gene expression and IL-8 release from bronchial epithelial cells (115-119). In addition, neutrophil elastase has also been

shown to induce mucus production by these cells (120-122). Elastase was also found to lower the ciliary beat frequency of epithelial cells, suggesting a role in impaired bacterial clearance in COPD (123). Mechanisms underlying the effects of elastase treatment appear to be complex and may involve activation of protease-activated receptors (PARs) (124, 125) or the proteolytic release of epidermal growth factor (EGF) from lung fibroblasts (126, 127). Interestingly, similar to CS, elastase was found to activate TLRs, supporting the importance of these receptors in the pathophysiology of COPD (128, 129).

Animal models of COPD

Animal models of disease are used for studies, which for ethical reasons cannot be performed in humans. The major disadvantage of these disease models is that they are per definition based on species different from human. However, once this limitation is considered when interpreting results, animal models can be used to gain insight into specific pathophysiological processes and putative therapeutic interventions. Each animal model has its own specific advantages and disadvantages; therefore, the suitability of a particular model will highly depend on the aims of the study.

In the last 30 years, modelling of COPD in animals has largely been performed in small laboratory animals, such as mice, rats and guinea pigs. Mice offer the advantage of a wide range of molecular and genetic tools, such as antibodies and knock-out mice. On the other hand, the lung and airway structures, the distribution of mucus glands and goblet cells, the autonomic innervation of the airways and airway vascularisation in guinea pigs closely resemble that of humans whereas in mice and rats they do not (130) . Despite the fact that several studies have used rats, these animals appear to be relatively resistant to the development of experimental COPD (131).

Cigarette smoke exposure

Cigarette smoke exposure is the major risk factor for the development of COPD and is therefore considered by many to be the ideal choice for modelling COPD (131). The protocols used for the exposure of animals vary greatly between the different studies. Thus, there is large variation in the length, frequency and number of exposures, the type of exposure (nose-only or whole-body exposure) and the type of cigarette used. Exposure of small animals to cigarette smoke has been performed in commercially available or home-made CS exposure apparatuses of varying degrees of complexity (131). The nose-only exposure system involves extensive handling and restraining of the animals, which may be

very stressful. Whole-body exposure results in deposition of CS particles on the pelt, which can be ingested during grooming; however, the animals are unrestrained and the exposure requires less animal handling. It has been demonstrated that animals suffered less weight loss after whole body exposure than after nose-only, suggesting this method may be preferred in order to avoid unwanted effects due to handling and restraining stress (131-133).Nevertheless, the nose-only cigarette smoke exposure has successfully been used for modelling COPD in animals (17, 134, 135) Because animals may change their breathing patterns as an avoidance reaction to inhalation of CS it has been suggested that monitoring of serum cotinine or blood carboxyhaemoglobin should be performed in order to quantify the exposure (131, 132, 136).

Despite these limitations and challenges, CS exposure has been shown to induce various features of COPD in animals, including pulmonary infiltration of macrophages and neutrophils, airway fibrosis and emphysema (32, 131, 134, 137-141). Although emphysema has consistently been reported after chronic CS exposure (131, 137-141), the degree varies between studies. This may be due to variation in the smoking protocol or species and strain differences (131, 142)
In guinea pigs, CS has also been shown to induce pulmonary hypertension (PH), associated with pulmonary vascular remodeling, both of which are observed in patients with COPD (135, 143-145). In addition, CS-induced PH has also been reported in rats but not in mice, although vascular remodeling does occur in the latter species (131, 146).

A major advantage of CS-models may be that the stimulus used is a major contributor to the development of COPD in human subjects. CS-exposure of experimental animals induces several pathological features of COPD. Another drawback is that CS-induced models are costly and time-consuming, as these models require 5 days per week CS-exposure for several hours, during 6 months. The severity of disease in these models is rated as GOLD stage 2, which indicates mild disease (131).

Elastase model of emphysema
This model consists of instillation of elastolytic enzymes in the lung resulting in tissue damage. A single instillation results in the development of emphysema. Early studies by Gross and colleagues (147), using papain instillation were instrumental in the establishment of the protease/anti-protease hypothesis and were a major contribution to the understanding of the role of α1-antitrypsin deficiency in the development of emphysema.

Although intuitively the mechanism of elastase-induced emphysema should be simple, involving destruction of tissue structure by enzymatic digestion, several lines of evidence suggest a much more complex mechanism. The half life of instilled elastases in the lung was found to be as short as 45-50 min (148), whereas the enlargement of alveolar spaces is progressive over a period of days and continues therefore after the exogenously applied elastases are no longer present in the lung (149-151). In accordance, treatment with elastase inhibitors prior to or immediately after instillation of elastase results in inhibition of airspace enlargement, whereas treatment 4 or 8 h post instillation does not (152-155). Elastase instillation induces an inflammatory response in the lung. Thus, increased expression of TNF-α, IL-1β, IL-6 and IL-8 as well as infiltration of macrophages and neutrophils have been reported, suggesting that inflammation might play a role in the development of elastase-induced emphysema (156, 157). Accordingly, mice lacking TNF-α- and/or IL-1β- receptors are strongly protected against the development of elastase-induced emphysema (156, 158).

Major advantages of the elastase model are the technical ease of inducing disease by a single instillation of the enzyme in the lung and the ability to control disease severity by adjusting the amount of enzyme (131). This model is particularly suited to study potential mechanisms of emphysema and regeneration processes in the lung. However, the model is not suitable for studying airway remodeling.

Starvation-induced model of emphysema

Starvation has been shown to induce emphysematous changes in the lung parenchyma. The study which revealed this phenomenon for the first time was conducted in the Warsaw Ghetto during World War II, where autopsy findings showed that a high percentage of people who died from starvation (13.5%) had emphysema (159). Subsequently, a number of studies reproduced this observation in rats, by subjecting the animals to severe caloric restriction for periods of several weeks. These studies confirmed that starvation induces emphysema-like changes as well as changes in lung mechanics. Reduced number of alveoli, increased alveolar volume and decreased alveolar surface area were reported (160-164). More recently, a study in anorexic subjects has shown that long-term caloric restriction is associated with loss of lung tissue and that in anorexic subjects the body mass index correlates with the diffusion capacity of carbon monoxide in the lung (165). However, despite similarities between the emphysematous changes induced by starvation, and emphysema in COPD, there are major differences in the pathology of these two conditions. The abnormal enlargement of airspaces and alveolar wall destruction observed in COPD is permanent, whereas the emphysema-like changes induced by starvation are

being reversed to normal levels after sufficient caloric intake (166, 167). Moreover, in rats with elastase-induced emphysema, calorie restriction induces a further increase in emphysema severity which is reversed by refeeding back to levels observed in animals treated with elastase alone (167). Furthermore, starvation-induced emphysematous changes, unlike emphysema in COPD, are not associated with airflow obstruction (160). In addition, this model lacks inflammatory cell influx in the lungs and structural changes in airways and pulmonary vasculature, which are observed in COPD (131). These data suggest that the starvation-induced emphysematous changes are likely the result of abnormal lung maintenance or growth. Therefore, this animal model is not well suited to study COPD.

LPS-induced model of COPD

LPS is a component of the cell wall of gram-negative bacteria, which is present as a contaminant in cigarette smoke, air pollution and organic dusts (168, 169). Exposure to environmental dusts containing LPS has been shown to induce chronic airflow obstruction and has been associated with the development of COPD in farmers (170, 171). In animals, a single LPS exposure induces pulmonary inflammation, characterized by infiltration of neutrophils and macrophages, as well as airway hyperresponsiveness (172-176). In addition, an increased number of airway mucus cells has also been observed after a single LPS exposure (177-181). Animals chronically exposed to LPS develop pulmonary inflammation as well as structural changes in the lung which are characteristic for COPD. Chronic LPS exposure induces increased numbers of macrophages and neutrophils (180, 182-188). Furthermore, CD8(+) T-cells as well as peribronchial and perivascular lymphocytic aggregates containing both CD8(+) and CD4(+) T-cells and B-cells were found to be increased in mice after chronic LPS exposure (188). Interestingly, 12 weeks of twice-weekly LPS exposure resulted in increased gene expression of the pro-inflammatory cytokines TNF-α, IFN-γ and IL-18 as well as increased numbers of macrophages and lymphocytic aggregates after 1- and 8-week recovery periods (188). This indicates that, similarly to COPD (189, 190), chronic LPS exposure induces inflammatory responses, which persist even after the stimulus is no longer present.

In addition to inflammation, chronic LPS exposure has been shown to induce airway remodelling. Increased numbers of Goblet cells have been observed in airways of both mice and guinea pigs after repeated LPS exposure (180, 188). Chronic LPS exposure also resulted in increased peribronchial deposition of collagen and thickening of the airway wall in mice (182, 184-188, 191). Although 12 weeks of twice weekly LPS exposure resulted in increased airway smooth

muscle mass in mice (188), such changes were not observed in guinea pigs after 3 weeks of 3 times per week LPS exposure (180). The cause of this discrepancy could be the difference in the number of exposures, but species differences might also play a role. Chronic LPS exposure has also been shown to induce emphysema in mice, guinea pigs and hamsters (188, 192-195). Vernooy and colleagues showed that the airway remodelling and emphysematous changes are still present in mice 8 weeks after the LPS exposure had ceased (188). In addition, Brass et al. showed that airways of mice exposed to LPS for 4 or 8 weeks, followed by 4 weeks of recovery, still show submucosal thickening and increased collagen deposition, associated with pro-fibrotic gene expression (186, 191). The numbers of goblet cells in the airways of LPS-exposed mice, although still above control levels, appeared to be decreased after 8 weeks of recovery, indicating this may be a reversible feature of LPS-induced disease (188). Collectively, these data indicate that chronic LPS-exposure induces persistent inflammatory responses in the lung as well as alterations of airway and lung structure, which closely resemble the pathological changes observed in COPD.

Several studies have shown that the LPS-mediated effects are dependent on the Toll-like receptor 4 (TLR4). Thus, features of LPS-induced disease, including AHR, inflammation and airway remodelling, were considerably impaired in mice expressing deficient TLR4 (182) or lacking LPS-binding protein (183) or CD14 (196), which are both required for the activation of TLR4 by LPS. In addition, LPS-induced AHR, inflammation and airway remodelling were inhibited by treatment of mice with a TLR4 antagonist, E5564 (Eritoran) (187).

The complex mechanisms underlying the LPS-induced inflammation and structural changes in the lung are not fully known. LPS induces the release of pro-inflammatory cytokines including TNF-α, IL-1β, IL-8, IL-6 and GM-CSF in the lung (175, 183, 188, 196-198). This leads to the influx and activation of inflammatory cells, which may contribute to the development of LPS-induced disease. In addition, LPS-induced activation of TLR4 in macrophages and/or neutrophils may perpetuate inflammation due to an additional IL-8 and TNF-α release (199, 200).

IL-1 appears to play an important role in LPS-induced pathophysiology. Thus, IL-1 receptor antagonist (IL-1Ra) was found to partially inhibit LPS-induced airway inflammation in rats, whereas IL-1R-knockout mice were protected against development of LPS-induced airway wall thickening and fibrosis, indicating a crucial role for this receptor in chronic LPS-induced airway inflammation and remodelling (184, 201).

Interestingly, TLR4 has also been shown to mediate CS-induced inflammation in vivo. Pulmonary infiltration of inflammatory cells as well as increase of pro-inflammatory cytokines in the BAL fluid after up to 5 weeks of CS exposure was strongly reduced in mice lacking a functional TLR4 (202, 203). In addition, IL-1R-knockout mice were similarly protected against CS-induced pro-inflammatory responses in the lung (202). These data indicate that signalling pathways required for the development of LPS-induced pathological changes also play an important role in the inflammatory responses to CS.

Major advantages of the LPS-induced COPD model are the wide range of pathological features which are induced over a relatively short period of time, requiring only 2-3 LPS exposures per week. Several studies have indicated that TLR4 may play a role in CS-induced inflammatory responses (89, 202, 203) as well as in the development of COPD (170, 171), suggesting that LPS is a relevant stimulus for modeling COPD. This model is also suitable for testing therapeutic interventions and is less labour intensive and less costly than CS-exposure. Like CS-exposure, chronic LPS is considered to induce relatively mild disease, up to now (131). Although LPS and CS may share common mechanisms to induce disease it is important to realize that there still may be differences in the etiology of disease.

Caenorhabditis elegans model of COPD?

Recently, one group has exposed Caenorhabditis elegans (C. elegans) to CS in order to study its effects on gene expression of the innate immune system while eliminating any involvement of the adaptive immune system, which is not present in this nematode (204). The authors showed that C. elegans tolerates up to 4 h of CS exposure, as this had no significant effect on the rate of mortality measured at 48 h post exposure. Furthermore, CS exposure had profound effects on gene expression and resulted in impaired bacterial clearance. Several genes, which had human orthologues were selected for further investigation using RNAi techniques. This approach lead to the conclusion that CS-induced downregulation of lbp-7 (human orthologue: fatty acid binding protein 5 (FAB5)) plays a major role in bacterial clearance in C. elegans. In order to determine the relevance of this finding for COPD, the authors demonstrated that FAB5 mRNA expression is increased by bacteria in primary human bronchial epithelial cells and that cells isolated from COPD patients show lower FAB5 mRNA levels than cells from healthy smokers.

Another recent study used a similar approach to investigate the role of hypercapnia - which may occur in COPD as a result of impaired gas exchange - in

gene expression as well as motility and muscle morphology (205). Exposure of C. elegans to increased CO_2 levels resulted in altered gene expression and decreased motility, which was associated with abnormal muscle fiber organization. These data support a role for hypercapnia in skeletal muscle wasting observed in COPD (46, 48, 49).

These two studies show that C. elegans can be used to study potential mechanisms involved in COPD. Exposure of C. elegans to conditions or factors relevant to human disease could be a powerful source of information due to its ease of use, low cost, fully sequenced genome and availability of RNAi tools, providing the obtained results can be translated to mammalian models and human disease. The use C. elegans as a model species for COPD does however have major drawbacks. Most importantly, since C. elegans is a nematode, it does not have lungs. Therefore, although C. elegans offers many possibilities for investigating functional implications of altered gene expression and could thus be used for studying specific processes related to human disease, it is not a disease model of COPD.

Cellular and molecular mechanisms

The understanding of cellular and molecular mechanisms underlying COPD is limited compared to other obstructive lung diseases such as asthma. A major goal of our investigations was to define specific mechanisms involved in inflammation and airway remodelling in this disease, using CSE- and LPS-exposed airway smooth muscle cells in vitro, as well as a newly developed guinea pig model of LPS-induced COPD in vivo and ex vivo. The in vitro studies were focused on signalling mechanisms involved in CSE-, LPS- and growth factor-induced changes in airway smooth muscle phenotype and function. The animal model studies addressed the role of the cholinergic system and the NO-arginase axis as two major, interrelated pathways that are likely to be involved in the inflammatory, structural and functional changes observed in COPD.

Cholinergic mechanisms

Increased cholinergic tone has been identified as the major reversible component of airflow limitation in COPD (206). Acetylcholine release from parasympathetic nerve endings results in contraction of airway smooth muscle and increased mucus release. This contribution of acetylcholine to airflow limitation is the basis for the use and effectiveness of anticholinergics as bronchodilators in COPD (207). However, recent studies have suggested that anticholinergics, in addition to bronchodilatation, may have other beneficial effects. Data from the UPLIFT

trial show that the long-acting anticholinergic tiotropium bromide reduces exacerbation frequency, all-cause mortality and the number of adverse respiratory and cardiac events in COPD patients (208, 209). In addition, a subgroup analysis indicated that tiotropium reduces the rate of lung function decline in patients with mild COPD (210), young patients (211) and in those not on other controller medication (212). Recent observations in an acute mouse model of CS exposure suggest that tiotropium may reduce pulmonary neutrophilia by reducing the expression of pro-inflammatory cytokines and chemokines in the lung (213). The effects of tiotropium on structural remodelling in COPD are still unknown.

However, tiotropium has been shown to inhibit allergen-induced airway eosinophilia as well as airway smooth muscle thickening and increased mucin expression, suggesting that acetylcholine contributes to airway inflammation and remodelling in asthma (214, 215). Muscarinic receptors are expressed in both inflammatory and structural cells in the lung (207). Muscarinic agonists have been shown to induce the release of pro-inflammatory chemokines from macrophages and epithelial cells (216, 217) and to augment CSE-induced IL-8 release from airway smooth muscle cells (62). In addition, the observation that muscarinic receptor stimulation induces proliferation and collagen synthesis in lung fibroblasts suggests a role for acetylcholine in fibrosis (218, 219). Interestingly, the acetylcholine synthesizing enzyme choline acetyltransferase (ChAT) is also widely expressed in the lung (207). The observations that muscarinic receptors are present on cells which have no parasympathetic innervation and that acetylcholine can be synthesized by non-neuronal cells indicate that non-neuronal acetylcholine may also be involved in lung physiology and pathophysiology. Indeed, ChAT expression is increased in lung fibroblasts from smokers and COPD patients, suggesting that non-neuronal acetylcholine may be involved in the pathogenesis of COPD (220).

Arginase

Arginase is an enzyme which converts L-arginine to L-ornithine and urea. It is involved in the urea cycle in the liver but is also expressed in extrahepatic tissues lacking a complete urea cycle (221). Two isoforms of arginase have been identified; arginase I is a cytosolic enzyme primarily expressed in the liver, whereas arginase II is a mitochondrial enzyme expressed in extrahepatic tissue (222). Both arginase isoforms are constitutively expressed in the lung, particularly in epithelial cells, (myo)fibroblasts and endothelial cells, as well as in macrophages and neutrophils (223-226). Arginase expression and/or activity was shown to be increased in animal models of asthma (221) as well as in

asthmatic patients (227-229). In a guinea pig model of allergic asthma, increased arginase activity was shown to contribute to allergen-induced airways obstruction, hyperresponsiveness, and inflammation (230). Increased arginase activity was found to result in a decreased L-arginine bioavailability for the enzyme nitric oxide synthase (NOS). This leads to a decreased production of the bronchodilatory, anti-inflammatory nitric oxide (NO) as well as an increased production of the pro-contractile, pro-inflammatory oxidant species, peroxynitrite (231-236). Decreased production of NO may contribute to airway obstruction, inflammation and hyperresponsiveness (235). Under inflammatory conditions, inducible NOS (iNOS) is upregulated and arginase-induced low L-arginine bioavailability results in concomitant production of NO and superoxide anions (O_2^-) by this enzyme, leading to rapid formation of peroxynitrite (237). Peroxynitrite production by iNOS may contribute to allergen-induced airway hyperresponsiveness as well as inflammation in allergic asthma (230, 238). Furthermore, peroxynitrite also induces MUC5A/C expression in airway epithelium and may therefore contribute to mucus hypersecretion (239). Arginase has also been shown to play a role in TGF-β-induced collagen synthesis, due to increased formation of the collagen precursor L-proline downstream of L-ornithine (222, 240, 241). Recent studies from our group indicate that increased arginase activity contributes to airway remodelling in asthma, which may involve increased synthesis of L-proline and polyamines downstream L-ornithine, as well as reduced production of NO (242).

Although many studies have indicated an important role of arginase in the pathophysiology of asthma, there are very few studies that have focused on its potential role in COPD. Only recently, increased arginase activity was demonstrated in BAL fluid from COPD patients (243). Remarkably, similar observations were made in sputum from patients with asthma or chronic bronchitis in the late 1970s (244, 245), but this was interpreted as leaking of hepatic arginase. Increased arginase gene expression in the lung has been observed in CS-exposed rats (246) and in LPS-exposed mice (191, 247). In addition, smoking was shown to further increase arginase gene expression and immunostaining in the airways of mild asthmatics (248). Increased arginase expression and/or activity has also been found in pulmonary endothelial cells and serum of patients with pulmonary arterial hypertension (249), which may occur as a co-morbidity of COPD (5, 41). Increased arginase activity may contribute to endothelial dysfunction in pulmonary arterial hypertension by causing reduced production of eNOS-derived vasodilatory NO (249). This is further supported by the observation that oral L-arginine or inhaled NO decrease pulmonary arterial pressures in this disease (250). Despite accumulating

evidence suggesting a potential increase of arginase activity or expression in COPD, no studies have thus far demonstrated a role for arginase in the pathophysiology of this disease.

TGF-β-activated kinase 1

TGF-β-activated kinase 1 (TAK1) is a serine/threonine kinase and a member of the MAP kinase kinase kinase family (MAP3K7). It was originally identified as a mediator of Smad-independent TGF-β signalling, but has since also been shown to play a key role in pro-inflammatory signalling pathways downstream of TLR, IL-1R and TNFR (Figure 1) (251-256). TAK1 is an important activator of the NF-κB pathway, as well as the ERK 1/2 and p38 MAP kinase pathways (251-255, 257-260). Activation of TAK1 contributes to the initiation of immune responses by inducing the release of pro-inflammatory cytokines, such as IL-8, IL-6 and TNF-α, and has also been shown to promote proliferation or survival of B-cells, T-cells and neutrophils (257, 261-264). In addition to stimuli of the above mentioned receptors, TAK1 is also activated by diesel exhaust particles as well as osmotic stress and hypoxia, indicating a role in stress-induced signalling (265-267). Considering the major role of TAK1 in TLR-signalling and the evidence suggesting a role for TLRs in the pathophysiology of COPD as well as in CS-induced signalling and inflammation, an important role for TAK1 in the development of COPD can be envisaged. However, no direct evidence has yet been reported.

In addition to its role in inflammation, several studies have indicated a major involvement of TAK1 in embryonal development (268-271). Interestingly, aberrant TAK1 signalling was shown to result, among others, in impaired development of the lung as well as abnormal vascular development lacking smooth muscle (269, 270). TAK1 has also been implicated in tissue remodelling. Increased TAK1 expression was associated with cardiac hypertrophy resulting from pressure overload in mice or with myocardial infarction in rats (272, 273). Expression of activated TAK1 in the mouse myocardium is sufficient to induce cardiac hypertrophy (273). Despite this evidence for the role of TAK1 in the development of the lung and vascular tissue as well as the remodelling of cardiac muscle tissue, there are no reports addressing the potential role of TAK1 in airway smooth muscle function.

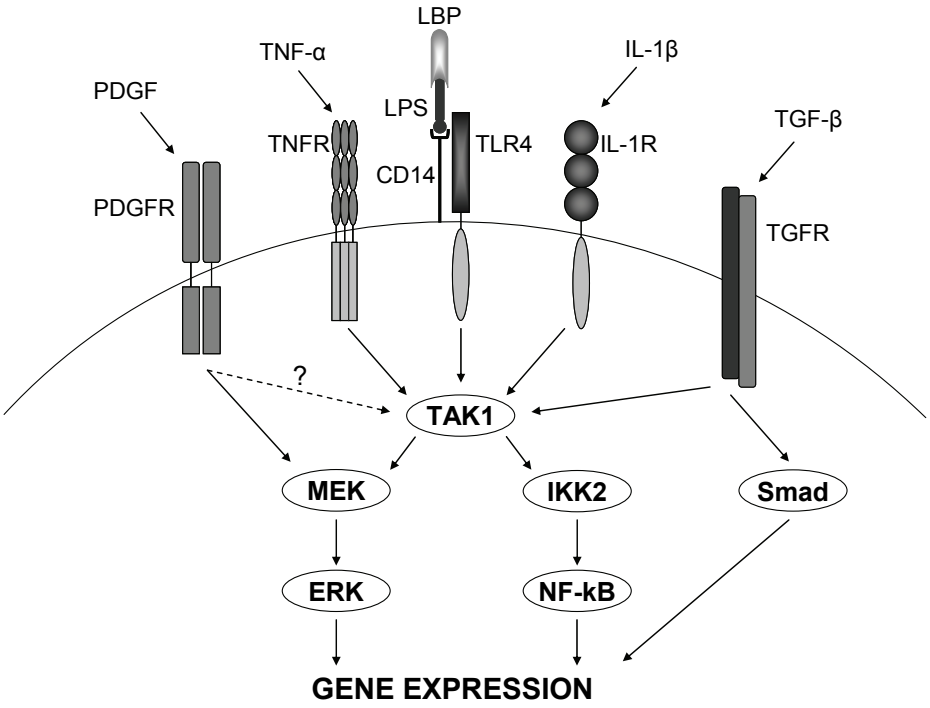

Figure 1: Pro-inflammatory and pro-fibrotic signalling in COPD.
Inhalation of cigarette smoke or other invoking factors of COPD, including lipopolysaccharide (LPS), leads to the release of pro-inflammatory cytokines and pro-fibrotic growth factors from structural and inflammatory cells in the lung. Pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), activate TNF- and IL-1-receptors (TNFR and IL-1R), respectively. LPS binds LPS binding protein (LBP) and CD14, thereby activating Toll-like receptor 4 (TLR4). Subsequently, intracellular adaptor proteins of the three receptors activate interleukin-1 receptor associated kinase (IRAK) and TNF-receptor-associated factor (TRAF), leading to activation of transforming growth factor-β (TGF-β)-activated kinase 1 (TAK1). TAK1 initiates NF-κB signalling via activation of IκB kinase 2 (IKK2) and extracellular signal-regulated kinase (ERK) signalling via activation of mitogen activated protein (MAP) kinase kinase (MEK). Among others, both signalling pathways are involved in the transcription of genes encoding for pro-inflammatory chemokines such as IL-8, which may perpetuate inflammation of the lung. Growth factors, including platelet-derived growth factor (PDGF), activate MAP kinase signalling via the small GTPase Ras and induce proliferative responses involved in airway remodelling. Although a link between PDGF and TAK1 has thus far not been demonstrated, we hypothesize that PDGFR stimulation may also lead to activation of TAK1. TGF-β activates the TGF-β receptor (TGFR), initiating both Smad and TAK1 signalling (either directly by TGFR or via the X-linked inhibitor of apoptosis (XIAP)), which may cause tissue remodeling by inducing proliferative and fibrotic responses.

Scope of the thesis

As indicated above, the objective of this thesis was to investigate mechanisms of pulmonary inflammation and remodeling in COPD by using *in vitro* and *in vivo* approaches. The focus of **Chapters 2 and 3** is phenotypic modulation of airway smooth muscle by stimuli involved in the pathogenesis of COPD. As discussed above, mitogens induce a proliferative, hypocontractile phenotype of airway smooth muscle. Proliferation of airway smooth muscle cells may cause increased airway smooth muscle mass, which has been demonstrated in COPD (18). Using cultured BTSM cells and tissue, the studies in **Chapter 2** explore the mitogenic capacity of CSE and LPS as well as the MAP kinase pathways potentially involved in the CSE- and LPS-induced responses. In **Chapter 3** the role of TAK1 in growth factor-induced ERK 1/2 signalling and modulation of airway smooth muscle cells and tissue to a proliferative, hypocontractile phenotype is investigated. The study was performed in BTSM cells and tissue as well as in primary human airway smooth muscle cells, the role of TAK1 being explored by the pharmacological TAK1 inhibitor LL-Z-1640-2 as well as the expression of dominant-negative TAK1. **Chapter 4** focuses on the potential role of airway smooth muscle cells as a source of pro-inflammatory cytokines in COPD. The studies described in this chapter investigate the role of TAK1, NF-κB and ERK 1/2 pathways in CSE-induced IL-8 release by cultured human airway smooth muscle cells. Studies described in **Chapter 5** address the role of endogenous acetylcholine in pulmonary inflammation and remodelling using an animal model of COPD. For this purpose, a guinea pig model of LPS-induced COPD was developed. Animals were intranasally instilled, twice weekly for 12 weeks, with sterile saline or LPS and pre-treated with either saline or the long-acting anticholinergic drug tiotropium. The effect of tiotropium on LPS-induced neutrophilia, MUC5A/C expression and changes in hydroxyproline content, mean linear intercept (MLI) and lung vasculature were evaluated. **Chapter 6** is a review of the literature describing the role of arginase in various pulmonary diseases. Evidence from animal models as well as from clinical studies is presented. **Chapter 7** is dedicated to unraveling the role of arginase in the pathophysiology of COPD. Using the guinea pig model of COPD described in **Chapter 5**, the effects of pretreatment by inhalation of the specific arginase inhibitor (2)S-amino-boronohexanoic acid (ABH) on LPS-induced pulmonary inflammation and remodelling were investigated. In addition to some of the parameters evaluated in **Chapter 5** (neutrophilia, MUC5A/C expression, hydroxyproline content and pulmonary vascular dimensions), arginase activity and IL-8 levels in the lung as well as right ventricle mass were evaluated in this disease model.

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Chapter 2

CIGARETTE SMOKE AND LIPOPOLYSACCHARIDE INDUCE A PROLIFERATIVE AIRWAY SMOOTH MUSCLE PEHENOTYPE

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Abstract

A major feature of chronic obstructive pulmonary disease (COPD) is airway remodelling, which includes an increased airway smooth muscle (ASM) mass. The mechanisms underlying ASM remodelling in COPD are currently unknown. We hypothesized that cigarette smoke (CS) and/or lipopolysaccharide (LPS), a major constituent of CS, organic dust and gram-negative bacteria, that may be involved in recurrent airway infections and exacerbations in COPD patients, would induce phenotype changes of ASM.
To this aim, using cultured bovine tracheal smooth muscle (BTSM) cells and tissue, we investigated the direct effects of CS extract (CSE) and LPS on ASM proliferation and contractility.
Both CSE and LPS induced a profound and concentration-dependent increase in DNA synthesis in BTSM cells. CSE and LPS also induced a significant increase in BTSM cell number, which was associated with increased cyclin D1 expression and dependent on activation of ERK 1/2 and p38 MAP kinase. Consistent with a shift to a more proliferative phenotype, prolonged treatment of BTSM strips with CSE or LPS significantly decreased maximal methacholine- and KCl-induced contraction.
Direct exposure of ASM to CSE or LPS causes the induction of a proliferative, hypocontractile ASM phenotype, which may be involved in airway remodelling in COPD.

Introduction

Chronic obstructive pulmonary disease (COPD) is an inflammatory lung disease characterized by a progressive and largely irreversible airflow obstruction, which involves structural changes of the lung, including emphysema and small airway remodelling (1). Small airway remodelling in COPD is characterized by adventitial fibrosis and mucus cell hyperplasia, and may involve increased airway smooth muscle (ASM) mass, particularly in severe disease (1-5). Small airway remodelling may contribute to the reduced lung function as well as to persistent airway hyperresponsiveness, which is present in most of the patients (6, 7).

Tobacco smoke exposure is considered to be the most important risk factor for COPD in developed countries. Lipopolysaccharide (LPS) - a constituent of the outer wall of gram-negative bacteria and a contaminant of tobacco smoke, organic dust and environmental pollution (8-11) - has been implicated in the development and progression of various pulmonary diseases, including COPD

(12-14). Cigarette smoke (CS) and LPS have previously been shown to induce features of airway remodelling in animal models, including airway wall thickening, increased ASM mass, goblet cell hyperplasia and collagen deposition (15-19).

Although the mechanisms involved in the development and progression of small airway remodelling in COPD are largely unknown, chronic inflammation of the airways is presumably of major importance. This is indicated by persistent infiltration of inflammatory cells, including macrophages, neutrophils and T- and B-lymphocytes, in the airway wall, which is correlated with the severity of airflow obstruction (3, 5). This inflammatory response is associated with the release of profibrotic cytokines and growth factors, which are linked to a repair and remodelling process that thickens the airway wall and narrows the airway lumen (20).

However, small airway remodelling could also result from direct effects of CS and LPS exposure on structural cells of the airway wall, independent of inflammation. Thus, studies using rat tracheal explants (21, 22) and a mouse model of CS exposure (23) have shown that CS exposure of the airway wall may lead to the release of TGF-β_1 and upregulation of platelet-derived growth factor (PDGF), connective tissue growth factor (CTGF) and procollagen gene expression independent of inflammatory cell infiltration. The inflammation-independent fibrotic response presumably involves an oxidant-driven mechanism, which may be reinforced by inflammatory cells such as macrophages and neutrophils, known to release oxidants in response to tobacco smoke (24). In addition, epithelial cells, fibroblasts, as well as ASM cells in culture have been shown to release pro-inflammatory and profibrotic cytokines in response to CS (25-29) or LPS (30-32).

As indicated above, various studies have indicated that increased airway smooth muscle mass may contribute to airway remodelling in COPD (2-5). Indeed, a direct correlation between the degree of smooth muscle mass and airflow obstruction in COPD has been reported (3, 5). Previous in vitro studies from our laboratory have demonstrated that growth factors, including PDGF, and extracellular matrix (ECM) proteins, including collagen I and fibronectin, induce a proliferative phenotype of bovine tracheal smooth muscle (BTSM), which is accompanied by reduced contractility of the muscle (33-35). PDGF-induced phenotypic modulation was shown to be mediated by ERK 1/2 and p38 MAP kinase, two signalling molecules that are importantly involved in mitogenic responses of ASM (33, 35). The direct effects of CSE and LPS on ASM

proliferation are, however, currently unknown. In this study, we present evidence that both CSE and LPS induce a proliferative, hypocontractile phenotype of ASM independent of inflammation, which could be important in the development and progression of ASM growth in COPD.

Methods

Isolation of Bovine Tracheal Smooth Muscle Cells

Bovine tracheae were obtained from local slaughterhouses and transported to the laboratory in Krebs-Henseleit buffer of the following composition (mM): NaCl 117.5, KCl 5.60, $MgSO_4$ 1.18, $CaCl_2$ 2.50, NaH_2PO_4 1.28, $NaHCO_3$ 25.00, and glucose 5.50, pregassed with 5% CO_2 and 95% O_2; pH 7.4. After dissection of the smooth muscle layer and removal of mucosa and connective tissue, tracheal smooth muscle was chopped using a McIlwain tissue chopper, three times at a setting of 500 μm and three times at a setting of 100 μm. Tissue particles were washed two times with Dulbecco's Modified Eagle's Medium (DMEM), supplemented with $NaHCO_3$ (7 mM), HEPES (10 mM), sodium pyruvate (1 mM), nonessential amino acid mixture (1:100), gentamicin (45 μg/ml), penicillin (100 U/ml), streptomycin (100 μg/ml), amphotericin B (1.5 μg/ml), and foetal bovine serum (FBS, 0.5%) (all purchased from GIBCO BRL Life Technologies, Paisley, UK). Enzymatic digestion was performed using the same medium, supplemented with collagenase P (0.75 mg/ml, Boehringer, Mannheim, Germany), papain (1 mg/ml, Boehringer), and Soybean trypsin inhibitor (1 mg/ml, Sigma Chemical, St. Louis, MO, USA). During digestion, the suspension was incubated in an incubator shaker (Innova 4000) at 37°C, 55 rpm for 20 min, followed by a 10-min period of shaking at 70 rpm. After filtration of the obtained suspension over a 50 μm gauze, cells were washed three times in supplemented DMEM containing 10% FBS. This isolation method results in a cell population positive for smooth muscle α-actin (95%) and smooth muscle myosin heavy chain (33, 36).

Cigarette Smoke Extract

Cigarette smoke extract was prepared by combusting 2 research cigarettes (University of Kentucky 2R4F; filters removed), using a peristaltic pump (Watson Marlow 323 E/D, Rotterdam, The Netherlands) and passing the smoke through 25 ml of FBS-free DMEM supplemented with penicillin and streptomycin at a rate of 5 minutes / cigarette. The obtained solution is referred to as 100% strength.

[^{3}H]-Thymidine Incorporation

BTSM cells were plated in 24-well cluster plates at a density of 50,000 cells per well, and were allowed to attach overnight in 10% FBS-containing DMEM at 37°C in a humidified 5% CO_2 incubator. Cells were washed two times with sterile phosphate-buffered saline (PBS, composition [mM] NaCl, 140.0; KCl, 2.6; KH_2PO_4, 1.4; $Na_2HPO_4.2H_2O$, 8.1; pH 7.4) and made quiescent by incubation in FBS-free medium, supplemented with apo-transferrin (5 µg/ml, human, Sigma), ascorbate (100 µM, Merck, Darmstadt, Germany), and insulin (1 µM, bovine pancreas, Sigma) for 72 h. Cells were then washed with PBS and stimulated with LPS (1–10,000 ng/ml), purified from *Escherichia coli* O55:B5 (Sigma) or PDGF (10 ng/ml) in FBS-free medium for 28 h. Treatment of cells with CSE (1-50 %) lasted 1 h, after which the cells were washed 3 times with PBS and incubated in FBS-free DMEM for another 27 h. [^{3}H]-thymidine (0.25 µCi/ml, Amersham, Buckinghamshire, UK) was present during the last 24 h of the incubations, followed by two washes with PBS at room temperature and one wash with ice-cold 5% trichloroacetic acid (TCA). Cells were incubated with TCA on ice for 30 min. Subsequently, the acid-insoluble fraction was dissolved in 0.5 ml NaOH (1 M). Incorporated [^{3}H]-thymidine was quantified by liquid-scintillation counting.

Cell number determination

BTSM cells were plated in 6-well cluster plates at a density of 100,000 cells / well in medium, containing 10% FBS. Cells were grown to 50% confluence after which they were serum-deprived for 72 h. Subsequently, cells were treated with CSE (15%) 2 times for 1 h, on day 0 and day 2, respectively, or with LPS (1µg/ml) or PDGF (10 ng/ml) for 4 days continuously. On day 4, the cells were washed twice with PBS and were trypsinized (0.25% Trypsin-EDTA (GIBCO); 15 min) and re-suspended in FBS-containing DMEM. Cells were then counted in duplicate, using a hemocytometer. When applied, the MEK inhibitors U0126 (3 µM; Tocris Cookson, Bristol, UK) or PD 98059 (30 µM, Sigma) and the p38 MAPK inhibitors SB 203580 (10 µM, Tocris) or SB 239063 (10 µM, Sigma) were added to the cells 30 min before stimulation and were present throughout the experiment.

Western blot analysis

BTSM cells were plated in 6-well cluster plates at a density of 200,000 cells / well in medium, containing 10% fetal bovine serum. Upon confluence, cells were washed two times with sterile PBS and made quiescent by incubation in serum-free medium, supplemented with apo-transferrin (5 µg/ml) and ascorbate (100 µM) for either 24 h, for ERK 1/2 and p38 MAP kinase phopsphorylation, or 72 h, for cyclin D1 expression. Cells were then washed with PBS and stimulated

in serum-free medium. To obtain total cell lysates, cells were washed once with ice-cold phosphate-buffered saline (PBS) and then lysed in ice-cold RIPA buffer (composition: 50 mM Tris, 150 mM NaCl, 1% Igepal CA-630, 1% deoxycholic acid, 1 mM NaF, 1 mM Na_3VO_4, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 7 μg/ml pepstatin A, 5 mM 2-glycerophosphoric acid, pH 8.0). Lysates were stored at –80 °C until further use. Cultured tissue strip homogenates were prepared by pulverizing the tissue under liquid nitrogen, followed by sonification in ice-cold RIPA buffer. Protein content was determined according to Bradford (37). Homogenates containing 50 μg of protein per lane were then subjected to immunoblot analysis using antibodies against cyclin D1, ERK 1/2, p38 MAP kinase or the phosphorylated forms of ERK 1/2 (Thr^{202}/Tyr^{204}) or p38 MAP kinase (Thr^{180}/Tyr^{182}) (Cell Signaling Technology, Beverly, MA, USA). The antibodies were visualized using enhanced chemiluminescence. Photographs of the blots were scanned and analyzed by densitometry (Totallab™; Nonlinear Dynamics, Newcastle, UK).

Tissue culture

After dissection of the smooth muscle layer and careful removal of mucosa and connective tissue, tracheal smooth muscle strips were prepared while incubated in gassed KH-buffer at room temperature. Care was taken to cut tissue strips with macroscopically identical length (1 cm) and width (2 mm). Tissue strips were washed once in sterile FBS-free DMEM, supplemented with apo-transferrin (5 μg/ml) and ascorbate (100 μM). Next, the tissue strips were transferred into suspension culture flasks containing a volume of 7.5 ml medium. CSE treated strips were exposed to 15 % CSE for 1 h daily during 8 days. LPS treatment was performed in the continuous presence of 1 μg/ml LPS during 8 days.

Isometric tension measurements

Tissue strips, collected from the suspension culture flasks, were washed with several volumes of KH buffer pregassed with 5% CO_2 and 95% O_2, pH 7.4 at 37°C. Subsequently, the strips were mounted for isometric recording (Grass force-displacement transducer FT03) in 20-ml water-jacked organ baths containing KH buffer at 37°C, continuously gassed with 5% CO_2 and 95% O_2, pH 7.4. During a 90-min equilibration period, with washouts every 30 min, resting tension was gradually adjusted to 3 g. Subsequently, the muscle strips were precontracted with 20 and 40 mM isotonic KCl solutions. Following two washouts, maximal relaxation was established by the addition of 0.1 μM (-)-isoprenaline (Sigma). In most of the experiments, no basal myogenic tone was detected. Tension was readjusted to 3 g, immediately followed by three washes with fresh KH buffer. After another equilibration period of 30 min, cumulative concentration response

curves were constructed using stepwise increasing concentrations of isotonic KCl (5.6–50 mM) or methacholine (1 nM–100 μM; ICN Biomedicals, Costa Mesa, CA, USA). When maximal tension was obtained, the strips were washed several times, and maximal relaxation was established using 10 μM (-)-isoprenaline.

Data analysis

All data represent means ± s.e.mean from separate experiments. The statistical significance of differences between data was determined by the Student's t-test for paired observations. Differences were considered to be statistically significant when $P<0.05$.

Results

CSE and LPS induce BTSM cell proliferation

Proliferative responses of isolated BTSM cells to CSE and LPS stimulation were investigated by [^{3}H]-thymidine incorporation and cell counting. A 1 h pulse treatment with CSE, followed by 27 h incubation in serum-free medium resulted in a significant and concentration-dependent increase in [^{3}H]-thymidine incorporation, reaching a maximum of 187 ± 13 % of control at a concentration of 15 % (Figure 1A). Similarly, LPS induced a concentration-dependent increase in [^{3}H]-thymidine incorporation of up to 254 ± 45 % of control, similar to that induced by a submaximal concentration of PDGF (10 ng/ml; 258 ± 64 %) (Figure 1B). Treatment of BTSM cells with 15 % CSE (two 1 h pulses, on day 0 and day 2), or 1 μg/ml LPS resulted in a significant increase in cell number as well, as determined 4 days after starting the treatment (Figure 1C). As a positive control, PDGF (10 ng/ml, 4 days) similarly increased BTSM cell number (Figure 1C). The combined treatment of cells with CSE (15%) and LPS (1 μg/ml) had no additional effect on cell numbers when compared to the separate treatments alone (data not shown). Collectively, these data indicate that both CSE and LPS induce proliferation of BTSM cells in a non-additive fashion.

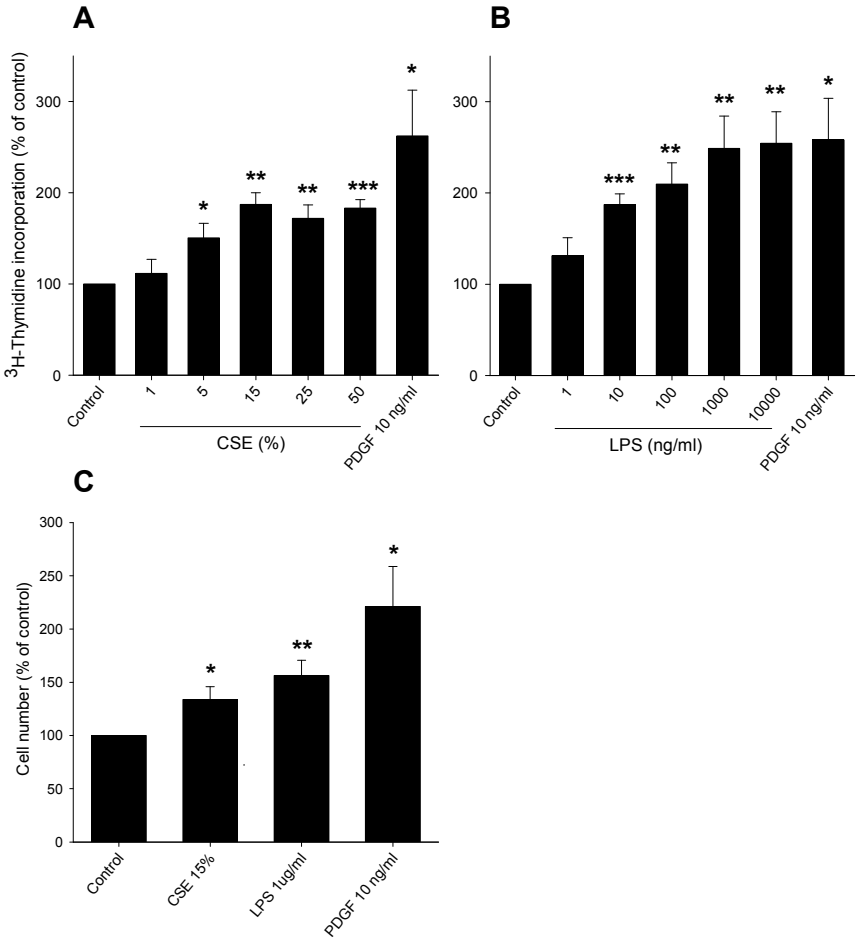

Figure 1: CSE and LPS induce BTSM cell proliferation. Subconfluent, serum-deprived BTSM cells were treated with increasing concentrations of CSE for 1 h (A) or increasing concentrations of LPS for 28 h (B). [3H]-Thymidine incorporation was determined 28 h after stimulation as described under methods. Data represent means ± S.E.M. of 5-7 experiments, each performed in triplicate. (C) Serum-deprived BTSM cells were treated 2 times (1 h, day 0 and day 2) with CSE (15%) or 4 days with LPS (1µg/ml) or PDGF (10 ng/ml). Cells were counted in duplicate on day 4, using a hemocytometer. Data represent means ± S.E.M. of 5-8 experiments. *P<0.05, **P<0.01, ***P<0.001 vs control

CSE and LPS induce ERK 1/2 and p38 MAP kinase phosphorylation and cyclin D1 expression

Western blot analysis was performed to investigate the effects of CSE (15 %) and LPS (1 μg/ml) on phosphorylation of ERK 1/2 and p38 MAP kinase, two major signalling pathways involved in ASM cell proliferation, and on the expression of cyclin D1, a key regulator of cell cycle progression downstream of ERK 1/2 and p38 MAP kinase. Both CSE and LPS induced a rapid phosphorylation of ERK 1/2 (Figure 2).

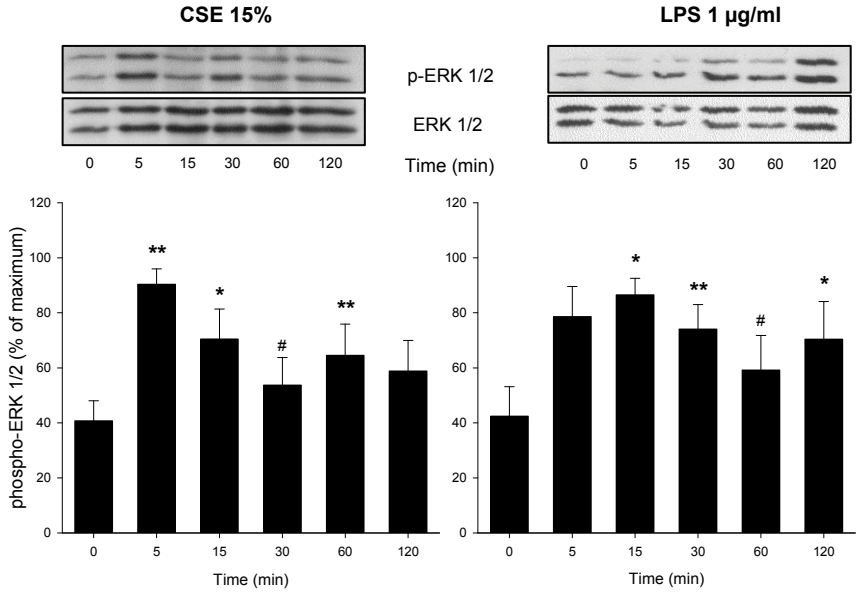

Figure 2: CSE and LPS induce ERK 1/2 phosphorylation. Serum deprived BTSM cells were treated with CSE (15%) or LPS (1 μg/ml) up to 2 h. Cell lysates were analyzed by immunoblotting for phospho-ERK 1/2 (Thr^{202}/Tyr^{204}) and total ERK 1/2 to correct for differences in protein loading. Phospho-ERK 1/2 was quantified using densitometry and normalized to the maximal response in each experiment. Representative blots are shown. Data represent means ± S.E.M. of 7 experiments. *P<0.05, #P<0.1 vs control at t=0

Both stimuli also induced a rapid phosphorylation of p38 MAP kinase, which, similarly to ERK 1/2 phosphorylation, was sustained (Figure 3). In addition, both CSE and LPS significantly increased the expression of cyclin D1, as assessed after 24 h, to a similar extent as 30 ng/ml PDGF (Figure 4), suggesting an important role for these signalling pathways in the proliferative response induced by CSE and LPS.

Role of ERK 1/2 and p38 MAP kinase in CSE- and LPS-induced proliferation

To test this hypothesis, the effect of CSE or LPS on cell number was determined in the presence or absence of U0126 (3 μM), an inhibitor of MEK, the upstream activator of ERK 1/2, or SB 203580 (10 μM), an inhibitor of p38 MAP kinase. As illustrated in Figures 5A and 5B, inhibition of MEK by U0126 and inhibition of p38 MAP kinase by SB 203580 completely abrogated the CSE- and LPS-induced increase in cell number. By contrast, no effect of the kinase inhibitors on basal cell numbers was observed. These findings were confirmed by using PD 98059 (30 μM) and SB 239063 (10 μM), alternative inhibitors for MEK and p38 MAP kinase, respectively (Figures 5C and 5D). Together with the CSE- and LPS-induced phosphorylation of ERK 1/2 and p38 MAP kinase described above, these data indicate that CSE- and LPS-induced proliferation is dependent on activation of the ERK 1/2 and p38 MAP kinase signalling pathways.

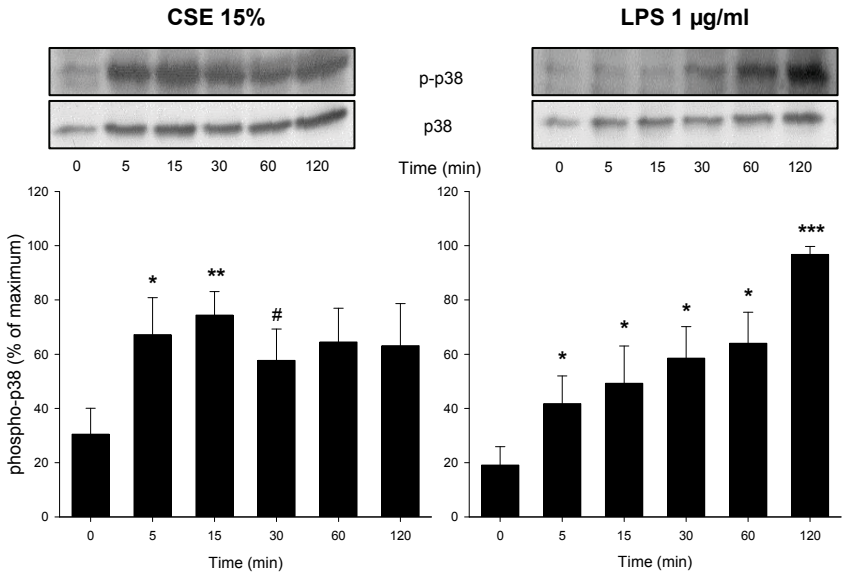

Figure 3: CSE and LPS induce p38 MAP kinase phosphorylation. Serum deprived BTSM cells were treated with CSE (15%) or LPS (1 µg/ml) up to 2 h. Cell lysates were obtained and analyzed by immunoblotting for phospho-p38 MAP kinase (Thr^{180}/Tyr^{182}) and total p38 MAP kinase to correct for differences in protein loading. Immunoblots were quantified using densitometry and the abundance of CSE- or LPS-induced p38 MAP kinase phosphorylation was normalized to the maximal response in each individual experiment. Representative blots are shown. Data represent means ± S.E.M. of 4-5 experiments. *P<0.05, **P<0.01, ***P<0.001, #P<0.1 vs control at t=0

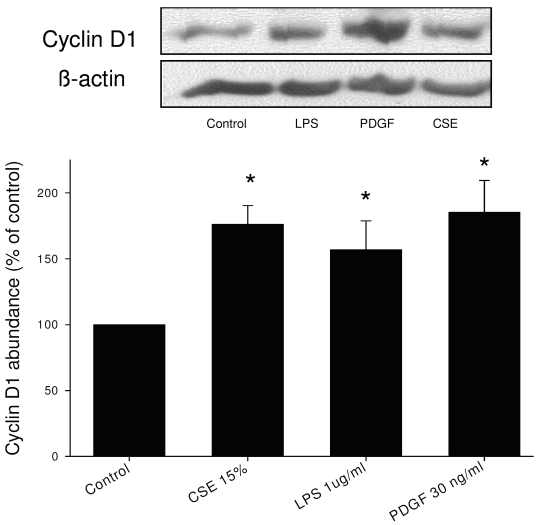

Figure 4: CSE and LPS increase cyclin D1 expression. Serum-deprived BTSM cells were treated with CSE (15%) for 1 h, or LPS (1µg/ml) or PDGF (30 ng/ml) for 24 h. Cell lysates were obtained 24 h after stimulation and analyzed by immunoblotting for cyclin D1 and β-actin to correct for protein loading. Cyclin D1 was quantified using densitometry and normalized to control expression. Data represent means ± S.E.M. of 4-7 experiments. *P<0.05 vs control

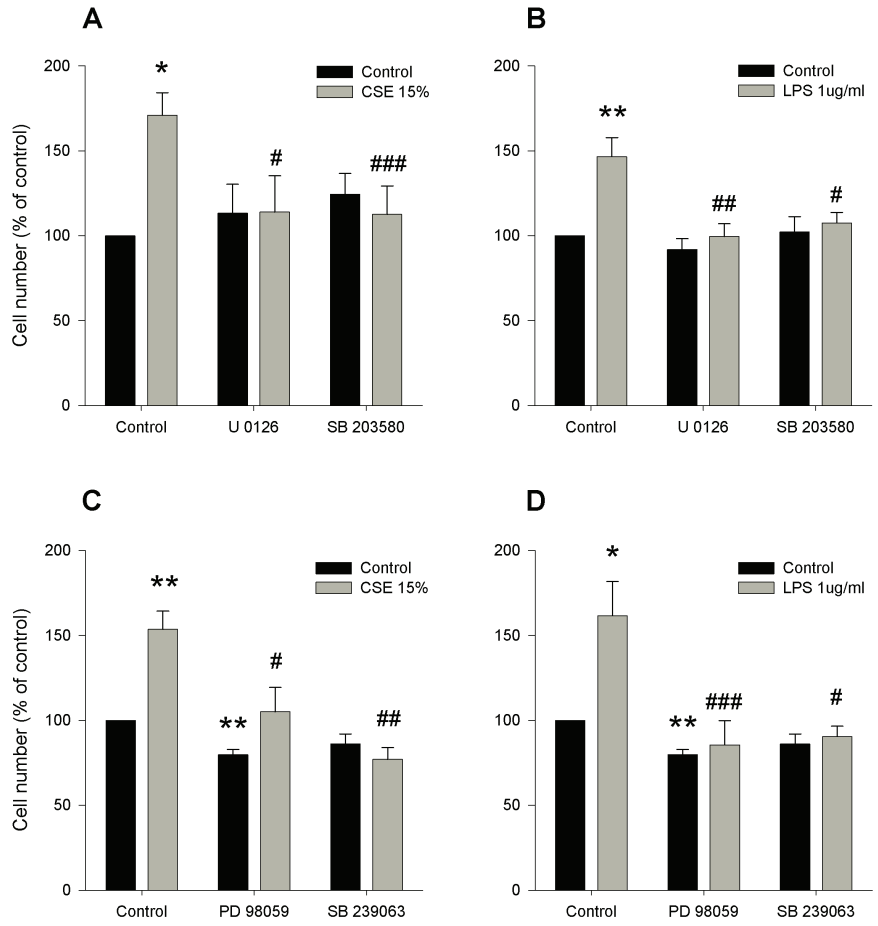

Figure 5: CSE- and LPS-induced proliferation is dependent on ERK 1/2 and p38 MAP kinase. Serum-deprived BTSM cells were treated 2 times (1 h, day 0 and day 2) with CSE (15%) or 4 days continuously with LPS (1 μg/ml) in the absence or presence of the MEK inhibitor U0126 (3 μM) and, the p38 MAP kinase inhibitor SB 203580 (10 μM) (panels A and B), as well as in the absence or presence of the MEK inhibitor PD 98059 (30 μM) and the p38 MAP kinase inhibitor SB 239063 (10 μM) (panels C and D). Cells were counted in duplicate on day 4, using a hemocytometer. Data represent means ± S.E.M. of 4-7 experiments. *P<0.05, **P<0.01 vs untreated control, #P<0.05, ##P<0.01, ###P<0.001 vs CSE or LPS treatment in the absence of inhibitor.

Effects of LPS and CSE on BTSM contractility

Previous studies have shown that the proliferative response of BTSM cells to growth factors and ECM proteins is linearly related to a decrease in contractility of BTSM tissue (33, 34). In order to investigate the effects of CSE and LPS on BTSM phenotype, strips were cultured for 8 days with 1 μg/ml LPS or were subjected to daily exposure to 15 % CSE for 1 h during 8 days. After both treatments, maximal contraction induced by methacholine or KCl was significantly reduced compared to untreated strips (Figures. 6A and 6B). No differences in the sensitivity (-log EC_{50}) to methacholine and KCl were found. These effects were associated with increased ERK 1/2 and p38 MAP kinase phosphorylation in the tissue (Figure 7). Collectively, these results indicate that both CSE and LPS induce a shift to a hypocontractile and proliferative ASM phenotype.

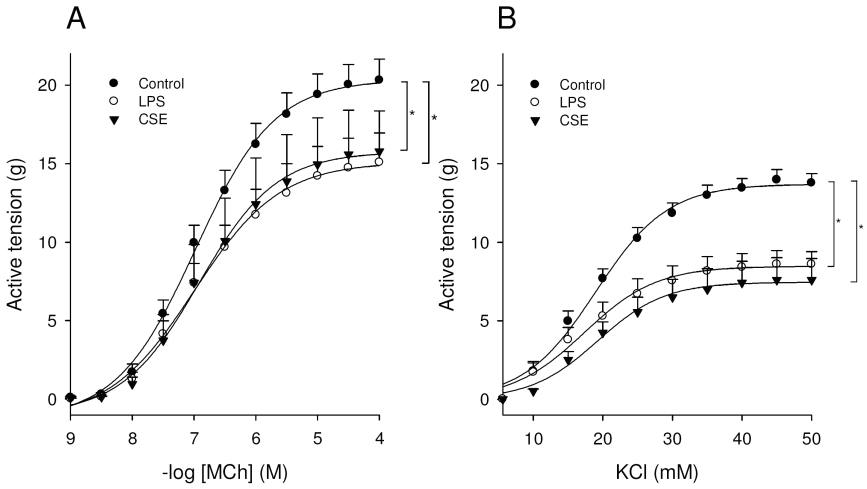

Figure 6: CSE and LPS induce BTSM hypocontractility. Methacholine (A)- and KCl (B)-induced contractions of BTSM strips cultured for 8 days with or without LPS (1 μg/ml) or exposed to 15 % CSE for 1 h daily during 8 days. Data represent means ± S.E.M. of 4-6 experiments, each performed in duplicate. *P<0.05 vs control.

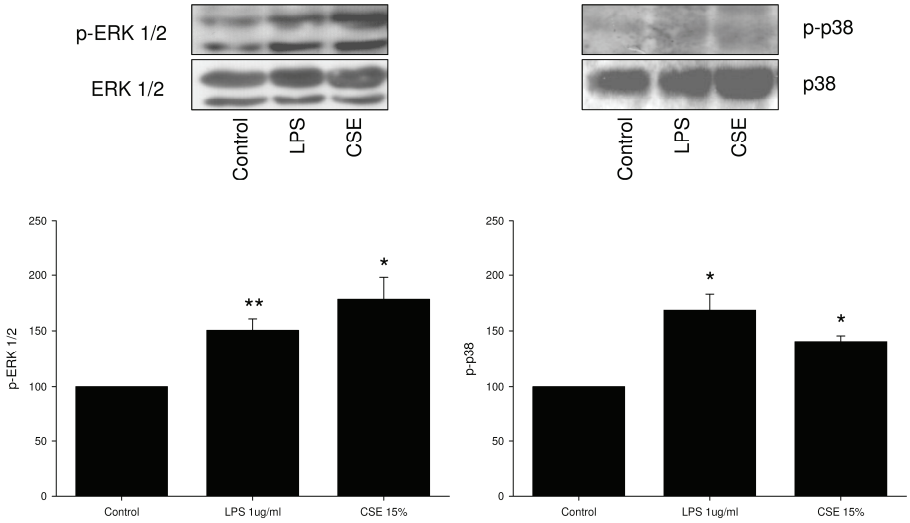

Figure 7: CSE and LPS induce ERK 1/2 and p38 MAP kinase phosphorylation in BTSM strips. BTSM strips were cultured for 8 days with or without LPS (1 µg/ml) or exposed to 15 % CSE for 1 h daily during 8 days. Tissue lysates were analyzed by immunoblotting for phospho-ERK 1/2 (Thr^{202}/Tyr^{204}) and phospho-p38 MAP kinase (Thr^{180}/Tyr^{182}). Differences in protein loading were corrected for by immunoblotting for total ERK 1/2 and total p38 MAP kinase. Immunoblots were quantified using densitometry. The abundance of CSE- and LPS-induced ERK 1/2 and p38 MAP kinase phosphorylation was normalized to controls from untreated strips. Data represent means ± S.E.M. of 3-6 experiments. *P<0.05, **P<0.01 vs control

Discussion

In this study, we demonstrated for the first time that CSE and LPS induce a profound and concentration-dependent increase in DNA synthesis and cell number of cultured ASM cells. The CSE- and LPS-induced proliferation is dependent on phosphorylation of ERK 1/2 and p38 MAP kinase and downstream mitogenic signalling. In addition, we demonstrated that CSE and LPS treatments reduce the maximal contraction of ASM preparations to methacholine and KCl, which is also associated with increased ERK 1/2 and p38 MAP kinase phosphorylation. Collectively, these data indicate that CSE and LPS induce a phenotype shift of ASM to a proliferative and less contractile phenotype that could be involved in airway remodelling in COPD.

Although small airway remodelling has been associated with cellular inflammation, evidence suggesting that direct action of cigarette smoke on the airway wall is involved in airway remodelling is accumulating. In rat tracheal explants, Wang and colleagues (21, 22) demonstrated direct effects of CS on the release of active TGF-β_1, with subsequent phosphorylation of Smad-2 and upregulation of CTGF and procollagen gene expression. In addition, in a cell-free system, cigarette smoke extract was found to release active TGF-β_1 from (recombinant) latent TGF-β_1 via an oxidative mechanism (22). Acute CS exposure of mice may also induce a transient increase in TGF-β_1-, CTGF-, procollagen- and PDGF-gene expression and Smad-2 phosphorylation (23). While the maximal response was observed 2 h after CS exposure, the increase in inflammatory cell numbers was only significant after 24 h, by which time the gene expression had subsided. This indicates that dissociation between pro-fibrotic remodelling responses and inflammatory cell responses may occur. Chronic CS exposure of these mice resulted in a persistent increase in gene expression of above-mentioned factors and an increase in airway wall collagen. Collectively, these data indicate that CS may initiate airway remodelling by inducing profibrotic growth factors in the airway wall, which can lead to increased deposition of matrix proteins. In addition, these observations imply that CS creates conditions which are strongly mitogenic to ASM, since both growth factors and collagen promote ASM proliferation, which may lead to an increase in ASM mass (33, 34, 38). Our present observations indicate that a direct effect of CS on ASM proliferation may also be involved in airway remodelling. To what extent autocrine processes, involving the release of growth factors and/or pro-proliferative ECM proteins by these cells (39, 40), may play a role, is currently unknown. Remarkably, previous reports (41) have indicated that CSE may also augment proliferation of passively sensitized human ASM cells.

Prolonged exposure of cultured airway structural cells, including ASM cells, to CSE may have cytotoxic effects on these cells by inducing apoptosis and necrosis in a concentration- and time-dependent manner (42-45). Thus, in human ASM cells, a time- and concentration-dependent induction of cell-cycle arrest, apoptosis and necrosis by exposure to 2,5 – 20 % CSE for 24 – 72 h has been demonstrated (42). Accordingly, the viability of our BTSM cells was reduced after 24 h continuous incubation of the cells with 15% CSE (not shown). However, it was found that short, pulsed exposures of ASM cells to 5 - 50 % CSE have a proliferative rather than a toxic effect on these cells. This is of major importance, as this approach seems to be a more suitable model for mimicking the *in vivo* effects of CS than continuous exposure to high concentrations of CSE for several hours. In addition, CSE exposure may be a more suitable approach for studying

the direct, epithelium-independent effects of CS on ASM, as during smoking ASM is not directly exposed to CS but indirectly, to components of CS after passing the epithelial barrier.

LPS activates the Toll-like receptor 4 (TLR4) signalling pathway, causing activation NF-κB and AP1, which results in transcription of pro-inflammatory cytokine genes and initiation of the innate immune response (46). In human subjects, acute experimental LPS inhalation leads to pulmonary and systemic inflammatory responses associated with airways obstruction and increased airway responsiveness (47, 48). Chronic exposure to LPS-containing dust or bio-aerosol in occupational or home environment has also been associated with persistent airway inflammation, decline of lung function and airway hyperresponsiveness (14, 49, 50). Moreover, LPS exposure may contribute to the severity of asthma (50). LPS may be importantly involved in bacterial infection-induced exacerbations of COPD, which contribute to the progression of the disease and diminish the quality of life (51-53). In animal models, exposure to LPS induces various inflammatory and pathological changes closely mimicking COPD, including airway remodelling and emphysema (17, 18, 54). Our present data provide evidence that a direct effect of LPS on ASM cell proliferation may contribute to airway remodelling. Although it has been reported that tobacco smoke is contaminated with LPS (8), LPS is unlikely to have contributed to the CSE-induced effects presented in this study, since LPS concentrations in the CSE were hardly detectable and far below the concentrations needed to induce ASM cell proliferation (not shown). This is in accordance with previous studies demonstrating that the LPS concentration in CSE is very low and that neutralisation of LPS in CSE, using polymyxin B, does not affect the CSE-induced IL-8 release by human macrophages (55). In addition, we investigated the effect of combined CSE and LPS treatment on ASM cell proliferation, since both factors may be involved simultaneously in exacerbations of COPD. However, no additive effects were observed, clearly indicating that both stimuli act via common pathways, as previously also suggested by others (55).

ASM cells display phenotypic plasticity, characterized by reversible changes in contractile, proliferative and synthetic characteristics, and governed by a variety of growth factors, cytokines, G-protein-coupled receptor agonists and ECM proteins (33-35, 38, 56-59). In vitro, smooth muscle-specific contractile protein expression is reduced in response to serum-rich media or growth factors, leading to a decrease in contractility, whereas the proliferative capacity is increased (33, 35, 57, 58). Previous studies have shown that ERK 1/2 and p38 MAP kinase are importantly involved in PDGF-induced proliferation and hypocontractility of

ASM (33, 35). Indeed, activation of ERK 1/2 has been shown to increase the expression of cyclin D1, a key regulator of G_1 phase cell cycle progression (60, 61) and to play a fundamental role in ASM cell proliferation (60, 62-64). p38 MAP kinase activation has also been shown to contribute to ASM cell cycle progression and proliferation (33, 35, 65-68), although this may depend on the mitogen used (65, 68). The present study demonstrated that both CSE and LPS induce phosphorylation of ERK 1/2 and p38 MAP kinase as well as increased expression of cyclin D1 in BTSM cells, whereas inhibition of ERK 1/2 and p38 MAP kinase prevented the CSE- and LPS-induced proliferation of these cells. As a possible mechanism that may be involved, CSE was recently shown to induce ERK 1/2 and p38 MAP kinase phosphorylation through NADPH oxidase-induced reactive oxygen species (ROS) formation in human ASM cells (69). NADPH oxidase has previously also been shown to be involved in proliferative effects of TGF-β_1 in these cells (70).

Expression of TLR4 receptors (31, 32) and LPS-induced ERK 1/2 and p38 MAP kinase phosphorylation (31, 71) in ASM cells have previously been reported as well. Remarkably, in rabbit ASM, it was shown that LPS-induced ERK 1/2 and p38 MAP kinase activation had opposing effects on LPS-induced hypercontractility (31). The LPS-induced hypercontractility of rabbit ASM preparations seems to be at variance with our observation of an LPS-induced hypocontractility of BTSM. Difference in duration of LPS treatment (24 h vs 8 days in our study) as well as species differences could possibly underlie this difference. Indeed, a previous study from our lab indicated that at least 4 days of treatment with FBS was required to induce a proliferative BTSM phenotype with a significant decrease in contractility (33). A hypocontractile ASM phenotype has also been observed after long-term incubation of ASM preparations with other growth factors, including PDGF and IGF-1 (33) as well as with pro-proliferative ECM proteins, such as collagen I and fibronectin (34). It has been demonstrated that the reduced contractility induced by growth factors and ECM proteins is accompanied by reduced expression of contractile proteins, such as sm-myosin, calponin and sm-α-actin (34). Such a mechanism could also underlie CSE- and LPS-induced hypocontractility of BTSM. Thus, CSE as well as LPS reduced the maximal contractile response to both a receptor-dependent (methacholine) and a receptor-independent (KCl) stimulus, indicating that post-receptor alterations such as reduced contractile protein expression are likely to be involved.

In conclusion, our in vitro data provide evidence that both CSE and LPS may contribute to airway remodelling in COPD through direct effects on ASM cells

causing a proliferative phenotype that may be involved in increased ASM mass in this disease.

Acknowledgements

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Chapter 3

TAK1 PLAYS A MAJOR ROLE IN GROWTH FACTOR-INDUCED PEHENOTYPIC MODULATION OF AIRWAY SMOOTH MUSCLE

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Abstract

Increased airway smooth muscle (ASM) mass is a major feature of airway remodeling in asthma and COPD. Growth factors induce an ASM phenotype, characterized by an increased proliferative state and a decreased contractile protein expression, reducing contractility of the muscle. TGF-β-activated kinase 1 (TAK1), a mitogen-activated protein kinase kinase kinase (MAP3K7), is a key enzyme in pro-inflammatory signaling in various cell types; however, its function in ASM is unknown. The aim of this study was to investigate the role of TAK1 in growth factor-induced phenotypic modulation of ASM. Using bovine tracheal smooth muscle (BTSM) strips and -cells, as well as human tracheal smooth muscle cells, we investigated the role of TAK1 in growth factor-induced proliferation and hypocontractility. Platelet-derived growth factor (PDGF; 10 ng/ml)- and fetal bovine serum (FBS; 5%)-induced increases in DNA synthesis and cell number in bovine and human cells were significantly inhibited by pretreatment with the specific TAK1 antagonist LL-Z-1640-2 (5*Z*-7-oxozeaenol; 100 nM). PDGF-induced DNA synthesis and ERK 1/2 phosphorylation in BTSM cells were strongly inhibited by both LL-Z-1640-2 pretreatment and transfection of dominant-negative TAK1. In addition, LL-Z-1640-2 inhibited PDGF-induced reduction of BTSM contractility and smooth muscle α-actin expression. The data indicate that TAK1 plays a major role in growth factor-induced phenotypic modulation of ASM.

Introduction

Airway smooth muscle (ASM) thickening is a pathological feature of asthma and chronic obstructive pulmonary disease (COPD) which may contribute to airflow limitation and airway hyperresponsiveness (1). The mechanisms underlying ASM remodeling have not been fully elucidated; however, there is evidence that ASM cell proliferation (2, 3), which can be induced by increased expression of growth factors in the airway wall (4, 5) may be involved. Growth factors induce a proliferative ASM phenotype, which is characterized by increased ASM cell proliferation and a decreased expression of contractile proteins, leading to decreased contractility (6-9). Previous studies have shown that extracellular signal-regulated kinase (ERK) 1/2 is a key enzyme in platelet-derived growth factor (PDGF)-induced proliferation (6, 7, 10, 11) and decrease in contractility of ASM (6, 7). Indeed, induction of sustained ERK 1/2 phosphorylation is required for cell cycle progression and proliferation of ASM cells (12, 13).

TGF-β-activated kinase 1 (TAK1), a serine/threonine kinase, is a member of the mitogen activated protein kinase kinase kinase (MAP3K) family (MAP3K7). Initially, TAK1 was identified as a mediator of transforming growth factor-β (TGF-β) and bone morphogenetic protein (BMP) signaling (14), but has since emerged as a key player in interleukin-1 (IL-1) –receptor (15), Toll-like receptor (TLR) (16) and tumor necrosis factor-α (TNF-α)-receptor (17) signaling. TAK1 activates both nuclear factor - kappa B (NF-κB) (17, 18) and MAP kinase (14) pathways, including the ERK 1/2 pathway (19-22).TAK1 has been found to play a major role in various immune responses (16, 23-25) and embryonal development (26-29). In addition, accumulating evidence suggests that TAK1 plays a role in cardiac muscle hypertrophy (30, 31), indicating that this enzyme may also be involved in the pathogenesis of tissue remodeling. The role of TAK1 in ASM function is currently unknown. In this study, we present evidence that TAK1 plays a major role in growth factor-induced proliferation and reduced contractility of ASM.

Materials and Methods

Isolation of tracheal smooth muscle cells

Bovine tracheae were obtained from local slaughterhouses and transported to the laboratory in Krebs-Henseleit (KH) buffer of the following composition (mM): NaCl 117.5, KCl 5.60, $MgSO_4$ 1.18, $CaCl_2$ 2.50, NaH_2PO_4 1.28, $NaHCO_3$ 25.00, and glucose 5.50, pregassed with 5% CO_2 and 95% O_2; pH 7.4. After dissection of the smooth muscle layer and removal of mucosa and connective tissue, tracheal smooth muscle was chopped using a McIlwain tissue chopper, three times at a setting of 500 μm and three times at a setting of 100 μm. Tissue particles were washed two times with Dulbecco's Modified Eagle's Medium (DMEM), supplemented with $NaHCO_3$ (7 mM), HEPES (10 mM), sodium pyruvate (1 mM), nonessential amino acid mixture (1:100), gentamicin (45 μg/ml), penicillin (100 U/ml), streptomycin (100 μg/ml), amphotericin B (1.5 μg/ml), and fetal bovine serum (FBS, 0.5%) (all purchased from GIBCO BRL Life Technologies, Paisley, UK). Enzymatic digestion was performed using the same medium, supplemented with collagenase P (0.75 mg/ml, Boehringer, Mannheim, Germany), papain (1 mg/ml, Boehringer), and Soybean trypsin inhibitor (1 mg/ml, Sigma Chemical, St. Louis, MO, USA). During digestion, the suspension was incubated in an incubator shaker (Innova 4000) at 37°C, 55 rpm for 20 min, followed by a 10-min period of shaking at 70 rpm. After filtration of the obtained suspension over a 50 μm gauze, cells were washed three times in supplemented DMEM containing 10% FBS. This isolation method results in a cell population positive for smooth muscle α-actin and smooth muscle myosin heavy chain (6, 32).

Human tracheal sections from anonymized lung transplantation donors were obtained from the Department of Cardiothoracic Surgery, University Medical Centre Groningen, and transported to the laboratory in ice-cold Krebs-Henseleit KH buffer. Human tracheal smooth muscle (HTSM) layer was prepared as described for BTSM and chopped using a McIlwain tissue chopper. Tissue slices were washed once with supplemented DMEM, placed in culture flasks and allowed to adhere. Upon reaching confluence, cells were passaged by trypsinization. Cells from passages 1-5 were used for the present study.

[^{3}H]-Thymidine incorporation

BTSM or primary HTSM cells were plated in 24-well cluster plates at a density of 30,000 cells per well, and were allowed to attach overnight in 10% FBS-containing DMEM in a humidified 5% CO_2 incubator at 37°C. Cells were washed twice with sterile phosphate-buffered saline (PBS, composition (mM) NaCl, 140.0; KCl, 2.6; KH_2PO_4, 1.4; Na_2HPO_4, 8.1; pH 7.4) and made quiescent by incubation in FBS-free DMEM supplemented with apo-transferrin (5 μg/ml, human, Sigma), ascorbate (100 μM, Merck, Darmstadt, Germany), and insulin (1 μM, bovine pancreas, Sigma), for 72 h. Cells were then washed with PBS and stimulated with PDGF (10 ng/ml) or FBS (5%) in FBS-free DMEM for 28 h, in the presence or absence of the TAK1 antagonist LL-Z1640-2 (100 nM, Bioaustralis, Smithfield NSW, Australia), which was added 30 min before stimulation. Treatment of cells lasted 28 h, the last 24 h in the presence of [^{3}H]-thymidine (0.25 μCi/ml, Amersham, Buckinghamshire, UK) after which the cells were washed twice with PBS and incubated with ice-cold 5% trichloroacetic acid (TCA) on ice for 30 min. Subsequently, the acid-insoluble fraction was dissolved in 0.5 ml NaOH (1 M). Incorporated [^{3}H]-thymidine was quantified by liquid-scintillation counting.

Alamar blue assay

BTSM cells were plated, cultured and made quiescent as described above. Cells were then stimulated with PDGF (10 ng/ml) or FBS (5%) for 4 days. After 4 days of stimulation, cells were washed twice with PBS and incubated with Hanks' balanced salt solution containing 5% Alamar blue (BioSource, Camarillo, CA) solution for 45 minutes. Proliferation was assessed by conversion of Alamar blue, as indicated by the manufacturer.

Transfection of plasmid DNA

For proliferation studies, cells were grown to 95% confluence on 24-well plates, washed twice with PBS and then transfected using a mixture of 2 μl Lipofectamine 2000 and 0.4 μg DNA encoding a hemmagluttinine (HA)-tagged,

kinase dead TAK1 mutant (TAK1 K63W) or GFP, as control, for 6 h in 200 µl DMEM without serum and antibiotics. After 6 h cells were washed twice with PBS and the medium was changed to DMEM supplemented with antibiotics and insulin (1 µM) and the cells were then cultured for another 72 h.

For western blotting, cells were grown to 95% confluence on 6-well plates and transfected using a mixture of 10 µl Lipofectamine 2000 and 2 µg DNA encoding TAK1 K63W or GFP for 6 h in 600 µl DMEM without serum and antibiotics. After 6 h cells were washed twice with PBS and the medium was changed to DMEM supplemented with antibiotics and 10% FBS and the cells were then cultured for another 18 h. Dominant-negative TAK1 was a generous gift from Dr. B.J.L. Eggen (33), (Department of Developmental Genetics, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, The Netherlands), with kind permission of Prof. K. Matsumoto, (Department of Molecular Biology, Graduate School of Science, Nagoya University, Japan).

Western blot analysis

BTSM cells were grown to confluence on 6-well cluster plates, using DMEM containing 10% FBS. Cells were then washed twice with sterile PBS and made quiescent by incubation in FBS-free DMEM, supplemented with insulin (1 µM), apo-transferrin (5 µg/ml) and ascorbate (100 µM) for 24 h. Cells were then washed with PBS and stimulated with PDGF (10 ng/ml), for up to 2 h, in FBS-free medium. Subsequently, cells were washed once with ice-cold PBS and then lysed in ice-cold RIPA buffer (composition: 50 mM Tris, 150 mM NaCl, 1% Igepal CA-630, 1% deoxycholic acid, 1 mM NaF, 1 mM Na_3VO_4, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 7 µg/ml pepstatin A, 5 mM 2-glycerophosphoric acid, pH 8.0). Lysates were stored at –80 °C until further use. Protein content was determined according to Bradford (34). Total cell homogenates were then subjected to immunoblot analysis using antibodies against p-ERK 1/2 (Thr^{202}/Tyr^{204}), ERK 1/2 (Cell Signaling Technology, MA, USA), smooth muscle α-actin (Sigma), GAPDH (Santa Cruz Biotechnology, CA, USA) or HA (Roche, IN, USA). The antibodies were visualized using enhanced chemiluminescence. Photographs of the blots were analyzed by densitometry.

Tissue culture

After dissection of the smooth muscle layer and careful removal of mucosa and connective tissue, BTSM strips were prepared while incubated in gassed KH-buffer at room temperature. Care was taken to cut tissue strips with macroscopically identical length (1 cm) and width (2 mm). Tissue strips were washed once in sterile FBS-free DMEM, supplemented with apo-transferrin (5

μg/ml) and ascorbate (100 μM). Next, the tissue strips were transferred into suspension culture flasks containing 7.5 ml FBS-free DMEM, and were cultured for 4 days in the absence or presence of PDGF (10 ng/ml) and/or LL-Z-1640-2 (100 nM).

Isometric tension measurements

Tissue strips, collected from the suspension culture flasks, were washed with several volumes of KH buffer pregassed with 5% CO_2 and 95% O_2, pH 7.4 at 37°C. Subsequently, the strips were mounted for isometric recording (Grass force-displacement transducer FT03) in 20-ml water-jacked organ baths containing KH buffer at 37°C, continuously gassed with 5% CO_2 and 95% O_2, pH 7.4. During a 90-min equilibration period, with washouts every 30 min, resting tension was gradually adjusted to 3g. Subsequently, the muscle strips were precontracted with 20 and 40 mM isotonic KCl solutions. Following two washouts, maximal relaxation was established by the addition of 0.1 μM (-)-isoprenaline (Sigma). In most of the experiments, no basal myogenic tone was detected. Tension was readjusted to 3 g, immediately followed by three washes with fresh KH buffer. After another equilibration period of 30 min, cumulative concentration response curves were constructed using stepwise increasing concentrations of isotonic KCl (5.6–50 mM) or methacholine (1 nM–100 μM; ICN Biomedicals, Costa Mesa, CA, USA). When maximal tension was obtained, the strips were washed several times, and maximal relaxation was established using 10 μM (-)-isoprenaline.

Data analysis

All data represent means ± s.e.mean from separate experiments. The statistical significance of differences between data was determined either by one-way ANOVA, followed by Bonferroni multiple comparison test, or by the Student's t-test, as appropriate. Differences were considered to be statistically significant when $P<0.05$.

Results

TAK1 regulates proliferation of BTSM and HTSM cells

In order to investigate the role of TAK1 in the proliferation of BTSM cells, [^{3}H]-thymidine incorporation and Alamar blue assays were performed. Stimulation of these cells with PDGF (10 ng/ml) or FBS (5%) resulted in an increase in [^{3}H]-thymidine incorporation (3.0- and 3.7-fold, respectively, after 28h Figure 1A), as well as an increase in cell numbers (1.4- and 2.4-fold, respectively, after 4 days; Figure 1B). Pretreatment of these cells with the specific TAK1 inhibitor, LL-Z-1640-2 (5Z-7-oxozeaenol; 100 nM) (19, 35), fully inhibited the PDGF-induced

increase in [^{3}H]-thymidine incorporation, whereas the FBS-induced response was inhibited by 69% (Figure 1A). Similarly, LL-Z-1640-2 pretreatment abolished the PDGF-induced increase in cell number and inhibited the FBS-induced increase by 55% (Figure 1B). As in BTSM cells, pretreatment with LL-Z-1640-2 abolished the PDGF-induced increase in [^{3}H]-thymidine incorporation (Figure 1C) and inhibited the FBS-induced increase in cell number by 62% (Figure 1D) in primary HTSM cells. Transfection of BTSM cells with dominant-negative TAK1 (TAK1 DN) resulted in a significant decrease of PDGF-induced [^{3}H]-thymidine incorporation, compared to GFP-transfected cells (Figure 2). Collectively, these data suggest that TAK1 plays a major role in growth factor-induced proliferation both in bovine and human ASM cells.

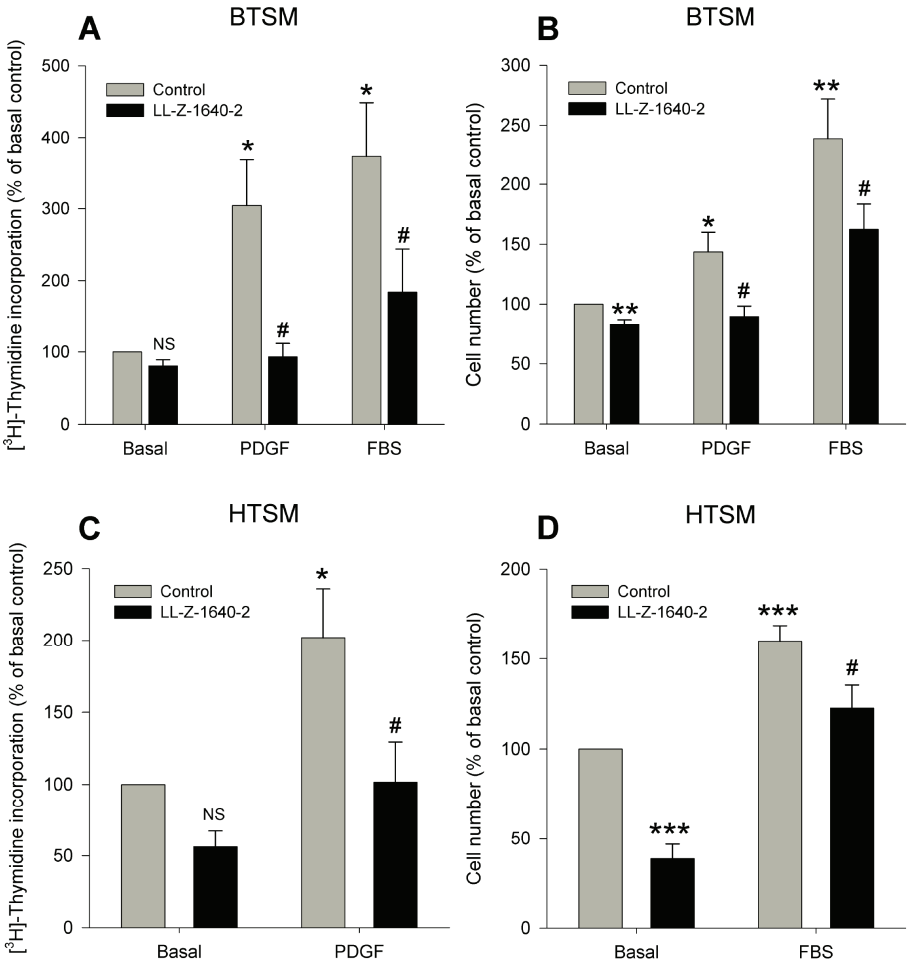

Figure 1: Growth factor-induced proliferation is inhibited by the TAK1 inhibitor LL-Z-1640-2. (A) [3H]-Thymidine incorporation in BTSM cells. (B) BTSM cell numbers determined by Alamar Blue. (C) [3H]-Thymidine incorporation in primary HTSM cells. Cells were stimulated with PDGF (10 ng/ml) or FBS (5%) in the presence or absence of LL-Z-1640-2 (100 nM). Data are expressed as % of basal controls and are means ± S.E.M. of 6-7 experiments, each performed in triplicate. *P<0.05, **P<0.01, ***P<0.001 vs untreated control, #P<0.05 vs treatment in the absence of inhibitor, NS: not significant vs untreated control.

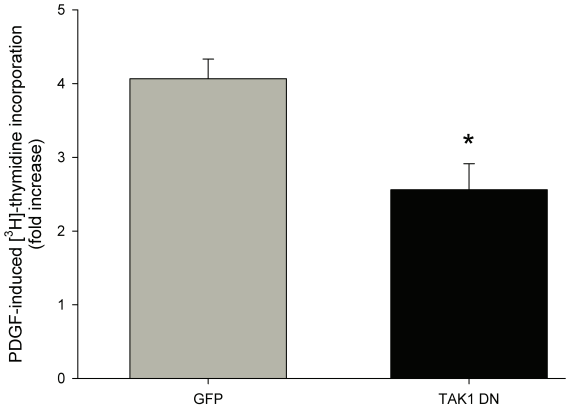

Figure 2: PDGF-induced [3H]-thymidine incorporation is inhibited by dominant-negative TAK1 (TAK1 DN). BTSM cells transfected with GFP (control) or TAK1 DN were stimulated with PDGF (10 ng/ml). Data are expressed as fold increase from respective unstimulated, transfected cells and are means of 5-7 experiments, each performed in triplicate. *P<0.05 vs GFP

TAK1 regulates ERK 1/2 phosphorylation in BTSM cells

In order to investigate the role of TAK1 in growth factor-induced ERK 1/2 phosphorylation, western blot analysis was performed. Stimulation of BTSM cells with PDGF for 5 min and 2 h resulted in a 6.4- and 3.7-fold increase in ERK 1/2 phosphorylation, respectively (Figure 3). LL-Z-1640-2 pretreatment inhibited the PDGF-induced increase at 5 min and 2 h by 68% and 73%, respectively (Figure 3).

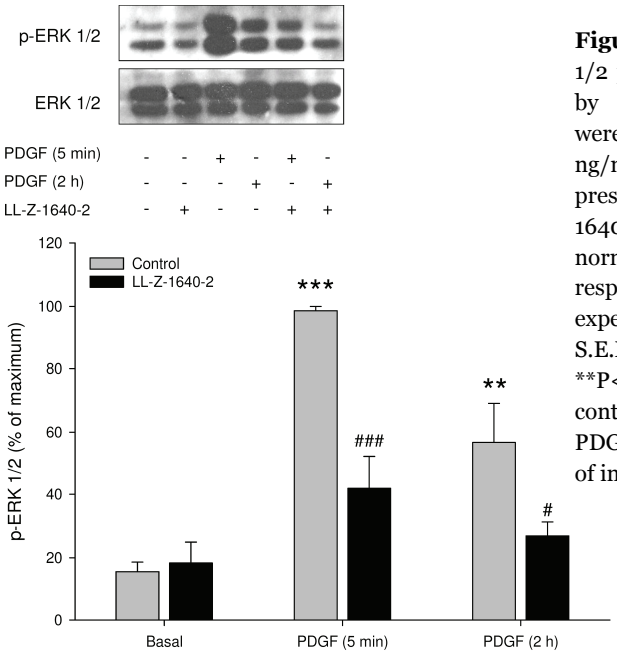

Figure 3: PDGF-induced ERK 1/2 phosphorylation is inhibited by LL-Z-1640-2. BTSM cells were treated with PDGF (10 ng/ml) for 5 min or 2 h in presence or absence of LL-Z-1640-2. Densitometry data are normalized to the maximal response in each individual experiment and are means ± S.E.M. of 5 experiments. **P<0.01, ***P<0.001 vs basal control, #P<0.05, ###P<0.001 vs PDGF treatment in the absence of inhibitor.

In addition, the ability of PDGF to induce ERK 1/2 phosphorylation was also evaluated in cells transfected with dominant-negative TAK1 or GFP, as a control. Stimulation of GFP-transfected cells with PDGF for 5 min and 2 h resulted in a 5.4- and 3.5-fold increase in ERK 1/2 phosphorylation. In dominant-negative TAK1-transfected cells, the PDGF-induced ERK 1/2 phosphorylation was inhibited by 55% at 5 min and 85% at 2 h, compared to GFP-transfected cells (Figure 4). Neither LL-Z-1640-2 pretreatment nor dominant negative TAK1 expression had a significant effect on basal ERK 1/2 phosphorylation. These data indicate that TAK1 plays a major role in PDGF-induced ERK 1/2 phosphorylation in ASM cells.

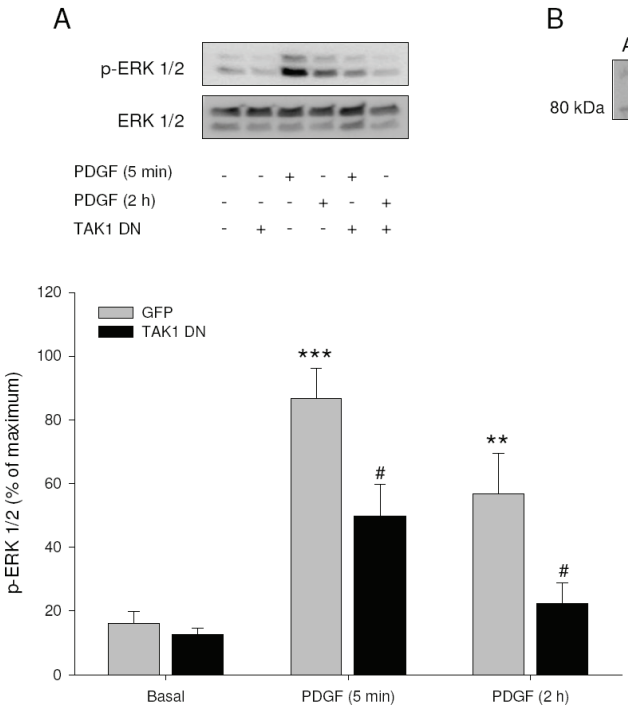

Figure 4: PDGF-induced ERK 1/2 phosphorylation is inhibited by dominant negative TAK1 (TAK1 DN) protein expression. BTSM cells transfected with TAK1 DN or GFP were treated with PDGF for 5 min or 2 h (A). Cell lysates were analyzed by immunoblotting for p-ERK 1/2 and total ERK 1/2. Expression of TAK1 DN was confirmed by immunoblotting for HA (B). Densitometry data are normalized to the maximal response in each individual experiment and are means ± S.E.M. of 7 experiments. **P<0.01, ***P<0.001 vs untreated GFP-transfected control cells, #P<0.05 vs PDGF-treated, GFP-transfected control cells.

TAK1 regulates contractility of BTSM tissue preparations

Previous studies have shown that growth factor-induced ASM cell proliferation strongly correlates with the capacity of growth factors to induce a decrease in contractility of ASM tissue preparations (6), which is associated with a decrease in contractile protein expression. In order to investigate the potential role of TAK1 in the development of growth factor-induced hypocontractile phenotype, we performed isometric contraction experiments using BTSM strip preparations. After 4 days of tissue culture in the presence of PDGF there was a significant decrease in maximal contraction to both methacholine (Figure 5A) and KCl (Figure 5B). The decrease in contractility to both stimuli was strongly inhibited in the presence of LL-Z-1640-2 (Figures 5A and 5B). Accordingly, LL-Z-1640-2 abolished the PDGF-induced decrease in smooth muscle α-actin expression in these tissue preparations. Collectively, our data indicate that TAK1 plays a major role in the induction of a proliferative, hypocontractile ASM phenotype by PDGF.

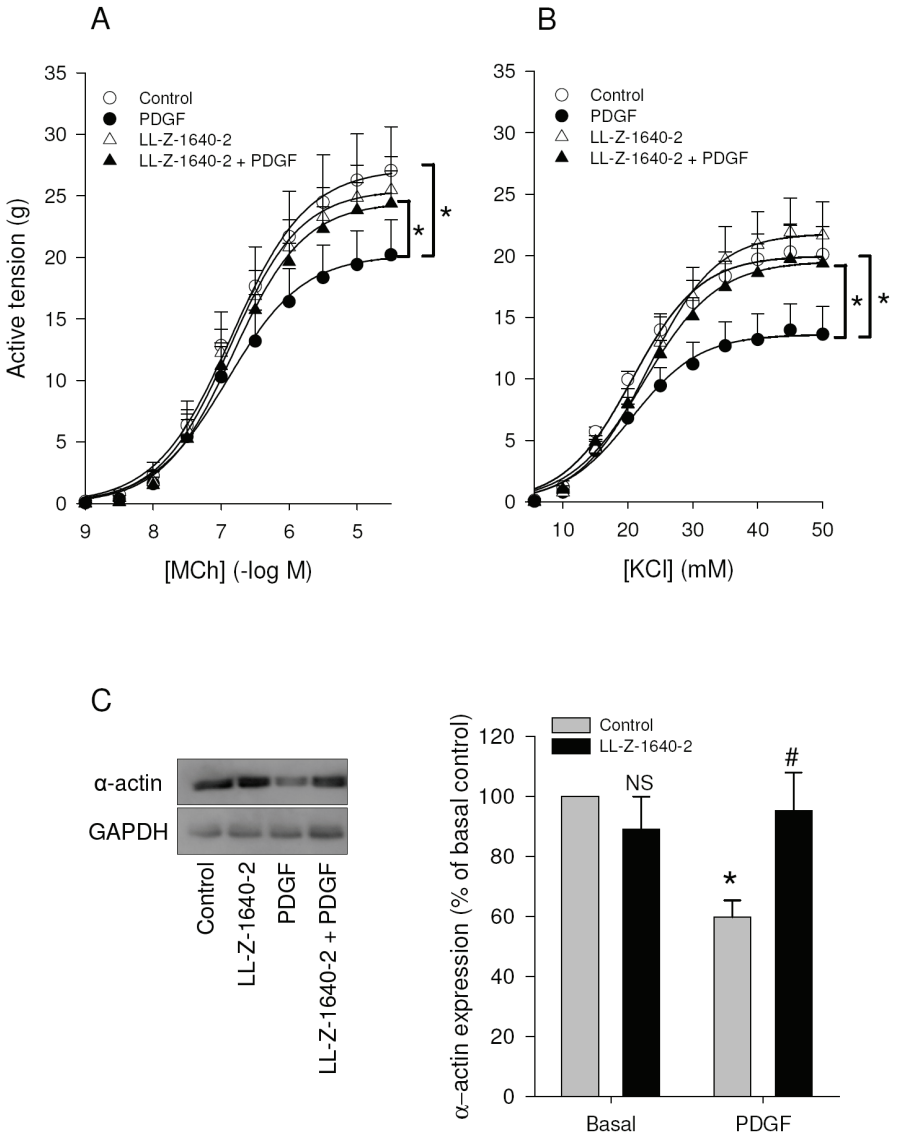

Figure 5: PDGF-induced decrease in contractility and contractile protein expression in BTSM tissue are inhibited by LL-Z-1640-2. Methacholine (MCh) (A)- and KCl (B)-induced contractions of BTSM strips cultured for 4 days in the absence or presence of PDGF (10 ng/ml), with or without LL-Z-1640-2 (100 nM). Contractile protein expression in cultured BTSM strips was determined by immunoblotting for α-actin and GAPDH as loading control (C). α-Actin blots were analyzed by densitometry. A representative blot is shown. Data are means ± S.E.M. of 4-6 experiments. *P<0.05 vs basal control, #P<0.05 vs PDGF, NS: not significant vs basal control.

Discussion

This is the first study addressing the role of TAK1 in ASM. In this study, we demonstrated that TAK1 is importantly involved in growth factor induced ASM cell proliferation, ERK 1/2 phosphorylation, hypocontractility and reduced contractile protein expression. Collectively, the data indicate that TAK1 plays a major role in the induction of a proliferative, hypocontractile ASM phenotype by growth factors such as PDGF.

Phenotypic modulation of ASM to a proliferative, hypocontractile phenotype occurs in response to a variety of mitogens, including growth factors (6, 7), G-protein-coupled receptor agonists (36-38), extracellular matrix proteins (39, 40) and, as recently shown, cigarette smoke and lipopolysaccharide (LPS) (41). The capacity of mitogens to induce ASM cell proliferation and decreased expression of contractile proteins has been found to correlate strongly with their capacity to decrease contractility of the ASM tissue preparations (6, 39). In addition, ERK 1/2 signaling induced by these mitogens has been shown to be fundamental to ASM cell proliferation (6, 7, 10-13) and the associated decrease in ASM contractility (6, 7). Induction of a proliferative phenotype of ASM may cause ASM thickening (42, 43) and thus contribute to decline in lung function and airway hyperresponsiveness in chronic pulmonary diseases like asthma and COPD (1). Our present data therefore indicate that TAK1 could be importantly involved in the progression of asthma and COPD.

TAK1 has previously been shown to play a role in cardiac hypertrophy as well. TAK1 activity was increased in mouse myocardium after pressure overload (31) as well as in the noninfarcted cardiomyocytes, in a rat model of myocardial infarction (30). In both cases, this was associated with the subsequent development of cardiac hypertrophy. Indeed, the sole expression of activated TAK1 in the myocardium of transgenic mice was sufficient to induce cardiac hypertrophy (31). Interestingly, in addition to airway and cardiac remodeling, TAK1 has also been involved in embryonal development. Thus, in the mouse embryo, TAK1 is highly expressed in a variety of tissues (26). TAK1-deficient mouse embryos are not viable (21, 27) and show an abnormal, undeveloped vasculature, lacking vascular smooth muscle (27). Moreover, *Tab1* gene mutant mouse embryos, which lack a functional TAK1-binding protein (TAB1) protein and TAK1 activity, also demonstrated abnormal development of the vasculature and the lung (28). Remarkably, a recent study in a Dutch birth cohort (44) has identified single nucleotide polymorphisms (SNPs) located in the *Tab1* and *Tab2* genes, encoding TAK1-binding proteins which regulate TAK1 activation, which

were associated with asthma. Although the functional consequence of these SNPs remains to be determined, this finding could implicate TAK1 signaling in the pathogenesis of asthma.

In addition to its inhibitory effect on ASM cell proliferation and ERK 1/2 phosphorylation, the TAK1 inhibitor LL-Z-1640-2 also inhibited the PDGF-induced decrease in maximal contraction to both a receptor-dependent stimulus, methacholine, and a receptor-independent stimulus, KCl. This suggests that PDGF induces a downstream effect and is confirmed by the decreased expression of the contractile protein, sm-α-actin. These data are consistent with the inhibition of a growth factor-induced shift to a proliferative, hypocontractile phenotype.

Our data identify TAK1 as a novel mediator of growth factor-induced proliferation of ASM cells. TAK1 has thus far not been implicated in PDGF-induced receptor tyrosine kinase signaling. In our study, PDGF-induced DNA synthesis and cell proliferation were fully inhibited, whereas the FBS-induced effects were only partially reduced by the TAK1 inhibitor LL-Z-1640-2. This observation suggests that mitogenic components of FBS may also increase cell proliferation via signaling pathways independent of TAK1. Although PDGF is one of the major mitogenic components of FBS, differences in pro-proliferative signaling induced by PDGF and FBS in ASM cells have been reported previously (7, 43, 45). For example, Rho-kinase was shown to play a role in FBS-induced proliferation of ASM cells (45), whereas it was not involved in proliferation or hypocontractility induced by PDGF (7).

TAK1 has been shown to play a role in ERK 1/2 phosphorylation induced by BMP in mouse chondrocytes (21, 22), IL-1β in mouse embryonal fibroblasts (21, 22), LPS and TNF-α in human neutrophils (19, 46) and LPS and phorbol-12-myristate-13-acetate (PMA) in human peripheral blood monocytes (19, 46). Interestingly, in HeLa cells TAK1 was shown to mediate TNF-α- but not EGF-induced ERK 1/2 phosphorylation (20), indicating that the contribution of TAK1 to ERK 1/2 signaling may be stimulus-specific. TAK1 was found to associate with Ras in caveolae of rat hepatic macrophages (47) and was also found to mediate resistance to apoptosis in H-Ras-transformed human bladder carcinoma T24 cells (48). However, at present it is still unknown at which signaling level PDGF may cross over to TAK1.

TAK1 has been shown to play a role in the proliferation of various cell types. Thus, in cultured TAK1-deficient mouse B cells, TLR- as well as B cell receptor- and

CD40-mediated proliferation and cell cycle progression were impaired (23). Similarly, in cultured TAK1-deficient mouse effector T cells, cytokine-induced proliferation and cell cycle progression proved also to be dependent on TAK1 (25). In a study using a xenograft model of breast cancer in mice, it was demonstrated that tumors originating from breast cancer cells expressing a dominant-negative TAK1 had a lower growth rate and a decreased expression of the proliferative marker Ki-67 compared to tumors originating form cells expressing wild type TAK1 (49). Studies in human hematopoietic stem cells have shown that a low concentration (10 pg/ml) of TGF-β - which was associated with TAK1 and ERK 1/2 activation but not SMAD or p38 MAP kinase pathway activation - did induce cell proliferation, whereas a high concentration of TGF-β (10 ng/ml), which strongly activated the SMAD and p38 MAP kinase pathways, inhibited cell proliferation (50, 51). These data clearly suggest that at low concentrations of TGF-β TAK1 may play a role in ERK 1/2-dependent, SMAD-independent signaling, causing cell proliferation.

In contrast, several studies have reported an inhibitory role for TAK1 in cell proliferation. Thus, expression of constitutively active TAK1 decreased DNA synthesis, cyclin D1 expression and cell cycle progression in porcine renal proximal tubular cells, whereas the expression of dominant negative TAK1 in these cells was shown to increase DNA synthesis and cyclin D1 expression (52). Dominant negative TAK1 expression in rat liver *in vivo* resulted in G_0 exit and cell cycle progression during regeneration after partial hepatectomy (53). These data indicate that the role of TAK1 in proliferation may be cell type specific.

In ASM cells, our data clearly indicate that TAK1 mediates PDGF-induced proliferation, since both proliferation and ERK 1/2 phosphorylation are inhibited by the TAK1 inhibitor LL-Z-1640-2. LL-Z-1640-2 has previously been identified as a specific inhibitor of TAK1 (35). In an *in vitro* kinase assay, this small molecule inhibitor was found to inhibit TAK1 with a high selectivity (IC_{50}=8 nM) over mitogen-activated protein kinase kinase 1 (MEK1; IC_{50}=411 nM) and MEK kinase 1 (MEKK1; IC_{50}=268 nM) (35). In other *in vitro* kinase assays, LL-Z-1640-2 (300 nM) inhibited TAK1-mediated MAP kinase kinase 6 (MKK6) phosphorylation, but had no effect on MEKK1-, MEKK4- or apoptosis signal-regulating kinase 1 (ASK1)-mediated phosphorylation (35). *In vitro* kinase assays performed on lysates from TNF-α-stimulated neutrophils have also indicated that LL-Z-1640-2 (1 μM) strongly inhibits TAK1, but does not affect MEKK1 or MEKK3 kinase activity (19). In accordance with the observation that EGF does not activate TAK1 (14), LL-Z-1640-2 (300 and 500 nM) had no effect on EGF induced ERK 1/2 phosphorylation in HEK293 (20) or HeLa cells (54), indicating

that the inhibitor is specific for TAK1 rather than a general inhibitor of MAP kinase signalling. In addition, LL-Z-1640-2 was shown to inhibit LPS-induced TNF-α, IL-1β and IL-6 production by human peripheral monocytes with an IC_{50} range (10 - 25 nM) similar to the values observed in the *in vitro* kinase assay mentioned above. Moreover, pretreatment of mice with LL-Z-1640-2 (ip injection; 3 - 30 mg/kg body mass) dose-dependently inhibited the LPS-induced increase of serum TNF-α concentration, indicating the potential for use of this inhibitor *in vivo*. In the present study, the specificity of LL-Z-1640-2 was confirmed by the inhibition of PDGF-induced [^{3}H]-thymidine incorporation and ERK 1/2 phosphorylation in cells transfected with dominant-negative TAK1.

In conclusion, our study has identified TAK1 as a novel regulator of growth factor-induced proliferation in ASM cells. TAK1 may therefore regulate phenotypic modulation of ASM and could contribute to the development of ASM remodeling in obstructive airways diseases.

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Chapter 4

ROLE FOR TAK1 IN CIGARETTE SMOKE-INDUCED PRO-INFLAMMATORY SIGNALLING AND IL-8 RELEASE BY HUMAN AIRWAY SMOOTH MUSCLE CELLS

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Abstract

Chronic obstructive pulmonary disease (COPD) is an inflammatory disease, characterized by a progressive decline in lung function. Airway smooth muscle (ASM) mass may be increased in COPD, contributing to airflow limitation and pro-inflammatory cytokine production. Cigarette smoke (CS), the major risk factor of COPD, causes ASM cell proliferation as well as interleukin-8 (IL-8)-induced neutrophilia. In various cell types, TGF-β-activated kinase 1 (TAK1) plays a crucial role in MAP kinase and NF-κB activation as well as IL-8 release induced by IL-1β, TNF-α and lipopolysaccharide. The role of TAK1 in CS-induced IL-8 release is not known. The aim of this study was to investigate the role of TAK1 in CS-induced NF-κB and MAP-kinase signaling and IL-8 release by human ASM cells. Stimulation of these cells with CS extract (CSE) increased IL-8 release and ERK 1/2 phosphorylation, as well as Iκ-Bα degradation and p65 NF-κB subunit phosphorylation. CSE-induced ERK 1/2 phosphorylation and Iκ-Bα degradation were both inhibited by pretreatment with the specific TAK1 inhibitor (LL-Z-1640-2; 100 nM). Similarly, expression of dominant-negative TAK1 inhibited CSE-induced ERK 1/2 phosphorylation. In addition, inhibitors of TAK1 and the NF-κB (SC-514; 50 μM) and ERK 1/2 (U 0126; 3 μM) signaling inhibited the CSE-induced IL-8 release by ASM cells. These data indicate that TAK1 plays a key role in CSE-induced ERK 1/2 and NF-κB signaling as well as in IL-8 release by human ASM cells, and identify TAK1 as a potential novel target for the inhibition of CS-induced inflammatory responses involved in the development and progression of COPD.

Introduction

Chronic obstructive pulmonary disease (COPD) is an inflammatory disease characterized by progressive airflow obstruction. Pulmonary infiltration of inflammatory cells, including neutrophils, may contribute to structural changes of the lung that are involved in the decline of lung function, including small airway remodeling (1). Small airway remodeling in COPD is characterized by peribronchiolar fibrosis and mucus cell hyperplasia, as well as increased airway smooth muscle (ASM) mass, particularly in severe disease (1-5).

By its contractile function, ASM is a key regulator of airway diameter. However, ASM cells can also act as synthetic cells, releasing inflammatory cytokines and contributing to inflammatory responses in the lung. Cigarette smoke (CS), the major risk factor of COPD, has been shown to induce the release of interleukin-8

(IL-8) from ASM cells, which may lead to increased numbers of neutrophils in the airways (6-8). The proportion of airways containing neutrophils - as well as other inflammatory cells - has been found to correlate with COPD severity (1). The mechanisms involved in IL-8 release by ASM cells have, however, not yet been elucidated.

The serine/threonine kinase TGF-β-activated kinase 1 (TAK1) is a member of the mitogen activated protein kinase kinase kinase (MAP3K) family (MAP3K7). Initially, TAK1 was identified as a mediator of transforming growth factor-β (TGF-β) and bone morphogenetic protein (BMP) signaling (9), but has since emerged as a key player in interleukin-1 (IL-1)- (10), tumor necrosis factor-α (TNF-α)- (11) and Toll-like receptor (TLR) (12) signaling. TAK1 activates both nuclear factor-kappa B (NF-κB) (13, 14) and MAP kinase (9) signaling pathways, including the ERK 1/2 pathway (15-18). TAK1 has been found to play a major role in various immune responses (12, 19-21); however, its role in pro-inflammatory cytokine release by ASM cells or in CS-induced signaling is currently unknown. In this study, we present evidence that TAK1 is importantly involved in CS-induced IL-8 release from ASM cells.

Materials and Methods

Cells

Primary human bronchial smooth muscle cells, immortalized by stable expression of human telomerase reverse transcriptase (hTERT) were cultured in Dulbecco's Modified Eagle Medium DMEM supplemented with 50 U/ml streptomycin, 50 μg/ml penicillin, 1.5 μg/ml amphotericin B and 10% vol/vol fetal bovine serum (FBS; Gibco BRL Life Technologies, Paisley, UK).

Cigarette Smoke Extract

Cigarette smoke extract (CSE) was prepared by combusting two University of Kentucky 3R4F research cigarettes (filters removed) using a peristaltic pump (Watson Marlow 323 E/D) and passing the smoke through 25 ml of FBS-free DMEM supplemented with penicillin, streptomycin and amphotericin B at a rate of 5 min / cigarette. The obtained solution is referred to as 100 % strength.

Interleukin-8 determination

hTERT immortalized human ASM cells were plated in 24-well cluster plates and grown to confluence. Upon confluence, cells were washed two times with sterile phosphate-buffered saline (PBS) and made quiescent by incubation in serum-

free medium, supplemented with ITS (5 µg/ml insulin, 5 µg/ml transferrin, and 5 ng/ml selenium) for 24 h. Cells were then washed with PBS and stimulated with CSE (5 or 15%) in serum-free medium. Inhibitors of TAK1 (LL-Z1640-2, 100nM), IκB kinase 2, IKK2 (SC-514; 50 µM) and mitogen-activated protein kinase kinase 1 MEK 1/2 (U 0126; 3 µM) were added 30 min before stimulation. Supernatants were collected 24 h after CSE stimulation and stored at -20°C until use. IL-8 levels were determined by using a specific sandwich enzyme-linked immunosorbent assay (ELISA) (Sanquin, Amsterdam, The Netherlands), according to the manufacturers' instructions.

Transfection of plasmid DNA

Cells were grown to 95% confluence on 6-well plates, washed twice with PBS and then transfected using a mixture of 10 µl Lipofectamine 2000 and 2 µg DNA encoding a hemmagluttinine (HA)-tagged, dominant-negative TAK1 mutant (TAK1 K63W) or GFP, as control, for 6 h in 600 µl DMEM without serum and antibiotics. After 6 h, cells were washed twice with PBS and the medium was changed to DMEM supplemented with antibiotics and 10% FBS. Subsequently, the cells were then cultured for another 18 h. Dominant-negative TAK1 was a kind gift from Dr. B.J. Eggen, (Department of Developmental Genetics, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, The Netherlands), with permission of Prof. K. Matsumoto, (Department of Molecular Biology, Graduate School of Science, Nagoya University, Japan)

Western blot analysis

Cells were plated in 6-well cluster plates and grown to confluence. Upon confluence, cells were washed two times with sterile PBS and made quiescent by incubation in serum-free medium, supplemented with ITS for 24 h. Cells were then washed with PBS and stimulated with 15% CSE in serum-free DMEM. To obtain total cell lysates, cells were washed once with ice-cold PBS and subsequently lysed in ice-cold RIPA buffer (composition: 50 mM Tris, 150 mM NaCl, 1% Igepal CA-630, 1% deoxycholic acid, 1 mM NaF, 1 mM Na_3VO_4, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 7 µg/ml pepstatin A, 5 mM 2-glycerophosphoric acid, pH 8.0). Lysates were stored at -20 °C until further use. Protein content was determined according to Bradford(22). Homogenates containing 20 µg of protein per lane were then subjected to immunoblot analysis, using specific antibodies against IκBα, p-p65, p-ERK 1/2 (Thr^{202}/Tyr^{204}), total ERK1/2, and β-actin or GAPDH as loading control. The antibodies were visualized using enhanced chemiluminescence. Photographs of the blots were analyzed by densitometry.

Data analysis

All data represent means ± S.E.M. from separate experiments. The statistical significance of differences between data was determined by one-way ANOVA, followed by a Bonferroni multiple comparison test. Differences were considered to be statistically significant when $P<0.05$.

Results

CSE-induced IL-8 production by ASM cells

First, we evaluated CS-induced IL-8 production by ASM cells, by determining the effects of CSE (5 and 15%) on IL-8 release by hTERT-immortalized human ASM cells. CSE induced a profound, concentration-dependent increase in IL-8 release by these cells (Figure 1). These findings were confirmed in primary human tracheal smooth muscle cells (data not shown).

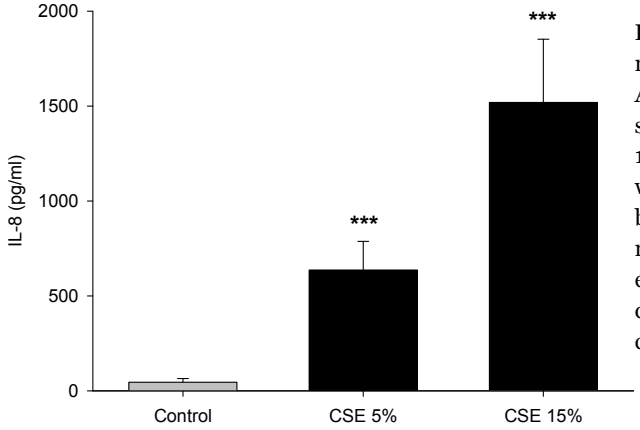

Figure 1: CSE induces IL-8 release by hTERT human ASM cells. Cells were stimulated with CSE (5 or 15%) for 24 h. Supernatants were collected and analyzed by ELISA. Data represent means ± S.E.M. of 8 experiments performed in duplicate. ***P<0.005 *vs* control.

CSE-induced NF-κB and ERK 1/2 pathway activation

To investigate the effects of CS on NF-κB and ERK 1/2 pathway activation, hTERT human ASM cells were stimulated with 15% CSE for 0.5 - 2 h (NF-κB) or for 5 min - 2 h (ERK 1/2). Cell lysates were analysed by western blotting for IκBα, and the phospho-p65 NF-κB subunit (NF-κB pathway) or phospho-ERK 1/2. CSE induced a significant decrease in IκBα abundance after 1 and 2 h of stimulation (Figure 2A) as well as an increase in phosphorylation of the p65 NF-κB subunit after 2h (Figure 2B). CSE induced phosphorylation of ERK 1/2, reaching significance after 60 min, which was sustained for up to at least 2 h (Figure 3). These data indicate that CSE induces activation of both the NF-κB and ERK 1/2 pathways.

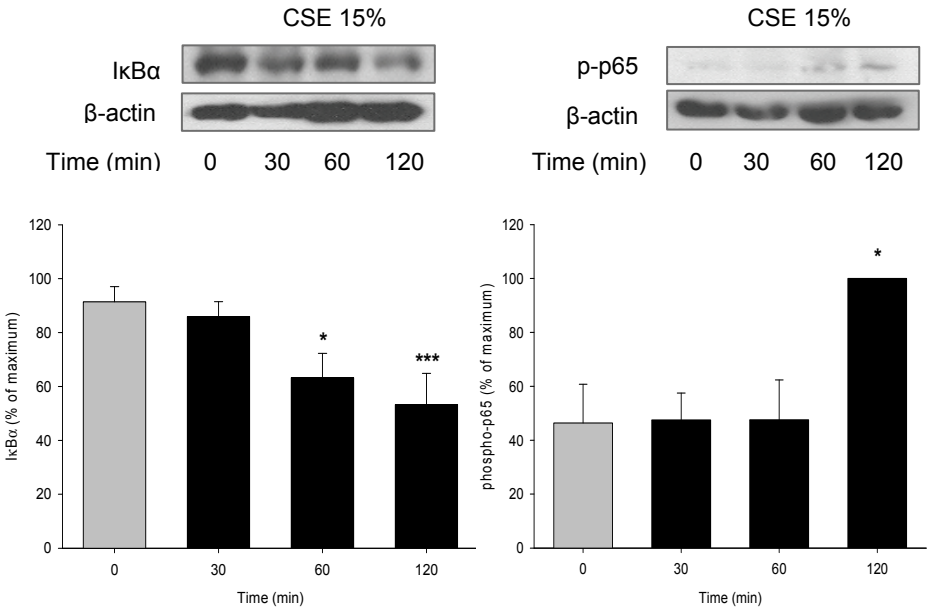

Figure 2: CSE induces IκBα degradation and p65 NF-κB phosphorylation in hTERT human ASM cells. Cells were treated with 15% CSE for up to 2 h. Cell lysates were analyzed by immunoblotting for IκBα and phospho-p65; β-actin was used to correct for protein loading. Densitometry data are normalized to the maximal response in each individual experiment and are means ± S.E.M. of 4-6 experiments. Representative blots are shown. *P<0.05; ***P<0.001 *vs* t=0.

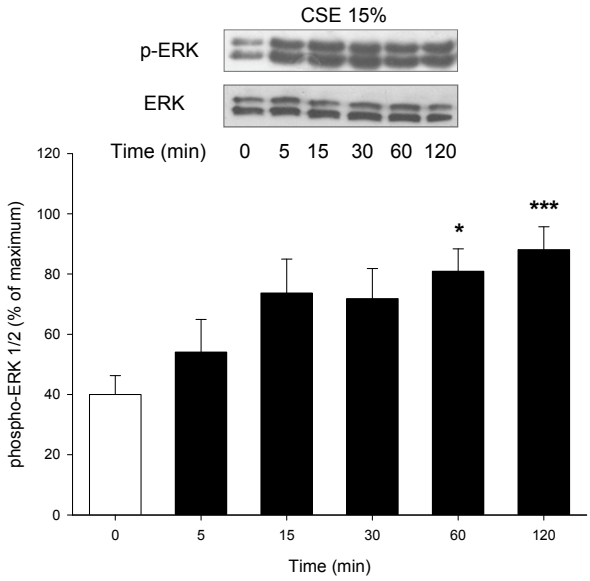

Figure 3: CSE induces ERK 1/2 phosphorylation in hTERT human ASM cells. Cells were treated with CSE 15% up to 2 h. Cell lysates were analyzed by immunoblotting for phospho-ERK 1/2; ERK 1/2 was used to correct for protein loading. Densitometry data are normalized to the maximal response in each individual experiment and are means ± S.E.M. of 3-5 experiments. Representative blots are shown. *P< 0.05; ***P< 0.001 *vs* t=0.

Involvement of TAK1 in CSE-induced NF-κB and ERK 1/2 pathway activation

In order to investigate the involvement of TAK1 in NF-κB and ERK 1/2 signaling activation, cells were stimulated with 15% CSE, in the absence or presence of the TAK1 inhibitor LL-Z-1640-2. In addition, cells were transfected with dominant negative TAK1 and stimulated with CSE as well. LL-Z-1640-2 profoundly inhibited the CSE-induced decrease in IκBα abundance (Figure 4A) as well as the increase in ERK 1/2 phosphorylation (Figure 4B). Similarly, transfection with dominant negative TAK1 abolished the CSE-induced increase in ERK 1/2 phosphorylation at t = 2h (Figure 5). These data indicate that TAK1 plays a major role in the activation of NF-κB and ERK 1/2 pathways by CSE.

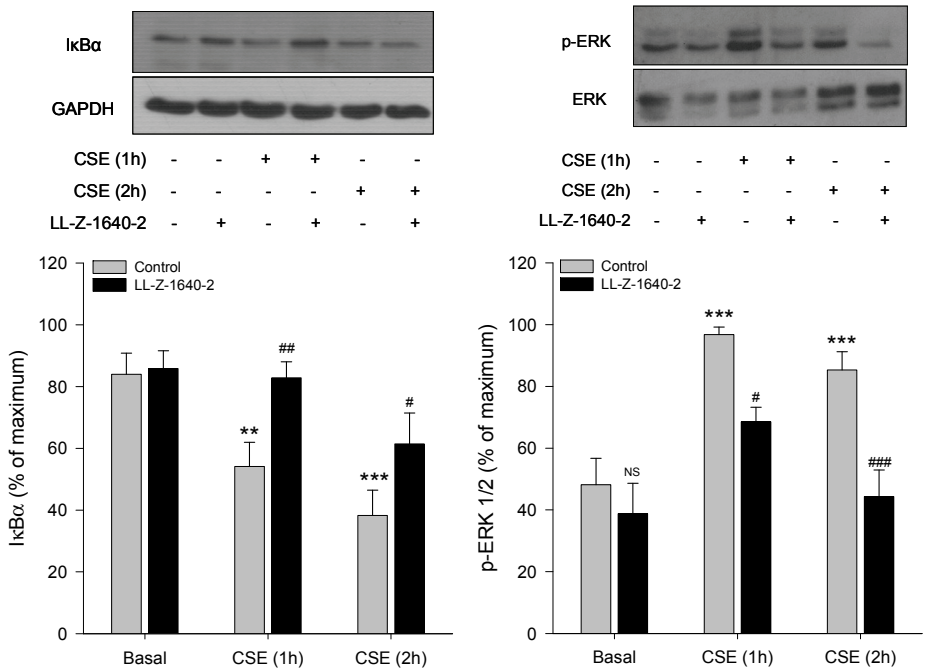

Figure 4: CSE-induced IκBα degradation (A) and ERK 1/2 phosphorylation (B) are inhibited by LL-Z-1640-2 (100nM) in hTERT human ASM. Cells were treated with CSE for 1 or 2 h in absence or presence of LL-Z-1640-2. Cell lysates were analyzed by immunoblotting for IκBα or phospho-ERK 1/2; GAPDH or ERK 1/2 was used to correct for protein loading, respectively. Densitometry data are normalized to the maximal response in each individual experiment and are means ± S.E.M. of 6-7 experiments. Representative blots are shown. **P<0.01, ***P<0.001 *vs* untreated basal, #P<0.05, ##P<0.01, ###P<0.001 vs respective CSE treatment in the absence of inhibitor.

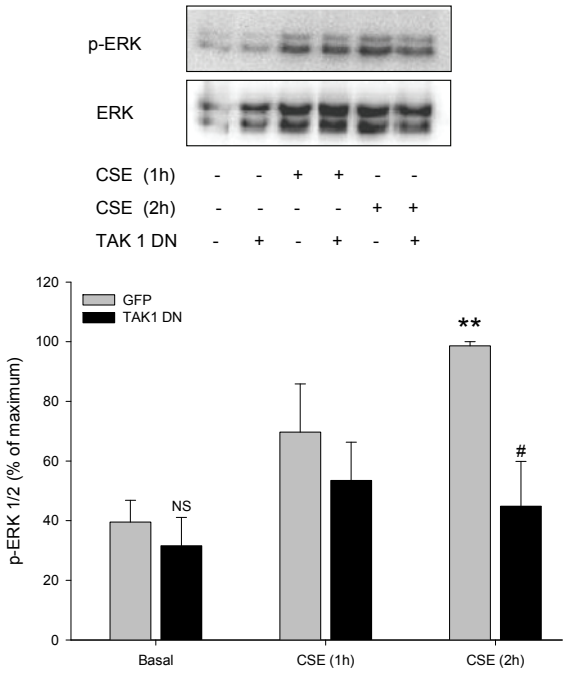

Figure 5: CSE-induced ERK 1/2 phosphorylation is inhibited by TAK1 DN protein expression in hTERT human ASM. Cells transfected with TAK1 DN or GFP were treated with CSE for 1 or 2 h. Cell lysates were analyzed by immunoblotting for phospho-ERK 1/2; ERK 1/2 was used to correct for protein loading. Densitometry data are normalized to the maximal response in each individual experiment and are means ± S.E.M. of 4-5 experiments. Representative blots are shown. **P<0.01, NS: not significant *vs* untreated GFP-transfected cells, #P<0.05 *vs* CSE treatment of GFP-transfected cells.

Inhibition of TAK1, IKK2 and ERK1/2 inhibits CSE-induced IL-8 release

To evaluate the functional roles of TAK1, NF-κB and ERK 1/2 in CSE-induced IL-8 release, cells were stimulated with 15% CSE in the absence or presence of LL-Z-1640-2 (100 nM), SC-514 (50 μM) and U0126 (3 μM), inhibitors of TAK1, IKK2 and MEK1/2, respectively. The CSE-induced IL-8 release was abolished by LL-Z-1640-2 (Figure 6) and strongly inhibited by SC-514 and U0126 (Figure 7), indicating that TAK1 and the downstream NF-κB and ERK 1/2 signaling pathways play a key role in CSE-induced IL-8 release.

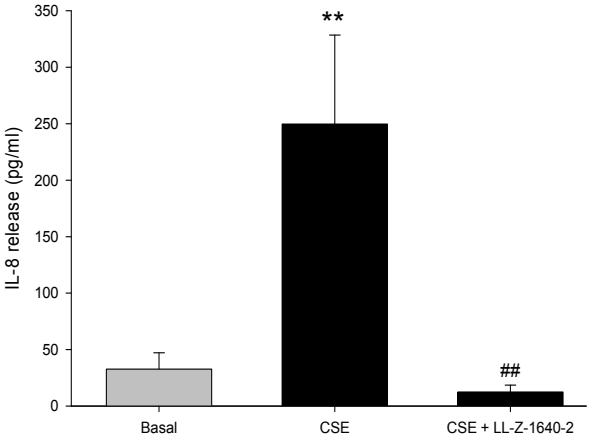

Figure 6: CSE-induced IL-8 release by hTERT human ASM cells is inhibited by LL-Z-1640-2 (100nM). Cells were treated with CSE for 24 h in the absence or presence of LL-Z-1640-2. Cell supernatants were analyzed by ELISA. Data are means ± S.E.M. of 6 experiments each performed in duplicate. **P<0.01 *vs* basal, ##P<0.01 *vs* CSE treatment in the absence of inhibitor.

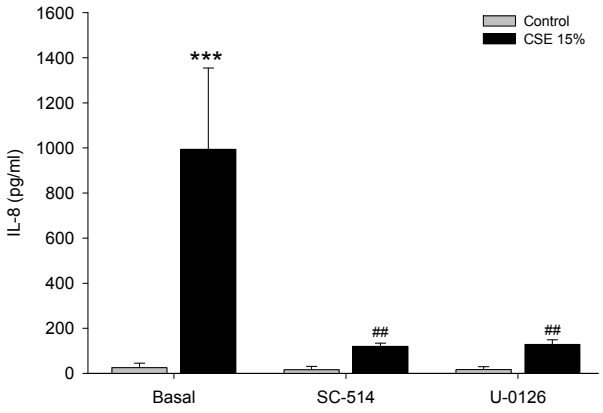

Figure 7: IKK2 and ERK 1/2 inhibitors suppress CSE-induced IL-8 release by hTERT human ASM cells. Cells were stimulated with CSE (15%) for 24 h, in presence or absence of IKK2 (SC-514, 50 μM) or ERK 1/2 (U-0126, 3 μM) inhibitors. Supernatants were collected and analyzed by ELISA. Data represent means ± S.E.M. of 4 experiments performed in duplicate. ***P<0.001 *vs* untreated control; ##P<0.01 *vs* untreated CSE.

Discussion

In this study, we demonstrated that TAK1 plays a key role in CSE-induced activation of NF-κB and ERK 1/2 and subsequent IL-8 release by human ASM cells. This is the first study implicating the involvement of TAK1 in CS-induced cellular signaling and pro-inflammatory cytokine release.

CS has previously been shown to induce IL-8 release from ASM cells (6-8). This may involve oxidative stress, as the CS-induced IL-8 release was inhibited by glutathione pre-treatment and associated with increased expression of heme oxygenase-1 (7). CS exposure has previously also been shown to induce NF-κB and MAP kinase pathways in various cells, including ASM cells (23-27). Although the IL-8 gene promoter region contains binding sites for NF-κB and AP1 (28), and these pathways were shown to be involved in CS-induced IL-8 release from human airway epithelial cells, lung fibroblasts and macrophages, as well as in neutrophils (23, 25, 29, 30), their contribution to CS-induced IL-8 release by ASM cells has only recently been demonstrated (26). Our data show that the CSE-induced activation of NF-κB and ERK 1/2 pathways is mediated by TAK1 and is crucial for IL-8 release in ASM cells.

TAK1 can be activated by a wide variety of stimuli. Pathogen-associated molecular patterns, such as lipopolysaccharide (LPS), and the cytokine IL-1β activate TAK1 via the adaptor protein MyD88. This leads to activation of IRAK4 and IRAK1 and subsequent formation of the TRAF6-TAK1 complex, which results in the activation of TAK1 (10, 31, 32). TNF-α receptor-induced TAK1 activation is mediated by the TRAF2/TRAF5 adaptor (11, 32). In addition to these receptor-specific stimuli, cellular stress, such as osmotic stress or hypoxia, have also been shown to activate TAK1 (33, 34).

TAK1 has been demonstrated to activate of NF-κB and MAP kinase pathways, which may lead to increased production of pro-inflammatory cytokines, including IL-6, IL-8 and TNF-α (19, 35-37). TAK1 has previously been shown to mediate IL-8 release form various cell types in response to pro-inflammatory stimuli, including IL-1β (38), TNF-α (15, 39) and LPS (15, 36). A role for TAK1 in CS-induced cellular responses has not previously been reported. However, it has been suggested that TLRs may mediate CS-induced signaling. Thus, CSE-induced IL-8 release was shown to be dependent on TLR4 and TLR9 activation in macrophages and neutrophils, respectively (24, 40). In addition, CS-induced neutrophilia and inflammatory cytokine production are attenuated in mice lacking TLR4 or the downstream MyD88 adapter (41, 42). These data suggest that TLRs may play a role in CS-induced inflammatory responses and provide a possible mechanism for the CS-mediated activation of TAK1. Although LPS was found to be present in CS (43), it may not be the cause of CSE-induced TLR4 activation since the LPS concentration in CSE was very low and CSE-induced cytokine release from macrophages was not affected by neutralization of LPS with polymyxin B (24, 27, 41).

Interestingly, diesel exhaust particles (DEP) have been shown to induce NF-κB activation in a TAK1-dependent manner in rat lung epithelial cells (44). DEP exposure, much like exposure to CS, induces an increase of lung neutrophils, macrophages and T-lymphocytes in experimental animals (45) and was recently shown to increase COPD mortality in railroad workers (46). Similarly, silica exposure, which has also been associated with the development of COPD (47), was shown to activate the NF-κB pathway in a TAK1-dependent manner in lung fibroblasts (48). Collectively, these and our data indicate that TAK1 may be importantly involved in inflammatory responses induced by environmental stimuli on various cell types that have been implicated in the development of COPD.

In conclusion, our observations indicate that TAK1 plays a major role in CS-induced IL-8 release by ASM cells, and identify this enzyme as a potential novel target for the inhibition of inflammatory responses that play a role in the development and progression of COPD.

Funding

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Chapter 5

TIOTROPIUM INHIBITS PULMONARY INFLAMMATION AND REMODELLING IN A GUINEA PIG MODEL OF COPD

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Abstract

Airway remodelling and emphysema are major structural abnormalities in COPD. In addition, pulmonary vascular remodelling may occur and contribute to pulmonary hypertension, a comorbidity of COPD. Increased cholinergic activity in COPD contributes to airflow limitation and, possibly, inflammation and airway remodelling.

This study aimed to investigate the role of acetylcholine in pulmonary inflammation and remodelling using an animal model of COPD. To this aim, guinea pigs were instilled intranasally with lipopolysaccharide (LPS) twice weekly for 12 weeks and were treated, by inhalation, with the long-acting muscarinic receptor antagonist, tiotropium.

Repeated LPS exposure induced airway and parenchymal neutrophilia and increased goblet cell numbers, lung hydroxyproline content, airway wall collagen and airspace size. Furthermore, LPS increased the number of muscularized microvessels in the adventitia of cartilaginous airways. Tiotropium abrogated the LPS-induced increase in neutrophils, goblet cells, collagen deposition and muscularized microvessels, but had no effect on emphysema.

In conclusion, tiotropium inhibits remodelling of the airways as well as pulmonary inflammation in a guinea pig model of COPD, suggesting that endogenous acetylcholine plays a major role in the pathogenesis of this disease.

Introduction

Chronic obstructive pulmonary disease (COPD) is an inflammatory disease, characterized by a progressive decline in lung function and airflow limitation that is not fully reversible. Structural features of COPD, which contribute to the airflow limitation, include emphysema and airway remodelling, characterized by mucous cell hyperplasia and airway fibrosis (1). In addition, pulmonary vascular remodelling has been observed, even in patients with mild COPD (2, 3). The remodelling of pulmonary vessels in COPD is characterized by smooth muscle proliferation, which contributes to thickening of the vessel wall of arteries (4, 5). Furthermore, there is evidence of muscularization of small vessels, which do not have a smooth muscle layer under healthy conditions (5, 6). Although the mechanisms of tissue remodelling in COPD are largely unclear, chronic pulmonary inflammation, characterized by infiltration of neutrophils, macrophages, CD4- and CD8-positive T lymphocytes and B cells, is presumably of major importance (7).

Anticholinergics are indicated as bronchodilator therapy in COPD (8). However, recent reports indicate that anticholinergics may have effects beyond bronchodilatation. Thus, the recent UPLIFT study (9-11) has shown that the use of the long-acting anticholinergic, tiotropium bromide, is associated with a reduction of the number of exacerbations, and of respiratory and cardiac morbidity and mortality of COPD patients (9, 10). In addition, prespecified subgroup analyses of the UPLIFT study have indicated protective effects of tiotropium on postbronchodilator FEV_1 decline in GOLD stage II COPD patients (11).

The airways are primarily innervated by parasympathetic cholinergic neurons, which regulate airway smooth muscle contraction and mucus secretion (12-14). Bronchomotor tone is increased in COPD, likely due to increased neuronal release of acetylcholine (ACh), which is the major reversible component of airflow obstruction in this disease (8, 12). Accumulating evidence suggests that in addition to neuronal ACh, non-neuronal ACh may also play a role in the pathogenesis of COPD (12). Thus, the ACh synthesizing enzyme choline acetyltransferase (ChAT) as well as muscarinic receptors, are expressed in both structural and inflammatory cells in the lung (12). Remarkably, increased ChAT expression was found in lung fibroblasts from smokers and COPD patients (15). Muscarinic M_3 receptor stimulation has been shown to increase the release of neutrophil chemotactic activity by alveolar macrophages (16), to induce interleukin-8 (IL-8) release by bronchial epithelial cells (17) and monocytes (18) and to augment cigarette smoke-induced IL-8 release by airway smooth muscle (ASM) cells (19). In addition, muscarinic receptor agonists were shown to stimulate or potentiate proliferation of lung fibroblasts (20) and ASM cells (21), as well as lung fibroblast collagen synthesis (22). Collectively, these data suggest a pro-inflammatory and pro-remodelling modulatory role for ACh in the lung. Evidence for such a role has also been found in a guinea pig model of chronic allergic asthma. In this model, it was demonstrated that tiotropium reduces airway eosinophilia as well as airway smooth muscle remodelling and goblet cell hyperplasia upon repeated allergen exposure (23, 24). In addition, tiotropium has recently been shown to inhibit airway inflammation and remodelling in a mouse model of gastro-oesophagal reflux disease (25).

Currently, the role of acetylcholine in the development and progression of COPD is unknown. In the present study, we addressed this question using a guinea pig model of lipopolysaccharide (LPS)-induced COPD. LPS is a component of the outer wall of gram-negative bacteria and a contaminant of organic dusts, environmental pollution and tobacco smoke (26), and has been associated with

the development of COPD (27). In addition, LPS may play an important role in bacterial infection-induced exacerbations of COPD, which contribute to the progression of the disease (28). Accordingly, in various animal models inhalation of LPS was shown to induce pathological features of COPD, such as neutrophilia, goblet cell hyperplasia, airway fibrosis and emphysema (29-32). In the present study, we present evidence that acetylcholine plays a major role in the development of pulmonary inflammation as well as in airway remodelling.

Methods

Animals

Outbred, male, specified pathogen-free Dunkin Hartley guinea pigs (Harlan, Heathfield, United Kingdom) weighing 350-400 g were used. All protocols described in this study were approved by the University of Groningen Committee for Animal Experimentation.

LPS instillation

Conscious guinea pigs were held in an upright position, while 200 µl LPS (5 mg/ml in sterile saline) was slowly instilled intranasally. After the intranasally instilled solution was aspirated, the animals were kept in the upright position for an additional 2 min to allow sufficient spreading of the fluid throughout the airways. Control animals were instilled with 200 µl of sterile saline.

Experimental protocol

Guinea pigs were challenged by intranasal instillation with either LPS or saline twice weekly, for 12 consecutive weeks. Thirty min prior to each instillation, animals received a nebulised dose of tiotropium bromide (Boehringer Ingelheim, Ingelheim, Germany) in saline (0.1 mM, 3 min) or saline (3 min), using a DeVilbiss nebulizer (type 646) as described previously (33). This dose of tiotropium has previously been shown to induce protection against inhaled methacholine that was measurable for approximately 96 h (34). Twenty-four h after the last instillation, the guinea pigs were sacrificed by experimental concussion, followed by rapid exsanguination. The lungs were immediately resected and kept on ice for further processing.

Tissue analysis

Transverse frozen cross-sections (4 µm) of the middle right lung lobe were used for histological and immunohistochemical analyses. To identify smooth muscle and goblet cells, sections were stained for sm-myosin heavy chain (sm-MHC) or

MUC5AC, respectively, as described previously (24). Neutrophils were identified by staining sections for TNAP (tissue non-specific alkaline phosphatase activity) (35). For immunohistochemical stainings, primary antibodies (Neomarkers; Fremont, CA, USA all) were visualised using horseradish peroxidase (HRP)-linked secondary antibodies (Sigma, St. Louis, MO, USA), diaminobenzidine (0.3 mg/ml) and ammonium nickel sulphate (25 mg/ml), adapted from Adams et al. (36). Sections were counterstained with haematoxylin. Airways within sections were digitally photographed and classified as cartilaginous or non-cartilaginous. All immunohistochemical measurements were carried out digitally using quantification software (ImageJ). For this purpose, the digital photographs were analysed at a magnification of 40-400x. Of each animal, 2 lung sections were prepared per staining, in which a total of 2 to 6 airways of each classification were analysed. Airway neutrophils were counted in the adventitia and sub-mucosa and expressed as number of positively stained cells per mm basement membrane length. For parenchymal neutrophil counts, positively stained cells were counted in five random microscopic fields using an eye-piece graticule and expressed as a percentage of total cell counts. MUC5AC-positive cells were counted in the epithelial layer and expressed as number of cells / mm basement membrane length. Microvessels which stained positively for sm-MHC were counted in the adventitia of cartilaginous airways and were expressed as number of vessels / mm^2 adventitia.

The upper right lung lobe was removed, inflated and fixed with formalin at a constant pressure of 25 cm H2O for 24 h, and embedded in paraffin. Four µm thick paraffin sections were cut. For evaluation of emphysema, paraffin sections were stained with haematoxylin and eosin. The mean linear intercept (MLI) was determined as a measure of alveolar airspace size, using 20-25 photomicroscopic images (magnification 200x) per animal, as described previously (37). For evaluation of airway wall collagen, paraffin sections were stained with Sirius Red and counterstained with haematoxylin. Non-cartilaginous airways were digitally photographed (magnification 100-200x) and the individual colour images were split into the red, green and blue channels. Using ImageJ software, the black and white images from the green channel were converted to binary images using the threshold function. The positively stained area in the airway wall, from the adventitial border to the basement membrane, was determined in 2 to 6 airways of each animal. The airway wall collagen area was normalised to the square of the basement membrane length. For evaluation of pulmonary vascular remodelling, paraffin sections were stained with Weigert's elastin (resorcin/fuchsin) and Van Gieson stain. The measurements were performed according to van Suylen et al. (38). Total vessel area of pulmonary arteries was defined as the area within the

lamina elastica externa, and lumen area was defined as the area within the lamina elastica interna. Medial area is the area between the lamina elastica interna and the lamina elastica externa. The medial area was normalized to the lumen area. Pulmonary arteries with an external diameter between 30 and 100 μm were analysed. The wall area of pulmonary arterioles was defined as the area between the lamina elastica externa and the lumen and was normalized to the lumen area. Only vessels with the longest / shortest diameter ratio < 2 were analysed.

Hydroxyproline assay

Lungs were analysed for hydroxyproline as an estimate of collagen content (39), as described previously (40). Lung homogenates were prepared by pulverizing tissue under liquid nitrogen, followed by sonification in PBS. Subsequently, homogenates were incubated with 5% v/v trichloroacetic acid on ice for 20 min. Samples were centrifuged and the pellet was resuspended in 10 ml of 12 N hydrochloric acid and heated overnight at 110 °C. The samples were reconstituted in 2 ml of water by incubating for 72 hr at room temperature, applying intermittent vortexing. Five μl samples were incubated with 100 μl chloramine T (1.4% chloramine T in 0.5 M sodium acetate/10% isopropanol) in a 96 well plate, for 30 min at room temperature. Next, 100 μl Ehrlich's solution (1.0 M 4-dimethylaminobenzaldehyde in 70% isopropanol / 30% perchloric acid) was added and samples were incubated at 65°C for 30 min. Samples were cooled to room temperature and the amount of hydroxyproline was quantified by colorimetric measurement (550 nm, Biorad 680 plate reader) of a pyrrole derivative of hydroxyproline, which forms a chromophore with Erlich's reagent. Concentrations were calculated using a standard curve. Data are expressed as mg hydroxyproline per lung.

Statistical analysis

Data are presented as mean ± SEM. Unless otherwise specified, statistical differences between means were calculated using one-way ANOVA, followed by Boniferroni or Newman Keuls multiple comparison test, as appropriate. Differences were considered significant at $p<0.05$.

Results

Inflammation

The neutrophil is a major inflammatory cell involved in the pathogenesis of COPD. Repeated LPS instillation induced significant increases in the numbers of neutrophils in both cartilaginous (2.0-fold) and non-cartilaginous (1.9-fold)

airways as well as in the parenchyma (2.1-fold) (fig. 1). Tiotropium treatment fully inhibited the LPS-induced neutrophilia in these compartments. Neutrophil numbers were not affected by tiotropium in the airways or the parenchyma of saline-challenged animals. These data indicate that tiotropium has a profound anti-inflammatory activity in chronically LPS-instilled guinea pigs.

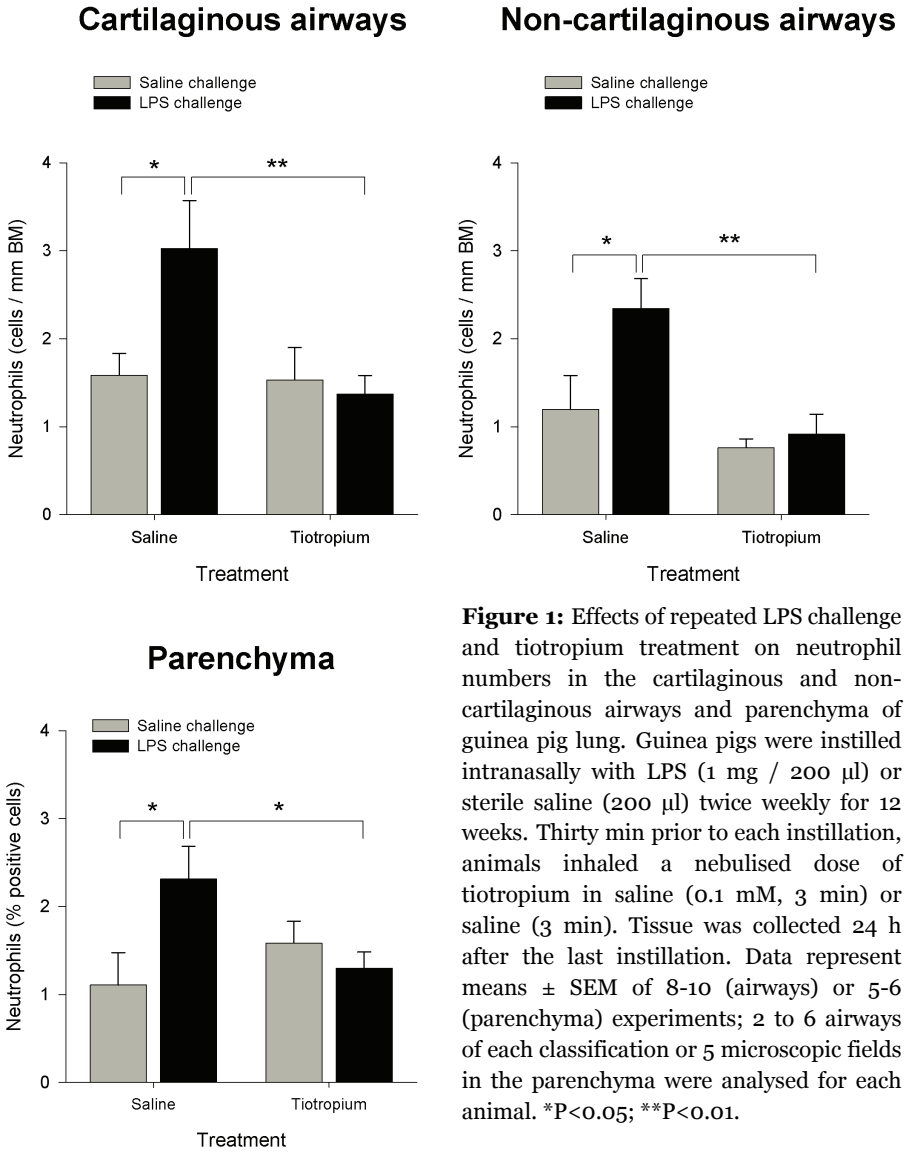

Figure 1: Effects of repeated LPS challenge and tiotropium treatment on neutrophil numbers in the cartilaginous and non-cartilaginous airways and parenchyma of guinea pig lung. Guinea pigs were instilled intranasally with LPS (1 mg / 200 µl) or sterile saline (200 µl) twice weekly for 12 weeks. Thirty min prior to each instillation, animals inhaled a nebulised dose of tiotropium in saline (0.1 mM, 3 min) or saline (3 min). Tissue was collected 24 h after the last instillation. Data represent means ± SEM of 8-10 (airways) or 5-6 (parenchyma) experiments; 2 to 6 airways of each classification or 5 microscopic fields in the parenchyma were analysed for each animal. *P<0.05; **P<0.01.

MUC5AC expression

In order to investigate the effects of LPS and tiotropium on mucus-producing goblet cells, sections were stained with a MUC5AC antibody. Repeated LPS instillation induced a significant 3.1-fold increase in the number of MUC5AC-positive cells in the epithelium of cartilaginous airways of the guinea pigs (fig. 2). Tiotropium treatment fully inhibited the LPS-induced increase, whereas it had no effect in saline-challenged animals.

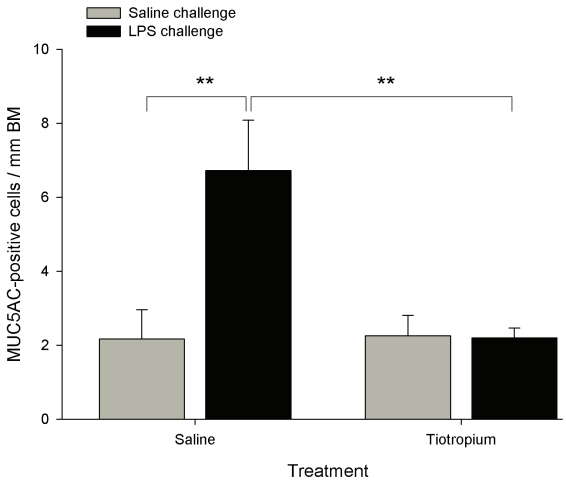

Figure 2: Effects of repeated LPS challenge and tiotropium treatment on MUC5AC-positive goblet cell number in guinea pig intrapulmonary cartilaginous airways. Guinea pigs were instilled intranasally with LPS (1 mg / 200 µl) or sterile saline (200 µl) twice weekly for 12 weeks. Thirty min prior to each instillation, animals inhaled a nebulised dose of tiotropium in saline (0.1 mM, 3 min) or saline (3 min). Tissue was collected 24 h after the last instillation. Data represent means ± SEM of 7 experiments; 2 to 5 airways were analysed for each animal. **P< 0.01.

Airway fibrosis

To evaluate fibrotic changes, lungs were analysed for hydroxyproline as an estimate of collagen content. Repeated LPS instillation induced a significant 1.3-fold increase in total lung hydroxyproline content (fig. 3). To confirm that collagen deposition was indeed increased in the airway compartment, Sirius Red staining was evaluated in the airway wall of non-cartilaginous airways. LPS induced a 1.7-fold increase in airway wall collagen content. Tiotropium fully inhibited the increase in hydroxyproline and airway wall collagen deposition induced by repeated LPS instillation, whereas it had no effect in saline-challenged animals.

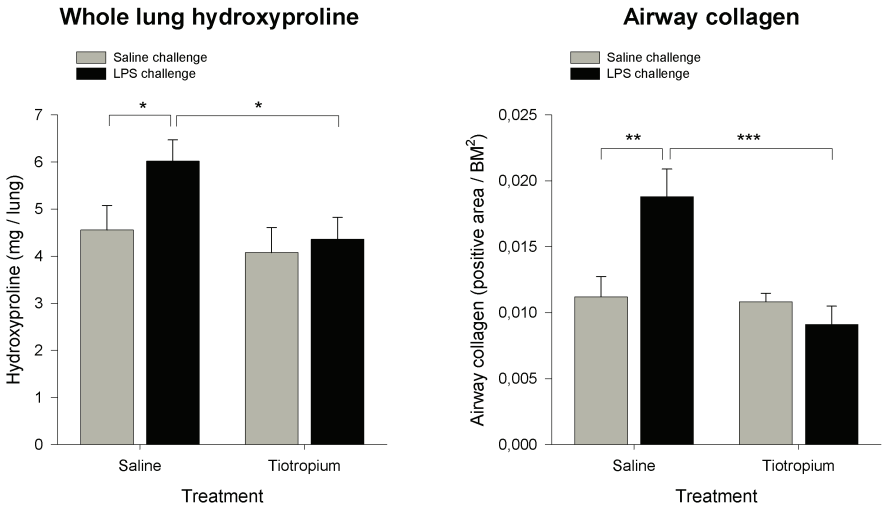

Figure 3: Effects of repeated LPS challenge and tiotropium treatment on guinea pig whole lung hydroxyproline content and collagen content in non-cartilaginous airways. Guinea pigs were instilled intranasally with LPS (1 mg / 200 µl) or sterile saline (200 µl) twice weekly for 12 weeks. Thirty min prior to each instillation, animals inhaled a nebulised dose of tiotropium in saline (0.1 mM, 3 min) or saline (3 min). Tissue was collected 24 h after the last instillation. Airway wall collagen was determined as Sirius Red-positive area and normalised to the square of the basement membrane length (BM^2). Data represent means ± SEM of 10-12 (hydroxyproline assay) or 5-6 (Sirius Red staining) experiments; for Sirius Red staining, 2 to 6 airways were analysed for each animal. *P<0.05; **P<0.01; ***P<0.001.

Emphysema

In order to evaluate the alveolar airspace size, MLI was determined in paraffin-embedded lung sections. A 7.3% increase in MLI was observed after 12 weeks of twice weekly LPS instillations (fig. 4). Tiotropium had no effect on the airspace size in either LPS- or saline-instilled animals.

Vascular remodelling

To evaluate pulmonary vascular remodelling, pulmonary artery medial area and pulmonary arteriole wall area were determined in formalin-fixed, paraffin-embedded guinea pig lung sections stained with Weigert's elastin and Van Gieson stain. Neither repeated LPS instillation nor tiotropium treatment had an effect on the medial area of pulmonary arteries or wall area of pulmonary arterioles (fig. 5). In addition, there was no evidence of intimal proliferation in

the pulmonary vessels of either classification. However, repeated LPS instillation increased the number of muscularized (sm-MHC-positive) microvessels in the adventitia of cartilaginous airways (2.4-fold) (fig. 6). This increase was fully inhibited by tiotropium.

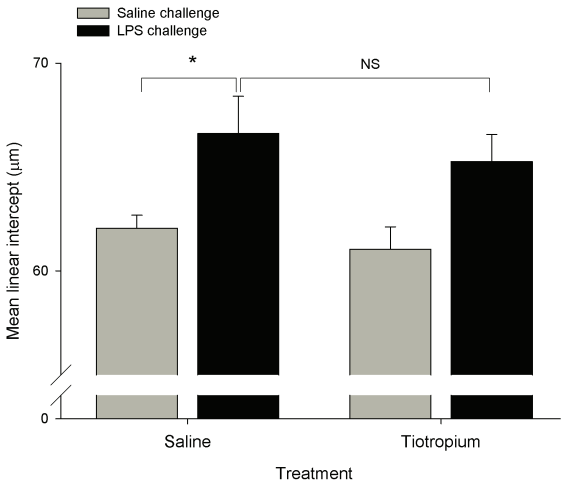

Figure 4: Effects of repeated LPS challenge and tiotropium treatment on alveolar airspace size (mean linear intercept) in guinea pig lung. Guinea pigs were instilled intranasally with LPS (1 mg / 200 µl) or sterile saline (200 µl) twice weekly for 12 weeks. Thirty min prior to each instillation, animals inhaled a nebulised dose of tiotropium in saline (0.1 mM, 3 min) or saline (3 min). Tissue was collected 24 h after the last instillation. Data represent means ± SEM of 4-5 experiments. *P<0.05; NS: not significant.

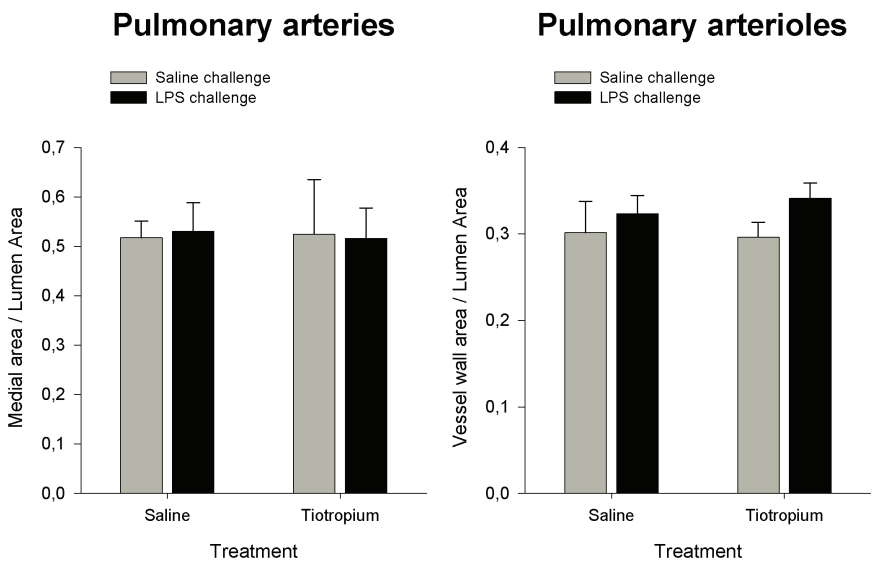

Figure 5: Effects of repeated LPS challenge and tiotropium treatment on the medial area of pulmonary arteries and the wall area of pulmonary arterioles. Guinea pigs were instilled intranasally with LPS (1 mg / 200 µl) or sterile saline (200 µl) twice weekly for 12 weeks. Thirty min prior to each instillation, animals inhaled a nebulised dose of tiotropium in saline (0.1 mM, 3 min) or saline (3 min). Tissue was collected 24 h after the last instillation. Data represent means ± SEM of 6-8 experiments.

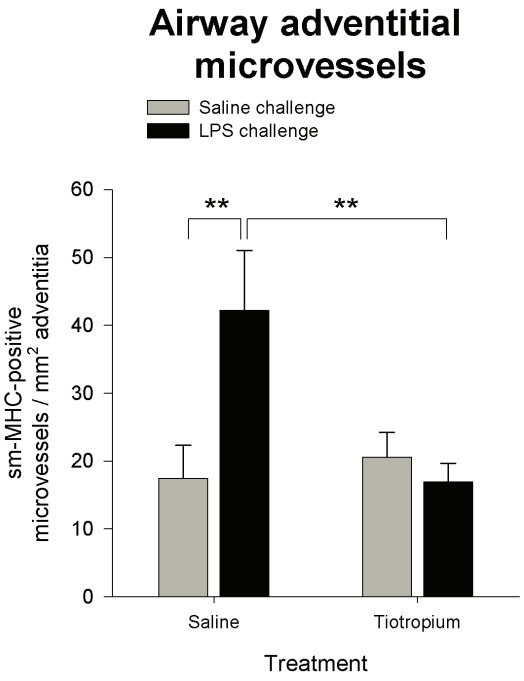

Figure 6: Effects of repeated LPS challenge and tiotropium treatment on the number of muscularized microvessels in the airway adventitia of cartilaginous airways in guinea pig lung. Guinea pigs were instilled intranasally with LPS (1 mg / 200 µl) or sterile saline (200 µl) twice weekly for 12 weeks. Thirty min prior to each instillation, animals inhaled a nebulised dose of tiotropium in saline (0.1 mM, 3 min) or saline (3 min). Tissue was collected 24 h after the last instillation. Data represent means ± SEM of 7-9 experiments. **P<0.01.

Discussion

In this study, we demonstrate for the first time that tiotropium inhalation inhibits neutrophilia, MUC5AC expression and airway fibrosis in an animal model of COPD, induced by repeated LPS exposure. In addition, we showed that repeated LPS instillation induced remodelling of the adventitial airway vasculature in this model, which was inhibited by tiotropium. Collectively, these data suggest that endogenous acetylcholine, acting through muscarinic receptors, plays a major role in pulmonary inflammation as well as in airway remodelling in COPD.

Neutrophil numbers are increased in sputum and bronchoalveolar lavage fluid of COPD patients (41). In addition, correlations between COPD severity and neutrophil numbers in the large airways and the percentage of neutrophil-containing small airways has been reported (41). ACh may contribute to neutrophilia as it was shown to stimulate the release of neutrophil chemotactic activity from isolated alveolar macrophages (16) and from isolated sputum cells of COPD patients (42). Furthermore, activation of muscarinic receptors

expressed in airway structural cells, including bronchial epithelial (17) and airway smooth muscle cells (19), may also contribute to neutrophil sequestration in the lungs by inducing or augmenting IL-8 release by these cells. In addition, the high capacity of neutrophils to synthesise ACh (43) implies that neutrophilia may result in increased non-neuronal ACh release in the lung. In our study, repeated LPS exposure increased neutrophil numbers in the airways and in the parenchyma. Because tiotropium inhalation fully inhibited this LPS-induced neutrophilic inflammation, a major role for ACh is inferred.

Mucus hypersecretion is a characteristic feature of COPD, which contributes significantly to airflow obstruction. MUC5AC expression can be upregulated by a variety of stimuli, including cigarette smoke, LPS and neutrophil elastase, and is increased in the airway epithelium of COPD patients (44). The present study indicates that endogenous ACh also plays a crucial role in LPS-induced MUC5AC expression. Interestingly, as early as in 1973, goblet cell hyperplasia has been observed following repeated administration of muscarinic agonists in experimental animals (45).

Airway fibrosis contributes to small airway thickening and airflow limitation in COPD (46). The role of ACh in airway fibrosis in COPD is currenly unknown. However, *in vitro* studies have indicated that ACh can induce proliferation of lung fibroblasts (20), and collagen synthesis by these cells (22). In addition, increased ChAT expression has recently been found in lung fibroblasts from healthy smokers and from COPD patients (15), suggesting that non-neuronal ACh may modulate fibroblast function in an autocrine fashion under these conditions. Our results demonstrated that tiotropium inhibits LPS-induced collagen accumulation in the lung and airway wall, indicating that ACh may be a key regulator of airway fibrosis. In addition to a possible direct effect of ACh on fibroblasts in this process, its effect on neutrophils could also play a role. Thus, increased neutrophil elastase activity, as observed in COPD (41), has also been associated with pulmonary fibrotic diseases, such as idiopathic pulmonary fibrosis (47). The potential role of neutrophil elastase in fibrosis is further supported by studies in mice, showing that neutrophil elastase inhibition (48) or gene deletion (49) inhibits bleomycin-induced pulmonary fibrosis in these animals.

LPS induced a significant increase in alveolar airspace size, which is indicative of emphysema. The observed increase in MLI after 12 weeks of twice weekly LPS instillation is of a similar magnitude as described previously for mice following 16 weeks exposure to cigarette smoke (37). In our study, no evidence for the

involvement of ACh in the development of LPS-induced emphysema was found. Thus, tiotropium had no significant effect on airspace size in either LPS- or saline-instilled animals. This could imply that neutrophilic inflammation is not essential for the development of LPS-induced emphysema, which corresponds with previous observations in patients with COPD (41). The mechanisms leading to the development of LPS-induced emphysema are not entirely clear, but presumably the release of elastolytic enzymes from neutrophils plays a role. Although chronic LPS-induced parenchymal neutrophilia in our model was inhibited by tiotropium, this does not necessarily mean that neutrophils have not contributed to alveolar destruction. A recent study in mice has shown that the acute LPS-induced BAL neutrophilia (at 4 h post LPS exposure) is not affected by tiotropium pretreatment (50). This finding indicates that tiotropium does not inhibit the acute LPS-induced signalling, which leads to the release of neutrophil-attracting chemokines, resulting in acute neutrophilia. Since a brief exposure to neutrophil elastase may already lead to emphysema, the acute tiotropium-resistent neutrophilic response after each LPS-instillation could be sufficient to initiate the development of emphysema. In addition, other inflammatory cell types such as macrophages, CD8-positive T-lymphocytes and B-cells may also contribute (37, 41).

Pulmonary vascular remodelling may occur in patients with COPD and contribute to pulmonary hypertension in these patients (51). Pulmonary vascular remodelling in COPD is characterized by thickening of the vessel wall as well as muscularization of microvessels that do not have a smooth muscle layer under healthy conditions (4, 6). The precise mechanisms underlying the pulmonary vascular remodelling in COPD are not known, but may involve hypoxia, inflammation and cigarette smoke constituents, leading to endothelial dysfunction and release of growth factors (52). The increase in vessel wall thickness is largely due to smooth muscle cell proliferation in the intima (4) as well as thickening of the media (6). Interestingly, thickening of pulmonary arteries and increased number of small vessels positive for smooth muscle actin have been found in guinea pigs after 3 or 6 months exposure to cigarette smoke (53). In contrast, in the present study no effect of LPS on pulmonary artery or arteriole thickness was found. Similarly, LPS did not affect airway smooth muscle mass under the same conditions (data not shown). However, LPS did increase the number of muscularized microvessels in the adventitia of cartilaginous airways. The strong inhibitory effect of tiotropium on this increase indicates a major role for endogenous ACh in this process. Although the mechanisms underlying these changes are presently unknown, airway inflammation might play a role (54). This is supported by the observation that

the neutrophil infiltration and its inhibition by tiotropium is particularly observed in the adventitial compartment of the airways (data not shown).

Recently, results of the UPLIFT trial have demonstrated that tiotropium treatment of patients with COPD reduces the number of exacerbations, the incidence of respiratory and cardiac adverse events as well as mortality (9, 10). A prespecified subgroup analysis also showed that in GOLD stage II COPD, tiotropium treatment reduces the rate of decline of postbronchodilator FEV_1 (11). Although the mechanisms underlying these effects remain to be established, data from our animal model suggest that anti-inflammatory and anti-remodelling properties of the drug could be involved.

In conclusion, our study has demonstrated that inhaled tiotropium inhibits pulmonary inflammation and airway remodelling in a guinea pig model of COPD, indicating that endogenous ACh may play a major role in the pathogenesis of this disease.

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Chapter 6

ARGINASE AND PULMONARY DISEASES

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Abstract

Recent studies have indicated that arginase, which converts L-arginine into L-ornithine and urea, may play an important role in the pathogenesis of various pulmonary disorders. In asthma, COPD and cystic fibrosis, increased arginase activity in the airways may contribute to airways obstruction and hyperresponsiveness by inducing reduced production of bronchodilatory nitric oxide (NO) due to competition with constitutive (cNOS) and inducible (iNOS) NO synthases for their common substrate. In addition, reduced L-arginine availability to iNOS induced by arginase may result in the synthesis of both NO and superoxide anion by this enzyme, thereby enhancing the production of peroxynitrite, which has procontractile and pro-inflammatory actions. Moreover, increased synthesis of L-ornithine by arginase might contribute to airway remodelling in these diseases. L-Ornithine is a precursor of polyamines and L-proline, which may promote cell proliferation and collagen production, respectively. Increased arginase activity may also be involved in other fibrotic disorders of the lung, including idiopathic pulmonary fibrosis. Finally, by inducing reduced levels of vasodilating NO, increased arginase activity has been associated with primary and secondary forms of pulmonary hypertension. Drugs targeting the arginase pathway could have therapeutic potential in these diseases.

Introduction

Arginase is the final enzyme of the urea cycle in the liver and is the key enzyme for the removal of highly toxic ammonium ions from the body. To this aim, L-arginine, which is produced by argininosuccinate lyase, is converted into L-ornithine and urea by the action of arginase. Besides in the liver, arginase is also expressed in cells and tissues that lack a complete urea cycle, including the airways and the lung.

Arginase exists in two distinct isoenzymes: arginase I and II, which are encoded by different genes. Arginase I is a cytosolic enzyme and is the predominant isoform in the liver, where it is highly expressed (1, 2). Although low levels of arginase II have been detected in liver as well (3), this mitochondrial enzyme is mainly expressed in extrahepatic tissue (1, 2). In the airways, both arginase I and II are constitutively expressed in bronchial epithelial cells, endothelial cells, (myo)fibroblasts and alveolar macrophages (3-5), while arginase II is also expressed in parenchymal epithelial cells (4). Although arginase expression in airway smooth muscle was below detection limit in some studies (4), other

studies have indicated that either isoform may be (conditionally) expressed in these cells (6, 7).

One of the biological functions of extrahepatic arginase may be regulation of the synthesis of nitric oxide (NO), via competition with NO synthase (NOS) for the common substrate, L-arginine (Fig. 1). In activated macrophages, for example, arginase activity limits the utilization of L-arginine by inducible NOS (iNOS) and suppress the cytotoxic response by these cells (8-10). The activity of arginase, on the other hand, is inhibited by N^{ω}-hydroxy-L-arginine (NOHA), an intermediate in the NO synthesis, showing a delicate balance between NOS and arginase activity in the control of NO production (11, 12). In addition, by the synthesis of L-ornithine extrahepatic arginase may be involved in tissue repair processes. Thus, L-ornithine is a precursor of polyamines and proline, which are involved in cell proliferation and collagen synthesis respectively (2, 13, 14).

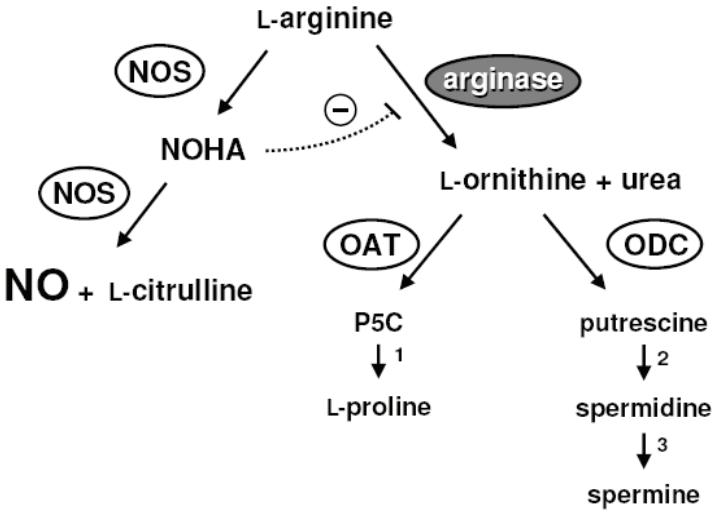

Figure 1: L-Arginine metabolism by arginase and NOS. L-Arginine is a substrate for NOS, yielding NO and L-citrulline, as well as for arginase, which produces L-ornithine and urea. Arginase regulates the production of NO via competition with NOS for their common substrate. On the other hand, NOHA, an intermediate in the NO synthesis by NOS, inhibits arginase activity. In addition, the arginase product L-ornithine is the precursor of the polyamines putrescine, spermidine and spermine, as well as of L-proline. Abbreviations: NO, nitric oxide; NOHA, N^{ω}-hydroxy-L-arginine; NOS, nitric oxide synthase; OAT, ornithine aminotransferase; ODC, ornithine decarboxylase; P5C, L-pyrroline-5-carboxylate; 1, pyrroline-5-carboxylate reductase; 2, spermidine synthase; 3, spermine synthase.

A functional role for constitutively expressed arginase in the airways has been established in guinea pig tracheal preparations, by using the specific arginase inhibitor N^{ω}-hydroxy-nor-L-arginine (nor-NOHA). Thus, nor-NOHA decreased methacholine-induced airway constriction by increasing the production of non-neural, presumably epithelium-derived, bronchodilating NO (15). Moreover, the arginase inhibitor increased NO-mediated airway smooth muscle relaxation induced by inhibitory nonadrenergic noncholinergic (iNANC) nerve stimulation (16). In both studies, the effects of nor-NOHA were quantitatively similar to the effects of exogenously applied L-arginine, supporting that arginase is involved in the control of airway responsiveness by attenuation of substrate availability to NOS (15-17).

Aberrant NO homeostasis as well as exaggerated tissue repair are involved in various inflammatory airway diseases associated with reduced lung function, airway hyperresponsiveness (AHR) and/or airway remodelling, such as allergic asthma (18, 19), chronic obstructive pulmonary disease (COPD; (20-22) and cystic fibrosis (23-25). In addition, reduced levels of NO have also been observed in lungs of patients with pulmonary arterial hypertension (PAH; (26), while dysregulated tissue repair and excessive fibrosis in the lung interstitium is observed in patients with idiopathic pulmonary fibrosis (27). This review will address the potential role of arginase in the pathophysiology of these diseases.

Allergic asthma

Role of NO in allergic asthma

Allergic asthma is a chronic inflammatory airways disease, characterized by allergen-induced early and late bronchial obstructive reactions and AHR to a variety of stimuli, including allergens, chemical irritants, cold air and pharmacological agents like histamine and methacholine (28). The development of bronchial obstructive reactions as well as of AHR is associated with infiltration and activation of inflammatory cells, particularly Th2 lymphocytes and eosinophils, in the airways (28). The cause of AHR may be multi-factorial, involving changes in the neurogenic and non-neurogenic control of airway smooth muscle function as well as structural changes in the airways such as epithelial damage, mucosal swelling and airway remodelling that is characterized by increased airway smooth muscle mass, subepithelial fibrosis, hyperplasia of mucous cells and angiogenesis (28). All these changes can be induced by a cascade of inflammatory reactions involving various mediators, including NO (19, 28, 29).

NO is produced by a family of NOS isoforms, which convert L-arginine into NO and L-citrulline, using oxygen and NADPH as cosubstrates (30). Three NOS isozymes are known: neuronal NOS (nNOS or NOS I), inducible NOS (iNOS or NOS II) and endothelial NOS (eNOS or NOS III). In the respiratory tract, nNOS and/or eNOS are constitutively expressed in iNANC neurons (nNOS), and in epithelial (nNOS and eNOS) and endothelial (eNOS) cells. These constitutive NOS (cNOS) isoenzymes are primarily involved in the neural and non-neural regulation of airway and vascular smooth muscle tone via both cGMP-dependent and -independent mechanisms (19). In addition, eNOS-derived NO has been shown to inhibit airway inflammation by suppressing the activation of NF-κB, thereby inhibiting the expression of iNOS as well as the production of inflammatory cytokines (31-35). It has been demonstrated that an impaired production of cNOS-derived bronchodilating NO contributes to the development of AHR in allergic asthma, both in animal models and in human asthma (36-42).

iNOS is induced in the airways by pro-inflammatory cytokines, particularly in inflammatory and epithelial cells (43-45). iNOS-derived NO may be involved in infiltration of inflammatory cells (46), mucosal swelling (47) and epithelial damage (46, 48), but may also have a beneficial bronchodilating action (46, 49), indicating a dualistic role in the airways. In contrast to cNOS, iNOS produces large amounts of NO, which causes increased concentrations of NO in the exhaled air of asthmatics (50). In experimental asthma, iNOS is induced in the airways during the allergen-induced late asthmatic reaction, similarly leading to increased levels of NO in the exhaled air (51, 52). In asthmatics, increased nitrotyrosine staining in the airways correlates well with iNOS expression, AHR and airway inflammation, suggesting that not iNOS-derived NO itself, but rather its reaction with superoxide to the highly reactive oxidant peroxynitrite may importantly account for the detrimental effects of iNOS in the airways (53). Indeed, peroxynitrite has procontractile and proinflammatory actions and is involved in the development of AHR after the late asthmatic reaction or after repeated allergen challenge (54-57).

Arginase in animal models of allergic asthma

In a guinea pig model of allergic asthma using ovalbumin-sensitized animals, it was demonstrated that a deficiency of NO underlies the development of AHR after the allergen-induced early asthmatic reaction (36, 39) and that this NO deficiency is caused by a decreased availability of L-arginine to cNOS (17, 37, 58). Using airway preparations from the same animal model, it was demonstrated that increased arginase activity may be involved in the reduced L-arginine bioavailability and AHR. Thus, after the early asthmatic reaction arginase activity

in the airways of allergen-challenged guinea pigs was 3.5-fold increased as compared to unchallenged animals, while incubation with the arginase inhibitor nor-NOHA completely reversed the allergen-induced AHR of perfused tracheal preparations from these animals by restoring NO production (58). L-Arginine limitation induced by increased arginase activity appeared also to underlie an impaired iNANC nerve-mediated airway smooth muscle relaxation after the early asthmatic reaction by inducing a deficiency of nNOS-derived NO (37). Collectively, these findings indicate a key role for arginase in the development AHR after the allergen-induced early asthmatic reaction by inducing a deficiency of both neuronal and non-neuronal NO (Fig. 2).

A second mechanism by which increased arginase activity may contribute to AHR in allergic asthma is via stimulation of peroxynitrite formation. Thus, studies in macrophages have indicated that under conditions of low L-arginine availability iNOS not only produces NO by its oxygenase moiety, but also synthesizes superoxide anions by its reductase moiety, which leads to an efficient formation of peroxynitrite (59). Increasing the L-arginine concentration in these cells stimulates NO production, while the formation of superoxide – and hence peroxynitrite – is reduced (60). In perfused guinea pig tracheal preparations obtained after the allergen-induced late asthmatic reaction, the AHR to methacholine was reduced by both the NOS inhibitor L-NAME and the superoxide anion scavenger superoxide dismutase (SOD), indicating the involvement of peroxynitrite in this process (54). Remarkably, the AHR was similarly diminished by the arginase inhibitor nor-NOHA and by exogenous L-arginine, highly suggesting that reduced L-arginine availability caused by increased arginase activity is involved in iNOS-induced production of peroxynitrite and AHR (61). This was underscored by the observation that the effect of nor-NOHA was fully reversed by L-NAME, indicating that arginase inhibition restores the production of bronchodilating NO. Moreover, the arginase activity in tracheal tissue as well as in bronchoalveolar lavage cells of the challenged animals was increased after the late asthmatic reaction (61).

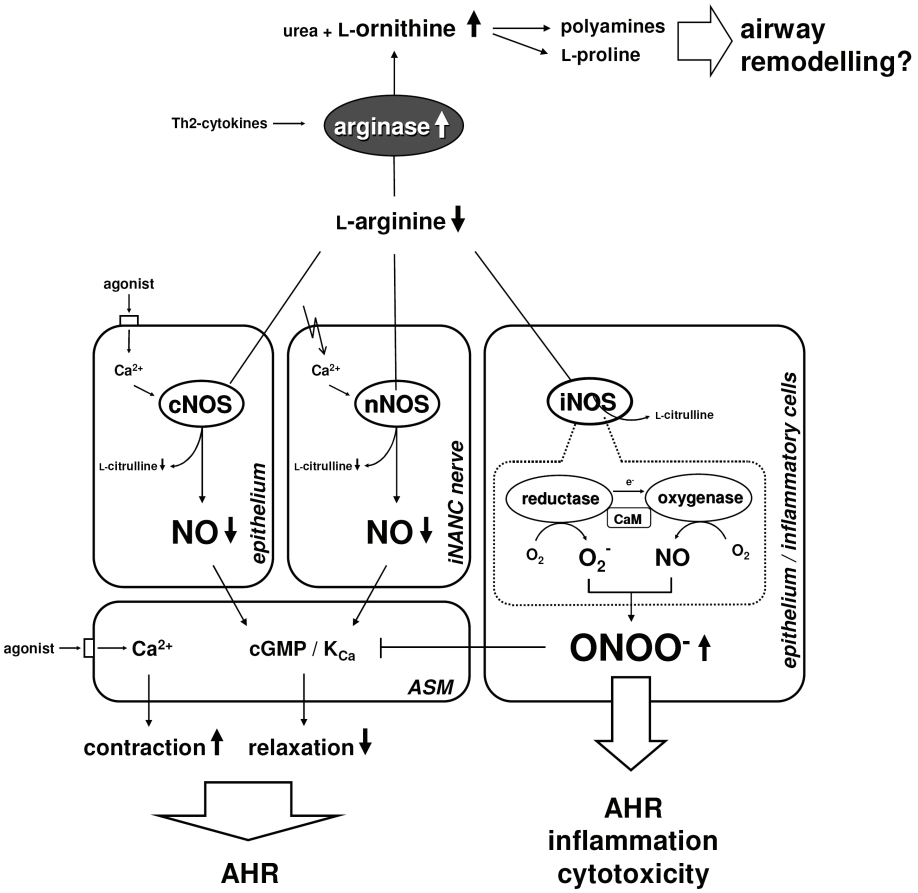

Figure 2: Role of increased arginase activity in the pathophysiology of allergic asthma. In allergic asthma, arginase expression and activity is increased by Th2-cytokines. Increased arginase activity limits the bioavailability of L-arginine to cNOS, leading to a reduced production of agonist-induced NO by the airway epithelium as well as of neuronal NO by iNANC nerves. Under basal conditions, NO induces airway smooth muscle relaxation by increasing the production of cGMP and/or by opening of calcium-activated potassium channels, thereby attenuating the airway responsiveness to contractile stimuli. Therefore, arginase-induced deficiency of cNOS-derived NO in allergic asthma contributes to AHR in this disease. Increased arginase activity also attenuates the availability of L-arginine to iNOS, which is induced during the late asthmatic reaction or in chronic asthma. The reduced L-arginine availability to iNOS results in the simultaneous production of NO and O_2^- by the oxygenase and reductase moieties of the enzyme, respectively, This leads to rapid formation of the highly reactive nitrogen species $ONOO^-$, which has procontractile, proinflammatory and cytotoxic actions in the airways. In addition, increased production of polyamines and L-proline downstream of L-ornithine may contribute to the process of

airway remodelling. Abbreviations: AHR, airway hyperresponsiveness; ASM, airway smooth muscle; CaM, calmodulin; e^-, electron; cGMP, cyclic 3',5'guanosine monophosphate; cNOS, constitutive nitric oxide synthase; iNANC nerve, inhibitory nonadrenergic noncholinergic nerve; iNOS, inducible nitric oxide synthase; K_{Ca}, calcium-activated potassium channel; nNOS, neuronal nitric oxide synthase; NO, nitric oxide, O_2^-, superoxide anion; $ONOO^-$, peroxynitrite.

The importance of increased arginase activity in the pathophysiology of asthma was confirmed in various animal models of allergic asthma, using different antigens. In BALB/c mice sensitized to ovalbumin and to *Aspergillus fumigatus* increased arginase activity in the lung was measured after allergen challenge (62). Notably, by microarray analysis of gene expression it was shown that among the 291 common genes that were induced by these allergens, enzymes involved in L-arginine metabolism, particularly arginase I and II, belonged to the most predominantly overexpressed genes. Northern blot analysis confirmed the increase in arginase I and II gene expression. In contrast to arginase I, arginase II was constitutively present, but induced to a much lesser extent. In situ hybridization and immunohistochemistry of ovalbumin-challenged animals demonstrated that high levels of arginase I were observed in the perivascular and peribronchial pockets of inflammation in asthmatic lung (62). In line with the microarray study mentioned above, proteomics of lung tissue from repeatedly ovalbumin-challenged C57BL/6 mice indicated considerable upregulation of arginase I (63).

According to previous studies in mouse macrophages (9, 64), lung arginase activity and mRNA expression of both arginase I and arginase II were strongly induced by the Th2 cytokines IL-4 and IL-13, which are abundant in allergic airway inflammation (62). Moreover, arginase I mRNA expression in mouse lung was also increased by IL-25, a novel member of the IL-17 family which induces Th2-like airway inflammation and AHR (65). Furthermore, increased arginase I gene expression was observed in Th2 polarized, but not in Th1 polarized, mice sensitized to and challenged with *Schistosoma mansoni* eggs (66), supporting the importance of Th2 cytokines in inducing arginase in asthma. Although IL-4 alone did not induce arginase activity in human alveolar macrophages, it greatly enhanced the response to cAMP-elevating agents (67). Recently, it was demonstrated that isolated human airway smooth muscle cells have low expression of particularly arginase II (6). Remarkably, the expression of arginase II, but not of arginase I, was increased after stimulation with IL-4 (6). Of note, in rat vascular smooth muscle cells the expression of arginase I was induced by IL-4 as well as by IL-13, while arginase II was not induced by these cytokines (68).

Several studies have indicated that cytokine-induced expression of arginase I is under important control of the transcription factors STAT6 (62, 68-71) and CCAAT-enhancer binding protein (69). However, IL-4-induced arginase II expression in the lung was hardly affected in STAT6$^{-/-}$ mice, indicating that the induction of arginase II is largely STAT6 independent (62).

Lung arginase activity and arginase I mRNA expression were also increased in BALB/c mice challenged trimellitic anhydride, although the induction was lower than in animals challenged with ovalbumin (72). In addition, lung arginase activity as well as mRNA and protein expression of both arginase I and II were shown to be increased in NC/Nga mice challenged with *Dermatophagoides farinae* (73). While arginase I was not detected in the lung of control animals, increased arginase I expression was observed in alveolar macrophages and infiltrating cells around the bronchioles in challenged mice (73). In different mouse models of Th2 cytokine-mediated inflammation, using ovalbumin (BALB/c and C57BL/6 mice), the parasite *Nippostrongylus brasiliensis* (BALB/c) or the fungus *Aspergillus fumigatus* (C57BL/6), as well as in IL-13 overexpressing mice, gene expression in the lung was studied to identify the genes that are commonly expressed in lung inflammation. Among the 26 characteristic transcripts of these 5 different models, arginase I was strongly increased in all (74). In another study in mice, instillation of IL-13 in the airways increased arginase activity and the expression of arginase I, but not arginase II, while NO synthesis was decreased (71). IL-13-induced arginase expression was also temporally correlated with the development, persistence, and resolution of IL-13-induced AHR to methacholine. *In vivo* treatment with RNA interference against arginase I abrogated the development of IL-13-induced AHR, supporting the importance of increased arginase activity for AHR in asthma (71). Further studies demonstrating the effects of specific arginase inhibitors and/or RNA interference against arginase I and II on allergen-induced AHR are clearly indicated.

Besides in guinea pigs and mice, increased arginase activity has also been observed in a rat model of allergic asthma. Thus, arginase activity was increased in lung homogenates of rats challenged with ovalbumin for three consecutive days (75).

Taken together, increased arginase induction has been observed in a wide variety of animal models of asthma, using different species and allergens (Table 1). The increased arginase activity may contribute to AHR by reducing the production of bronchodilating NO as well as by stimulation of the formation of procontractile

and proinflammatory peroxynitrite (Fig. 2). Interestingly, in lung epithelial cells it was recently demonstrated that overexpression of arginase may increase NF-κB activation by decreased production of NO, suggesting that increased arginase activity in allergic asthma could also promote airway inflammation and AHR by increased production of inflammatory cytokines (76).

Arginase in human asthma

The significance of increased arginase expression and activity may for the pathophysiology of human asthma was first demonstrated by Zimmermann et al. (2003), who demonstrated that arginase I protein expression is increased in BAL cells of asthmatic patients. Moreover, in bronchial biopsies of these patients, enhanced mRNA expression of arginase I was observed in inflammatory cells as well as in the airway epithelium. Surprisingly, arginase activity was increased in serum of asthmatic patients experiencing an exacerbation, which was associated with reduced plasma L-arginine levels, indicating that changes in arginase expression in asthma are not confined to the airways and that reduced levels of circulating L-arginine could contribute to NO deficiency and hyperresponsiveness of the airways (77). Moreover, in some of these patients arginase activity declined and L-arginine concentrations increased after improvement of symptoms (77). Quite remarkably, enhanced arginase activity was already found in expectorated sputum of asthmatic patients over two decades ago (78).

The effect of smoking on arginase expression in human airways has recently also been studied (7). Endobronchial biopsy specimens from steroid-naïve mild asthmatics were investigated for changes in immunoreactivity for arginase I, ornithine decarboxylase (ODC, the rate limiting enzyme in polyamine synthesis, Fig. 1) and iNOS in smoking versus non-smoking patients. Interestingly, increased immunoreactivity for arginase I and ODC was observed in both the epithelium and smooth muscle layers of the smokers, while iNOS-immunoreactivity was similar in both groups. In addition, arginase I mRNA expression was increased in the epithelium and smooth muscle bundles of smoking asthmatics as compared to the non-smoking patients. To investigate which component of cigarette smoke may attribute for the increase in arginase expression, the effect of nicotine on arginase and ODC expression in cultured airway epithelial and smooth muscle cells and fibroblasts was studied. Nicotine significantly increased arginase I mRNA in the epithelial cells, while a trend towards increased arginase I mRNA expression was observed in airway smooth muscle cells and fibroblast after nicotine treatment. In addition, nicotine significantly increased ODC mRNA expression in fibroblasts and epithelial cells,

but not in the airway smooth muscle cells (7). These observations might also be of relevance for the pathogenesis of COPD (see below).

Interestingly, single nucleotide polymorphisms (SNPs) in arginase I and arginase II have recently found to be associated with atopy and risk of childhood asthma, respectively (79).

COPD

COPD is an inflammatory disease characterized by a progressive, irreversible decline in lung function. The leading cause of COPD is cigarette smoke (80). Smoking initiates pulmonary inflammation characterized by prominent infiltration of neutrophils, macrophages and T-lymphocytes, particularly of the $CD8^+$ subset (81, 82). Major features of COPD are airway hyperresponsiveness and progressive decline in lung function associated with structural changes of the peripheral lung, including small airway remodelling - characterized by mucus cell hyperplasia, airway fibrosis and increased airway smooth muscle mass - and alveolar wall destruction (20-22, 81, 82).

Using the single expiratory flow technique measuring NO derived from the (predominantly larger) airways, COPD has been associated with increased levels of exhaled NO (eNO) in patients with severe stage disease and during exacerbations (83-85). However, using this method, eNO is often low or in the normal range in patients with stable COPD (86). This has been attributed to the effect of tobacco smoking, which down-regulates eNOS (87) and iNOS (88). Indeed, smokers exhale lower amounts of NO compared to nonsmokers (89). Reduced levels of NO may also result from smoking-induced increased oxidative stress, by formation of peroxynitrite from NO and superoxide anions. Indeed, peroxynitrite generation is considerably increased in sputum macrophages of COPD patients, which is negatively correlated with FEV_1 in these patients (90). Recently, methods for measuring eNO at multiple respiratory flows have indicated that, while airway NO is relatively low in COPD, there is an increase in alveolar NO that is related to disease severity and not affected by smoking (91).

Table 1: Changes in arginase activity and the expression of arginase I and II in animal models and human pathology of pulmonary diseases

	Species	Condition/ stimulus	Localization	Arg I	Arg II	Activity	References
Asthma							
	Human		Sputum	n.d.	n.d.	+	Kochanski, 1978
			Serum	n.d.	n.d.	+	Morris, 2004
			BAL cells, epithelium, lung, MΦ	+	n.d.	n.d.	Zimmermann, 2003
		Cigarette smoke	Smooth muscle, epithelium	+	n.d.	n.d.	Bergeron, 2007
	Guinea pig	Ovalbumin	BAL cells, trachea	n.d	n.d.	+	Maarsingh, 2004; Meurs, 2002
	Mouse	*A. fumigatus*	Lung	+	+	+	Lewis, 2007; Zimmermann, 2003
		D. farinae	Lung, serum, MΦ	+	+	+	Takemoto, 2007
		IL-4	Lung	+	+	n.d.	Zimmermann, 2003
		IL-13	Lung	+	=	+	Lewis, 2007; Yang, 2006; Zimmermann, 2003
		N. brasiliensis	Lung	+	=	n.d.	Lewis, 2007
		Ovalbumin	Lung, MΦ	+	+	+	Fajardo, 2004; Greene, 2005; Lewis, 2007; Zimmermann, 2003
		S. mansoni eggs	Lung	+	n.d.	n.d.	Sandler, 2003
		TMA	Lung	+	+	+	Greene, 2005
	Rat	Ovalbumin	Lung	n.d.	n.d.	+	Abe, 2006

COPD							
	Human		Sputum	n.d.	n.d.	+	Chachaj, 1978; Kochanski, 1980
		GOLD 0, I, II-A	Lung	=	=	n.d.[1]	Tadie, 2007
	Rat	Cigarette smoke	Lung	+	n.d.	n.d.	Gebel, 2006
CF							
	Human		Plasma, sputum,	n.d.	n.d.	+	Grasemann, 2005b; 2006b
Fibrosis							
	Human	IPF	Lung	-	=	=	Kitowska, 2007
		IPF	Epithelium, fibroblasts, lung, MΦ	+	n.d.	n.d.	Mora, 2006
	Mouse	Bleomycin	Epithelium, fibroblasts, lung, MΦ	+	+	n.d.	Endo, 2003; Kitowska, 2007
		Herpes virus	Lung, MΦ	+	n.d.	+	Mora, 2006
		Silica	BAL cells, lung, MΦ	+	n.d.	+	Misson, 2004
	Rat	Silica	BAL cells, lung	+	=	+	Nelin, 2002; Poljakovic, 2007; Schapira, 1998
PH							
	Human	Primary PH	PAEC, serum	=	+	+	Xu, 2004
		Secondary PH in sickle cell disease	Erythrocytes, plasma, serum	n.d.	n.d.	+	Morris, 2003; 2005
	Mouse	Hemoglobin$^{-/-}$	Lung	n.d.	n.d.	+	Hsu, 2007
	Rat	Monocrotaline	PAEC	n.d.	n.d.	+	Sasaki, 2007

-, decreased; +, increased; =, unchanged, n.d., not determined; [1]arginase inhibition decreases the sensitivity to acetylcholine in COPD patients compared to control subjects.
Arg I, arginase I; Arg II, arginase II; CF, cystic fibrosis; COPD, chronic obstructive pulmonary disease; IPF, idiopathic lung fibrosis; MΦ, macrophages; PAEC, pulmonary arterial endothelial cells; PH, pulmonary hypertension; TMA, trimilletic anhydride

Quite interestingly, microarray analysis of gene expression, followed by reverse transcription real-time quantitative PCR in lungs from smoking rats revealed a marked time- and dose-dependent up-regulation of arginase I expression during 2-13 weeks of smoke exposure (2 times 1 h/day, 5 days/week; (92). Smoke-induced expression of arginase I might be involved in the relatively low NO production in the airways from COPD patients as well as in peroxynitrite production and AHR found in these patients. The latter was supported by the recent observation that increased arginase activity may be involved in the enhanced sensitivity to methacholine of bronchial preparations from patients with mild COPD (93). Interestingly, subcutaneous injection (5 weeks, once daily) of cigarette smoke extract in rabbits increased expression of arginase I and an increase in arginase activity in cavernous tissue, while NOS activity and nNOS expression were significantly decreased (94). Moreover, electric field stimulation-induced neurogenic and NO mediated cavernous smooth muscle relaxation was attenuated by cigarette smoke extract administration (94). Whether cigarette smoke-induced increase in arginase activity in the airways also leads to reduced iNANC-mediated NO production and airway smooth muscle relaxation has not been studied yet.

Cystic fibrosis

Cystic fibrosis (CF) is a progressive disease, characterized by pulmonary inflammation and bacterial infection, chronic airway obstruction, airway remodelling and AHR (23-25). Despite the inflammatory nature of CF, levels of eNO are decreased (98, 99). The reduced NO levels in the airways of CF patients might contribute to microbial infection and colonization as well as to functional changes of the airways. Several mechanisms may contribute to low pulmonary levels of NO in CF, including reduced expression of iNOS (100), polymorphisms of nNOS (101, 102) and eNOS (103), mechanical retention of NO in airway secretions (104), increased metabolism to peroxynitrite (105), consumption of NO by denitrifying bacteria (106) and increased arginase activity in the airways (107, 108).

In a mouse model of cystic fibrosis, an impaired electrical field stimulation-induced airway smooth muscle relaxation was found, which was reversed by L-arginine and NO (109). This indicates that a deficiency of NO due to substrate limitation to nNOS compromises airway relaxation and contributes to airway obstruction. Indeed in CF patients a positive correlation was found between pulmonary function and exhaled NO and NO metabolite concentrations in sputum (98, 104). Moreover, inhalation of L-arginine increased exhaled NO

levels and improved lung function in these patients (110). However, oral L-arginine treatment increased L-arginine levels in sputum and plasma as well as levels of exhaled NO, but failed to improve pulmonary function (111). Increased consumption by arginase may account for the L-arginine limitation in CF, since sputum arginase activity in CF is markedly increased as compared to controls and is even further increased during pulmonary exacerbation (108). Interestingly, increased arginase activity negatively correlated with lung function (FEV_1), while increased eNO and improvement of lung function by antibiotic treatment were associated with a decrease in arginase activity (108). At least some portion of the increased arginase activity in the above-mentioned study could have been derived from microorganisms in the sputum. However, increased arginase activity was also observed in plasma of CF patients during pulmonary exacerbation, while plasma L-arginine levels were decreased (107). Treatment with antibiotics decreased the arginase activity and restored the L-arginine levels in these patients, confirming the close relationship between increased arginase activity and decreased levels of L-arginine (107). Taken together, these findings indicate that increased arginase activity in CF contributes to the NO deficiency and pulmonary obstruction in CF by limiting the availability of L-arginine to NOS.

Arginase in airway remodelling and fibrotic pulmonary disorders

Increased arginase activity in asthma, COPD and cystic fibrosis might also contribute to the airway remodelling observed in these diseases by increased production of the L-proline and the polyamines putrescine, spermidine and spermine from L-ornithine (Fig. 1, 2). L-Proline, the precursor of collagen, is synthesized from L-ornithine in a two step reaction involving ornithine aminotransferase and pyrroline-5-carboxylate reductase, while ODC initiates the synthesis of polyamines that could be involved in proliferation of structural cells in the airways (2, 18, 112, 113) Fig. 1). In support of this concept, transfection of rat vascular smooth muscle cells with arginase I induced increased polyamine levels as well as enhanced proliferation of these cells (114). The involvement of arginase in airway remodelling remains to be established, however. In support of a potential role of arginase in airway fibrosis in asthma, IL-4 and IL-13 increased arginase I and II expression and arginase activity in cultured rat fibroblasts (5). Moreover, IL-4 induced increased arginase II expression in human airway smooth muscle cells (6). In addition, increased levels of polyamines have been observed in mouse lung after allergen challenge (62) and in serum of asthmatic patients (115), respectively. Notably, growth factors like EGF and PDGF, known to be enhanced in asthma, may be involved in the induction of arginase and of

enzymes of the polyamine synthetic pathway (116-118). Polyamines can stimulate the expression of genes implicated in cell proliferation by promoting histone acetyltransferase activity resulting in chromatin hyperacetylation (119). Interestingly, in bronchial biopsies of asthmatic patients, the activity of histone acetyltransferase is increased (120).

As mentioned above, exposure to cigarette smoke and/or nicotine may induce increased expression of arginase I and ODC in human airway structural cells (7). In support, long-term exposure to mainstream smoke increased ODC activity in rat trachea and lung (121). That nicotine could account for the effect of cigarette smoke was shown by the observation that a single subcutaneous injection of nicotine also induces a transient increase in ODC activity in rat trachea (122). These findings suggest that smoking may contribute to airway remodelling in asthma as well as in COPD.

Expression of collagen I mRNA as well as arginase I and II mRNA and protein was increased in bleomycin-induced lung fibrosis in mice (123). Arginase II expression colocalized with the collagen-specific chaperone Hsp47, indicating a prominent role for arginase in collagen synthesis in lung fibrosis (123). In another study, bleomycin induced a time-dependent increase in arginase I and II expression in mouse lung, which was accompanied by decreased levels of L-arginine (124). Arginase was localized to macrophages and epithelial cells as well as in interstitial fibroblasts, especially in fibrotic lesions (124). Increased arginase expression in fibrotic areas was also observed in herpes virus-induced lung fibrosis in mice (125). Interestingly, arginase I and II expression was induced by TGF-β in primary mouse fibroblasts and treatment with the non-specific arginase inhibitor NOHA prevented TGF-β-induced increase in collagen collagen content in a post transcriptional manner (124). In several animal models of silicosis, another inflammatory lung disease characterized by fibrosis, arginase activity and arginase I expression were also enhanced in lung and alveolar macrophages (126-129). However, a direct relationship between increased arginase activity and expression and fibrosis was not always found (127). Thus, arginase I expression and activity in lung of silica-exposed mice were increased at 3 days, but not 30 or 60 days, after silica exposure, while levels of hydroxyproline – a marker of fibrosis – were increased at all three time points (127). Treatment with an arginase inhibitor could clarify whether (increased) arginase activity is indeed involved in silica-induced fibrosis.

In line with the above findings in mouse models of lung fibrosis, arginase I expression was also increased in patients with idiopathic pulmonary fibrosis

(IPF), especially in alveolar macrophages, epithelial cells and areas with pleura thickening and interstitial fibrosis (125). However, in another study lung arginase I expression in IPF patients was decreased, while no differences were observed in arginase II expression and arginase activity in lung, nor in arginase I and II expression in cultured fibroblasts from these patients (124). Therefore, more investigation is needed to establish the role of arginase in human pulmonary fibrotic disease.

Arginase and pulmonary hypertension

Reduced levels of NO have been observed in lungs of patients with pulmonary hypertension (PH; (26). These reduced NO levels may be caused by changes in L-arginine metabolism, since low levels of plasma L-arginine have been found in patients with primary and secondary forms of hypertension (130-132). Inhalation of NO as well as L-arginine supplementation have shown to be of potential benefit in the treatment of PH (131-133). The decreased L-arginine levels may result from increased arginase activity. Thus, increased arginase activity in serum has been detected in patients with primary PH (PPH), which was associated with reduced L-arginine to L-ornithine levels in these patients (130). Moreover, increased arginase II expression has been observed in PPH patients, especially in the endothelium of arteries and arterioles, as well as in cultured pulmonary arterial endothelial cells from these patients (130). Increased arginase activity was also observed in a mouse model of PH. Thus, in mice with monocrotaline-induced PH arginase activity was increased, while NOS activity and cGMP production were reduced (134). Of note, reduced eNOS expression and increased accumulation of endogenous NOS inhibitors, such as monomethylarginine and asymmetric dimethylarginine, did also contribute to reduced NO production in this model (134).

Decreased plasma levels of L-arginine and NO metabolites are also observed in infants with persistent PH (135); however, whether this is caused by increased arginase activity is presently unknown. Interestingly, a recent study indicated that arginase II expression in pulmonary arteries is regulated developmentally, with maximal expression and activity during fetal life (136). It is therefore tempting to speculate that developmentally high levels of arginase activity may be involved in the pathogenesis of PPH of the newborn.

Reduced plasma L-arginine levels and increased serum and plasma arginase activity have also been observed in patients with sickle cell anemia and associated secondary PH (131, 132). Oral treatment with L-arginine increased

plasma levels of both L-arginine and L-ornithine and reduced pulmonary artery systolic pressure (131). The increased plasma arginase activity in patients with sickle cell anemia and PH may be primarily caused by release of the enzyme from erythrocytes during intravascular hemolysis. In addition, hemolysis may contribute to reduced NO bioavailability and endothelial dysfunction via release of erythrocyte hemoglobulin, which scavenges NO (132). Increased arginase activity in the lung and in plasma as well as reduced eNOS activity in the lung were observed in a mouse model of sickle cell disease and PH (137). Similar pathobiology was observed in a nonsickle mouse model of acute alloimmune hemolysis, indicating that hemolysis is sufficient to cause these changes (137).

In conclusion, increased arginase activity appears to be importantly involved in the pathophysiology of both primary and secondary PH by limiting the bioavailability of L-arginine to eNOS in the pulmonary vasculature.

Conclusions

It has been well established that changes in L-arginine metabolism by NO synthases are involved in a variety of diseases, including diseases of the respiratory system. During the last few years there is growing interest in the potential role of arginases as a key regulators of the synthesis of NO, as well as of polyamines and L-proline, in these diseases. Animal model studies have indicated that arginase is importantly involved in the regulation of airway responsiveness, and pharmacological studies using specific arginase inhibitors have revealed a pathophysiological role of the enzyme in the pathogenesis of allergic asthma. Moreover, remarkable increases in pulmonary arginase activity and/or expression associated with altered NO and L-arginine homeostasis have been observed in animal models and human pathology of asthma, COPD, cystic fibrosis, idiopathic pulmonary fibrosis and pulmonary hypertension. Although the functional role of arginase overexpression in most of these diseases has not yet fully been established, there is accumulating evidence that arginase may be an important novel target for drug therapy of these diseases.

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Chapter 7

INCREASED ARGINASE UNDERLIES INFLAMMATION AND REMODELLING IN A GUINEA PIG MODEL OF COPD

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Abstract

Airway inflammation and remodelling are major features of COPD, while pulmonary hypertension is a common comorbidity associated with a poor disease prognosis. Recent studies in animal models have indicated that increased arginase activity contributes to features of asthma, including allergen-induced airway inflammation and remodelling. Although cigarette smoke and lipopolysaccharide (LPS), major risk factors of COPD, may increase arginase expression, the role of arginase in COPD is unknown.
This study aimed to investigate the role of arginase in pulmonary inflammation and remodelling using an animal model of COPD.
Guinea pigs were instilled intranasally with LPS or saline twice weekly for 12 weeks and pretreated by inhalation of the arginase inhibitor 2(S)-amino-6-boronohexanoic acid (ABH) or vehicle.
Repeated LPS exposure increased lung arginase activity, resulting in increased L-ornithine/L-arginine and L-ornithine/L-citrulline ratios. Both ratios were reversed by ABH. ABH inhibited the LPS-induced increases in pulmonary IL-8, neutrophils and goblet cells as well as airway fibrosis. Remarkably, LPS-induced right ventricular hypertrophy, indicative of pulmonary hypertension, was prevented by ABH.
In conclusion, increased arginase activity contributes to pulmonary inflammation, airway remodelling and right ventricular hypertrophy in a guinea pig model of COPD, indicating therapeutic potential for arginase inhibitors in this disease.

Introduction

Chronic obstructive pulmonary disease (COPD) is characterized by a progressive decline in lung function and airflow limitation that is not fully reversible. Chronic inflammation, characterized by increased numbers of neutrophils, macrophages, CD8+ and CD4+ T lymphocytes and B cells in the lung, could contribute to structural changes underlying the airflow limitation, including emphysema and airway remodeling (1). Airway remodeling in COPD is predominantly characterized by mucus cell hyperplasia and peribronchiolar fibrosis (2). In addition, pulmonary hypertension, a comorbidity present in a large proportion of COPD patients, may lead to right ventricular hypertrophy and pulmonary vascular remodeling (3-5).

Recent studies in animal models (6-11) and in patients (8, 10, 12-16) have indicated a major role for increased arginase activity in the pathophysiology of asthma. Increased activity of arginase, which converts L-arginine to L-ornithine and urea, decreases the L-arginine bioavailability to constitutive and inducible isoforms of nitric oxide synthase (NOS) in the airways. This results in decreased production of bronchodilatory NO as well as increased synthesis of proinflammatory and procontractile peroxynitrite, which contribute to the development of allergen-induced airway hyperresponsiveness (AHR) (6, 9, 11). Treatment with inhaled arginase inhibitors strongly protected against allergen-induced airway obstruction, AHR and airway inflammation in guinea pig (7) and mouse (14, 17) models of acute allergic asthma *in vivo*. Using repeatedly allergen-challenged guinea pigs, we recently demonstrated that increased arginase activity also has a major role in airway remodeling in chronic asthma, as indicated by effective inhibition of these features by the inhaled arginase inhibitor 2(*S*)-amino-6-boronohexanoic acid (ABH) (18). In addition to changes in NO metabolism, this may involve increased production of L-ornithine downstream products such as polyamines and L-proline, that cause cell proliferation and collagen synthesis, respectively (10).

Although several studies have revealed the important role of arginase, particularly arginase I, in the pathophysiology of asthma, little is known about its role in COPD (8). However, increased arginase activity was already demonstrated in the late 1970s in sputum from patients with chronic bronchitis (19, 20) and more recently in bronchoalveolar lavage (BAL) fluid (21) and platelets (22) from COPD patients. Interestingly, cigarette smoke has been shown to induce arginase I expression in rat lung (23) and in airways from patients with mild asthma (24). High constitutive expression of arginase I has been demonstrated in azurophilic granules from human neutrophils (25), which are known to be released in COPD (26). Increased arginase activity and decreased NO synthesis have also been implicated in pulmonary arterial hypertension (26), a comorbidity of COPD.

In the present study we investigated the role of arginase in features of pulmonary inflammation, airway remodeling and pulmonary hypertension in a guinea pig model of lipopolysaccharide (LPS)-induced COPD (Chapter 5). LPS, a contaminant of cigarette smoke and environmental pollution, has been implicated in the development of COPD (27-31), and LPS exposure of experimental animals may induce various features of this disease, including inflammation, airway remodeling and emphysema (32-34). In addition, LPS has been shown to induce increased arginase expression in alveolar macrophages (35, 36) and lung tissue (37, 38).

Materials and Methods

Animals

Outbred, male, specified pathogen-free Dunkin Hartley guinea pigs (Harlan, Heathfield, United Kingdom) weighing 350-400 g were used. All protocols were approved by the University of Groningen Committee for Animal Experimentation.

Experimental protocol

Guinea pigs were challenged by intranasal instillation with either 200 µl LPS (5 mg/ml in saline) or 200 µl saline twice weekly, for 12 consecutive weeks (Chapter 5). Thirty min before each instillation, animals received a nebulised dose of the arginase inhibitor ABH in phosphate-buffered saline (PBS) (25 mM nebulizer concentration, 15 min) or PBS (15 min), using a DeVilbiss nebulizer (39). Twenty-four h after the last instillation, the guinea pigs were humanely euthanized by experimental concussion, followed by rapid exsanguination. Heart and lungs were immediately resected and kept in Krebs-Henseleit buffer or on ice, respectively, for further processing.

Arginase activity assay

Arginase activity, expressed as pmol urea produced per mg protein per min, was determined in lung homogenates, by measuring the conversion of [^{14}C]-L-arginine to [^{14}C]-urea at 37°C (9).

Amino acid quantification

Frozen lung tissue was homogenized in Tris-HCl buffer (50 mM Tris-HCl, 150 mM NaCl; pH 7.5) and centrifuged (12 000 x g; 20 min; 4°C) to remove insoluble material. In the supernatants, concentrations of the amino acids L-ornithine, L-arginine and L-citrulline were determined using high performance liquid chromatography followed by tandem mass spectrometry (HPLC-MS/MS) as described recently (18).

Interleukin-8 determination

Interleukin-8 (IL-8) was determined in lung homogenates using an enzyme-linked immunosorbent assay (ELISA) for guinea pig IL-8 according to manufacturer's instructions (Cusabio Biotech, Wuhan, China).

Tissue analysis

Transverse frozen cross-sections (4 µm) of the middle right lung lobe were used for histological and immunohistochemical analyses. Neutrophils were identified by staining sections for TNAP (tissue non-specific alkaline phosphatase activity)

(40). MUC5A/C antibody (Neomarkers; Fremont, CA, USA) was used to identify MUC5A/C-expressing goblet cells (41). Sections were counterstained with haematoxylin. Airways within sections were digitally photographed (40-200x magnification) and classified as cartilaginous or non-cartilaginous. Measurements were performed using ImageJ or NIS (Nikon) quantification software. Neutrophils in the airway adventitia and sub-mucosa were expressed as number of positively stained cells/mm basement membrane length (34). Parenchymal neutrophils were expressed as a percentage of total cell counts (34). MUC5A/C-positive cells in the epithelium were expressed as number of cells/mm basement membrane length (Chapter 5).

The upper right lung lobe was inflated and fixed with formalin at 25 cm H_2O constant pressure for 24 h, and embedded in paraffin. For evaluation of pulmonary vascular dimensions, sections (4 μm) were stained with Weigert's elastin (resorcin/fuchsin) and Van Gieson stain (42). Pulmonary vessel dimensions were determined as described in Chapter 5. For evaluation of airway wall collagen, sections were stained with Sirius Red and counterstained with haematoxylin. The positively stained area in the airway wall, from the adventitial border to the basement membrane, of non-cartilaginous airways was determined as described in Chapter 5. The airway wall collagen area was normalized to the square of the basement membrane length.

To evaluate right ventricular hypertrophy, Fulton's index, *i.e.* the ratio of the right ventricle weight and the sum of the septum and left ventricle weights, was determined.

Hydroxyproline assay

Lungs were analysed for hydroxyproline as an estimate of collagen content, using chloramine T and Erlich's solution (Chapter 5).

Statistical analysis

Data are presented as mean ± SEM. Statistical differences between means were calculated using an unpaired two-tailed Student's t-test or one-way ANOVA, followed by a Boniferroni or Newman Keuls multiple comparison test, as appropriate. Differences were considered significant when $P<0.05$.

Results

Arginase activity and amino acid concentrations in the lung

LPS induced a 2.2-fold increase in arginase activity in lung homogenates *ex vivo* (Figure 1A). Both in LPS- and in saline-challenged animals *in vivo* treatment with inhaled ABH did not significantly change the arginase activity measured *ex vivo* (Figure 1A). Repeated LPS challenge also increased the L-ornithine/L-arginine and L-ornithine/L-citrulline ratio's in the lung (Figure 1B&C), indicating that the endogenous arginase activity is increased and that the balance between arginase and NOS activity is shifted towards arginase. ABH treatment did not affect the amino acid ratios in saline-challenged animals. However, in the LPS-challenged animals, ABH treatment reduced both the L-ornithine/L-arginine ratio and the L-ornithine/L-citrulline ratio to levels below those observed in the PBS-treated, saline-challenged animals (Figure 1B&C). Collectively, these data indicate that LPS instillation induces increased arginase activity in the lung, which is inhibited by ABH *in vivo*.

Table 1: Levels of L-arginine, L-ornithine and L-citrulline in lung homogenates of guinea pigs following repeated saline or LPS challenge and treatment with either inhaled PBS or ABH.

	PBS-treated		**ABH-treated**	
	Saline challenged	LPS challenged	Saline challenged	LPS challenged
L-Arginine (µmol/mg protein)	3.77±1.02	3.85±0.42	3.36±0.66	4.34±0.44
L-Ornithine (µmol/mg protein)	3.99±1.08	4.13±0.40	2.66±0.70	1.29±0.52*,†
L-Citrulline (µmol/mg protein)	2.54±0.51	1.92±0.12	1.92±0.39	2.57±0.27

Data represent means ± SEM of 5-8 experiments. *P<0.05 *vs* saline-challenged control; †P<0.05 vs LPS-challenged control.

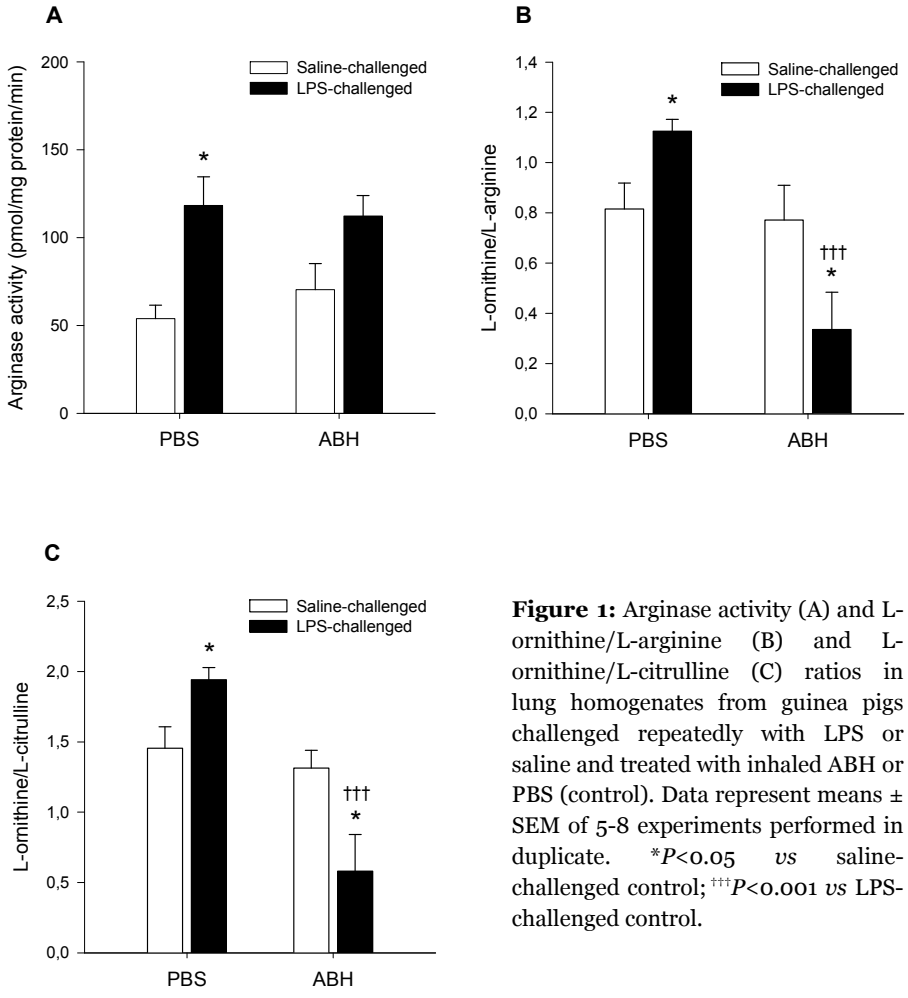

Figure 1: Arginase activity (A) and L-ornithine/L-arginine (B) and L-ornithine/L-citrulline (C) ratios in lung homogenates from guinea pigs challenged repeatedly with LPS or saline and treated with inhaled ABH or PBS (control). Data represent means ± SEM of 5-8 experiments performed in duplicate. $^{*}P<0.05$ *vs* saline-challenged control; $^{\dagger\dagger\dagger}P<0.001$ *vs* LPS-challenged control.

Inflammation

Neutrophils are a major inflammatory cell type involved in COPD pathogenesis and are a rich source of arginase (25). Repeated LPS instillation increased the neutrophil number in both cartilaginous (2.9-fold) and non-cartilaginous (3.2-fold) airways as well as in the parenchyma (2.0-fold) (Figure 2). ABH treatment reduced the neutrophil numbers in these compartments by 83%, 60% and 56%, respectively (Figure 2). ABH treatment did not affect neutrophil numbers in saline-challenged animals. In order to assess potential mechanisms involved in arginase-induced neutrophilia in the LPS-challenged animals, we determined

levels of the neutrophil chemoattractant IL-8 in lung homogenates. Figure 3 indicates that the induction of neutrophil infiltration by repeated LPS instillation is associated with a significant increase of IL-8 in the lung, which was fully inhibited by inhalation of ABH. As with neutrophilia, no effects were observed in saline-challenged animals (Figure 3). These data indicate that LPS-induced arginase activity contributes to neutrophilia by increasing IL-8 levels.

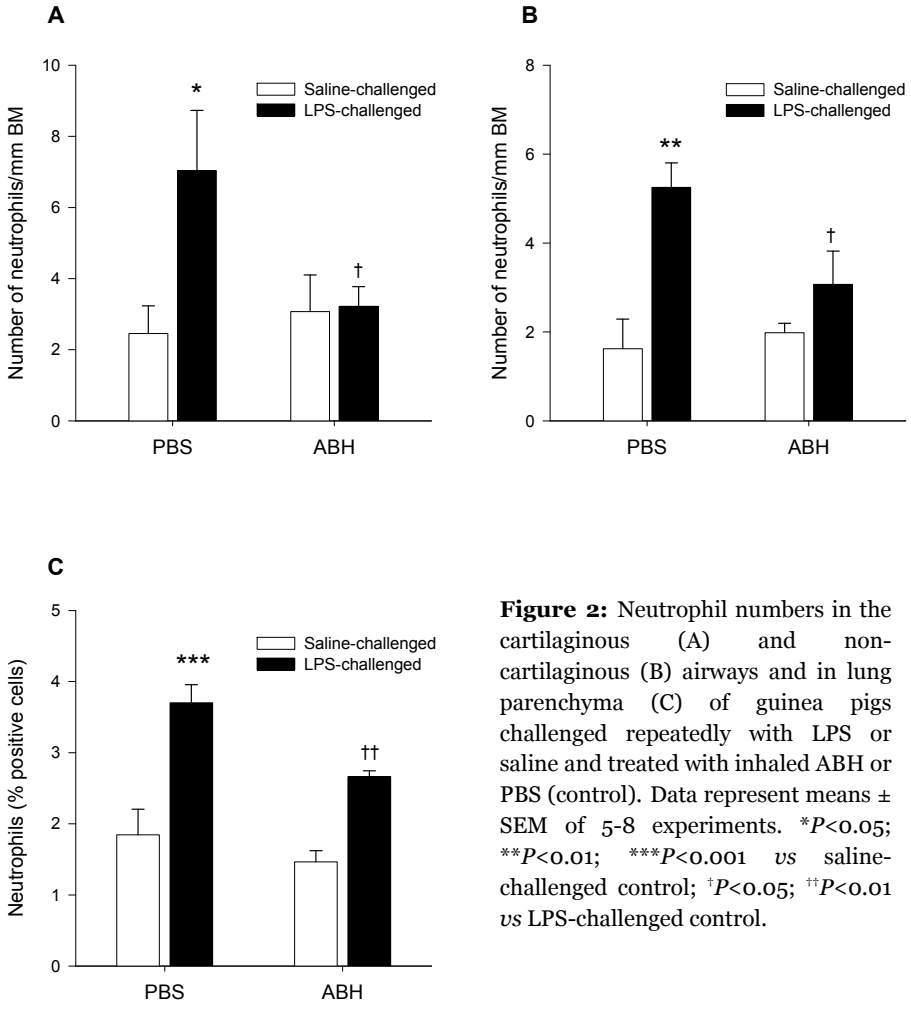

Figure 2: Neutrophil numbers in the cartilaginous (A) and non-cartilaginous (B) airways and in lung parenchyma (C) of guinea pigs challenged repeatedly with LPS or saline and treated with inhaled ABH or PBS (control). Data represent means ± SEM of 5-8 experiments. **P*<0.05; ***P*<0.01; ****P*<0.001 *vs* saline-challenged control; †*P*<0.05; ††*P*<0.01 *vs* LPS-challenged control.

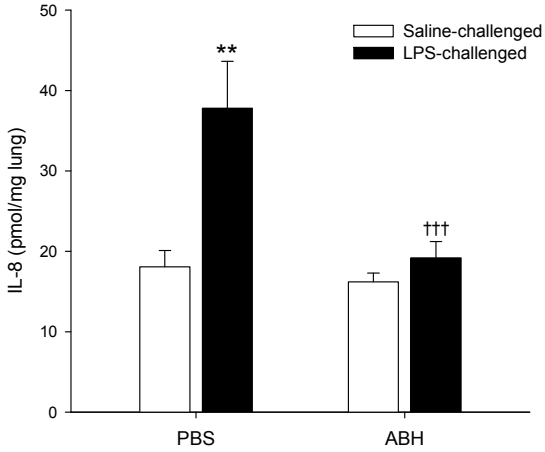

Figure 3: IL-8 levels in lung homogenates from guinea pigs challenged repeatedly with LPS or saline and treated with inhaled ABH or PBS (control). Data represent means ± SEM of 5-8 experiments performed in duplicate. $^{**}P<0.01$ *vs* saline-challenged control; $^{\dagger\dagger\dagger}P<0.001$ *vs* LPS-challenged control.

MUC5A/C expression

Repeated LPS instillation induced a significant 2.2-fold increase in the number of MUC5AC-positive cells in the epithelium of cartilaginous airways (Figure 4), indicating mucus hypersecretion. ABH treatment fully inhibited the LPS-induced MUC5A/C expression, whereas it had no effect in saline-challenged animals (Figure 4).

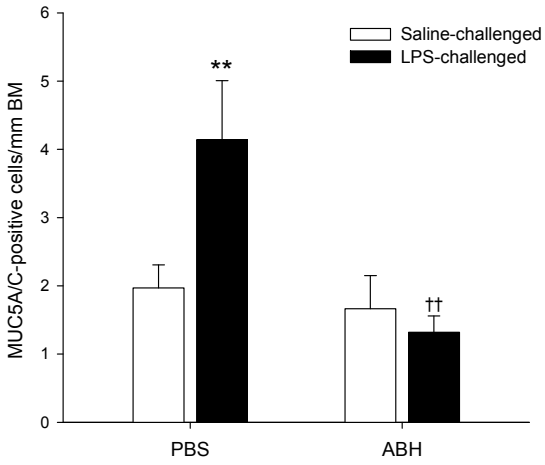

Figure 4: MUC5A/C-positive goblet cell number in intrapulmonary cartilaginous airways of guinea pigs challenged repeatedly with LPS or saline and treated with inhaled ABH or PBS (control). Data represent means ± SEM of 5-7 experiments. $^{**}P<0.01$ *vs* saline-challenged control; $^{\dagger\dagger}P<0.001$ *vs* LPS-challenged control.

Airway fibrosis

To evaluate fibrotic changes, lungs were analysed for hydroxyproline as an estimate of collagen content. Repeated LPS instillation induced a significant 1.7-fold increase in total lung hydroxyproline content (Figure 5A). ABH treatment inhibited the LPS-induced increase in hydroxyproline by 75%, whereas it had no effect on the hydroxyproline content in saline-challenged animals (Figure 5A).

To assess changes in collagen deposition in the airway compartment, Sirius Red staining was evaluated in the airway wall of non-cartilaginous airways. Similar to the increase in hydroxyproline content, LPS induced a 1.9-fold increase in airway wall collagen content (Figure 5B). ABH fully inhibited the LPS-induced collagen deposition in the airway wall, whereas it did not affect the collagen content in the airway wall of saline-challenged animals (Figure 5B).

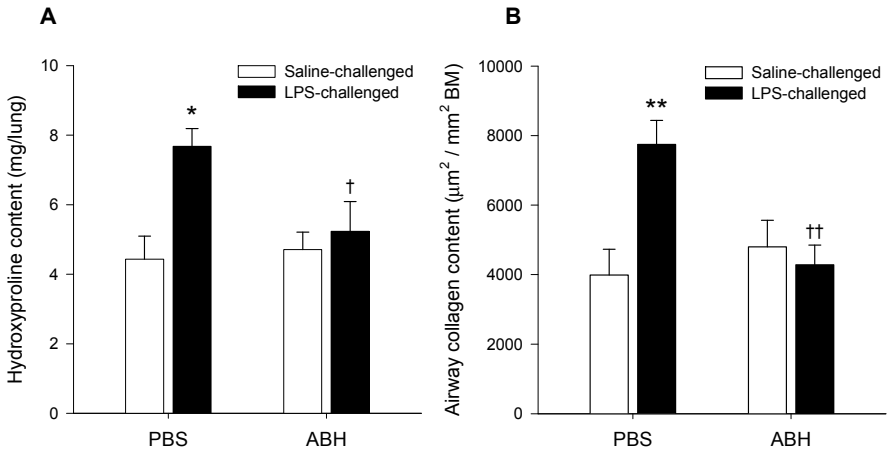

Figure 5: Whole lung hydroxyproline content (A) and collagen content in the airway wall (Sirius red; B) in guinea pigs challenged repeatedly with LPS or saline and treated with inhaled ABH or PBS (control). Data represent means ± SEM of 5-8 experiments. Hydroxyproline determinations were performed in triplicate and 2 to 6 airways were analysed for each animal for the Sirius red staining. **P*<0.05, ***P*<0.01 *vs* saline-challenged control; †*P*<0.05, ††*P*<0.01 *vs* LPS-challenged control.

Right ventricular hypertrophy

Repeated LPS challenge induced right ventricular hypertrophy as indicated by a significant 1.4-fold increase in Fulton index (Figure 6). ABH treatment fully inhibited the LPS-induced right ventricular hypertrophy, whereas ABH had no effect in saline-challenged animals (Figure 6).

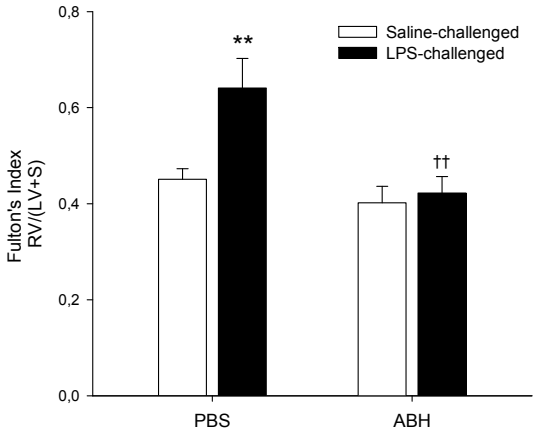

Figure 6: Fulton's index in guinea pigs challenged repeatedly with LPS or saline and treated with inhaled ABH or PBS (control). Data represent means ± SEM of 4-8 experiments. $^{**}P<0.01$ *vs* saline-challenged control; $^{††}P<0.01$ vs LPS-challenged control.

Pulmonary vessel wall dimensions

To evaluate pulmonary vessel wall dimensions, pulmonary artery medial area and pulmonary arteriole wall area were determined in formalin-fixed, paraffin-embedded guinea pig lung sections stained with Weigert's elastin and Van Gieson stain. Neither repeated LPS instillation nor ABH treatment affected the medial area of pulmonary arteries or wall area of pulmonary arterioles (Figure 7). In addition, there was no evidence of intimal proliferation in the pulmonary vessels of either classification.

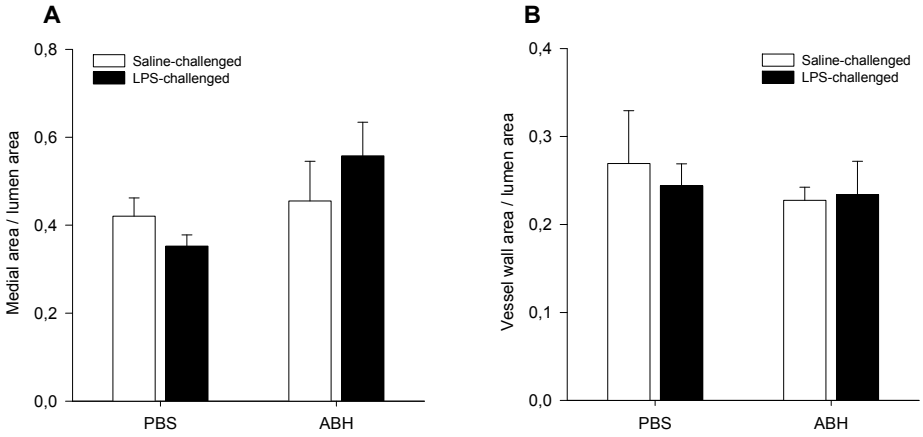

Figure 7: Pulmonary artery medial area (A) and pulmonary arteriole wall area (B) in guinea pigs challenged repeatedly with LPS or saline and treated with inhaled ABH or PBS (control). Data represent means ± SEM of 4-8 experiments for the arteries and 3-4 experiments for the arterioles.

Discussion

This is the first study to demonstrate the effectiveness of an (inhaled) arginase inhibitor in preventing indices of pulmonary inflammation, airway remodeling and pulmonary hypertension in an animal model of COPD. Thus, inhaled ABH protected against neutrophil infiltration, mucus hypersecretion and airway fibrosis induced by repeated intranasal LPS instillation in guinea pigs. In addition, repeated LPS challenge induced right ventricular hypertrophy, which was similarly inhibited by inhalation of the arginase inhibitor.

It was found that repeated LPS challenge *in vivo* increased arginase activity in guinea pig lung homogenates determined *ex vivo*. This presumably reflects increased arginase expression induced by the LPS challenge, as arginase is a constitutively active enzyme. Unfortunately, due to lack of specific antibodies against (subtypes of) guinea pig arginase it was not possible to determine arginase protein expression in a direct manner; however, increased arginase gene expression in the lung induced by inhalation of LPS has previously been observed in mice (37, 38). The lack of effect of ABH inhalation on the induction of increased arginase activity by LPS as measured *ex vivo* (ABH not being present in the assay) suggests that arginase is not involved in the regulation of its own expression. This is in contrast with allergen-induced increase in arginase activity, that can be inhibited by arginase inhibitors (17, 68). This is presumably due to potentiation of allergen-induced IL-13 production by constitutive arginase activity present in the airways, which in turn may enhance arginase expression (10). The LPS-induced increase in arginase activity determined *ex vivo* was reflected by increased L-ornithine/L-arginine and L-ornithine/L-citrulline ratio's in the lung. The LPS-induced increase in L-ornithine/L-citrulline ratio indicates that the increased arginase activity competes with NOS for L-arginine. Treatment with inhaled ABH prevented the enhanced L-ornithine/L-arginine ratio induced by LPS, indicating that endogenous arginase activity was indeed inhibited by inhaled ABH. Moreover, the reduction in the L-ornithine/L-citrulline ratio by ABH indicates restoration of NOS activity by the arginase inhibitor. Interestingly, ABH treatment of the LPS-challenged animals resulted in attenuation of both ratios below the levels observed for saline-challenged animals. The reduced L-ornithine/L-citrulline ratio below baseline might be explained by LPS-induced iNOS activity, which results in increased L-citrulline production, as also reflected by the trend towards an increase of the L-citrulline concentration in the lungs of ABH-treated, LPS-challenged animals (Table 1). Indeed, induction of iNOS by LPS is well established (43). Increased expression of iNOS could also account for the decreased L-ornithine/L-arginine ratio, as recycling of L-citrulline is an

important source of L-arginine, under inflammatory conditions (44, 45). This is also supported by the observation that argininosuccinate synthetase, an enzyme which plays a key role in the conversion of L-citrulline to L-arginine, is upregulated in the lung by LPS treatment *in vivo* (38, 46). Taken together, our findings indicate that LPS induces increased arginase activity in the lung *in vivo,* which is inhibited by inhaled ABH, thereby favoring NOS activity and increasing NO production. Since NO has anti-inflammatory and anti-fibrotic actions (47), such a mechanism may well be involved in the inhibition of LPS-induced neutrophil influx, collagen synthesis and mucus production by the arginase inhibitor, as is also discussed below.

Increased arginase activity has previously been found in BAL fluid (21) and platelets (22) of COPD patients. In addition to LPS, arginase expression is also induced by cigarette smoke as shown in rat lung (23) and rabbit cavernous tissue (48). Moreover, arginase expression in the airways is further increased in patients with mild asthma who smoke, compared to non-smoking asthmatics (24).

Neutrophils are involved in the pathogenesis of COPD. Pulmonary neutrophils are increased in COPD and correlations between airway neutrophil numbers and COPD severity have been found (26). ABH inhalation strongly inhibited LPS-induced neutrophilia in our model, indicating that induction of arginase by LPS importantly contributes to the neutrophilic inflammation. To investigate possible mechanisms underlying this anti-inflammatory effect of ABH, we determined concentrations of the major neutrophil-attracting chemokine IL-8 in whole lung homogenates. LPS-induced neutrophilia was associated with a increased IL-8 in the lung, while neutrophil influx and increase in IL-8 were both inhibited by ABH, suggesting that increased arginase activity may contribute to neutrophilia by increasing IL-8 levels in the lung.

One of the mechanisms underlying enhanced IL-8 production and airway inflammation by arginase might be via promoting NF-κB by attenuating the synthesis of NO, which inhibits this process via nitrosylation of the transcription factor (49). In addition, increased arginase activity causes uncoupling of iNOS and subsequent production of the pro-inflammatory oxidant species peroxynitrite (8), which induces IL-8 expression in various cell types (50, 51). Accordingly, breakdown of this oxidant reduces smoke-induced IL-8 levels in sheep lung (52).

Mucus hypersecretion contributes to airflow limitation in COPD. Increased MUC5AC expression is observed in the airway epithelium of COPD patients and can be induced by cigarette smoke and LPS, as well as by neutrophil elastase and peroxynitrite (53). ABH fully inhibited the LPS-induced MUC5AC expression in the guinea pig airway epithelium, indicating a major role for increased arginase activity in this process. The effect of ABH may be the result of the inhibition of IL-8 production and airway neutrophilia, which may both contribute to increased MUC5AC expression (54, 55). Moreover, ABH could decrease the LPS-induced MUC5AC expression by inhibiting peroxynitrite formation and restoring NO production (56).

Airway fibrosis is a characteristic feature of COPD, which contributes to airway wall thickening and airflow limitation (57). Previous studies have shown that increased arginase expression contributes to bleomycin-induced lung fibrosis in mice (58), lung allograft fibrosis in rats (59) and repeated allergen challenge-induced fibrosis in guinea pigs (68). The present study indicates that increased arginase activity also contributes to LPS-induced fibrosis in the lung, particularly in the airway wall. This may involve increased production of L-ornithine and its downstream product L-proline, which is a precursor of collagen (60). In accordance, TGF-β, a major pro-fibrotic factor, has been shown to induce arginase activity in the rat lung and fibroblasts (59) and TGF-β-induced collagen synthesis was reduced by inhibitors of arginase in lung fibroblasts of rats and mice (61, 62). In addition, the inhibition of fibrosis by ABH may also be due to the increased production of NO and decreased formation of peroxynitrite (63, 64).

Our data show that repeated LPS-challenge induces right ventricular hypertrophy, a feature of pulmonary hypertension, a known co-morbidity of COPD (3). The LPS-induced right ventricular hypertrophy was prevented by ABH. Our data therefore suggest that repeated LPS challenge results in pulmonary hypertension via induction of arginase. Pulmonary hypertension and right ventricular hypertrophy may result from (a combination of) vascular remodeling and functional changes in the vessel wall, both leading to increased resistance in the pulmonary vasculature (3). In our model, we did not observe changes in pulmonary vessel dimensions after repeated LPS instillation or by treatment with inhaled ABH, suggesting that increased resistance in the pulmonary vessels and subsequent right ventricular hypertrophy in this model are due to exaggerated constriction of the vessels rather than remodeling. In this respect, endothelial dysfunction caused by reduced activity of eNOS has been proposed as a potential mechanism (65). Indeed, pulmonary hypertension has

been associated with reduced L-arginine and NO levels (66), whereas inhalation of NO and oral therapy with L-arginine decrease pulmonary arterial pressure in primary or secondary pulmonary hypertension (67). Increased consumption of L-arginine by enhanced arginase II expression and activity in the endothelium has been shown to contribute to the reduced L-arginine and NO levels (66, 67). In addition, hypoxia, which is considered to play a major role in COPD-related pulmonary hypertension, upregulates arginase in human lung microvascular endothelial cells (68). Our data would suggest that increased arginase activity may contribute to pulmonary hypertension and right ventricular hypertrophy in COPD, possibly by inducing endothelial dysfunction, and that this process can be effectively targeted by inhalation of arginase inhibitors.

In conclusion, our study demonstrates that increased arginase activity plays a major role in pulmonary inflammation, airway remodeling and right ventricular hypertrophy in a guinea pig model of COPD and that arginase inhibitors may have therapeutic potential in the treatment of this disease.

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Chapter 8

General Discussion and Summary

Effects of cigarette smoke extract and lipopolysaccharide on airway smooth muscle phenotype

Chronic obstructive pulmonary disease (COPD) is an inflammatory lung disease characterized by a progressive and largely irreversible airflow obstruction, which involves structural changes of the lung, including emphysema and airway remodelling (1). Various studies have indicated that increased airway smooth muscle (ASM) mass may contribute to airway remodelling in COPD (1-5). Mitogens, including growth factors and extracellular matrix proteins, induce proliferation of ASM and cause induction of a proliferative, hypocontractile ASM phenotype, that may be involved in thickening of the muscle (6, 7). Although the exact mechanisms leading to ASM thickening in COPD are not known, inflammation presumably plays an important role (7, 8).

Interestingly, several studies have indicated that cigarette smoke (CS) exposure may also initiate airway remodelling by direct action on the airway wall, without the need for inflammatory cell infiltration. *In vitro* experiments demonstrated that CS exposure of rat tracheal explants results in increased expression of pro-fibrotic growth factors in the airway wall (9, 10). In addition, *in vivo* CS-exposure of mice was found to increase the expression of procollagen, connective tissue growth factor (CTGF), transforming growth factor-β (TGF-β) and platelet-derived growth factor (PDGF) in the airway wall, prior to the onset of inflammatory cell infiltration (11). These studies clearly demonstrate the potential for a direct, inflammation-independent contribution of CS to airway wall remodelling.

The studies described in **Chapter 2** support this contention and indicate that CS extract (CSE) and lipopolysaccharide (LPS) induce a profound and concentration-dependent increase in bovine tracheal smooth muscle (BTSM) cell proliferation. For CSE, short pulsatile stimulation of cells is required in order to avoid cell death induced by prolonged exposure to this stimulus. In addition, similar to previous observations with PDGF (12, 13), we demonstrated that CSE- and LPS-induced proliferation of BTSM cells is mediated by ERK 1/2 and p38 MAP kinase. Moreover, BTSM cell proliferation was associated with increased expression of cyclin D1. Consistent with a shift to a more proliferative phenotype, prolonged treatment of BTSM strips with CSE or LPS reduced the contractility of BTSM tissue. Accordingly, CSE- or LPS-induced hypocontractility of BTSM was associated with increased phosphorylation of MAP kinases in the tissue. Collectively, our data indicate that CSE and LPS may induce a proliferative, hypocontractile ASM phenotype, independent of an effect on other structural or inflammatory cells in the airway wall. Concentrations of LPS in the CSE were

hardly detectable and far below the concentrations needed for ASM cell proliferation. In addition, there was no additional effect of the combination of CSE and LPS on the proliferative responses in ASM cells, indicating that LPS does not mediate the CSE-induced proliferation and that common pathways may be involved, as has previously also been shown by others (14-16).

The role of TAK1 in the regulation of ASM phenotype and synthetic function

The studies described in **Chapters 3 and 4** are the first to demonstrate a role of TGF-β-activated kinase 1 (TAK1) in ASM. TAK1 has previously been identified as a key component of Toll-like receptor, IL-1 receptor and TNF-α receptor signalling (17-20), and was found to play a major role in embryonal development through the TGF-β/BMP signalling pathway (21-24). In addition, TAK1 has been shown to regulate proliferation of various cell types and has been implicated in cardiac remodelling as well as vascular smooth muscle development (21, 22, 25, 26).

The data in **Chapter 3** indicate that in BTSM cells as well as in human tracheal smooth muscle cells, TAK1 regulates growth factor-induced proliferation. Thus, PDGF- or foetal bovine serum-induced DNA-synthesis and increased ASM cell number were strongly inhibited by the specific TAK1 inhibitor, LL-Z1640-2. PDGF-induced ERK 1/2 phosphorylation was attenuated by LL-Z1640-2, as well as by expression of a dominant-negative TAK1, indicating the involvement of TAK1 in PDGF-induced ERK 1/2 signalling. In addition, the PDGF-induced hypocontractility and decreased expression of contractile proteins were inhibited by LL-Z-1640-2. Collectively, these data identify TAK1 as a novel mediator of PDGF-induced signalling in ASM and indicate that TAK1 plays a major role in growth factor-induced phenotypic modulation of ASM.

In addition to their role in the regulation of airway diameter, ASM cells also have a synthetic function. They are a source of pro-inflammatory cytokines as well as pro-fibrotic growth factors and extracellular matrix proteins, and may therefore contribute to the development of both airway inflammation and remodelling in COPD (6, 7). CSE exposure of ASM cells has previously been shown to induce release of the neutrophil chemokine IL-8 (27-30). Although the molecular mechanisms underlying this effect have not yet been fully elucidated, NF-κB and ERK 1/2 signalling pathways were recently shown to be involved (28). Since TAK1 is a major upstream regulator of both NF-κB and ERK 1/2 activation (19, 31-34), it could act as a regulator of CSE-induced pro-inflammatory signalling.

The studies described in **Chapter 4** confirm that CSE induces IL-8 release by human ASM cells. The importance of NF-κB and ERK 1/2 signalling was demonstrated by the strong inhibition of the CSE-induced IL-8 release by pharmacological inhibitors of IκBα kinase 2 (IKK2) and mitogen activated protein kinase kinase (MEK), direct activators of NF-κB and ERK 1/2 signalling, respectively. TAK1 was shown to play a major role in the CSE-induced activation of NF-κB and ERK 1/2, as expression of dominant-negative TAK1 and/or pretreatment with LL-Z-1640-2 inhibited IκBα degradation and ERK 1/2 phosphorylation, whereas LL-Z-1640-2 also inhibited the CSE- induced IL-8 release by the ASM cells. These results show that TAK1 plays a key role in CSE-induced IL-8 release by human ASM cells through NF-κB and ERK 1/2 signalling.

Collectively, the data presented in **Chapters 3 and 4** identify TAK1 as a regulator of pro-proliferative and pro-inflammatory signalling in ASM cells and indicate that TAK1 may be a novel target for the inhibition of inflammation and remodelling in obstructive airways diseases like COPD.

Guinea pig model of LPS-induced COPD

As described in **Chapter 1**, several approaches have been used to develop an animal model of COPD (35). To investigate mechanisms as well as pharmacological treatment of COPD *in vivo* and *ex vivo*, we established a guinea pig model of LPS-induced COPD. LPS is a relevant stimulus for the development as well as for exacerbations of COPD (14-16, 36, 37) because it induces a wide variety of inflammatory responses and structural changes involved in COPD, both in patients and in animal models (38-45). Moreover, pulmonary inflammation and remodelling induced by repeated LPS exposure are maintained for prolonged periods of time when LPS is no longer administered, indicating persistence of the disease, which is also a characteristic of COPD (42, 45, 46).

The studies described in **Chapters 5 and 7** show that 12 weeks of twice weekly intranasal instillations of LPS (1 mg / 200 μl) in conscious guinea pigs results in pulmonary neutrophilia, increased IL-8 levels in the lung, increased epithelial MUC5A/C expression, airway fibrosis, emphysema and right ventricular hypertrophy, all characteristic features of COPD.

Cholinergic mechanisms in COPD

Increased cholinergic tone is the primary reversible component of airflow obstruction in COPD, as evidenced by the effectiveness of anticholinergic bronchodilator therapy in this disease. However, recent findings, including the

UPLIFT trial (47), indicate that the long-acting anticholinergic tiotropium may have additional benefits other than bronchodilation. Thus, tiotropium was shown to reduce the number of exacerbations and overall mortality. Although no effect on lung function decline in the whole study population was observed, the rate of lung function decline was decreased by tiotropium in patients not on other controller medication, patients with moderate COPD and young patients (47-50). Mechanisms underlying the non-bronchodilator effects of tiotropium are currently not fully understood, but various studies have indicated that muscarinic receptor stimulation may promote the release of pro-inflammatory chemokines from airway structural cells, including epithelial and ASM cells, as well as macrophages (27, 51, 52). Furthermore, muscarinic receptor stimulation increases collagen production by and proliferation of lung fibroblasts and augments growth-factor induced proliferation of ASM cells, suggesting a role for acetylcholine in fibrosis and ASM remodelling (53-56). Recently, it has been shown that tiotropium pretreatment reduces CS-induced pro-inflammatory cytokine expression and inflammatory cell numbers in BAL from mice, indicating a role for endogenous acetylcholine in CS-induced inflammation (57). Previous studies in an animal model of chronic asthma further support a potential role for acetylcholine in airway inflammation and remodelling, as tiotropium pretreatment reduced airway eosinophilia as well as ASM remodelling and goblet cell hyperplasia induced by repeated allergen exposure (58, 59).

The studies described in **Chapter 5** demonstrate that tiotropium inhalation inhibits neutrophilia, epithelial MUC5AC expression and airway fibrosis in a guinea pig model of LPS-induced COPD, indicating that endogenous acetylcholine plays a major role in airway inflammation and remodelling in this disease. In addition, airway vascular remodelling was also demonstrated in the COPD model, as evidenced by the increased numbers of muscularized microvessels in the adventitia of cartilaginous airways. Neither the cause nor the consequence of this type of remodelling is clear at present. Tiotropium inhibited the increased muscularization of the microvessels, indicating for the first time that acetylcholine may also be involved in vascular remodelling. No changes in pulmonary vascular dimensions were observed in this disease model.

Repeated LPS exposure also increased the alveolar airspace size in this study, indicating that emphysema is induced. The development of emphysema was, however, not affected by tiotropium, suggesting that acetylcholine does not contribute to alveolar remodelling in this model. Neutrophils are considered to play a major role in the development of emphysema. Although *chronic* LPS-induced parenchymal neutrophilia in our model was inhibited by tiotropium, this

does not necessarily imply that neutrophils are not involved in alveolar destruction.It has recently been shown that tiotropium does not inhibit acute BAL neutrophilia induced by a *single* LPS exposure in mice (57). Since it has been shown that brief exposure of the lung to neutrophil elastase may already induce emphysema, it is possible that the acute neutrophilic response after each LPS exposure is sufficient to induce emphysema. However, other inflammatory cell types may also contribute to tissue breakdown in the parenchyma (60).

Collectively, these data suggest that endogenous acetylcholine, acting through muscarinic receptors, plays a major role in pulmonary inflammation and airway remodelling in COPD, which could underlie the beneficial non-bronchodilator effects of tiotropium in this disease.

The role of arginase in COPD

Nitric oxide (NO) is synthesized from the amino acid L-arginine by nitric oxide synthase (NOS) isoenzymes, and has potent bronchodilatory and anti-inflammatory actions (61). Under pathophysiological conditions, including asthma, increased expression and activity of the enzyme arginase - which converts L-arginine to L-ornithine and urea - can lead to a decreased bioavailability of L-arginine to NOS. This may result not only in decreased NO production but also in increased formation of the pro-inflammatory and pro-contractile oxidant species, peroxynitrite (62). Both the decrease of NO and the increase of peroxynitrite contribute to airway hyperresponsiveness and airway inflammation in asthma (63-69). In addition, increased arginase activity has recently been shown to contribute to airway remodelling in chronic asthma, which may involve altered NO metabolism as well as increased production of polyamines and L-proline, downstream products of L-ornithine that may cause cell proliferation and collagen synthesis, respectively (70). Although a recent study has indicated that arginase activity is increased in the BAL from COPD patients (71), its role in the pathophysiology of COPD is currently unknown.

The studies described in **Chapter 7** focus on the role of arginase in COPD, using the guinea pig model. These studies showed that chronic LPS exposure increased arginase activity in lung homogenates, indicating increased expression of the enzyme. This is in accordance with previous studies showing that a single LPS exposure increases arginase gene expression in mouse lung (42, 72). The LPS-induced increase of arginase activity *in vivo* in our study was also reflected by an increased L-ornithine/L-arginine ratio in the lung tissue. The enhanced arginase activity was associated with increased IL-8 levels, neutrophils, epithelial MUC5A/C expression and with airway fibrosis in the lung.

Pretreatment with the arginase inhibitor 2(S)-amino-6-boronohexanoic acid (ABH) by inhalation effectively inhibited the LPS-induced increase of arginase *activity*, as indicated by a decrease of the L-ornithine/L-arginine ratio in the lung. LPS-induced arginase *expression* was not affected by ABH, as ABH pretreatment *in vivo* did not affect the LPS-induced arginase activity in the *ex vivo* assay, performed in the absence of ABH. This is consistent with previous observations indicating that LPS is a direct stimulus for arginase expression (73, 74).

ABH pretreatment also inhibited the LPS-induced increase in IL-8, neutrophils, MUC5A/C expression and airway fibrosis in the lung, indicating a major contribution of the increased arginase activity to pulmonary inflammation and remodelling. One potential mechanism underlying the role of arginase in these processes is increased synthesis of peroxynitrite induced by uncoupling of iNOS by the low L-arginine availability, which causes simultaneous production of NO and superoxide anions by the enzyme (62). Peroxynitrite has previously been shown to induce IL-8 expression in various cell types and may therefore contribute to neutrophilia (75, 76). In addition, peroxynitrite may directly induce MUC5A/C expression, although elastase derived from activated neutrophils as well as increased IL-8 could also contribute (77-79). Increased peroxynitrite formation as well as decreased NO production has previously been implicated in fibrotic processes. In addition, as mentioned above, arginase-derived L-ornithine may be converted to L-proline, and thus enhance collagen synthesis (61). Accordingly, arginase was found to mediate TGF-β-induced collagen synthesis in lung fibroblasts (80, 81).

In addition to inflammation and structural changes in the lung, right ventricle mass was found to be increased in the LPS-exposed animals. This indicates that pulmonary hypertension develops in our disease model. Pulmonary hypertension is present in a large proportion of COPD patients and is associated with poor prognosis (82). Chronic inflammation and hypoxia may cause endothelial dysfunction of the pulmonary arteries, by inducing decreased endothelial NOS expression (83), reduced NO production (84) and enhanced release of vasoconstrictors such as endothelin-1 (82), which may increase contractile tone of the vessels. In addition, vascular remodelling, characterized by intimal proliferation and thickening of the vessel wall, may also contribute to pulmonary arterial hypertension (82). The rise in pulmonary afterload, due to the increased pulmonary vascular pressure, results in right ventricular hypertrophy (85). Increased arginase activity has been demonstrated in endothelial cells from patients with pulmonary arterial hypertension, which is associated with decreased bioavailability of L-arginine and reduced NO synthesis (84). The

observations that oral L-arginine and inhaled NO therapy decrease pulmonary arterial pressure in this disease indicate an important role for the aberrant NO homeostasis (86, 87). Pulmonary arterial wall dimensions were not altered in our study, suggesting that exaggerated vasoconstriction rather than vascular remodelling underlies the right ventricular hypertrophy observed in our model. Our data demonstrated that ABH pretreatment inhibits the LPS-induced development of right ventricular hypertrophy, indicating a major role for increased arginase activity in this process.

In conclusion, the present studies have demonstrated that increased arginase activity contributes to pulmonary inflammation, airway remodelling and right ventricular hypertrophy in our animal model of COPD, indicating that arginase inhibitors may have therapeutic potential in the treatment of this disease.

Summarizing, the main findings from the studies described in this thesis are:

- Cigarette smoke and LPS induce a proliferative, hypocontractile phenotype of ASM. This effect is mediated by activation of ERK 1/2 and p38 MAP kinase, and may result from a direct action of the stimuli on ASM, without involvement of other airway structural or inflammatory cells (**Chapter 2**).

- Short, pulsatile exposure of cells or tissue to CSE is a suitable approach for *in vitro* modelling of *in vivo* CS exposure (**Chapter 2**).

- CSE and LPS share common signalling pathways in ASM proliferation, but LPS is unlikely to mediate the CSE-induced effect (**Chapter 2**).

- TAK1 is a key intermediate in PDGF-induced ERK 1/2 signalling in ASM and plays a major role in growth factor-induced phenotypic modulation of ASM (**Chapter 3**).

- TAK1 plays a major role in CSE-induced NF-κB and ERK 1/2 signalling as well as IL-8 release by ASM cells (**Chapter 4**).

- TAK1 is a novel target for the inhibition of airway inflammation and remodelling in obstructive airways diseases such as COPD (**Chapters 3 and 4**).

- Repeated LPS exposure in a guinea pig model of COPD results in the development of neutrophilia, increased Il-8 levels, emphysema, increased epithelial MUC5A/C expression and airway fibrosis in the lung as well as right ventricular hypertrophy (**Chapters 5 and 7**). The induction of these major characteristics of COPD indicates that this model is suitable for studying pathogenic processes and therapeutic treatment of this disease.

- Endogenous acetylcholine contributes to pulmonary neutrophilia, increased epithelial MUC5A/C expression, airway fibrosis and airway microvessel remodelling in an animal model of COPD (**Chapter 5**). The potential involvement of acetylcholine in inflammation and airway remodelling may underlie beneficial non-bronchodilator effects of tiotropium in COPD patients.

- Increased arginase activity plays a major role in pulmonary inflammation, airway remodelling and right ventricle hypertrophy in a guinea pig model of LPS-induced COPD. Inhalation of arginase inhibitors may therefore be a useful therapeutic intervention in this disease (**Chapter 7**).

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Nederlandse samenvatting

Chronic obstructive pulmonary disease (COPD), voornamelijk veroorzaakt door roken, wordt gekenmerkt door een progressieve afname van de longfunctie. Ontsteking in de long leidt tot de ontwikkeling van structurele afwijkingen, zoals emfyseem en luchtwegremodelling, die bijdragen aan de luchtwegobstructie en longfunctieafname bij deze ziekte. Het doel van de studies beschreven in dit proefschrift was het ontrafelen van nieuwe potentiële mechanismen voor de pathofysiologie van COPD.

Effecten van sigarettenrookextract en lipopolysaccharide op luchtweg- gladde spiercellen

Verschillende studies hebben aangetoond dat toename van de luchtweg-gladde spier massa bij kan dragen aan luchtwegremodelling in COPD. Groeifactoren en extracellulaire matrix eiwitten induceren proliferatie van luchtweg-gladde spiercellen waardoor deze een proliferatief, hypocontractiel fenotype kunnen aannemen, dat mogelijk betrokken is bij de verdikking van de spierlaag. De exacte mechanismen die een rol spelen bij de toename van luchtweg-gladde spiermassa in COPD zijn niet volledig bekend, maar ontsteking van de luchtwegen speelt hierbij vermoedelijk een belangrijke rol.

Enkele studies hebben aangetoond dat ook het blootstellen van luchtwegen aan sigarettenrook remodelling van de luchtwegwand kan induceren, onafhankelijk van de effecten van inflammatoire celinfiltratie. Sigarettenrook-blootstelling van tracheale preparaten van ratten resulteerde in verhoogde expressie van pro-fibrotische groeifactoren in de luchtwegwand. Daarnaast resulteerde sigarettenrook-blootstelling van muizen in verhoogde expressie van procollageen, connective tissue growth factor (CTGF), transforming growth factor-β (TGF-β) en platelet-derived growth factor (PDGF) in de luchtwegwand, nog voordat de infiltratie van ontstekingscellen was opgetreden. Deze studies tonen aan dat blootstelling aan CS remodelling kan induceren door een direct effect op de luchtwegwand, zonder een associatie met ontsteking.

De studies beschreven in **Hoofdstuk 2** ondersteunen deze stelling en geven aan dat sigarettenrookextract en lipopolysaccharide (LPS) een sterke en concentratie-afhankelijke toename van celproliferatie van de runder tracheale gladde spiercellen induceren. Voor sigarettenrookextract, is korte pulsatiele stimulatie van de cellen noodzakelijk om celproliferatie te induceren, aangezien langdurige blootstelling aan deze stimulus celdood induceert. Bovendien werd aangetoond dat, net als voor PDGF, sigarettenrookextract-en LPS-geïnduceerde proliferatie

van runder tracheale gladde spiercellen gemedieerd wordt door activatie van ERK 1/2 en p38 MAP kinase. Ook ging de celproliferatie gepaard met een verhoogde expressie van cycline D1 en resulteerde langdurige behandeling van runder tracheale gladde spierpreparaten met sigarettenrookextract of LPS in een verlaagde contractiliteit van het weefsel, wat consistent is met de inductie van een proliferatief fenotype. Samengevat tonen onze bevindingen aan dat sigarettenrookextract en LPS rechtsreeks een proliferatief, hypocontractiel fenotype van luchtweg-gladde spier kunnen induceren, wat niet afhankelijk is van effecten op andere structurele of ontstekingscellen in de luchtwegen. LPS was nauwelijks detecteerbaar in het sigarettenrookextract, en in veel lagere concentraties dan nodig om proliferatie van luchtweg-gladde spiercellen te induceren. Toch werd geen extra effect van de combinatie van sigarettenrookextract en LPS op de celproliferatie waargenomen. Dit suggereert dat LPS niet bijdraagt aan de sigarettenrookextract-geïnduceerde celproliferatie, terwijl overeenkomstig eerdere waarnemingen sigarettenrookextract en LPS mogelijk wel een gemeenschappelijke signaaltransductieroute activeren.

De rol van TAK1 in de regulatie van fenotype en synthetische functie van de luchtweg-gladde spier

De studies beschreven in **Hoofdstuk 3 en 4** zijn de eerste die een functionele rol voor TGF-β-geactiveerd kinase 1 (TAK1) in luchtweg-gladde spier aantonen. TAK1 werd eerder geïdentificeerd als een belangrijke component van Toll-like receptor, IL-1 receptor en TNF-α receptor signaaltransductieroutes. Ook speelt TAK1 een belangrijke rol in de embryonale ontwikkeling, door de betrokkenheid van dit kinase bij de TGF-β/BMP signaaltransductie. In verschillende celtypes is aangetoond dat TAK1 proliferatie reguleert. Bovendien werd aangetoond dat TAK1 betrokken is bij cardiale remodelling en de ontwikkeling van de vasculaire gladde spier.

Uit de studies in **Hoofdstuk 3** blijkt dat zowel in BTSM cellen als in humane tracheale gladde spiercellen, TAK1 de groeifactor-geïnduceerde proliferatie reguleert. De PDGF- of serum-geïnduceerde DNA-synthese en toename in celproliferatie werden sterk geremd door de specifieke TAK1 remmer, LL-Z-1640-2. Ook werd de PDGF-geïnduceerde ERK 1/2 fosforylatie sterk verlaagd door LL-Z-1640-2 of door de expressie van een dominant-negative TAK1 mutant (TAK1 DN). Daarnaast werd ook de PDGF-geïnduceerde hypocontractiliteit en afname van contractiele eiwitexpressie geremd door LL-Z-1640-2. Deze bevindingen identificeren een nieuwe regulerende rol voor TAK1 bij PDGF-geïnduceerde ERK 1/2- activatie en geven aan dat TAK1 hiermee een belangrijke

rol speelt in de groeifactor-geïnduceerde fenotypische modulatie van de luchtweg-gladde spier.

Naast hun rol in de regulatie van de luchtwegdiameter, hebben luchtweg-gladde spiercellen ook een synthetische functie. Zij zijn een bron van pro-inflammatoire cytokines, pro-fibrotische groeifactoren en extracellulaire matrix eiwitten, en kunnen daardoor bijdragen aan luchtwegontsteking en remodelling bij COPD. Eerder werd al aangetoond dat sigarettenrookextract de productie van het chemokine IL-8 door humane luchtweg-gladde spiercellen induceert. Hoewel de moleculaire mechanismen die hieraan ten grondslag liggen nog niet volledig opgehelderd zijn, suggereert recent onderzoek dat activatie van NF-kB en ERK 1/2 hierbij een rol speelt. Aangezien TAK1 NF-kB en ERK 1/2 kan activeren, zou het betrokken kunnen zijn bij de sigarettenrookextract-geïnduceerde IL-8 productie.

De studies beschreven in **Hoofdstuk 4** bevestigen dat sigarettenrookextract IL-8-productie door humane luchtweg-gladde spiercellen induceert. De bijdrage van NF-kB en ERK 1/2 aan dit proces wordt ondersteund door de sterke remming van de sigarettenrookextract-geïnduceerde IL-8-productie door farmacologische remmers van NF-kB- en ERK 1/2-activatie. Door expressie van de TAK1 DN mutant of door voorbehandeling met LL-Z-1640-2 kon zowel de IκBα afbraak als de ERK 1/2 fosforylering door sigarettenrookextract-worden geremd. LL-Z-1640-2 remde tevens de sigarettenrookextract-geïnduceerde IL-8-productie. Deze resultaten tonen aan dat TAK1 een belangrijke rol speelt bij de sigarettenrookextract-geïnduceerde NF-kB- en ERK 1/2 activatie in humane luchtweg-gladde spier cellen, hetgeen tot een verhoogde IL-8-productie door deze cellen leidt.

Samengevat duiden de bevindingen uit **Hoofdstuk 3 en 4** op een belangrijke rol voor TAK1 bij de proliferatieve en ontstekingsbevorderende signaaltransductie in luchtweg-gladde spiercellen en geven daarmee aan dat TAK1 een potentieel nieuw doelwit is voor de behandeling van ontsteking en luchtwegremodelling bij obstructieve longziekten, zoals COPD.

Caviamodel voor LPS-geïnduceerde COPD

Zoals beschreven in **Hoofdstuk 1**, kan de ontwikkeling van een diermodel voor COPD op verschillende manieren worden benaderd. Voor ons onderzoek naar de pathofysiologische mechanismen en farmacologische behandeling van COPD, hebben wij een caviamodel voor COPD ontwikkeld, waarbij gebruik is gemaakt van LPS als ziekte-inducerende stimulus. LPS is een relevante stimulus voor

zowel de ontwikkeling als de exacerbaties van COPD. Chronische toediening van LPS in de luchtwegen induceert een breed scala aan ontstekingsreacties en structurele veranderingen in de long die kenmerkend zijn voor COPD. Bovendien houden, net als bij COPD, de LPS-geïnduceerde ontsteking en remodelling langdurig aan, ook wanneer de longen niet meer aan LPS worden blootgesteld. De studies beschreven in **Hoofdstuk 5 en 7** tonen aan dat herhaalde intranasale instillatie van LPS (1 mg / 200 µl; tweemaal per week gedurende 12 weken) in de cavia resulteert in neutrofiele ontsteking van de longen, verhoogde IL-8-expressie in het longweefsel, verhoogde MUC5A/C-expressie door het luchtwegepitheel, luchtwegfibrose, longemfyseem en rechterventrikel hypertrofie. Dit zijn karakteristieke kenmerken van COPD.

Verhoogde cholinerge tonus is de primaire reversibele component van luchtwegobstructie bij COPD, zoals blijkt uit de effectiviteit van anticholinergica als bronchusverwijders bij deze ziekte. Recente bevindingen, onder andere uit de UPLIFT studie, geven echter aan dat het langwerkende anticholinergicum tiotropium ook gunstige effecten bij COPD-patiënten kan hebben die niet berusten op bronchusverwijding. Zo werd aangetoond dat tiotropium het aantal exacerbaties en de sterfte onder COPD-patiënten vermindert. Hoewel er op de longfunctie-afname in de gehele studiepopulatie geen significant effect werd waargenomen, werd deze afname wel vertraagd door tiotropium bij patiënten die geen andere medicatie gebruikten, bij patiënten met matig (stadium II) COPD en bij jonge patiënten met versneld longfunctieverlies. De onderliggende mechanismen van deze effecten van tiotropium zijn nog maar ten dele bekend.

Verschillende studies hebben aangetoond dat muscarinereceptorstimulatie de productie van ontstekingsbevorderende chemokines door diverse cellen in de luchtwegen, waaronder epitheel- en luchtweg-gladde spiercellen en macrofagen, bevordert. Bovendien bevordert muscarinereceptorstimulatie de productie van collageen door en de proliferatie van longfibroblasten en potentieert het de groeifactor-geïnduceerde proliferatie van luchtweg-gladde spiercellen. Deze bevindingen suggereren een rol voor acetylcholine bij ontsteking, fibrose en remodelling van de luchtweg-gladde spier. Onlangs is aangetoond dat voorbehandeling met tiotropium de sigarettenrook-geïnduceerde ontstekingsbevorderende cytokine-expressie en infiltratie van ontstekingscellen in de bronchoalveolaire lavagevloeistof (BAL) van muizen remt, hetgeen erop wijst dat endogeen acetylcholine aan de sigarettenrook-geïnduceerde ontsteking bijdraagt. Ook hebben eerdere studies in een diermodel voor chronisch astma een potentiële rol van acetylcholine bij luchtwegontsteking en –remodelling aangetoond. Voorbehandeling met tiotropium remde in dit model de door

allergeen geïnduceerde eosinofiele ontsteking van de luchtwegen, remodelling van de luchtweg-gladde spier en slijmbekercelhyperplasie.

De studies beschreven in **Hoofdstuk 5** tonen aan dat in het caviamodel voor COPD de door LPS geïnduceerde neutrofiele ontsteking in luchtwegen en longparenchym, verhoogde MUC5A/C-expressie in het luchtwegepitheel en luchtwegfibrose geremd worden door tiotropium. Deze bevindingen suggereren dat endogeen acetylcholine een belangrijke rol speelt bij de pulmonale ontsteking en luchtwegremodelling in deze ziekte. Daarnaast werd in dit model remodelling van de luchtwegmicrovasculatuur aangetoond. Het aantal gemuscullariseerde microvaten in de adventitia van de kraakbeenhoudende grote luchtwegen nam als gevolg van de LPS-blootstelling toe. Zowel de mechanismen als het gevolg van dit type remodelling zijn nog onduidelijk, maar de bevinding dat tiotropium de muscularisatie van de microvaten remt is een eerste aanwijzing dat acetylcholine ook aan vasculaire remodelling bij kan dragen. In dit model werden geen veranderingen van de dimensies van de pulmonale bloedvaten waargenomen. Herhaalde blootstelling aan LPS resulteerde eveneens in de ontwikkeling van emfyseem, waarop tiotropium geen effect bleek te hebben. Dit suggereert dat acetylcholine niet bijdraagt aan alveolaire remodelling in dit model.

Samengevat suggereren deze bevindingen dat endogeen acetylcholine, via activatie van muscarinereceptoren, een belangrijke rol speelt bij de pulmonale ontsteking en luchtwegremodelling in COPD. Dit zou een verklaring kunnen zijn voor de gunstige niet-bronchusverwijdende effecten van tiotropium bij deze ziekte.

De rol van arginase bij COPD

Stikstofmonoxide (NO), een molecuul dat sterke bronchusverwijdende en ontstekingsremmende eigenschappen heeft, wordt gesynthetiseerd uit het aminozuur L-arginine door stikstofmonoxide synthase (NOS) iso-enzymen. Onder pathofysiologische condities, zoals bijvoorbeeld bij astma, leidt verhoogde expressie en activiteit van het enzym arginase - dat L-arginine omzet in L-ornithine en ureum - tot een verminderde biologische beschikbaarheid van L-arginine voor NOS. Dit leidt niet alleen tot een verminderde NO-productie, maar ook tot een verhoogde aanmaak van de ontstekings- en contractiebevorderende NO-metaboliet peroxynitriet. In experimentele modellen voor astma werd aangetoond dat zowel de daling van de NO-concentratie als de toename van de peroxynitrietconcentratie bij kunnen dragen aan luchtweghyperreactiviteit en -ontsteking bij astma. Daarnaast werd in een recente studie aangetoond dat verhoogde arginase-activiteit eveneens een belangrijke rol kan spelen bij

luchtwegremodelling in chronisch astma. Hoewel recent een verhoogde arginase-activiteit werd aangetoond in de BAL van COPD-patiënten, is de rol van arginase in de pathofysiologie van deze ziekte nog onbekend.

De studies beschreven in **Hoofdstuk 7** waren toegespitst op het ontrafelen van de rol van arginase bij COPD. In het caviamodel voor COPD werd aangetoond dat chronische blootstelling aan LPS via de luchtwegen leidt tot een toename van de arginase-activiteit in de longen, hetgeen wijst op een verhoogde expressie van het enzym. Dit is in overeenstemming met eerdere studies in muizen, die aantoonden dat een enkele LPS-blootstelling een toename van de arginase-genexpressie induceert in de long. De LPS-geïnduceerde toename van de arginase-activiteit in onze studie resulteerde eveneens in een verhoogde L-ornithine/L-arginine ratio in het longweefsel. De verhoogde arginase-activiteit was eveneens geassocieerd met een verhoogde long IL-8-concentratie, neutrofiele ontsteking, verhoogde MUC5A/C-expressie en luchtwegfibrose.

Voorbehandeling met de arginaseremmer 2(S)-amino-6-boronohexanoic acid (ABH) door middel van inhalatie remde de LPS-geïnduceerde toename van de arginase-activiteit, wat bleek uit een daling van de L-ornithine/L-arginine ratio in het longweefsel. De LPS-geïnduceerde arginase-expressie werd niet beïnvloed door ABH, aangezien de ABH voorbehandeling *in vivo* geen effect had op de LPS-geïnduceerde arginase-activiteit *ex vivo*, gemeten in afwezigheid van de arginaseremmer. Dit is in overeenstemming met resultaten van eerdere studies, waaruit bleek dat LPS een directe stimulus is voor de arginase-expressie.

ABH voorbehandeling remde ook de door LPS geïnduceerde toename van IL-8-concentratie in de long, de neutrofiele ontsteking, de verhoogde MUC5A/C-expressie in het luchtwegepitheel en de luchtwegfibrose, wat wijst op een belangrijke bijdrage van de toegenomen arginase-activiteit aan de luchtwegontsteking en -remodelling. Een potentieel onderliggend mechanisme voor de bijdrage van arginase aan deze processen is verhoogde vorming van peroxynitriet. Dit is het gevolg van ontkoppeling van induceerbaar NO-synthase (iNOS) bij een lage beschikbaarheid aan L-arginine, waarbij gelijktijdig NO en superoxide anionen gesynthetiseerd worden. Eerder is aangetoond dat peroxynitriet IL-8-expressie in verschillende celtypen induceert; hiermee zou het kunnen bijdragen aan de neutrofiele ontsteking. Daarnaast kan peroxynitriet direct MUC5A/C-expressie induceren in luchtwegepitheelcellen. Verhoogde peroxynitrietvorming en verminderde NO-productie werden al eerder in verband gebracht met fibrotische processen. Bovendien kan het door arginase gesynthetiseerde L-ornithine worden omgezet in L-proline, een aminozuur dat

essentieel is voor de synthese van collageen, en daarmee bijdragen aan fibrose. Arginase werd eerder geïdentificeerd als een belangrijke speler bij TGF-β-geïnduceerde collageensynthese in longfibroblasten.

Naast ontsteking en remodelling in de long, werd in **Hoofdstuk 7** ook aangetoond dat LPS rechterventrikel hypertrofie induceert. Dit impliceert dat in dit model sprake is van de ontwikkeling van pulmonale arteriële hypertensie. Pulmonale arteriële hypertensie is aanwezig in een groot aantal COPD-patiënten en wordt geassocieerd met een slechtere ziekteprognose. Chronische ontsteking en hypoxie kunnen tot endotheliale dysfunctie van de pulmonale arteriën leiden, door verminderde endotheliale NOS-expressie en NO-productie alsmede een toegenomen productie van contractiele agonisten, zoals endotheline-1. Bovendien kan vasculaire remodelling, gekenmerkt door intimaproliferatie en verdikking van de vaatwand, bijdragen aan pulmonale arteriële hypertensie. De stijging van de pulmonale weerstand, als gevolg van verhoogde pulmonale vasculaire druk, resulteert in rechterventrikel hypertrofie. Verhoogde arginase-activiteit is aangetoond in endotheelcellen van patiënten met pulmonale arteriële hypertensie, en wordt geassocieerd met een verminderde biologische beschikbaarheid van L-arginine en een verminderde NO synthese. Orale L-arginine therapie en NO inhalatie leiden tot een daling van de pulmonale arteriële druk bij deze ziekte. Dit wijst erop dat de verstoorde NO-homeostase een belangrijke rol speelt bij pulmonale arteriële hypertensie. De afmetingen van de pulmonale arteriële vaatwand werden niet beïnvloed door de LPS-instillatie in onze studie, hetgeen suggereert dat toegenomen vasoconstrictie ten grondslag ligt aan de rechterventrikel hypertrofie in ons model. Onze bevindingen tonen tevens aan dat ABH voorbehandeling de LPS-geïnduceerde ontwikkeling van rechterventrikel hypertrofie remt, waarmee een belangrijke rol voor verhoogde arginase-activiteit in dit proces wordt gesuggereerd.

Samengevat hebben de studies beschreven in **Hoofdstuk 7** aangetoond dat verhoogde arginase-activiteit bijdraagt aan pulmonale ontsteking, luchtwegremodelling en rechterventrikel hypertrofie in ons caviamodel voor COPD. Dit suggereert dat arginaseremmers therapeutisch potentieel hebben voor de behandeling van deze ziekte.

Conclusies
De belangrijkste conclusies van dit proefschrift zijn:

- Sigarettenrook en LPS leiden tot een proliferatief, hypocontractiel fenotype van de luchtweg-gladde spier. Dit wordt gemedieerd door activering van ERK 1/2 en p38 MAP-kinase, en kan het gevolg zijn van een direct effect van deze stimuli op de gladde spier, zonder betrokkenheid van andere structurele of ontstekingscellen in de luchtweg (**Hoofdstuk 2**).

- Korte, pulsatiele stimulatie van cellen of weefsel met sigarettenrookextract is een geschikte methode voor het *in vitro* modelleren van sigarettenrook-blootstelling *in vivo* (**Hoofdstuk 2**).

- Sigarettenrookextract en LPS hebben een gemeenschappelijke signaaltransductieroute bij de proliferatie van luchtweg-gladde spiercellen, maar het is onwaarschijnlijk dat LPS de sigarettenrookextract-geïnduceerde effecten medieert (**Hoofdstuk 2**).

- TAK1 speelt een belangrijke rol bij de PDGF-geïnduceerde ERK 1/2-activatie in luchtweg-gladde spier en bij de groeifactor-geïnduceerde fenotypische modulatie van luchtweg-gladde spier (**Hoofdstuk 3**).

- TAK1 speelt een belangrijke rol bij de sigarettenrookextract-geïnduceerde NF-kB- en ERK 1/2-activatie in luchtweg-gladde spiercellen, alsmede in de hiermee geassocieerde IL-8-release door deze cellen (**Hoofdstuk 4**).

- TAK1 is een nieuw doelwit voor de inhibitie van luchtwegontsteking en -remodelling bij obstructieve longziekten, zoals COPD (**Hoofdstuk 3 en 4**).

- Herhaalde LPS-blootstelling in een caviamodel voor COPD resulteert in de ontwikkeling van neutrofiele ontsteking, verhoogde IL-8 productie, emfyseem, verhoogde epitheliale MUC5A/C-expressie en luchtwegfibrose in long, alsmede in rechterventrikel hypertrofie (**Hoofdstuk 5 en 7**). De inductie van deze belangrijke kenmerken van COPD geeft aan dat dit model geschikt is voor het bestuderen van pathofysiologische processen en therapeutische behandeling van deze ziekte.

- Endogeen acetylcholine draagt bij aan neutrofiele ontsteking in de long, verhoogde MUC5A/C-expressie door het luchtwegepitheel, luchtwegfibrose en remodelling van de luchtwegmicrovasculatuur in een diermodel voor COPD (**Hoofdstuk 5**). De potentiële betrokkenheid van acetylcholine bij pulmonale ontsteking en luchtwegremodelling kan ten grondslag liggen aan de niet-luchtwegverwijdende effecten van tiotropium bij COPD-patiënten.

- Verhoogde arginase-activiteit speelt een belangrijke rol bij pulmonale ontsteking, luchtwegremodelling en rechterventrikel hypertrofie in een caviamodel voor COPD. Inhalatietherapie met arginaseremmers is daarom een interessante nieuwe invalshoek voor de behandeling van deze ziekte (**Hoofdstuk 7**).

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Dit is het moment om even terug te blikken, want in zo veel tijd is ook heel veel gebeurd. Ik zou hierbij een aantal mensen willen bedanken voor hun directe of indirecte bijdrage aan dit proefschrift.

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Herman, jij was mijn primaire begeleider tijdens dit project en zonder jouw inzet was het allemaal niet mogelijk geweest. Aan jouw legendarische oog voor detail en jouw enthousiasme heb ik heel veel gehad. Onze besprekingen riepen altijd nieuwe vragen op waardoor ik steeds weer richting kreeg om verder te zoeken. Jouw kritische revisie van manuscripten heeft mijn schrijfvaardigheid aangescherpt en heeft er ook voor gezorgd dat mijn kennis van de materie snel groeide. Een nieuwe onderzoekslijn starten en een nieuw diermodel ontwikkelen is niet niks. Daarom ben ik jou zeer dankbaar voor het vertrouwen en de vrijheid die ik heb gekregen heb om aan dit project zelf invulling te geven. Dit was heel motiverend en was uiteindelijk een beslissende factor in mijn keuze om na mijn promotie in de wetenschap door te gaan. In het begin zei jij al: “wij gaan gelijk de diepte in”, en dat hebben wij zeker gedaan. Ik heb ontzettend veel van jou geleerd! Naast het werk op de afdeling hebben wij ons op congressen en borrels goed vermaakt en ook dat heb ik altijd gewaardeerd. Herman, bedankt!

Beste Hans, jij hebt significant bijgedragen aan de discussies tijdens dit project en ook heb jij de nodige puntjes op de i gezet met manuscripten. Jij bent altijd bereid om jouw encyclopedische kennis van receptorfarmacologie, die blijft verbazen, met de jongere generaties te delen. Jouw deur stond altijd open voor vragen en gaf jij vaak gevraagd en ongevraagd advies over de beste manier om dingen aan te pakken. Toen ik tweedejaars student farmacie was, heb jij als docent mij geïntroduceerd aan de wereld van farmacologie. Mede door jouw enthousiasme heb ik besloten om eerst mijn onderzoeksproject en later mijn promotieonderzoek bij de vakgroep Moleculaire Farmacologie te doen. Hans, bedankt!

Natuurlijk wil ik ook graag mijn copromotor Dr. Reinoud Gosens bedanken.
Dr. Gosens, beste Reinoud, het is eindelijk zover! Na jouw terugkomst uit Canada kwam er meer vaart in mijn project; dat had alles te maken met jouw praktische en theoretische expertise en jouw enthousiasme. Ik kon met jou altijd even snel overleggen, en binnen vijf minuten kon jij voor elke uitdaging een aanpak

bedenken. Tijdens onze discussies richtte jij je op de kansen en oplossingen waardoor ik gefocust verder kon. Onze discussies hebben wij natuurlijk ook voortgezet tijdens de vrijdagmiddagsessies. Daar ging het voornamelijk over wetenschap en niet zozeer over werk, ook al zag niet iedereen het verschil daartussen was. Op een gegeven moment waren wij als enige twee op deze sessies overgebleven; de harde kern waar ze bij De Pintelier elke vrijdag op konden rekenen. Uiteraard hebben wij onze reputatie als doorzetters ook verdiend. Tijdens de ATS congressen hebben we ook steeds tijd gemaakt om uitgebreid met onze *buddies* te discussiëren over de laatste ontwikkelingen, tot in de late uren. Wij hebben ons goed vermaakt! Jouw bijdrage aan de totstandkoming van dit proefschrift is ontzettend groot geweest. Jij hebt mij enorm veel geholpen in deze periode en zonder jouw betrokkenheid was het allemaal veel lastiger geweest. Reinoud, mijn dank is groot!

Uiteraard wil ik ook de leden van de leescommissie, Prof. dr. G.G. Brusselle
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Dr. Harm Maarsingh: Beste Haarmsingh, jij en ik hebben ook goed samengewerkt tijdens mijn onderzoek. Onze samenwerking heeft onder andere geleid tot hoofdstuk 7 van dit proefschrift. Na al deze jaren is de rol van arginase eindelijk in een model voor COPD aangetoond. Harm, bedankt voor de goede samenwerking en de leuke gesprekken. Wij hebben het wel vaker gehad over de functionele bijdrage en de activiteit van verschillende pathologische factoren; voor arginase hebben wij gelukkig een remmer, met de andere moeten wij helaas vooralsnog leren leven. Succes met de voortzetting van jouw onderzoek!

Dr. Ad Nelemans: Beste Ad, ook jou wil ik graag bedanken voor jouw bijdrage aan de ontwikkeling van mij als jonge wetenschapper. Als student heb ik heel veel aan jouw begeleiding gehad. Jouw enthousiasme en de bereidheid om nieuwe ideeën uit te proberen hebben ervoor gezorgd dat ik als student al eigen

experimenten mocht bedenken. Juist in die tijd is het mij duidelijk geworden dat het wetenschappelijk onderzoek iets voor mij was. Ook hebben wij samen een leuk project over TRP-kanalen in de luchtwegen geschreven; helaas hebben wij daarvoor geen financiering gekregen, anders had dit proefschrift er heel anders uitgezien. Ad, bedankt, en succes met jouw nieuwe baan bij het Opleidingsinstituut Levenswetenschappen!

Dr. Dedmer Schaafsma: Beste Dedmer, ook jij hebt mij enige tijd begeleid tijdens mijn masterproject. Maar waar het tussen ons vooral goed klikte waren de vrijdagmiddagsessies. Jij hebt mij kennis laten maken met deze geweldige MF traditie, die als alternatieve opvoeding voor jonge wetenschappers dient. Deze opvoeding was dan ook van grote waarde op de ATS congressen, waar wetenschap tot diep in de nacht wordt bedreven. Dit heeft ons een goede reputatie opgeleverd in bepaalde wetenschappelijke kringen. Jouw passie voor de wetenschap heeft ook bijgedragen aan mijn overtuiging dat dit de juiste afdeling voor mij was. Dedmer, bedankt!

Prof. dr. Martina Schmidt: Beste MarTina, bedankt voor de scherpe discussies, door deze interacties met jou heb ik ook veel geleerd, vooral over controle-experimenten en het belang van goed overdragen van protocollen. Ook op congressen, borrels en in de auto was het altijd heel gezellig.

Sara (Dr. Roscioni), you were a great roomy! I'm glad you survived sharing the office with me, and after all these years I'm sure that all you remember are the good times. Sorry if I drove you crazy with the music. Remember the cool stuff we did in the beginning; the dinners, Friday afternoon drinks (where is my bike?) and the movies?! I loved your tiramisu and the pasta carbonara. Yes, those were the days! We should have kept it up longer, but I guess at some stage there's just too little time to do everything. It really was fun while it lasted. I wish you lots of luck, and I'm sure you will make the best of it.

Bart Dekkers, Dr. Dekkers! Jij hebt mij met de allereerste experimenten voor dit proefschrift geholpen. Natuurlijk waren wij ook congress-roomies. Op de BCN, NVF en ATS meetings hebben wij meerdere malen een kamer (en een enkele keer een bed) gedeeld. Wij konden goed met elkaar opschieten, en jij een ik op een kamer stond garant voor een leuke tijd en veel imitaties van enkele (niet nader te noemen) bekenden. Wij hebben ons tijdens congressen altijd erg goed vermaakt; San Diego staat mij nog goed bij, met de Old Town Trolley, Balboa Park en natuurlijk de zwaardvis en de oesters. Goede tijden! Op de vrijdagmiddagsessies was je vroeger ook vaak aanwezig, de laatste tijd wat minder. Maar je kunt ook

niet alles hebben. Aangezien jij de matrix expert bent hoef ik het jou waarschijnlijk niet te vertellen, maar…..*there is no spoon*. Succes met alles!

Loes, ontzettend bedankt dat jij mijn paranimf wilt zijn. Ik heb jou tijdens jouw bachelorproject (C&O) leren kennen. Toen hebben de studenten lootjes getrokken om te bepalen wie aan mijn project zou mogen werken; jij en Marieke zijn het geworden. Maar eigenlijk heb ik het winnende lot getrokken omdat ik twee goede studentes mocht begeleiden, die bruikbare resultaten hebben behaald. Uiteindelijk ben jij na jouw masteronderzoek ook aio geworden op de afdeling. Bij jou is het ACh onderzoek in goede handen. Gelukkig ben jij ook op de borrels vaak aanwezig, want de traditie moet voortgezet worden. Succes!

Marieke Smit: Beste Marieke, in de eindfase van mijn onderzoek heb jij ook een bijdrage geleverd aan dit proefschrift, vooral op het gebied van histochemie en morfometrie. Bedankt voor jouw hulp tijdens deze drukke periode.

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Ik wil graag ook al mijn masterstudenten bedanken voor hun geweldige inzet en grote bijdrage die zij aan dit proefschrift hebben geleverd:

Dries Lesterhuis: jaargenoot, masterstudent, paranimf. Toen jij bij de afdeling kwam om bij mij jouw masteronderzoek te verrichten, had ik weinig verwachtingen. Maar jij had bewust gekozen voor deze nieuwe onderzoekslijn, en jij bleek een hele gemotiveerde en zeer capabele student te zijn. Jij werkte snel en nauwkeurig waardoor jij vrij snel mooie resultaten hebt verkregen. Jij hebt dan ook een grote bijdrage geleverd aan Hoofdstuk 2 van dit proefschrift. Wat mij nog meer vrij snel duidelijk werd, was dat wij het op persoonlijk vlak ook heel goed met elkaar konden vinden. Dat heeft natuurlijk ook geresulteerd in een heel gezellige periode op het lab, en daarbuiten. De regelmatige vrijdagmiddagsessies en de proeverijen staan mij nog goed bij. Wij hebben ons goed vermaakt! Jij hebt natuurlijk ook enorm geholpen met het inwerken van nieuwe studenten; met jouw praktische vaardigheden was jij prima in staat om ze de ins en outs van experimenteren bij te brengen. Ik denk dat voor ons beiden meer uit deze samenwerking is gekomen dan wij van tevoren hadden verwacht. Ten slotte wil

ik je ook bedanken voor het optreden als mijn paranimf. Laten wij hopen dat dit ook een leuke vrijdagmiddagsessie wordt.

Marieke van der Vegt: Beste Marieke, ook jij hebt een grote bijdrage geleverd, met name aan Hoofdstuk 4. De experimenten wilden niet altijd lukken, maar aan jouw inzet en praktische vaardigheid lag het niet. Als bachelorstudent viel jij al op als een goede kandidaat voor een masterproject. Ook buiten het lab was jij heel actief, maar dan op het gebied van sport. Door al jouw inspanningen en prestaties op het gebied van zwemmen en triathlon heb jij inmiddels de status van een topsportster gekregen. Het is enorm hard werken maar jij weet de topsport goed te combineren met je studie. Jij hebt mij ook een beetje geïnspireerd om te gaan zwemmen en weer te beginnen met hardlopen; daarvoor ben ik je ontzettend dankbaar. Vanwege jouw sportverplichtingen was jij niet zo vaak aanwezig bij de vrijdagmiddagsessies, maar die paar keer dat jij er wel bij was…was het heel gezellig. Bedankt voor al jouw inzet en alle leuke momenten in de koffiekamer. Succes met alles!

Joana Valadas: Hey Jo, how's life? You came over as a Pharmacy student from Portugal because you wanted to get some experience in performing scientific research. I am really glad, and very lucky you came to our lab. You were a very hardworking student and your efforts resulted in a significant contribution to the characterisation of our new model of COPD, as can be seen in Chapter 5. You had a great attitude and you quickly became a very valued member of the lab. We also spent a lot of time together outside the lab, you organised quite a few dinners and parties at your place and you attended our Friday afternoon meetings regularly. The period during which you were here was a very special one; the right people met at the right time and it was a lot of fun. Thank you for all your hard work and the good times. Good luck with everything!

Claudia Atmaj: Beste Claudia, jij heb heel hard gewerkt op het lab en zelfs toen er geen studiepunten meer te verdienen waren wou jij doorgaan om dat ene, laatste proefje te doen. Dat heeft uiteraard geresulteerd in een aanzienlijke bijdrage aan Hoofdstuk 4. Ook met jou was het altijd heel leuk om in de pauze even bij te kletsen. Weet je nog wat ik tegen jou zei? Je moet vaker koffie gaan drinken! Ik hoop dat je mijn advies ter harte hebt genomen. Ik wens jou heel veel succes met je studie en de periode daarna. Maak er iets leuks van!

Riham Sami: Halaw Riham, jij bent coauteur op twee hoofdstukken (2 en 3) van dit proefschrift. Dat gaat natuurlijk niet vanzelf, ook al lijkt het bij jou soms wel zo. Jij was gemotiveerd, efficiënt en onafhankelijk, en jouw resultaten waren

altijd van topkwaliteit! Altijd in voor een grap en nooit in een slechte bui. Ook met jou kon ik het dus heel goed vinden. Succes met je toekomst, wat je ook gaat doen!

Ook mijn bachelorstudenten Willeke en Sjoerd wil ik bedanken voor hun inzet en de gezelligheid.

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Prof. dr. Hoarau; Galice, it really was a great experience living in that old house with you. We lived in a terrible place, yet I only have good memories of it. Weird, isn’t it? We certainly did have fun! All the crazy adventures: the mice in your kitchen, the vomit in the toilet (as well as other stuff), the once-a-year dishwashing expedition, strange neighbours, your homemade “chocolate”, the French music, the arthouse films, the old Mazda and the moving out (nearly broke the windows on the house and almost decapitated a passer-by). Yes, those were definitely good times! I hope you and Hin are doing well in Norway. You certainly know how to have fun and I’m sure that no matter where you are, you will enjoy life to the fullest.

En nu wil ik graag familie Mitrović bedanken. Darko, ik heb jou als de allereerste persoon op de allereerste introductiedag van de studie farmacie ontmoet. Toen bleek dat wij uit hetzelfde regio afkomstig waren. Dat kon geen toeval zijn! Jouw familie heeft zo ontzettend veel voor mij gedaan; ik had geen kamer in Groningen en jij bood mij aan om bij jullie te komen logeren. Jullie hebben mij als volwaardig lid in jullie familie opgenomen en ik heb in Siddeburen ook een hele leuke tijd gehad. Dit zal ik nooit vergeten. Beba, Mijo, Igor en Darko, ik wil jullie hartelijk bedanken voor jullie vriendschap en gastvrijheid. Darko, succes met je opleiding tot ziekenhuisapotheker; met jouw instelling en motivatie gaat dat helemaal goed komen. Veel geluk met jouw meiden.

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Ten slotte wil ik mijn vriendin Olga bedanken:

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Tonio
Groningen, juni 2011

Curriculum Vitae

The author of this thesis was born in Zagreb, Croatia, on the 13th of April 1981. After finishing his pre-university education (International Baccalaureate, Rotterdam International Secondary School) in 2000, he studied Pharmacy at the University of Groningen, obtaining a BSc degree in 2003 and MSc (PharmD) in 2006. His Master's thesis on the effects of endocannabinoids on intracellular $[Ca^{2+}]$ homeostasis in bronchial epithelial cells and the effects of growth factors on airway smooth muscle contractility was completed at the Department of Molecular Pharmacology, University of Groningen. During his studies he was a student assistant in the Pharmacology practical course and a member of the Education Committee for Pharmacy at the University of Groningen (2004-2005). In addition, he was a member of the Quality Assurance Netherlands Universities (QANU) Review Committee for Pharmaceutical Sciences (2005-2006). After graduation, he initiated his PhD-study at the Department of Molecular Pharmacology, on a research project entitled: "Development and properties of a new animal model of COPD", the results of which are presented in this thesis.

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