Inflammation and remodelling in experimental models of COPD - Mechanisms and therapeutic perspectives

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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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