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Muscarinic receptors in airway smooth muscle

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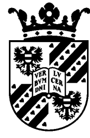
Muscarinic receptors in airway smooth muscle

Roles in inflammation and remodelling



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Muscarinic receptors in airway smooth muscle
Roles in inflammation and remodelling

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1 General introduction

1.1 Chronic airway diseases - asthma and COPD

Chronic airway diseases, including asthma and chronic obstructive pulmonary disease (COPD) are a major global health problem. The global incidence of asthma and COPD is rising, and predicted to reach epidemic proportions in 2020 for COPD (1). Asthma is one of the most common chronic diseases, which affects approximately 300 million individuals worldwide, especially children. According to the Global Initiative for Asthma (2), asthma is defined as "*a chronic inflammatory disorder of the airways in which many cells and cellular elements play a role. The chronic inflammation causes an associated increase in airway hyperresponsiveness that leads to recurrent episodes of wheezing, breathlessness, chest tightness, and coughing, particularly at night or in the early morning. These episodes are usually associated with widespread but variable airflow obstruction that is often reversible either spontaneously or with treatment*" (2). From the definition, it is clear that airway inflammation and obstruction play a substantial role in the pathophysiology and pathogenesis of asthma. An early onset and genetic inheritance, affecting individuals in early childhood or adolescence are key features of asthma (3). Although current drug therapy can adequately control the disorder in most patients, a subgroup of patients with difficult-to-treat severe asthma exists, characterized by chronic symptoms, underscoring the need for the development of novel drug therapy (4, 5).
COPD is commonly caused by long-term exposure to toxic gases and particles, in particular by cigarette smoking (6). According to the Global Initiative for Chronic Obstructive Lung Disease (GOLD) guidelines (7), COPD is defined as "*a common preventable and treatable disease, characterised by persistent airflow limitation that is usually progressive and associated with an enhanced chronic inflammatory response in the airways and the lung to noxious particles or gases. Exacerbations and comorbidities contribute to the overall severity in individual patients*" (7). In contrast to most patients with asthma, the airflow limitation in COPD is progressive and not fully reversible (8).

1.1.1 Pathogenesis and pathophysiology of asthma and COPD

1.1.1.1 Asthma

Asthma is characterised by a chronic Th2-type inflammation of the airways, associated with widespread and variable airway obstruction. Most patients are allergic and develop IgE-mediated reactions to inhalational allergens,

characterised by early and late asthmatic reactions. The early asthmatic reaction is induced by IgE-mediated mast cell activation, causing the release of various bronchoconstricting and pro-inflammatory mediators, including histamine, leukotrienes and cytokines. This results in airway smooth muscle contraction, increased vascular permeability and mucus secretion, all contributing to the acute airway obstruction (8). The late asthmatic reaction is caused by chemokine and cytokine-induced recruitment and activation of inflammatory cells, particularly eosinophils. In addition to the release of contractile and pro-inflammatory mediators by these cells, eosinophil-derived cationic proteins like major basic protein are involved in the development of acute and reversible airway hyperresponsiveness to non-allergic stimuli (9). In addition, chronic airway hyperresponsiveness as well as irreversible decline in lung function can be induced by airway remodelling due to recurrent inflammatory reactions in the airways, which may involve mediators, cytokines and growth factors released from both inflammatory and structural airway cells (8-10).

Structural changes observed in asthmatics airways are epithelial damage, thickening of the basement membrane, subepithelial fibrosis, goblet cell hyperplasia, submucosal gland enlargement, increased airway smooth muscle mass, decreased cartilage integrity and increased airway vascularity (11, 12). A particularly important feature of airway remodelling is the increased airway smooth muscle mass, as in addition to contraction, airway smooth muscle cells can participate in inflammatory and remodelling processes by the release of specific cytokines, extracellular matrix proteins and growth factors, thereby communicating with structural and inflammatory cells in the airways in an autocrine and paracrine fashion (13-16). This will be further discussed below.

1.1.1.2 COPD

COPD is characterised by pulmonary inflammation associated with progressive loss of lung function. COPD includes both emphysema and chronic bronchitis and these conditions often coexist (17). Chronic inflammation in COPD patients can trigger structural alterations and narrowing of particularly the small airways, as well as emphysema, characterised by parenchymal and alveolar destruction. The loss of lung function may result from both airway remodelling, characterised by peribronchiolar fibrosis, increased airway smooth muscle mass and mucus cell hyperplasia, as well as loss of elastic recoil by parenchymal damage (18, 19). In addition to long-term exposure to tobacco smoke and other environmental

factors like chemicals and dusts, genetic predisposition seems to be an important risk factor for the development of COPD as only 15 to 20% of the smokers develop COPD (20). Accordingly, genome-wide association studies found several loci which have been associated with COPD susceptibility (21-26). COPD has a slow and late onset, is progressive in nature, and primarily affects the middle-aged and the elderly with a smoking history. The highest prevalence is in patients over 65 years of age (27).

Neutrophils, macrophages and $CD8^+$ T lymphocytes are predominantly recruited to the airways of COPD patients, which is largely driven by cytokines and mediators such as interleukin (IL)-1β, IL-8, tumour necrosis factor-α (TNF-α), leukotriene B_4 (LTB_4), interferon-γ and matrix metalloproteinases (28). The inflammatory response in COPD occurs predominantly in the small airways and the lung parenchyma (28), and is involved in the structural changes mentioned above.

1.1.2 Current drug therapy

Inhaled β_2-adrenoceptor agonists and glucocorticosteroids are the mainstay therapy for asthma (2). Short-acting β_2-agonists, like salbutamol and terbutaline, are used as reliever therapy for episodes of dyspnoea, whereas inhaled glucocorticosteroids, with or without long-acting β_2-agonists such as formoterol and salmeterol, are used as controller therapy for persistent asthma. According to the current guidelines, short-acting anticholinergics, like ipratropium and oxitropium, may be used as alternative bronchodilators to relieve exacerbations in uncontrolled severe asthma.

Since cholinergic tone appears to be the major reversible component of airways obstruction in COPD, short-acting ipratropium and oxitropium as well as long-acting tiotropium are mainstay bronchodilator therapies in this disease, together with short- and long-acting β_2-agonists (7). In contrast to patients with asthma, most patients with COPD are relatively resistant to anti-inflammatory therapy with inhaled glucocorticosteroids. Therefore, effective treatment of patients with COPD is a major unmet need.

The guidelines for the treatment of asthma and COPD (2, 7) advocate anticholinergics for their bronchodilator properties. Interestingly, recent findings also suggest non-bronchodilator actions of these drugs, which could involve anti-inflammatory and anti-remodelling properties (29). Moreover, experimental models showed that muscarinic receptors could be involved in inflammation and

remodelling (30-38). In this thesis, potential novel mechanisms underlying muscarinic receptor-mediated airway inflammation and remodelling will be described, with particular focus on the role of the airway smooth muscle cells in these processes.

1.2 Airway smooth muscle and its role in disease

The primary role of airway smooth muscle is constriction of the airways, regulating bronchial tone. In asthma and to a lesser extent in COPD, increased airway smooth muscle mass is a major contributor to chronic airway narrowing (39). In addition, recent studies demonstrate that airway smooth muscle cells can communicate with their environment to modulate local airway inflammation and remodelling.

1.2.1 Airway smooth muscle remodelling

Airway smooth muscle remodelling occurs in both asthma and COPD. In asthma, airway smooth muscle mass may be increased in both large and small airways, whereas smooth muscle thickening of the small airways is predominantly observed in COPD patients (40-42). A correlation between smooth muscle mass and the severity of the disease has been found both in asthma and in COPD (43, 44). Both hyperplasia and hypertrophy of smooth muscle cells occur in the airway smooth muscle bundle in asthma, and possibly in COPD (43, 45-48). The relative contribution of these processes to airway smooth muscle thickening in asthma is still under debate (43, 46, 47) and could be dependent on the disease phenotype (48, 49). Furthermore in asthma an increase in extracellular matrix deposition is observed within and surrounding the smooth muscle bundles (45, 50, 51), which results in airway wall thickening, but also in increased stiffness of the tissue, limiting the ability of airway smooth muscle to induce airway narrowing as detailed below (52). The mechanisms underlying the increase in airway smooth muscle mass in asthma and COPD have not completely been identified. Secreted growth factors and cytokines from both the airway epithelium and infiltrated inflammatory cells may contribute to the increased airway smooth muscle mass, as well as the thickening of the airway smooth muscle towards the epithelium (53, 54). Furthermore, airway smooth muscle cells produce pro-remodelling, but also pro-inflammatory and pro-angiogenic mediators, such as eotaxin, IL-8, vascular

endothelial growth factor (VEGF), connective tissue growth factor (CTGF) and extracellular matrix proteins (55-57), which may be enhanced in smooth muscle of asthma an COPD patients (28, 45). Mechanical stimuli can also stimulate the expression of cytokines as well as of contractile proteins in airway smooth muscle cells (58). Furthermore, exposure to cigarette smoke drives airway smooth muscle cells to proliferate (59). The mechanisms involved and their pathophysiological implications will be discussed in detail below.

1.2.2 Communication with inflammatory cells

Although airway inflammation is associated with cellular and structural changes in the airways (40, 60), there is as yet no consensus about the causal role of inflammation in airway remodelling. It has been proposed that chronic inflammation drives the airway remodelling in asthmatic patients, as mediators including TGF-β, IL-1β and IL-6, produced by both inflammatory and structural cells, can induce airway remodelling (14, 61). In addition, the airway smooth muscle may directly facilitate the inflammatory processes in asthma and COPD. For example in asthma, the expression of the chemokine eotaxin by airway smooth muscle cells allows eosinophils to be attracted to the airways, leading to local release of cytotoxic mediators, pro-inflammatory cytokines, and TGF-β (62). Airway smooth muscle cells also express several receptors and mediators, including cellular adhesion molecules (CAMs), cytokine receptors, Toll-like receptors, chemokines, proteases, and growth factors, facilitating inflammatory and remodelling processes within the airways (63-65). For instance, both autocrine and paracrine secretion of IL-1β, LTB4, IL-6, IL-8, eotaxin and RANTES by airway smooth muscle cells has been reported, enhancing inflammation (Figure 1.1, (66)). Inflammatory cells can also release numerous mediators, including IL-6, IL-8, and TGF-β, allowing activation of the airway smooth muscle cells (45, 53, 67), and contributing to airway smooth muscle remodelling (Figure 1.1). Overall, this underlines that anti-inflammatory treatment could be a potential strategy to treat airway remodelling in chronic disease.

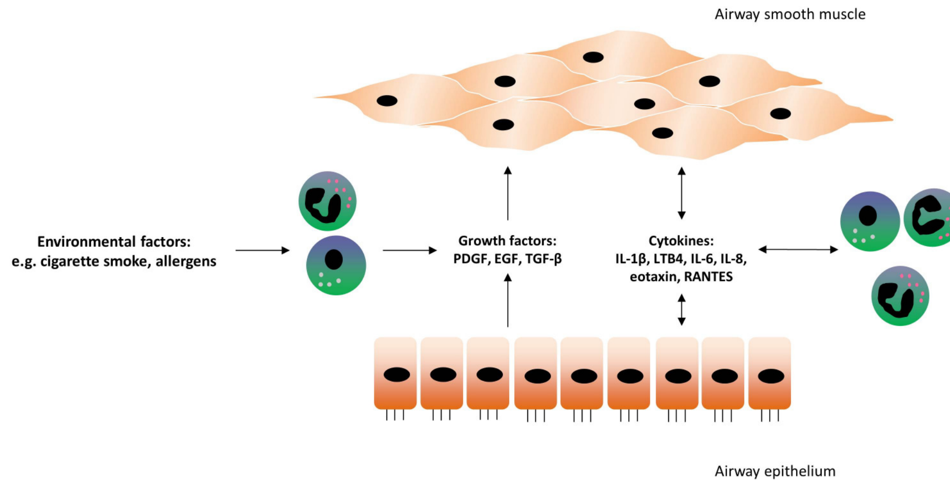

Figure 1.1. Bidirectional cellular communication in the airway wall. Growth factors can be released by epithelial cells or inflammatory cells in response to environmental factors, inducing airway smooth muscle cells to proliferate and release specific cytokines to communicate with other structural or inflammatory cells. Epithelial cells can be triggered by proteolytic allergens, environmental pollutants or Th2 cytokines. See text for further details. EGF: epidermal growth factor; PDGF: platelet-derived growth factor; TGF-β: transforming growth factor-β; LTB_4: leukotriene B_4; IL: interleukin.

1.2.3 Communication with structural cells

Bidirectional communication between the airway smooth muscle and other components of the airway wall, including different structural and inflammatory cell types and the extracellular matrix, is involved in various physiological and pathophysiological effects of the airways (16). The airway epithelium is the interface between the external environment and the airways (68). Injured or stressed airway epithelium by environmental factors, such as allergens and tobacco smoke, increases the release of fibroproliferative and fibrogenic growth factors. Indeed, in asthmatic airway epithelial cells, the release of numerous pro-inflammatory cytokines (e.g. IL-6 and IL-8) and mediators (e.g. LTB_4) as well as pro-remodelling factors, including growth factors like platelet-derived growth factor (PDGF), epidermal growth factor (EGF), TGF-β and VEGF is enhanced compared to normal epithelium (68-70) (Figure 1.1). Similar responses of the airway epithelium are observed in COPD patients. The airway epithelium is also altered in COPD patients, particularly due to the exposure to cigarette smoke, which stimulates protection and repair processes by releasing cytokines and mediators, including

TGF-β (45, 71, 72). Overall, the release of these cytokines and growth factors will affect the airway smooth muscle cells by promoting inflammatory responses, but also remodelling responses, including deposition of extracellular matrix proteins and airway smooth muscle growth. For example, the secretion of IL-8, IL-6 and MCP-1 by damaged airway epithelium promotes smooth muscle cell proliferation (73). The secretion of several cytokines including IL-1β, IL-6 and IL-8 by airway smooth muscle cells can in turn affect the epithelial layer, but also attract inflammatory cells (45, 63). Further, mechanical stimulation of bronchial epithelial cells by constricted airways can induce the release of TGF-β, endothelin-1, early growth response-1 and the extracellular matrix protein fibronectin (74-76).

1.2.4 Extracellular matrix and integrins

Extracellular matrix, the non-cellular component within tissues, provides airway structure and function. The amount and composition of extracellular matrix are altered in patients with asthma and COPD compared with healthy subjects (16, 77). In asthma, the basement membrane is thickened and there is deposition of extracellular matrix beneath the basement membrane as well as within and surrounding the airway smooth muscle bundles in both large and small airways. In COPD, the thickness of the subepithelial basement membrane is usually unchanged; however peribronchial fibrosis occurs in particularly the small airways (78). Increased deposition of collagen I, III, and V, fibronectin, tenascin, hyaluronan, versican, biglycan, lumican and laminin α2/β2-chains, and decreased deposition of collagen IV, decorin and elastin were observed in the airway wall of asthmatics compared to healthy subjects (79-81). Also in COPD patients, increased expression of collagen I, III and IV, fibronectin and laminin, and decreased expression of decorin compared to healthy subjects has been reported (82). In asthma, deposition of extracellular matrix is correlated with the severity of the disease, but not with age or duration of the disease (83). The expression of the extracellular matrix protein elastin within the bronchial wall is inversely related to airway hyperresponsiveness in asthmatic patients (51).

In addition to their structural role, extracellular matrix proteins have been reported to be involved in the regulation of various cellular processes, including migration, proliferation, attachment, cytokine release and contraction (79, 80, 84-89). Additionally, extracellular matrix proteins play a role in the maturation of airway smooth muscle cells (90). Airway smooth muscle cells grown on fibronectin and collagen I have reduced expression of contractile proteins such as sm-α-actin,

calponin and sm-MHC, which is associated with modulation of the airway smooth muscle cell phenotype towards a proliferative state (80, 87). Indeed, collagen I and fibronectin enhanced growth factor-induced airway smooth muscle proliferation (90), which requires interaction with α2β1, α4β1 and α5β1 integrins through the arginine-glycine-aspartic acid (RGD) sequence within these extracellular matrix proteins (85, 91). Integrins are major cell-surface receptors for extracellular matrix proteins, including collagens, fibronectin and laminins (92). These heterodimeric transmembrane glycoproteins interact with specific amino acid sequences within the extracellular matrix proteins, including the RGD sequence. The human repertoire of the integrin superfamily is constituted of 24 α/β heterodimers (92), of which the α5β1 integrin is a major regulator of airway smooth muscle cell function (79, 85, 91, 93-95). Accumulation of extracellular matrix within the airway smooth muscle bundles could have both protective and detrimental effects. Thus, it may protect against excessive bronchoconstriction due to the increased stiffness leading to greater internal resistance against shortening (96, 97). On the other hand, *in vivo* studies also support detrimental effects of extracellular matrix proteins in airway smooth muscle remodelling. In a guinea pig model of chronic asthma, treatment with an integrin blocking peptide containing the RGD binding motif inhibited allergen-induced airway smooth muscle remodelling, including airway smooth muscle hyperplasia and contractility (91), suggesting a potentially beneficial role for RGD-specific inhibitors in the treatment of airway remodelling.

1.2.5 Airway smooth muscle phenotype switching

Phenotype switching of airway smooth muscle cells contributes to the increased airway smooth muscle mass observed in asthmatics and COPD patients. Phenotype switching is defined as a dynamic process whereby differentiated airway smooth muscle cells can reversibly change their phenotype to a proliferative/synthetic state (modulation) or a contractile state (maturation), depending on their environment (98, 99). In presence of specific extracellular matrix proteins, including fibronectin and collagen I, or growth factors, including PDGF, airway smooth muscle cells can undergo modulation from a contractile state into a proliferative/synthetic state, among others characterised by the synthesis of organelles for protein and lipid synthesis and mitochondria (100). This process is reversible, as airway smooth muscle cells can undergo re-maturation by re-expression of specific smooth muscle contractile protein markers in the

absence of mitogens, or by laminins and TGF-β (101, 102). Paradoxically, growth factors like insulin and TGF-β may have both mitogenic and pro-contractile properties in airway smooth muscle cells. The mechanism behind this differentiated regulation is still unknown. In this thesis, the role of TGF-β in airway smooth muscle phenotype regulation will be investigated in detail.

To distinguish between both phenotypic states of airway smooth muscle cells, unique markers have been identified. Modulation of airway smooth muscle cells to a proliferative/synthetic phenotype is associated with downregulation of sm-α-actin, calponin, sm-myosin, desmin, myosin light chain kinase and caldesmon and reduced expression of the Ca^{2+} handling and growth repressing functions of caveolae, implicating that cells in this state lose their response to contractile agonists (103-106). As indicated above, cells undergoing this modulation are characterised by abundant organelles involved in synthesis and secretion including the Golgi apparatus and mitochondria. Protein markers for the proliferative state include non-muscle myosin heavy chain, vimentin, CD44 homing cellular adhesion molecule, l-caldesmon, protein kinase C α/β and β-catenin, which are associated with decreased responsiveness to contractile agonists (104). Maturation of airway smooth muscle is associated with re-expression of sm-α-actin, calponin, SM22, sm-MHC, desmin, h-caldesmon and increased responsiveness to contractile agonists (98, 102, 104). The amount of synthetic organelles is decreased in this state, whereas caveolae expression is increased, implicating that cells can respond to contractile agonists (106, 107). Transcription and translation of these specific airway smooth muscle phenotype markers occur under regulation of specific intracellular pathways as outlined below.

1.2.6 Signalling mechanisms associated with phenotype switching of airway smooth muscle cells

Extracellular stimuli, including peptide growth factors, extracellular matrix proteins and contractile agonists acting on G protein-coupled receptors can induce modulation or maturation of airway smooth muscle cells by activating specific intracellular signalling mechanisms (80, 87, 108). The RhoA/Rho kinase, mitogen-activated protein kinase (MAPK/ERK) and phosphoinositol 3-kinase (PI3K) pathways have been proposed to be key intracellular mechanisms in maturation and modulation of airway smooth muscle cells, by regulating transcription and translation of specific smooth muscle genes (109-112).

The RhoA/Rho kinase pathway is one of the central players in smooth muscle specific gene transcription by regulating the nuclear localisation of serum response factor (SRF). Polymerisation of sm-actin in response to RhoA/Rho kinase pathway activation induces the loss of monomeric globular actin (g-actin), allowing the release of the co-activator myocardin-related transcription factor A (MAL), which associates only with sm-α-actin in its monomeric form. Binding of two SRF molecules to a CArG box element in the promoter region of smooth muscle specific genes and recruitment of the co-activators myocardin and MAL to the SRF complex facilitates promoter activation of these genes. By contrast, when SRF is bound to ternary complex factors (TCFs), including phospho-Elk-1, the *c-fos* promoter is activated to facilitate transcription of proliferative genes (113). The MAPK pathway is responsible for the phosphorylation of Elk-1, indicating an important role of this pathway in determining the fate of SRF-mediated transcription and therefore the regulation of smooth muscle plasticity (113). In this way, the presence of specific transcriptional co-activators is crucial in the regulation of transcription of either smooth muscle specific genes or proliferative genes. In contrast to the MAPK/ERK pathway, the PI3K pathway is involved in the activation of smooth muscle specific gene transcription by disruption of the interaction of SRF with the inhibitory Fox04 transcription factors, allowing the co-activator myocardin to bind to SRF (Figure 1.2) (114). Smad-dependent signalling can also enhance smooth muscle specific gene transcription after association with co-factors and transcription factors (109, 115).

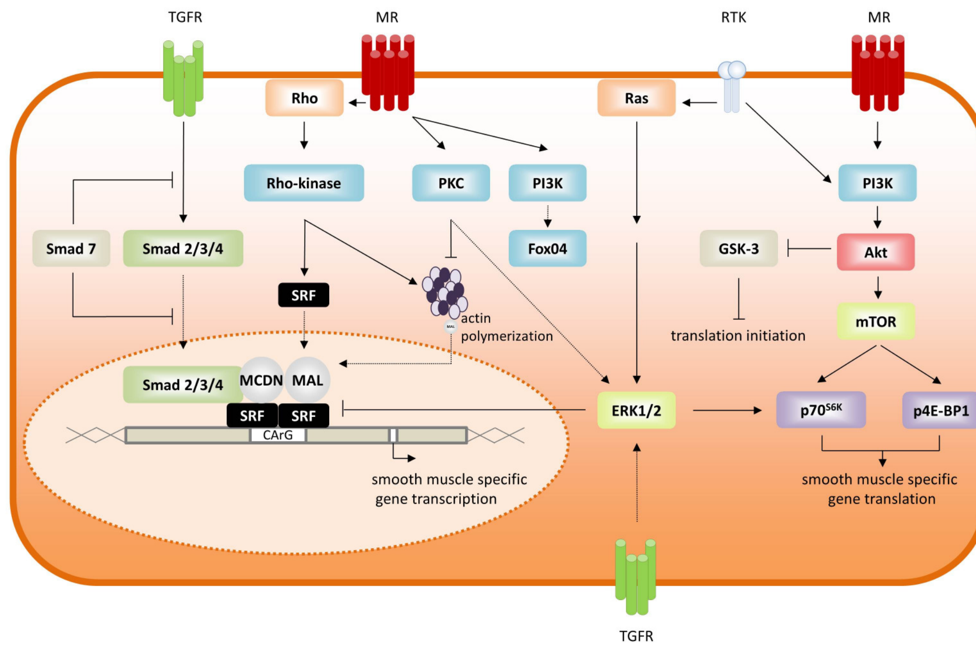

Figure 1.2. Mechanisms of airway smooth muscle maturation. Maturation of airway smooth muscle cells requires transcription and translation of contractile and contraction regulatory proteins. Activation of Smad-independent pathways through TGF-β signalling is also common. For example, RhoA can be activated followed by activation of downstream target proteins, such as Rho-kinase, to prompt rearrangement of the cytoskeletal elements associated with cell spreading, cell growth regulation, and cytokinesis. See text for further details. MR: muscarinic receptor; TGFR: TGF receptor; SRF: serum response factor; MCDN: myocardin; PKC: protein kinase C; PI3K: phosphatidyl inositide 3-kinase; GSK-3: glycogen synthase kinase-3, Akt: protein kinase B, mTOR: mammalian target of rapamycin; TCE: TGF-β control elements, 4E-BP1: eukaryotic translation initiation factor 4E-binding protein 1.

Translation of the transcripts from smooth muscle specific genes is under control of the PI3K/Akt and mTOR/p70S6K pathways. PI3K signalling induces activation of Akt1 followed by mammalian target of rapamycin (mTOR). Active mTOR phosphorylates eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) allowing the activation of the eukaryotic initiation factor eIF4. Subsequently, protein translation and contractile protein accumulation is initiated (111, 112). Activation of mTOR also allows the activation of p70S6K, responsible for ribosomal activation and the accumulation of contractile proteins (111) (Figure

1.2). Another downstream target of the PI3K/Akt signalling is glycogen synthase kinase (GSK)-3, whose repressive effects on translational processes are reversed after phosphorylation by Akt (116, 117).

1.2.7 TGF-β signalling and airway smooth muscle phenotype switching

TGF-β is an important mediator of tissue remodelling in patients with asthma and COPD. In the lung, TGF-β can be expressed by various cell types, including eosinophils, macrophages, epithelial cells, fibroblasts and airway smooth muscle cells (52). Increased levels of TGF-β have been found in asthmatics, COPD patients and tobacco smokers (67, 118-121). Both the airway epithelium and airway smooth muscle cells of patients with COPD overexpress TGF-β compared to healthy smokers (120, 122). In addition, genetic studies have reported strong associations between polymorphisms within genes of the TGF-β superfamily and COPD development (25, 123). In response to epithelial damage, excessive production of TGF-β occurs, triggering airway remodelling (124). During inflammation, neutrophil elastase and mast cell tryptase promote the release of TGF-β_1 from airway smooth muscle cells (125, 126). Plasmin regulates the release and the conversion of biologically active TGF-β from the extracellular matrix (127). The functional impact of TGF-β on airway smooth muscle cells is significant. This growth factor can induce airway smooth muscle modulation and maturation depending on its concentration, by activation of the Smad signalling followed by the regulation of SRF-dependent gene transcription or by inducing autocrine release of growth factors, including PDGF (115, 117, 128). The binding of TGF-β to the TGF-β receptor-type II allows recruitment and phosphorylation of the TGF-β type I receptor, followed by both phosphorylation and nuclear translocation of the "regulatory" Smads-2, -3 and -4, and activation of Smad-independent pathways. In turn, Smad-2, -3, and -4 can bind with the nuclear SRF to regulate smooth muscle specific gene transcription, and thereby promoting cell function including inducing a contractile phenotype (129) (Figure 1.2). As part of a negative feedback loop, Smad-6 and -7 antagonise TGF-β signalling by counteracting the induction of smooth muscle specific target genes (Figure 1.2) (115, 129).

Maturation of smooth muscle cells is promoted by Smad-dependent pathways activating TGF-β control elements (TCE) on the promoters of smooth muscle specific target genes. In this way, TGF-β augments the expression of contractile proteins in airway smooth muscle cells, including sm-α-actin and calponin (130, 131). TGF-β also activates Smad-independent pathways, including MAPK,

RhoA/Rho kinase and PI3K/Akt pathways, thereby promoting airway smooth muscle cell proliferation. For example, activation of the PI3K/Akt pathway by TGF-β can lead to airway smooth muscle proliferation by up-regulating the expression of cyclin D1 (132). Phosphorylation of p38 and ERK1/2 by TGF-β_1 also enhances airway smooth muscle cell proliferation (133). Cooperative regulation between Smad-dependent and Smad-independent pathways has been reported (134). For example, crosstalk between the MAPK pathway and Smad-pathway can enhance cell proliferation and fibrosis (135). Moreover, RhoA can be activated via Smad-dependent and -independent pathways to induce actin stress fibre formation (134).

In fibroblasts, TGF-β triggers the deposition of extracellular matrix including collagen and fibronectin by promoting the expression of extracellular matrix genes and suppressing the activity of matrix metalloproteinases genes, which degrades extracellular matrix (136, 137). Similar observations were done in airway smooth muscle cells (127, 130). Expression of the pro-fibrotic factor CTGF is also induced by TGF-β; moreover, TGF-β is enhanced in airway smooth muscle cells of asthmatics (55). The induction of the matrix proteins requires activation of the Smad signalling, especially Smad-3, but also of the MAPK pathway (138). Although TGF-β is predominantly involved in airway remodelling, TGF-β can also regulate inflammatory responses by inducing the release of cytokines and chemokines, including IL-8, IL-6 by airway smooth muscle (64). Overall, TGF-β can regulate the gene/protein expression of extracellular matrix proteins, contractile proteins, cytokines, growth factors, and enzymes in airway smooth muscle cells, suggesting a major role for TGF-β in airway remodelling and inflammation (16, 64).

1.3 Cholinergic system of the airways

Classically, acetylcholine is considered a neurotransmitter of the peripheral and the central nervous system. Activation of the neuronal cholinergic system in the airways induces airway smooth muscle contraction and mucus secretion, thereby causing airway obstruction. The activity of the parasympathetic system in asthma and COPD is increased by airway inflammation. However, more than half a century ago already, acetylcholine was reported present in bacteria, protozoa, yeast, algae, fungi, and plants (139), supporting the existence of a non-neuronal cholinergic system. In humans, the non-neuronal cholinergic system was found to

be ubiquitously present, including in the airways. A growing body of evidence now supports the notion that the non-neuronal cholinergic system also plays an important role in the pathogenesis and pathophysiology of asthma and COPD.

1.3.1 Cholinergic system

In neurons and in non-neuronal cells, acetylcholine is synthesised by a single-step reaction catalysed by the enzymes choline acetyltransferase (ChAT) or carnesylacetyltransferase (CarAT) using their two precursors choline and acetyl-CoA. The uptake of choline is the rate-limiting step in acetylcholine synthesis and is regulated by specific choline transporters, including the high affinity choline transporter (CHT)1 and choline-specific transporter-like proteins (CTL1-4). In neurons, acetylcholine is stored in and released from synaptic vesicles by the vesicular acetylcholine transporter (VAChT). Although VAChT was found to be expressed in several epithelial cell types (secretory cells, neuroendocrine cells and brush cells), airway epithelial cells also have an alternative release mechanism for acetylcholine by active transport via organic cation transporters (OCT) (140-142). Rapid hydrolysis by the enzyme acetylcholinesterase (AChE) or butyrylcholinesterase (BChE) allows the termination of the actions of acetylcholine once released by neuronal and non-neuronal cells (143). In the airways, ChAT and CarAT are expressed in airway smooth muscle cells, fibroblasts, epithelial cells and inflammatory cells, such as macrophages, mast cells, lymphocytes and granulocytes (144). However, the release of acetylcholine by the majority of these cell types has not been proven yet. Remarkably, most of these cells also express cholinergic receptors (see for overview (144-146)).

1.3.2 Muscarinic receptors subtypes in the airways

Two types of cholinergic receptors exist, the G protein-coupled muscarinic receptors and the nicotinic receptors, which are ligand-gated cation channels (146, 147). The clinically used anticholinergics for asthma and COPD are specific for muscarinic receptors; the role of nicotinic receptors in the airways will not be further discussed here (for a review see Racke et al. (148)).

Five muscarinic receptor subtypes (M_1-M_5) have been identified, which are expressed in various tissues (149). Muscarinic M_2 and M_4 receptors are both G_i-coupled receptors, inducing inhibition of adenylyl cyclase and closure of Ca^{2+}-dependent K^+-channels. Muscarinic M_1, M_3 and M_5 receptors are G_q-coupled receptors, activating phospholipase C (PLC)β and thereby inducing an increase in

the cytoplasmatic calcium (Ca^{2+}) concentration through the production of inositol 1,4,5-trisphosphate (IP_3) and subsequent mobilization of Ca^{2+} from the endoplasmatic reticulum (149). In addition, diacylglycerol (DAG) is produced, causing activation of protein kinase C (PKC).

As mentioned, muscarinic receptor expression is found in many cell types, including neurons and a variety of non-neuronal cells (144). In the airways, muscarinic M_1, M_2 and M_3 receptors are widely expressed and are involved in different functional responses, including the regulation of smooth muscle contraction, mucus secretion, cell proliferation and neurotransmitter, mediator and cytokine release (see Table 1.1 for an overview).

Table 1.1. Muscarinic receptor subtypes in the airways – localisation and function

Muscarinic receptor subtype	Localisation	Function
M_1	Parasympathetic ganglia	Facilitation of neurotransmission (150)
	Epithelial cells	facilitation of cytokine and chemotactic factor release (36, 151)
	Submucosal glands	Electrolyte and water secretion (152-154)
	Mast cells	Regulation of inflammatory mediator production, inhibition of histamine release (39, 139)
	T lymphocytes	Increased cytotoxicity (139, 155, 156)
	B lymphocytes	Proliferation (139, 156)
	Neutrophils	Chemotaxis (69)
	Eosinophils	Unidentified(69)
	Macrophages/ monocytes	Chemotactic factor release (e.g. LTB_4)
M_2	Pre- and postganglionic cholinergic nerves	Inhibition of ACh release (157-159)
	Airway smooth muscle cells	Functional antagonism of β-agonist-induced relaxation (160-164)
	Fibroblasts	Proliferation, extracellular matrix production (37, 165)
	Epithelial cells	Facilitation of cytokine and chemotactic factor release (36, 151)
	Sympathetic nerves	Inhibition of noradrenaline release (166)
	T lymphocytes	Increased cytotoxicity (139, 155, 156)
	B lymphocytes	Proliferation (139, 156)
	Neutrophils	Chemotaxis (69)
	Macrophages/ monocytes	Chemotactic factor release (139)
M_3	Airway smooth muscle cells	Contraction, proliferation, cytokine release, functional antagonism of β-agonist induced relaxation (30, 34, 139, 162, 167-171)
	Epithelial cells	Facilitation of cytokine and chemotactic factor release (36, 151)
	Submucosal glands	Mucus secretion (154) Electrolyte and water secretion (152, 153)
	Goblet cells	Mucus secretion (172)
	T lymphocytes	Increased cytotoxicity (139, 155, 156)
	B lymphocytes	Proliferation (139, 156)
	Neutrophils	Chemotaxis (69)
	Macrophages/ monocytes	Chemotactic factor release (139, 173)

1.3.3 Regulation of airway smooth muscle contraction by muscarinic receptors

Airway smooth muscle expresses both muscarinic M_2 and M_3 receptors, in a ratio of 4:1 (167). Although muscarinic M_2 receptors are predominantly expressed, the muscarinic M_3 receptor is responsible for contraction. In airway smooth muscle, activation of muscarinic M_3 receptors induces the release of Ca^{2+} from intracellular stores not only by activating PLCβ and subsequent formation of IP_3, but also by activation of the CD38/cyclic ADP-ribose pathway via opening of ryanodine receptor channels in the sarcoplasmatic reticulum (174, 175). The release of Ca^{2+} activates Ca^{2+}-calmodulin-dependent myosin light chain kinase to phosphorylate myosin light chain, followed by airway smooth muscle contraction by interaction of the myosin light chain with actin.

Muscarinic M_3 receptors also activate the Rho/RhoA pathway to facilitate airway smooth muscle contraction. Contraction is induced by inhibition of myosin light chain phosphatase (MLCP) leading to Ca^{2+}-sensitisation, which is defined as an augmented contractile response at a given Ca^{2+} concentration (176). MLCP can be directly inhibited by phosphorylation by RhoA-kinase, but also by binding with the phosphoprotein CPI-17, which can be phosphorylated by Rho-kinase and PKC (177).

1.3.4 Regulation of inflammation and remodelling by muscarinic receptors

There is recent evidence that muscarinic receptors on both structural and inflammatory cells in the airways may be involved in airway inflammation and remodelling.

Muscarinic receptor activation may contribute to structural changes in the airways by airway smooth muscle phenotype modulation, and differentiation and activation of fibroblasts. Thus, muscarinic M_3 receptor stimulation may profoundly enhance growth factor-induced airway smooth muscle proliferation (34, 178-181). Cross-talk between muscarinic M_3 receptors and receptor tyrosine kinases, like epidermal growth factor (EGF) receptors and PDGF receptors causes a synergistic increase in airway smooth muscle cell proliferation through PI3K/Akt- and mTOR/p70S6K-dependent signalling pathways (178, 180, 181). Moreover, muscarinic M_3 receptor stimulation induces PKC-dependent GSK-3 inhibition, also promoting PDGF-induced airway smooth muscle proliferation (34, 179).

As indicated above, the regulation of airway smooth muscle cell maturation is under control of the RhoA/Rho-kinase signalling pathway through the expression

of smooth-muscle specific genes (98, 102, 111, 182). Muscarinic agonists regulate the promoter activity of smooth muscle specific genes, including sm-22, sm-MHC and SRF-dependent genes, both by RhoA and PI3K signalling (110, 181, 183-184). RhoA-dependent promoter activity of sm-22 and sm-MHC is upregulated in response to inhibition of PKC (Figure 1.2) (182). Overall, this suggests an important role for muscarinic M_3 receptor activation in the maturation of airway smooth muscle cells, and thus in airway remodelling.

A clear role for muscarinic M_2 receptors on airway smooth muscle cells in airway remodelling has not yet been reported. However, in fibroblasts muscarinic M_2 receptor activation induces cell proliferation through activation of the MAPK pathway (38). Furthermore, carbachol, a muscarinic agonist, induces the production of collagen by fibroblasts (37, 165). Interestingly, collagen is also known to induce cell proliferation (80), which might suggest a role for muscarinic M_2 receptor-induced collagen production in the regulation of cell proliferation. Muscarinic receptors are also involved in the transition of fibroblasts into myofibroblasts, through an increase in ERK1/2 phosphorylation, RhoA activation, inhibition of cAMP signalling and the release of autocrine TGF-β (185).

The role of muscarinic receptors in airway remodelling and the mechanism(s) involved need further clarification, particularly in concert with TGF-β as a key player in airway remodelling.

Functional roles for acetylcholine in inflammation have also emerged. In bronchial epithelial cells, acetylcholine triggers eosinophil, monocyte and neutrophil chemotactic activity (186, 187). Both IL-8 and LTB_4 release by epithelial cells were found dependent on the ERK1/2 and NF-κB pathways (36, 69, 151). In bovine tracheal smooth muscle, upregulation of cyclooxygenase (COX)-2, COX-1, IL-8 and urokinase type plasminogen activator (PLAU) gene expression was reported in response to the muscarinic agonist carbachol (168). Muscarinic receptor agonists induce modest release of IL-6 and IL-8 by airway smooth muscle cells (Figure 1.3, (30)). Exposure to cigarette smoke extract can significantly enhance these properties (30). Nonetheless, the mechanism behind this interaction is still unknown. Several inflammatory cells are also activated by acetylcholine, for example promoting lymphocyte proliferation, chemotaxis of neutrophils and chemotactic factor release by macrophages (Figure 1.3, table 1.1 (145)). Inflammatory mediators can also trigger the upregulation of muscarinic receptor signalling. Indeed, both G_i and G_q protein expression and associated intracellular

signalling were upregulated in airway smooth muscle cells in response to TNF-α and IL-1β (160, 188). Collectively, these findings indicate that muscarinic receptors may be importantly involved in regulating inflammatory responses, suggesting an anti-inflammatory role of anticholinergics in obstructive airways diseases.

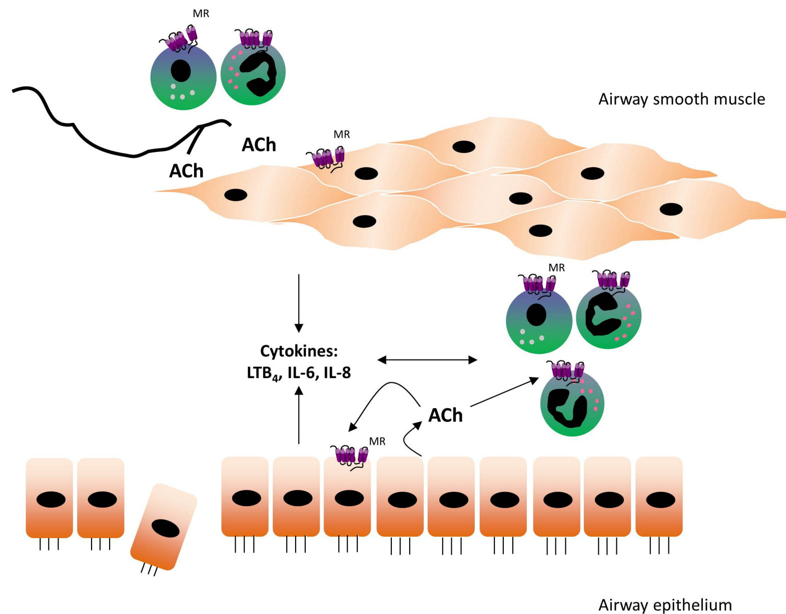

Figure 1.3. Role of neuronal and non-neuronal acetylcholine release in airway inflammation. Stimulation of muscarinic receptors on inflammatory cells by neuronal or non-neuronal acetylcholine may directly activate these cells. Moreover, muscarinic receptor activation on epithelial and airway smooth muscle cells promotes the secretion of cytokines by these cells, causing infiltation and activation of inflammatory cells. See text for further details. LTB_4: leukotriene B_4; IL: interleukin; ACh: acetylcholine; MR: muscarinic receptor.

In vivo studies support the role of muscarinic receptors in inflammation and remodelling. In a guinea pig model of chronic asthma, the allergen-induced airway smooth muscle remodelling, including increased contractile protein expression and airway smooth muscle thickening, but also mucous gland hypertrophy, increased MUC5AC-positive goblet cell numbers and eosinophilia, were inhibited by tiotropium bromide (33, 189, 190). Furthermore, in a murine model of asthma infiltration of macrophages and eosinophils and expression of IL-4, IL-5, IL-13 and TGF-β_1, all measured in the bronchoalveolar lavage fluid (BALF), as well as airway

smooth muscle thickening were significantly inhibited by tiotropium bromide (191). The allergen-induced infiltration of eosinophils in the BALF was also inhibited by the novel muscarinic receptor antagonist, aclidinium bromide (192). Also in a cigarette smoke-induced mouse model of COPD, tiotropium bromide exerted anti-inflammatory activity, not only by inhibiting total cell number and neutrophils, but also by reducing the expression of cytokines, including IL-6, KC, TNF-α, and LTB_4 in the BALF (193). A recent study using muscarinic receptor subtype deficient mice, showed a pro-inflammatory role for the muscarinic M_3 receptor in cigarette smoke-induced neutrophilia and cytokine release, and an anti-proinflammatory role for muscarinic M_1 and M_2 receptors (194).

In a guinea pig model of LPS-induced COPD, pulmonary neutrophilia and increased peribronchial collagen deposition were also inhibited by tiotropium bromide (195). Likewise, the short-acting anticholinergic ipratropium had inhibitory effects in a cadmium-induced rat model of pulmonary inflammation (196). In a diesel particle-induced rat model of acute lung injury, bilateral vagotomy, but also atropine reduced neutrophilia (197). Collectively, these studies indicate a profound role for muscarinic receptor activation in airway remodelling and inflammation in asthma and COPD.

1.4 Aims of the studies

Besides its role as a major regulator of airway constriction and mucus secretion, acetylcholine can also regulate remodelling and inflammation of the airways. The mechanisms involved have only partially been identified. The primary aim of this thesis is **to investigate the functional interactions of the cholinergic system with inflammatory and remodelling processes in the airways**. To this aim, *in vitro* studies were designed to assess the effects of muscarinic receptor stimulation alone or in concerted action with cigarette smoke extract or TGF-β_1 on key features of inflammation and remodelling, using human airway smooth muscle cells and precision-cut lung slices.

Airway smooth muscle cells are capable of expressing and releasing cytokines and growth factors, which suggests an important role of this cell type in pro-inflammatory responses (45, 63). In particular, cigarette smoke (extract) can provoke inflammatory responses, including IL-8 secretion, in human airway smooth muscle cells, which can be enhanced by G_q-coupled muscarinic M_3

receptors (30, 45, 145). The mechanism(s) by which muscarinic receptors regulate these pro-inflammatory responses are still unknown. In **chapter 2**, we investigated the intracellular signalling mechanisms involved in the release of IL-8 in response to muscarinic receptor stimulation and cigarette smoke extract in human airway smooth muscle cells. In this study, the role of PKC and its downstream effectors NF-κB and ERK1/2 was investigated.

In addition to its role in pro-inflammatory responses, muscarinic receptor stimulation has been shown to be involved in airway smooth muscle remodelling, including increased contractile protein expression, cell proliferation and extracellular matrix deposition (145, 198). The pro-fibrotic cytokine TGF-β has also been reported to be involved in these processes; however, the functional relationship between muscarinic receptors and TGF-β_1 has thus far not been described. **Chapter 3** and **4** address the potential interaction between muscarinic receptor stimulation and TGF-β_1 in processes involved in airway remodelling. **Chapter 3** discusses the effect of muscarinic receptor stimulation on TGF-β_1-induced contractile protein expression in human airway smooth muscle cells and elucidates the intracellular signalling mechanisms involved. In **chapter 4**, the cooperative regulation of airway smooth muscle cell proliferation by muscarinic receptors and TGF-β_1 through autocrine regulation of extracellular matrix proteins is described. The roles of integrins and muscarinic receptor subtypes in this process were investigated by using integrin-blocking peptides and specific antagonists (**chapter 4**).

During bronchoconstriction, mechanical forces are induced in the airways, which can trigger features of airway remodelling (74, 199, 200). The functional importance of muscarinic agonist-induced bronchoconstriction on airway remodelling was studied in **chapter 5**. This study describes mechanisms involved in the induction of airway remodelling by bronchoconstriction, using precision-cut lung slices as an *in vitro* model. Since a potential role for TGF-β in the induction of airway remodelling by bronchoconstriction was recently indicated by a clinical study in mild asthma patients (201), the involvement of endogenous TGF-β release by bronchoconstriction was studied using selective inhibitors.

Finally, **chapter 6** reviews the role of the cholinergic system in remodelling and inflammatory processes within the airways.

This thesis provides new insights into the regulatory role of muscarinic receptors in airway smooth muscle function.

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2 Pro-inflammatory mechanisms of muscarinic receptor stimulation in airway smooth muscle

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Abstract

Background: Acetylcholine, the primary parasympathetic neurotransmitter in the airways, plays an important role in bronchoconstriction and mucus production. Recently, it has been shown that acetylcholine, by acting on muscarinic receptors, is also involved in airway inflammation and remodelling. The mechanism(s) by which muscarinic receptors regulate inflammatory responses are, however, still unknown.

Methods: The present study was aimed at characterizing the effect of muscarinic receptor stimulation on cytokine secretion by human airway smooth muscle cells (hASMc) and to dissect the intracellular signalling mechanisms involved. hASMc expressing functional muscarinic M_2 and M_3 receptors were stimulated with the muscarinic receptor agonist methacholine, alone, and in combination with cigarette smoke extract (CSE), TNF-α, PDGF-AB or IL-1β.

Results: Muscarinic receptor stimulation induced modest IL-8 secretion by itself, yet augmented IL-8 secretion in combination with CSE, TNF-α or PDGF-AB, but not with IL-1β. Pretreatment with GF109203X, a protein kinase C (PKC) inhibitor, completely normalized the effect of methacholine on CSE-induced IL-8 secretion, whereas PMA, a PKC activator, mimicked the effects of methacholine, inducing IL-8 secretion and augmenting the effects of CSE. Similar inhibition was observed using inhibitors of IκB-kinase-2 (SC514) and MEK1/2 (U0126), both downstream effectors of PKC. Accordingly, western blot analysis revealed that methacholine augmented the degradation of IκBα and the phosphorylation of ERK1/2 in combination with CSE, but not with IL-1β in hASMc.

Conclusions: We conclude that muscarinic receptors facilitate CSE-induced IL-8 secretion by hASMc via PKC dependent activation of IκBα and ERK1/2. This mechanism could be of importance for COPD patients using anticholinergics.

2.1 Introduction

Chronic obstructive pulmonary disease (COPD) is an inflammatory lung disease characterized by airflow limitation that is not fully reversible (1). The pathophysiology of COPD is mainly caused by cigarette smoke. COPD is associated with an increase in local and systemic inflammatory cytokines including TNF-α and IL-1β (2). Furthermore, clinical studies reported that the levels of IL-8 (3) and

leukotriene B_4 (4) are correlated to the proportion of neutrophils present and are increased in induced sputum of COPD patients. Additionally, during exacerbations periods, IL-8 levels are increased (3). Attracted by IL-8, neutrophils play a significant role in the pathogenesis of COPD. Neutrophils promote tissue inflammation and injury by inducing the release of mediators including elastase, metalloproteases and reactive oxygen species (4).

Acetylcholine, the primary parasympathetic neurotransmitter in the airways plays an important role in COPD, by regulating bronchoconstriction and mucus production (5). Parasympathetic tone may be increased in COPD (5). Therefore, anticholinergics -including tiotropium bromide, a long-acting bronchodilator- are often used as a mainstay therapy for COPD (6). Recently, however, it has been established that activation of the cholinergic system may also contribute to inflammatory responses in the lung. For example, the release of IL-8 and leukotriene B_4 by bronchial epithelial cells (7, 8) and alveolar macrophages (9) *in vitro* appears to be induced by acetylcholine, resulting in increased neutrophil, monocyte, and eosinophil chemotactic activities, an effect that may be enhanced in COPD. Also, animal studies showed that anticholinergics are capable of reducing neutrophilic and eosinophilic inflammation induced by inhaled diesel-soot (10), inhaled allergen (11), or LPS (12). Furthermore, it has been reported that airway vascular leakage is mediated by muscarinic receptors (13). Collectively, these findings suggest a role in pro-inflammatory responses for muscarinic receptors. Nonetheless, it is still undefined what the potential anti-inflammatory effects of muscarinic antagonists are in the lungs of patients with COPD (14), which is in part due to the unknown mechanisms behind the regulation of inflammatory responses by muscarinic receptors.

Human airway smooth muscle (ASM) has been attributed an important role in pro-inflammatory responses in COPD (5). These cells are capable of expressing and releasing cytokines and growth factors, including IL-6 and IL-8 (15). Furthermore, it has been reported that ASM cells express cell surface molecules, which can directly interact with immune cells, suggesting an immunomodulatory role of these cells in COPD (16). Increased pro-inflammatory cytokine release is induced by stimulating human ASM cells (hASMc) with G-protein-coupled receptors, growth factors and extracellular matrix proteins (15, 16). Additionaly, cigarette smoke can evoke inflammatory responses in human hASMc, such as IL-8 secretion (17). Muscarinic M_2 and M_3 receptors, both G-protein-coupled receptors, are expressed in abundance in hASMc, suggesting that acetylcholine regulates

inflammatory responses by ASM (18). Indeed, we recently reported that muscarinic receptor stimulation augments cigarette smoke extract (CSE)-induced IL-8 secretion by hASMc, which was mediated by the muscarinic M_3 receptor subtype (19).

Although these observations illustrate the potential role for acetylcholine in regulating airway inflammation, the mechanism(s) by which muscarinic receptors regulate inflammatory responses are still unknown. In the present study, we investigated the regulation of cytokine secretion from hASMc by muscarinic receptors, alone and in concerted action with various pro-inflammatory stimuli involved in the pathogenesis of COPD. In addition, we investigated the intracellular signalling mechanisms involved, in particular the role of protein kinase C (PKC) and downstream pathways.

2.2 Materials and methods

2.2.1 Antibodies and reagents

Methacholine chloride (MCh) was purchased from ICN Biomedicals (Zoetermeer, the Netherlands). GF109203X and U0126 were both from Tocris Cookson Inc. (Bristol, UK). SC514 was obtained from Calbiochem (Amsterdam, The Netherlands). PMA, mouse anti-ß-actin antibody, horseradish peroxidase (HRP)-conjugated rabbit anti-mouse antibody, HRP-conjugated goat anti-rabbit, recombinant human TNF-α, and IL-1β were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). Human recombinant platelet-derived growth factor-AB (PDGF-AB) was from Bachem (Weil am Rhein, Germany). Phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204) antibody and p44/42 MAPK (ERK1/2) antibody were obtained from Cell Signalling Technology (Beverly, CA, USA). Rabbit anti-IκBα (clone-15) was purchased from Santa Cruz Biotechnology, INC (Santa Cruz CA, USA). All other chemicals were of analytical grade.

2.2.2 Cell culture

Human bronchial smooth muscle cell lines immortalized by stable expression of human telomerase reverse transcriptase (hTERT) were prepared as described previously (20). The primary cultured human bronchial smooth muscle cells used to generate these cell lines were prepared from macroscopically healthy segments of 2nd-to-4th generation main bronchus obtained after lung resection

surgery from patients with a diagnosis of adenocarcinoma. All procedures were approved by the Human Research Ethics Board of the University of Manitoba. Cells were grown to confluence using DMEM supplemented with 10% FBS, 100 µg/mL streptomycin, 100 U/mL penicillin and 1.5 µg/mL amphotericin B. Cultures were maintained in a humidified incubator at 37°C-5% CO2, and media was changed every 2-3 days.

2.2.3 Cytokine release

Cells were cultured in 24 well plates and grown until confluence followed by serum-deprivation for 1 day in DMEM supplemented with antibiotics (100 µg/mL streptomycin, 100 U/mL penicillin and 1.5 µg/mL amphotericin B) and ITS (5 µg/mL insulin, 5 µg/mL transferrin, and 5 ng/mL selenium) before each experiment. The cells were stimulated with the muscarinic receptor agonist methacholine chloride (MCh, 10 µM), alone and in combination with either CSE (5%), TNF-α (1 ng/mL), PDGF-AB (30 ng/mL) or IL-1β (1 ng/mL) for 24 hrs to determine cytokine secretion in cell-free supernatant. 100 % strength CSE was prepared by combusting two 3R4F research cigarettes (without filter) (University of Kentucky, Kentucky, USA) using a peristaltic pump and passing the smoke through 25 mL of FBS-free medium at the rate of one cigarette per 5 min. CSE was freshly prepared before every experiment and was used within 15 min after preparation. Additionally, where appropriate, hASMc were pre-incubated with either the PKC inhibitor GF109203X (3 µM), the IKK-2-inhibitor SC514 (50 µM) or the MEK inhibitor U0126 (3 µM) for 30 min. Cells were also treated with the PKC activator PMA (0.1 µM). Cytokine levels were quantified using enzyme-linked immunosorbent assays (ELISA), according to the manufacturer's instructions (Sanquin Pharmaceutical services, Amsterdam, The Netherlands). The detection limit was 1 pg/ml for IL-8 and 0.2 pg/ml for IL-6. We diluted samples were needed to remain in the range of the standard curve.

2.2.4 Preparation of whole cell lysates

HASMc were cultured in 6 well plates and grown until confluence followed by serum-deprivation for 1 day in DMEM supplemented with antibiotics (100 µg/mL streptomycin, 100 U/mL penicillin and 1.5 µg/mL amphotericin B) and ITS before each experiment. The cells were stimulated with the muscarinic receptor agonist MCh (10 µM), alone and in combination with either CSE (5 %) or IL-1β (1 ng/mL) for 60 or 120 min. To obtain whole cell lysates, cells were washed once with ice-

cold PBS (NaCl 140 mM, KCl 2.6 mM, KH_2PO_4 1.4 mM, $Na_2HPO_4.2H_2O$ 8.1 mM, pH 7.4), followed by lysis using ice-cold RIPA buffer (Tris 40 mM, NaCl 150 mM, Igepal 1%, deoxycholic acid 1%, NaF 1 mM, Na_3VO_4 1 mM, aprotinin 10 μg/mL, leupeptin 10 μg/mL, pepstatin A 7 μg/mL, β-glycerophosphate 1.08 mg/mL, pH 8.0). Sonicated lysates were assayed for protein content according to Bradford and stored at -20°C until further use.

2.2.5 Western Blotting

Equal amounts of protein were separated on 10 % polyacrylamide-SDS gels and transferred to nitrocellulose membranes. To avoid non-specific binding, membranes were blocked with blocking buffer (Tris-HCl 50 mM, NaCl 150 mM, TWEEN-20 0.1%, non-fat dried milk powder 5 %) for 1 hour at room temperature. The membranes were then incubated with specific primary antibodies, all diluted in blocking buffer, for one hour at room temperature. After washing the membranes three times with TBS-T 0.1% (Tris-HCl 50 mM, NaCl 150 mM, TWEEN-20 0.1%) for 10 min, incubation with the secondary antibody conjugated to HRP was performed during 1 h at room temperature, followed by additional three washes with TBS-T 0.1%. Bands were subsequently visualized on film using enhanced chemiluminescence reagents and analysed by densitometry (Totallab^{TM}, Nonlinear dynamics, Newcastle, UK). All bands were normalized to either β-actin for IκBα or to total ERK1/2 for phospho ERK1/2.

2.2.6 Data analysis

Data are presented as mean values ± SE. Statistical significance of differences between means was determined by a Student's *t* test or by one-way ANOVA, where appropriate. Data were considered statistically significant when $p < 0.05$.

2.3 Results

2.3.1 Muscarinic receptor stimulation facilitates cytokine secretion induced by CSE, TNF-α and PDGF-AB

Recently, it has been reported that stimulation of muscarinic receptors induces the release of IL-8 from human bronchial epithelial cells and facilitates the release of IL-8 from hASMc induced by CSE (8, 19). We evaluated the pro-inflammatory properties of muscarinic receptor stimulation in hASMc, alone and in concerted

action with CSE (5 %), PDGF-AB (30 ng/mL), TNF-α (1 ng/mL) or IL-1β (1 ng/mL) (Figure 2.1). Previous findings indicated that the effects of muscarinic receptor stimulation on ASM cytokine secretion were most profound for IL-6 and IL-8 (19), with maximal effects seen at a concentration of 10 μM MCh. Therefore, we used 10 μM MCh and focused on IL-6 and IL-8 cytokines for our measurements. We observed a minor increase in IL-8 induced by MCh alone. CSE alone induced a significant increase of both IL-8 and IL-6 secretion, which was significantly and synergistically amplified by co-stimulation with MCh. In addition, MCh induced a synergistic increase in both IL-8 and IL-6 secretion in combination with TNF-α. Furthermore, a synergistic effect was also observed with the combination of MCh and PDGF-AB for IL-8 secretion. However, the effect of IL-1β, which induced a very high IL-8 and IL-6 production by its own, was not significantly augmented by MCh (Figure 2.1). IL-8 release in response to IL-1β was found concentration dependent, but treatment with MCh had no additional effects regardless of the concentration IL-1β used (data not shown).

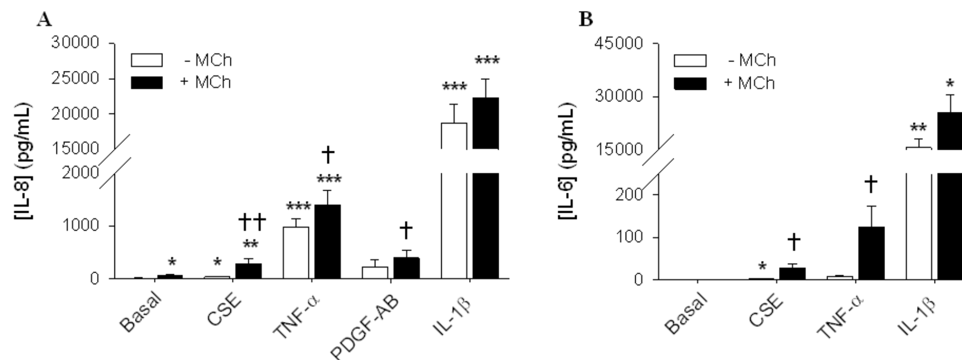

Figure 2.1. Muscarinic receptor stimulation augments cytokine secretion induced by CSE, PDGF-AB and TNF-α, but not by IL-1β. hASMc were stimulated with CSE (5%, n=22 and n=6 for IL-8 and IL-6, respectively), TNF-α (1 ng/mL, n=17 and n=5 for IL-8 and IL-6, respectively), IL-1β (1 ng/mL, n=17 and n=6 for IL-8 and IL-6, respectively) or PDGF-AB (30 ng/mL, n=6 for IL-8), in the absence or presence of MCh (10 μM) for 24 hours. Supernatants were analyzed for the presence of IL-8 (A) or IL-6 (B). Data shown are the means ± SE of n independent experiments. *p< 0.05, **p< 0.01 and ***p< 0.001 compared to basal; †p< 0.05 and ††p< 0.01 compared to the absence of MCh (Student's t-test for paired observations).

2.3.2 PKC is involved in the synergistic effect of muscarinic receptor stimulation with CSE

PKC plays an important role as a signalling intermediate in pro-inflammatory cytokine secretion by inducing the activation of several downstream pathways, including the IKK-2/IκBα/NF-κB and Raf-1/MEK/ERK1/2 pathways (21). The stimulation of muscarinic receptors induces the activation of PKC in ASM (22, 23). We hypothesized therefore, that PKC could play a central role in the synergism between CSE and MCh in IL-8 secretion. HASMc were pretreated with GF109203X (3 μM), a specific PKC inhibitor, and subsequently stimulated with MCh, CSE and their combination (Figure 2.2). GF109203X significantly inhibited the synergistic effect of MCh on CSE-induced IL-8 secretion, demonstrating a requirement for PKC in this synergism. Remarkably, in the absence of the muscarinic agonist, GF109203X tended to increase the CSE-induced IL-8 secretion.

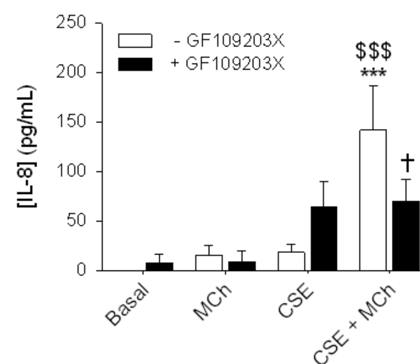

Figure 2.2. Involvement of PKC in the potentiation of CSE-induced IL-8 release by muscarinic receptor stimulation. hASMc were stimulated with CSE (5%) in the absence or presence of MCh (10 μM) and/or GF109203X (3 μM) for 24 hours. Supernatants were analyzed for the presence of IL-8. Data represent means ± SE of 7 independent experiments each performed in duplicate. ***$p < 0.001$ compared to basal; $^{\$\$\$}p < 0.001$ compared to CSE; †$p < 0.05$ compared to the absence of GF109203X (One-way ANOVA followed by Newman-Keuls multiple comparisons test).

To investigate whether PKC activation was sufficient for a synergistic IL-8 secretion in combination with CSE, we used PMA (0.1 μM) as a PKC activator. Indeed, CSE-induced IL-8 secretion was highly augmented in the presence of PMA, which could be abolished to the level of CSE-induced IL-8 secretion when pre-treated with GF109203X (Figure 2.3A). These data indicate that PKC activation is

sufficient for a synergistic interaction with CSE, which is in support of a central role for PKC in regulating the synergy between MCh and CSE. In contrast to MCh, however, PMA induced a considerable IL-8 secretion by itself, which was abolished when the cells were pre-treated with GF109203X.

PKC has been shown to induce activation of the NF-κB and ERK1/2 pathways in different cells (21). Moreover, it has been reported that the stimulation of muscarinic receptors through acetylcholine mediates the release of IL-8 in human bronchial epithelial cells by NF-κB- and ERK1/2-dependent mechanisms (8). To test the involvement of the NF-κB and ERK1/2 pathways as a result of PKC activation, hASMc were stimulated with PMA after pre-treatment with either an IKK-2 inhibitor, SC514, or a MEK1/2 inhibitor, U0126. IL-8 secretion induced by PMA was significantly decreased in presence of these pharmacological inhibitors (Figure 2.3B for SC514 and Figure 2.3C for U0126, respectively). Moreover, western blot analysis indicated that the activation of PKC by PMA induced the phosphorylation of ERK1/2 and the degradation of IκBα in hASMc. Collectively, these data indicate that PKC is able to activate the IκBα/NF-κB and MEK/ERK1/2 pathways, leading to IL-8 secretion from hASMc (Figure 2.3D).

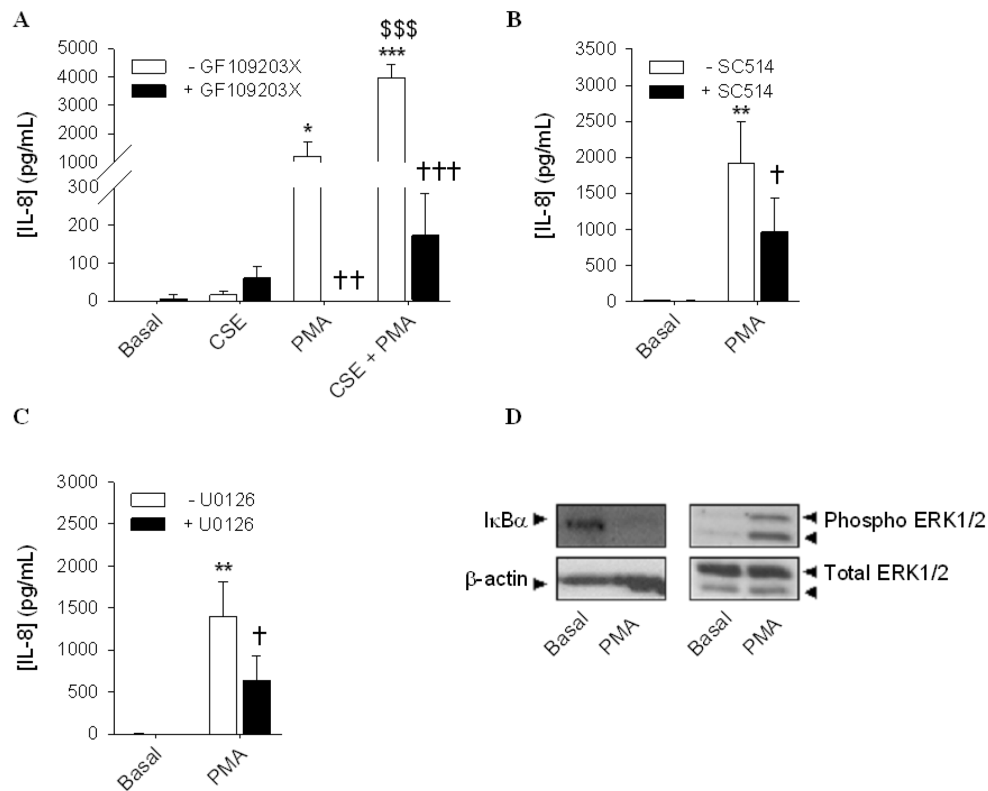

Figure 2.3. PKC activation is sufficient to facilitate CSE-induced IL-8 secretion in hASMc. hASMc were stimulated with PMA (0.1 µM), in the absence or presence of CSE (5%) and GF109203X (3 µM) (A), SC514 (50 µM) (B) or U0126 (3 µM) (C) for 24 hours. Supernatants were analyzed for the presence of IL-8. Data represent means ± SE of 4-6 independent experiments each performed in duplicate. *p < 0.05, **p< 0.01 and ***p< 0.001 compared to basal; $^{\$\$\$}$p< 0.001 compared to PMA; †p< 0.05, ††p< 0.01 and †††p < 0.001 compared to the absence of inhibitor (One-way ANOVA followed by Newman-Keuls multiple comparisons test). (D) hASMc were stimulated with PMA (0.1 µM) for 1 hour. Cell lysates were analyzed for IκBα breakdown and phosphorylation of ERK1/2 by western blot. β-actin and total ERK1/2 were used as loading controls. Western blots shown are representative of 4 experiments.

2.3.3 Involvement of the IκBα/NF-κB pathway in the synergistic effect of muscarinic receptor stimulation with CSE

HASMc were pretreated with the IKK-2 inhibitor SC514 to test the involvement of this pathway in the synergistic IL-8 secretion by MCh and CSE (Figure 2.4A). SC514 completely inhibited the MCh- and CSE-induced IL-8 secretion. Furthermore, the synergistic effect of the combination of MCh and CSE was abolished (Figure 2.4A).

These results confirm the involvement of the IκBα/NF-κB pathway in the observed IL-8 secretion. Therefore, we next investigated the effects of muscarinic receptor stimulation on IκBα degradation, alone and in combination with CSE at different time points (60 and 120 min of treatment). IκBα degradation was measured by western blot analysis. Although MCh did not induce significant IκBα degradation by itself, it augmented the response induced by CSE; particularly after 120 min of incubation (Figure 2.4D). Overall, these results indicate that muscarinic receptor stimulation promotes the activation of the IκBα/NF-κB pathway induced by CSE, which likely contributes to the synergistic IL-8 secretion. Interestingly, and in line with the lack of effect of MCh on IL-1β-induced cytokine secretion, MCh did not augment maximal IL-1β-induced IκBα degradation at t = 60 and 120 min (Figure 2.4E). However, IL-1β-induced IL-8 secretion in presence or absence of MCh, was significantly inhibited by SC514 (Figure 2.4B).

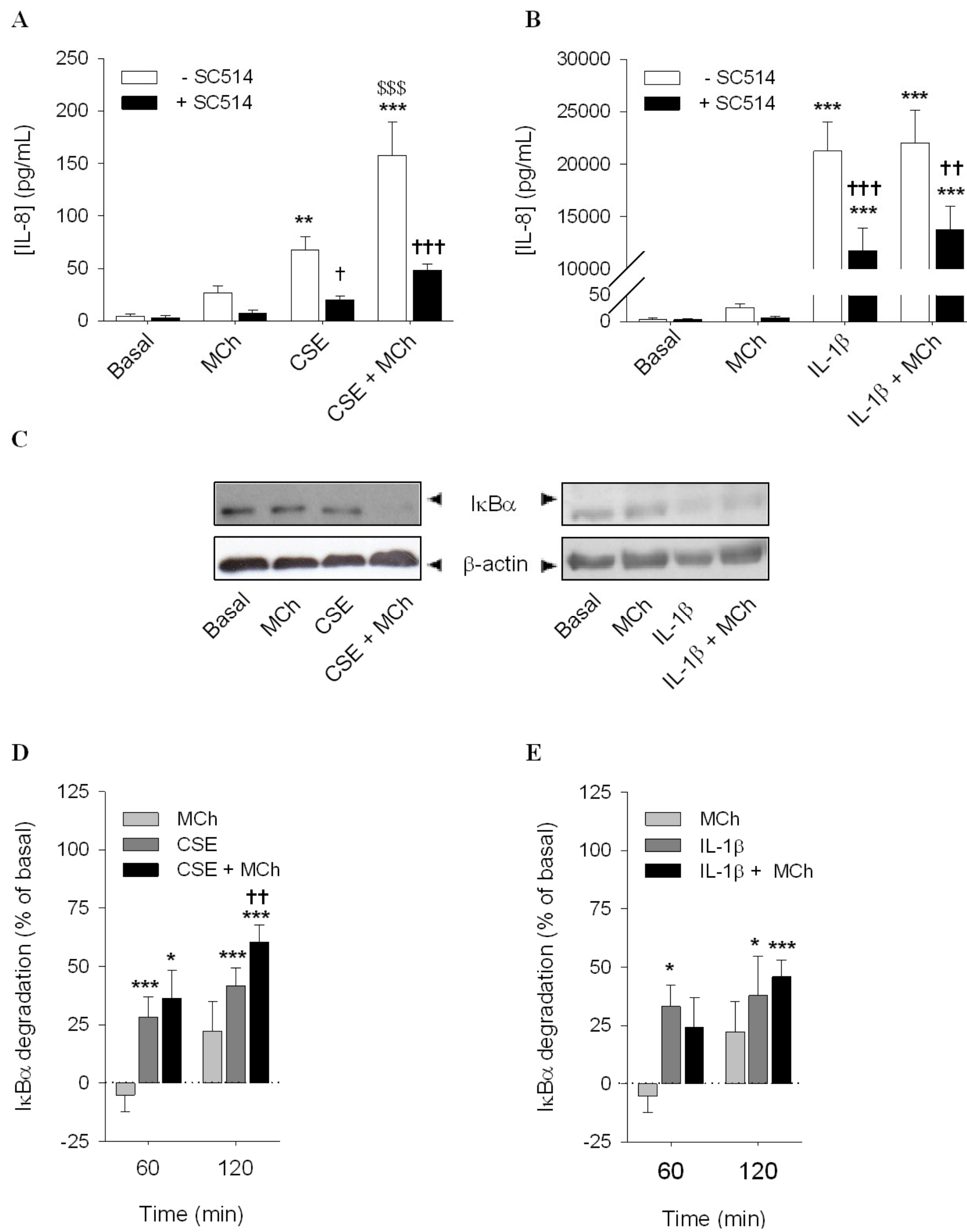

Figure 2.4. Involvement of the IκBα/NF-κB pathway in IL-8 secretion induced by CSE, IL-1β and MCh. hASMc were stimulated with CSE (5%) (A) or IL-1β (1 ng/mL) (B) in the absence or presence of MCh (10 µM) and/or SC514 (50 µM) for 24 hours. Supernatants were analyzed for the presence of IL-8. Data represent means ± SE of 5 independent experiments each performed in duplicate. **p< 0.01 and ***p< 0.001 compared to basal; †p< 0.05, ††p< 0.01 and †††p< 0.001 compared to the absence of SC514, $^{\$\$}$p< 0.01

compared to CSE (One-way ANOVA followed by Newman-Keuls multiple comparisons test). (C-E) hASMc were stimulated with CSE (5%) (C-D) or IL-1β (1 ng/mL) (C-E) in the absence or presence of MCh for 60 min and 120 min (representative blots shown in C) as indicated. IκBα degradation was determined by western blot and corrected for the expression of β-actin, which was used as a loading control. Data represent means ± SE of 9-10 experiments. *p< 0.05 and ***p< 0.001 compared to basal and ††p< 0.01 compared the absence of MCh (Student's *t*-test for paired observations).

2.3.4 Involvement of the MEK/ERK1/2 pathway in the synergistic effect of muscarinic receptor stimulation with CSE

To test the involvement of the MEK/ERK1/2 pathway in IL-8 secretion induced by MCh and CSE, we pretreated the cells with the MEK1/2 inhibitor, U0126 (3 μM) (Figure 2.5A). In the presence of U0126, IL-8 secretion induced by co-stimulation of CSE with MCh was significantly decreased (Figure 2.5A). These results confirm the involvement of the MEK/ERK1/2 pathway in the observed IL-8 secretion. Therefore, we next assessed phosphorylation of ERK1/2 induced by MCh and CSE (Figure 2.5C-D). Although, ERK1/2 phosphorylation was not significantly increased when cells were stimulated with MCh alone after one hour of incubation, 15 min incubation is sufficient to induce significant ERK1/2 phosphorylation (23). In combination with CSE, MCh induced a significant increase in the phosphorylation of ERK1/2 at this time point (one hour). These results support the involvement of the ERK1/2 pathway in the synergism between CSE and MCh at the level of IL-8 secretion. In contrast, IL-1β induced ERK1/2 phosphorylation was not increased by MCh and also pre-treatment with U0126 had no effect (Figure 2.5B-D). These results are in agreement with the results of Orsini, et al., demonstrating that IL-1β can induce a transient phosphorylation of ERK1/2 in human airway smooth muscle cells (24).

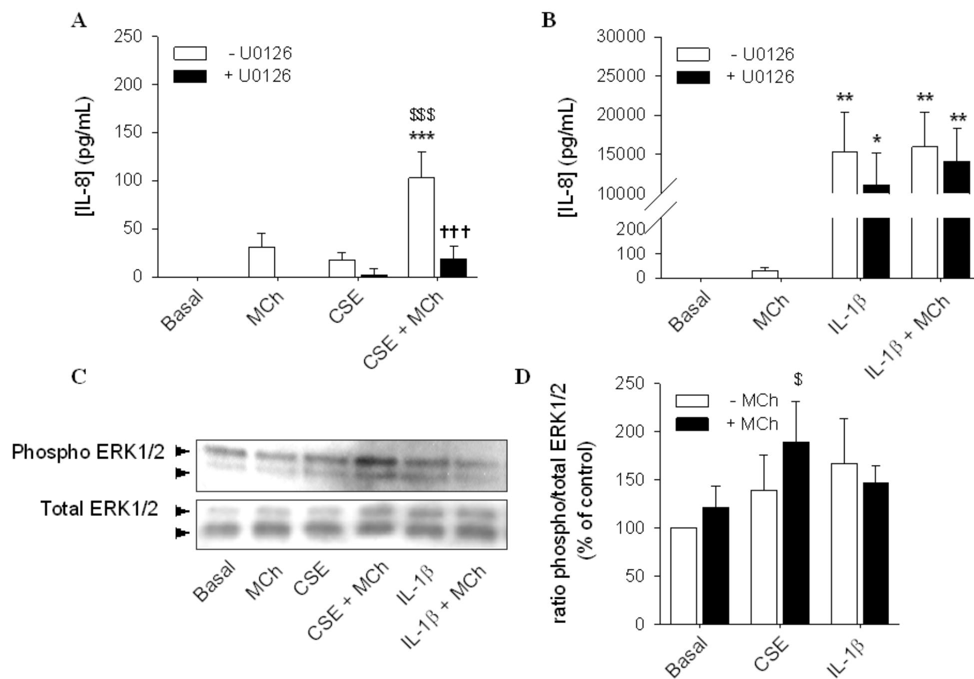

Figure 2.5. Involvement of the MEK/ERK1/2 pathway in IL-8 release induced by MCh. hASMc were stimulated with CSE (5%) (A) or IL-1β (1 ng/mL) (B), in the absence or presence of MCh (10 µM) and/or U0126 (3 µM) for 24 hours. Supernatants were analyzed for the presence of IL-8. *p< 0.05 and **p< 0.01 compared to basal; $^{\$\$\$}$p< 0.001 compared to CSE alone, †††p<0.001 compared to the absence of U0126. (One-way ANOVA followed by Newman-Keuls multiple comparisons test). (C-D) hASMc were stimulated with CSE (5%) or IL-1β (1 ng/mL) in the absence or presence of MCh (10 µM) for 60 min. Cell lysates were analyzed for phospho-ERK1/2 by western blot and corrected for the expression of total ERK1/2, which was used as a loading control. Data represent means ± SE of 5 independent experiments each performed in duplicate. $^{\$}$p< 0.05 compared to CSE alone (Student's t-test for paired observations).

2.4 Discussion

In the present study, we demonstrate that muscarinic receptors stimulate the secretion of the pro-inflammatory cytokine IL-8 from hASMc, and augment the response induced by TNF-α, CSE and PDGF-AB. Furthermore, we dissected the underlying mechanism of the synergistic IL-8 production. To permit the release of the pro-inflammatory cytokine IL-8 after activation of the muscarinic receptors and CSE, activation of PKC is required, which is followed by the breakdown of IκBα.

In parallel, the activation of PKC leads to the stimulation of MEK1/2 inducing the phosphorylation of ERK1/2. Both pathways regulate IL-8 secretion, which, as previously described, is dependent on NF-κB and AP-1 IL-8 promoter activation (25).

Our current and previously published data (19) indicate that the activation of muscarinic receptors in hASMc facilitates the secretion of the pro-inflammatory cytokines IL-6 and IL-8. Functional muscarinic receptors are expressed on the majority of inflammatory cells (5). Also, the endogenous muscarinic receptor ligand acetylcholine and its synthesizing enzyme choline acetyltransferase (ChAT) are present in several extraneuronal cell types, including airway epithelial cells, lymphocytes, eosinophils, neutrophils, macrophages, and mast cells (5, 26). Furthermore, animal models showed that atropine reduces lung inflammation induced by diesel-soot in rats (10), and that tiotropium bromide inhibits several aspects of airway inflammation and remodelling in ovalbumin-sensitized guinea pigs (11, 27). Additionally, it has been reported that carbachol, by activation of muscarinic receptors, is able to increase inflammatory gene expression in ASM, including IL-6, IL-8 and cyclooxygenase-2 (COX-2) (28). Furthermore, acetylcholine (ACh) can induce leukotriene B_4 (LTB_4) release from sputum COPD cells (4), also indicating a regulatory role for ACh in inflammatory cells. Taken together, this indicates that acetylcholine is importantly involved in the regulation of pro-inflammatory responses. Our current results provide new insights as we demonstrate that the activation of muscarinic receptors interacts with several cytokines and growth factors, in particular with TNF-α, PDGF-AB and CSE to enhance their inflammatory response in hASMc.

HASMc produce a variety inflammatory mediators (15, 16, 29). This suggests an important role for ASM in inflammatory responses in COPD. Indeed, hASMc are a source of chemokines and cytokines that play a role in chronic pulmonary diseases like COPD and asthma, including IL-8 and IL-6. The levels of IL-8 are correlated with the degree of neutrophilic inflammation and are increased in sputum in COPD patients (3, 30). Several pro-inflammatory stimuli, including IL-17 (31-33), gram-positive and gram-negative bacteria (34), β-tryptase (35), IL-1β (32) and TNF-α (17) are able to induce IL-8 secretion from human ASM. Moreover, CSE synergizes with TNF-α to enhance IL-8 secretion by ASM (17). We previously demonstrated that CSE and muscarinic M_3 receptor stimulation leads to a

synergistic increase in IL-8 secretion by hASMc (19), which as demonstrated in this study, is dependent on downstream signalling to PKC and the IκBα/NF-κB and MEK/ERK1/2 pathways. Nicotinic receptors and muscarinic M_2 receptors are not involved in this synergism, as gallamine had no effect on IL-8 release induced by either CSE or MCh (19). This indicates that acetylcholine may also play an important role in the inflammatory/immunomodulatory processes driven by human ASM.

Using the PKC inhibitor GF109203X, we demonstrate that the synergism of MCh and CSE-induced IL-8 secretion is mediated by PKC in hASMc. In fact, activation of PKC was sufficient to induce synergistic IL-8 secretion in combination with CSE, which was confirmed by the use of the PKC activator, PMA. These observations correspond with an earlier study from our group demonstrating that MCh augments PDGF-induced cell proliferation via the activation of PKC (23) and appear to suggest that muscarinic M_3 receptors exert their facilitatory effects on remodelling and inflammation to an important extent via the activation of PKC. Downstream, we demonstrated that PKC is able to induce the activation of IκBα/NF-κB and MEK/ERK1/2 pathways in hASMc and that these pathways are involved in the secretion of IL-8 induced by the co-stimulation of muscarinic receptors and CSE. Interestingly, the co-stimulation with CSE and MCh appeared required to reveal the importance of PKC, as stimulation with either CSE or MCh alone was not sufficient to demonstrate an involvement of PKC. This indicates that PKC stimulation by MCh is not sufficient to induce an IL-8 or IL-6 response by itself, but augments pro-inflammatory signalling to NF-κB and ERK1/2 induced by CSE. However, synergistic functional interactions with IL-1β, an important cytokine in COPD pathogenesis (36), were not observed, both for IL-8 secretion and for activation of the signalling pathways investigated, indicating that the mechanism of the synergistic interaction is stimulus specific. Lower concentrations of IL-1β were also tested and were found to be similarly unaffected by MCh (data not shown).

The combination of MCh and CSE likely triggers PKC to activate IKK-2. This kinase allows the phosphorylation and degradation of IκBα leading to the translocation of NF-κB into the nucleus to regulate NF-κB gene transcription (37). Furthermore, PKC has been shown to be critically involved in the activation of the ERK1/2 pathway in human aortic smooth muscle cells (38). PKC induces the phosphorylation of Raf-1, an upstream regulator of ERK1/2 activation, which is

followed by the regulation of AP-1 dependent gene transcription. The IL-8 gene contains both NF-κB and AP-1 binding sites in its promoter region (25). Epithelial cells are also able, to induce IL-8 secretion through the activation of ERK1/2 and NF-κB in response to pro-inflammatory stimuli, including acetylcholine (8, 39, 40). Taken together, these findings and our previous findings (19) indicate that the synergism between muscarinic M_3 receptors and CSE is mediated by PKC dependent activation of the downstream pathways NF-κB and ERK1/2, to induce the secretion of IL-8.

It is unclear whether the pro-inflammatory effects of muscarinic receptor stimulation and CSE, as observed in our current work, are relevant to the COPD patient. Nonetheless, several clinical studies demonstrated that short-term therapy with tiotropium bromide improves airflow and hyperinflation (41, 42). Moreover, long-term use (up to 6 to 12 months) of this anticholinergic drug improved exercise tolerance, quality of life, rates of dyspnoea but also the exacerbation frequency in COPD patients, which are associated with periods of increased inflammatory cell influx (41, 43). The Understanding Potential Long-Term Impacts on Function with Tiotropium (UPLIFT) study concluded that COPD patients treated with tiotropium bromide during a 4-year period improved their quality of life, frequency of exacerbations and lung function, but tiotropium bromide did not reduce the decline in FEV_1 over the treatment period (44). Nonetheless, in a subgroup of COPD patients of the UPLIFT study, which were not on other controller medication, a reduction in the accelerated FEV_1 decline was observed in the tiotropium bromide arm (post-hoc analysis of the UPLIFT study (44)). This was also observed in the subgroup of stage II COPD patients (45). Collectively, besides the well described bronchodilatory effects, these findings suggest additional, non-bronchodilator properties for tiotropium bromide (6). An anti-inflammatory role for anticholinergics is in agreement with animal and cell culture studies showing a role for acetylcholine in cell proliferation, extracellular matrix protein secretion and inflammation (5, 46, 47) and with our present findings showing that the inflammatory response induced by CSE, TNF-α and PDGF-AB can be augmented by muscarinic receptor stimulation in hASMc. It should be emphasized, however, that the hypothesis “tiotropium bromide may exert anti-inflammatory effects in COPD patients” still needs to be tested in clinical studies.

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3 Muscarinic receptor stimulation augments TGF-β_1-induced contractile protein expression by airway smooth muscle cells

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Abstract

Acetylcholine (ACh) is the primary parasympathetic neurotransmitter in the airways. Recently, it was established that ACh, via muscarinic receptors, regulates airway remodelling in animal models of asthma and COPD. The mechanisms involved are not well understood. Here, we investigated the functional interaction between muscarinic receptor stimulation and transforming growth factor (TGF)-β_1 on the expression of contractile proteins in human airway smooth muscle (ASM) cells. ASM cells expressing functional muscarinic M_2 and M_3 receptors, were stimulated with methacholine (MCh), TGF-β_1 or their combination for up to 7 days. Western Blot analysis revealed a strong induction of sm-α-actin and calponin by TGF-β_1, which was increased by MCh in ASM cells. Immunocytochemistry confirmed these results and revealed that the presence of MCh augmented the formation of sm-α-actin stress fibres by TGF-β_1. MCh did not augment TGF-β_1-induced gene transcription of contractile phenotype markers. Rather, translational processes were involved in the augmentation of TGF-β_1-induced contractile protein expression by muscarinic receptor stimulation, including phosphorylation of GSK-3β and 4E-BP1which was enhanced by MCh. In conclusion, muscarinic receptor stimulation augments functional effects of TGF-β_1 in human ASM cells on cellular processes that underpin airway smooth muscle remodelling in asthma and COPD.

3.1 Introduction

Acetylcholine is the primary parasympathetic neurotransmitter in the airways that is associated with the regulation of bronchoconstriction and mucus secretion (1). Therefore, therapy with anticholinergics, such as tiotropium bromide, is often prescribed to patients with chronic obstructive pulmonary disease (COPD) and, to a lesser extent, with asthma. More recent evidence indicates that acetylcholine (either neuronal or non-neuronal) also regulates airway inflammation and airway remodelling, which might contribute to the therapeutic effectiveness of these drugs (1). Acetylcholine is synthesized by the enzyme choline acetyltransferase (ChAT) in different cell types including structural cells such as neurons, epithelial cells, airway fibroblasts and airway smooth muscle cells (2). It can act as a paracrine or autocrine mediator to induce cell proliferation and cytokine release

by epithelial cells and human lung fibroblasts. The expression of ChAT is increased in epithelial cells and fibroblasts from COPD patients, and therefore the increased release of acetylcholine may promote airway inflammation and remodelling (3, 4). Several studies using animal models of allergic asthma demonstrated that tiotropium bromide inhibits increased airway smooth muscle thickness, myosin expression, eosinophilic airway inflammation, airway fibrosis and airway hyperresponsiveness induced by repeated allergen exposure (5-7). In addition, in animal models of COPD tiotropium reduced LPS-induced airway neutrophilia, collagen deposition and muscularization of microvessels (8), inhibited neutrophil elastase-induced goblet cell metaplasia (9), and reduced cigarette smoke-induced pulmonary inflammation (10). Anti-inflammatory and anti-remodelling properties of tiotropium have also been demonstrated in a mouse model of gastro-oesophageal reflux disease (11).

The multifunctional cytokine TGF-β_1 plays an important role in remodelling of the airways in various chronic airway diseases. This pro-fibrotic cytokine is highly expressed in many cell types of the airways of patients with these diseases (12). TGF-β_1 has been reported to induce proliferation and maturation of airway smooth muscle (ASM) cells, depending on its concentration (12, 13). In ASM cells, TGF-β_1 promotes the expression of contractile phenotype markers, including sm-α-actin, through both transcriptional and translational control (13). Transcriptional regulation requires signalling to RhoA and Smad2/3, which promotes the nuclear presence and transcriptional activity of serum response factor (SRF) at smooth muscle specific genes (13-16). In parallel, TGF-β_1 signals to PI3K, which results in downstream phosphorylation of proteins that control protein translation, including p70S6K, glycogen synthase kinase 3 (GSK-3) and 4E-binding protein1 (4E-BP1), a cellular response that requires the presence of caveolae (13, 17-19). Furthermore, TGF-β_1 regulates cell proliferation, including airway epithelial cell and fibroblast proliferation, cell differentiation, including myofibroblast differentiation, and the synthesis of extracellular matrix proteins such as fibronectin and collagen (20, 21).

Activation of the PI3K pathway by muscarinic receptor stimulation has previously been reported to enhance platelet-derived growth factor (PDGF)- and epidermal growth factor- (EGF)-induced ASM cell proliferation (22-25). Functional crosstalk between the cholinergic system and TGF-β_1 in ASM is, however, still unknown. We hypothesized that muscarinic receptors contribute to ASM remodelling by enhancing TGF-β_1 function. Therefore, in human ASM cells, we investigated the

effect of muscarinic receptor stimulation on TGF-β_1-induced expression of the contractile phenotype markers calponin and sm-α-actin. In addition, we studied potential mechanisms of interaction, in particular at the level of transcriptional and translational processes, including ChAT and contractile protein mRNA expression and phosphorylation of GSK-3β and 4E-BP1.

3.2 Materials and methods

3.2.1 Antibodies and reagents

Methacholine chloride (MCh) was purchased from ICN Biomedicals (Zoetermeer, the Netherlands). Human recombinant TGF-β_1 was obtained from R&D systems (Abingdon, UK). Mouse anti-α smooth muscle actin (sm-α-actin) antibody, mouse anti-calponin antibody, horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG antibody, HRP-conjugated goat anti-rabbit IgG antibody, and interleukin-1ß (IL-1ß) were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). Phospho-GSK-3-α/β (Ser21/9) antibody and phospho-4E-BP1 (Thr37/46) antibody were obtained from Cell signalling Technology (Beverly CA, USA). GAPDH antibody and total GSK-3 antibody were purchased from Santa Cruz Biotechnology, INC (Santa Cruz CA, USA). Cy3-conjugated secondary antibody was obtained from Jackson ImmunoResearch (West Grove PA, USA). The inhibitor LY294002 purchased from Tocris Biosciences (Bristol, UK). Tiotropium bromide was obtained from Boehringer Ingelheim Pharma GmbH (Biberach an der Riss, Germany). All other chemicals were of analytical grade.

3.2.2 Cell culture

Human bronchial smooth muscle cell lines immortalized by stable expression of human telomerase reverse transcriptase (hTERT) were prepared as described previously (26). The primary cultured human bronchial smooth muscle cells used to generate hTERT ASM cells were prepared from macroscopically healthy segments of 2nd to 4th generation main bronchus obtained after lung resection surgery from patients with a diagnosis of adenocarcinoma. All procedures were approved by the Human Research Ethics Board of the University of Manitoba. Cells were grown to confluence using DMEM supplemented with 10% foetal bovine serum, streptomycin 50 U/mL, penicillin 50 µg/mL and amphotericin B 1.5 µg/mL. Cultures were maintained in a humidified incubator at 37°C-5% CO_2, and

media were changed every 2-3 days. The expression and function of muscarinic M_2 and M_3 receptors in these cells has previously been described (27).

3.2.3 Stimulation of human ASM cells

Cells were cultured in 6-well plates and grown until confluence. After serum deprivation for 24 hours in DMEM supplemented with antibiotics (100 µg/mL streptomycin, penicillin 100 U/mL and amphotericin B 1.5 µg/mL) and 1% ITS (insulin 5 µg/mL, transferrin 5 µg/mL, and selenium 5 ng/mL), the cells were stimulated with the muscarinic receptor agonist methacholine (MCh, 10 µM), TGF-β_1 (2 ng/mL), or the combination of MCh (10 µM) and TGF-β_1 (2 ng/mL) for 2 hours, 1 day, 4 days or 7 days. Where mentioned, cells were pre-incubated with LY294002 (10 µM) or tiotropium bromide (10 nM) for 30 min.

3.2.4 Western Blotting

To obtain whole cell lysates, stimulated cells were washed once with ice-cold phosphate-buffered saline (PBS, composition: NaCl 140 mM, KCl 2.6 mM, KH_2PO_4 1.4 mM, Na_2HPO_4 8.1 mM, pH 7.4), followed by lysis using ice-cold SDS-lysis buffer (62.5 mM Tris-HCl, 2% SDS, NaF 1 mM, Na_3VO_4 1 mM, aprotinin 10 µg/mL, leupeptin 10 µg/mL, pepstatin A 7 µg/mL at pH 8.0). Equal amounts of protein were separated on polyacrylamide SDS gels and transferred to nitrocellulose. To avoid non-specific binding, membranes were blocked with blocking buffer (Tris-HCl 50 mM, NaCl 150 mM, TWEEN-20 0.1%, non-fat dried milk powder 5%) for 1 hour at room temperature. Afterwards, the membranes were incubated with the specific primary antibody, all diluted in blocking buffer, for 1 hour at room temperature. After washing the membranes 3 times with Tris Buffered Saline Tween20 0.1% (TBS-T 0.1%: Tris-HCl 50 mM, NaCl 150 mM, TWEEN-20 0.1%) for 10 min, incubation with the secondary antibody labelled with HRP was performed for 1 hour at room temperature, followed by an additional 3 washes with TBS-T 0.1%. Bands were subsequently visualized on film using enhanced chemiluminescence reagents and analysed by densitometry (TotallabTM, Nonlinear dynamics, Newcastle, UK). All bands were normalized to either GAPDH for phospho-4E-BP1 or to total GSK-3 for phospho-GSK-3-α/β.

3.2.5 Immunofluorescence

Cells were cultured on Labtek IITM chamber slides and grown until confluence. After serum deprivation for 24 hours in DMEM supplemented with antibiotics and

1% ITS, the cells were stimulated with the muscarinic receptor agonist methacholine (MCh, 10 μM), alone and in combination with TGF-β_1 (2 ng/mL) for 1, 4 or 7 days. After washing the cells twice with cytoskeleton buffer (CB: MES 10 mM, NaCl 150 mM, EGTA 5 mM, MgCl2 5 mM, and glucose 5 mM at pH 6.1), the cells were fixed with CB containing 3% paraformaldehyde (PFA) for 15 min. The cells were then incubated with CB buffer containing 3% PFA and 0.3% Triton-X-100 for 5 min, followed by an additional 2 washes with CB. Cells were then blocked for 1 hour in cyto-TBS (Tris-base 20 mM, NaCl 154 mM, EGTA 2.0 mM, and MgCl2 2.0 mM at pH 7.2) with BSA 1% and normal donkey serum 2%. After that, cells were stained with mouse sm-α-actin antibody overnight at 4°C. After washing 3 times with cyto-TBS containing 0.1% Tween-20 (cyto-TBS-T) for 10 min, incubation with the secondary antibody Cy3-mouse (dilution 1:50 in cyto-TBS-T) was performed during 3 hours at room temperature. Cells were then washed 4 times for 15 min in cyto-TBS-T and the nuclei were stained with Hoechst 33342 (dilution 1: 1000 in cyto-TBS-T) (Invitrogen, Breda, the Netherlands) for 1 min. Before mounting the slides with ProLong Gold anti-fade reagent (Invitrogen, Breda, The Netherlands), cells were washed 4 times with ultra-pure water. After staining, the slides were analysed using an Olympus AX70 microscope equipped with digital image capture system (ColorView Soft System with Olympus U CMAD2 lens).

3.2.6 RNA isolation and real-time quantitative RT-PCR of sm-α-actin, calponin, ChAT and ribosomal subunit 18S

Total cellular RNA was isolated using the Rneasy mini kit (Qiagen, Venlo, The Netherlands). RNA concentration was determined by Nanodrop ND1000 (Wilmington DE, USA). By reverse transcription, cDNA was synthesized using the Promega cDNA synthesis kit. Real-time quantitative PCR for ChAT, sm-α-actin and calponin was performed using an IQ5 real time detection system (Biorad, Veenendaal, The Netherlands). The specific primer sets used to detect ribosomal subunit 18S (18S rRNA), ChAT, sm-α-actin and calponin are illustrated in Table 3.1. The abundance of gene expression for sm-α-actin and calponin was adjusted for the expression of 18S rRNA and normalized to the expression found in control cultures using the $2^{-\Delta\Delta}$Cq method.

Table 3.1. Primer sequences of sm-α-actin, calponin, ChAT, and 18S rRNA used for real-time quantitative PCR

Substance	Sequences	
Sm-α-actin	Forward	5′-GACCCTGAAGTACCCGATAGAAC-3′
	Reverse	5′-GGGCAACACGAAGCTCATTG-3′
Calponin	Forward	5′-TGTTTGAGAACACCAACCATACACA-3′
	Reverse	5′-GTTTCCTTTCGTCTTCGCCAT-3′
ChAT	Forward	5′-TTTTGTGAGAGCCGTGACTG-3′
	Reverse	5′-CACAGGACCATAGCAGCAGA-3′
18S rRNA	Forward	5′-CGCCGCTAGAGGTGAAATTC-3′
	Reverse	5′-TTGGCAAATGCTTTCGCTC-3′

3.2.7 Data analysis

Data are presented as mean values ± SEM. Statistical significance was determined by one-way ANOVA or by two-way ANOVA, where appropriate, followed by a post-hoc Student-Newman-Keuls multiple comparisons test for paired observations. Data were considered statistically significant if $p \leq 0.05$.

3.3 Results

3.3.1 Effect of muscarinic receptor stimulation on contractile protein expression induced by TGF-β_1

We first analysed the effects of MCh (10 µM), alone and in combination with TGF-β_1 (2 ng/mL) on contractile protein expression (calponin and sm-α-actin) by human ASM cells. TGF-β_1 alone induced a significant increase in calponin and sm-α-actin expression in these ASM cells after 7 days (Figure 3.1A-C). Interestingly, the induction of sm-α-actin occurred considerably later than the induction of calponin. MCh had no significant effect on contractile protein expression by itself. However, both TGF-β_1-induced calponin and sm-α-actin expression were significantly increased by 7 days co-stimulation with MCh, to a similar extent (1.4-fold; Figure 3.1A-C). The response of MCh was concentration dependent to induce the expression of contractile proteins (Figure 3.1D).

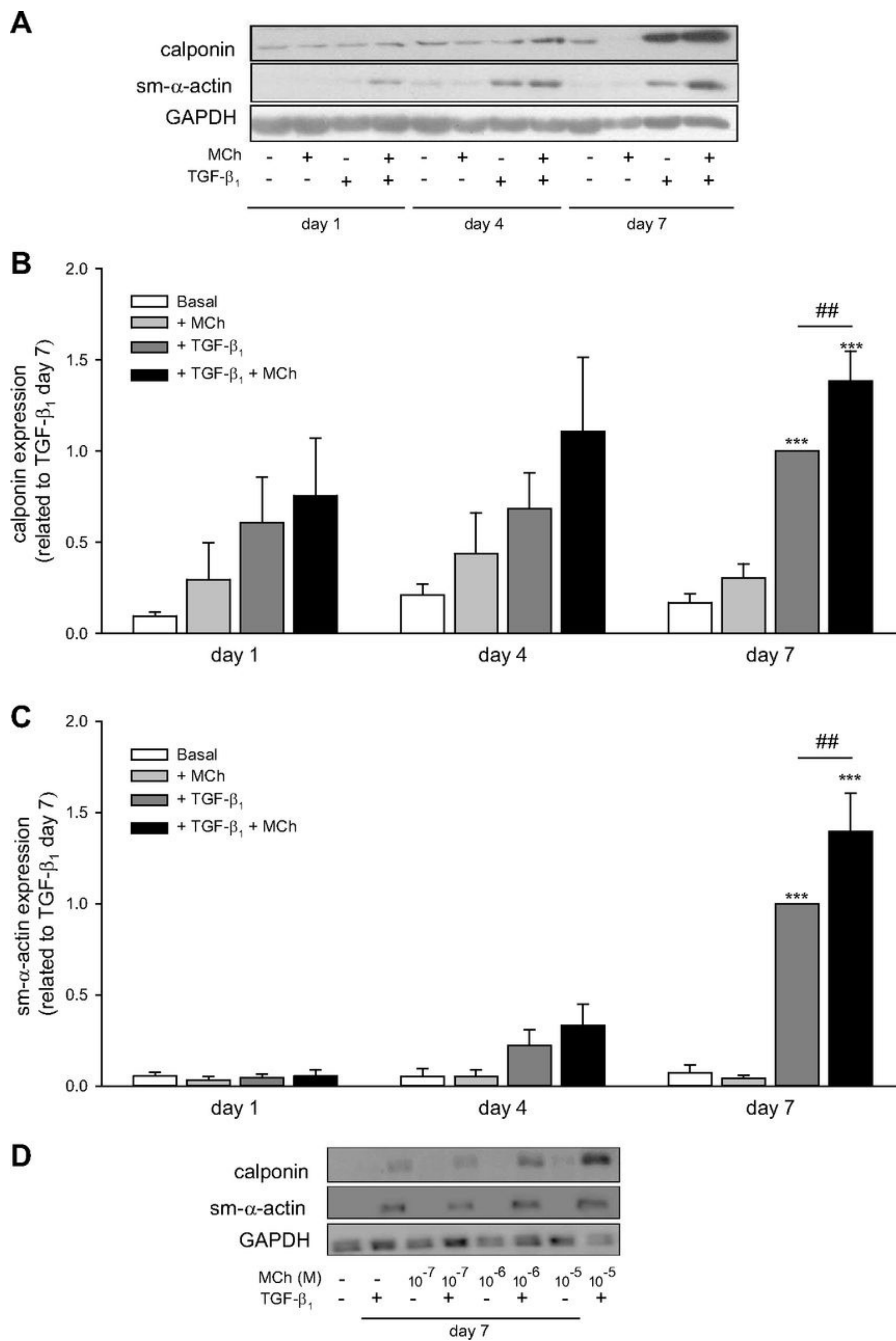
A
calponin
sm-α-actin
GAPDH
MCh
TGF-β1
day 1
day 4
day 7
B
calponin expression
(related to TGF-β1 day 7)
Basal
+ MCh
+ TGF-β1
+ TGF-β1 + MCh
##
***
2.0
1.5
1.0
0.5
0.0
day 1
day 4
day 7
C
sm-α-actin expression
(related to TGF-β1 day 7)
Basal
+ MCh
+ TGF-β1
+ TGF-β1 + MCh
##
***
2.0
1.5
1.0
0.5
0.0
day 1
day 4
day 7
D
calponin
sm-α-actin
GAPDH
MCh (M)
TGF-β1
day 7

Figure 3.1. Muscarinic receptor stimulation augments contractile protein expression induced by TGF-β_1. Human ASM cells were stimulated with TGF-β_1 (2 ng/mL), in the absence or presence of methacholine (MCh; 10 µM) for 1, 4 or 7 days. Cells lysates were analysed for the presence of calponin (B) or sm-α-actin (C). Representative blots are shown in (A). Data shown are the means ± SE of 4-7 independent experiments. ***p≤ 0.001 compared to basal; ##p≤ 0.01 compared to the absence of methacholine. A dose-response for MCh was performed using the concentration 10^{-7} M, 10^{-6} M, 10^{-5} M in presence or absence of TGF-β_1 (2 ng/mL) for 7 days. Cell lysates were analyzed for the presence of calponin or sm-α-actin. Data shown of 1 experiment (D).

As we expected alterations in the morphology of the cells due to the increased contractile phenotype marker protein expression after treatment, immunocytochemistry was performed. Interestingly, increased sm-α-actin stress fibre formation was observed after 7 days of treatment with TGF-β_1 (Figure 3.2). Co-stimulation with MCh clearly amplified this effect, whereas the muscarinic receptor agonist had no effect by itself (Figure 3.2). Collectively, these data indicate that human ASM cells acquired a contractile phenotype by TGF-β_1 treatment, which could be enhanced by muscarinic receptor stimulation.

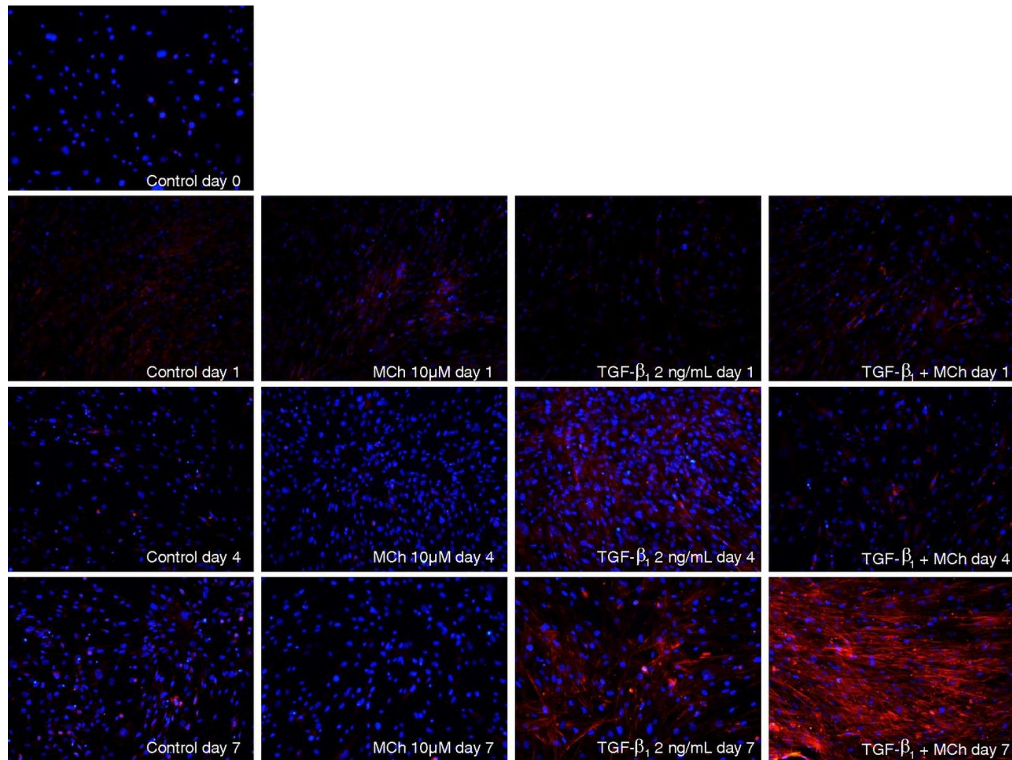

Figure 3.2. Methacholine augments TGF-β$_1$-induced sm-α-actin stress fibre expression after 7 days of stimulation. Human ASM cells were grown to confluence. After 24 hours of serum deprivation, cells were treated with TGF-β$_1$ (2 ng/mL) in absence or presence of MCh (10 μM) for 1, 4 or 7 days, as indicated. Cells were stained for α-actin (red) and the nuclei were stained with Hoechst 33342. The images shown are representative of 2 experiments. Magnification 10x40.

3.3.2 Autocrine ACh secretion is not involved in TGF-β$_1$ induced contractile protein expression

To investigate whether human ASM cells express ChAT in response to TGF-β$_1$, we measured mRNA levels of ChAT induced by TGF-β$_1$ and/or MCh after 1 (Figure 3.3A) and 7 days (Figure 3.3B) of treatment. Baseline mRNA expression of ChAT in ASM was low (Cq = 31.06 and 10.42 for ChAT and 18S rRNA, respectively). Also, we observed no induction of ChAT expression in response to either TGF-β$_1$, MCh or their combination. Next, we pre-incubated ASM cells with tiotropium bromide for 30 min, followed by stimulation with TGF-β$_1$ for 7 days. TGF-β$_1$ induced an increase in the expression of calponin and sm-α-actin; however, pre-treatment with tiotropium bromide did not counteract the expression of these contractile

proteins. These data suggest that the increase in contractile protein expression induced by TGF-β_1 stimulation is not dependent on the autocrine production of acetylcholine by ASM.

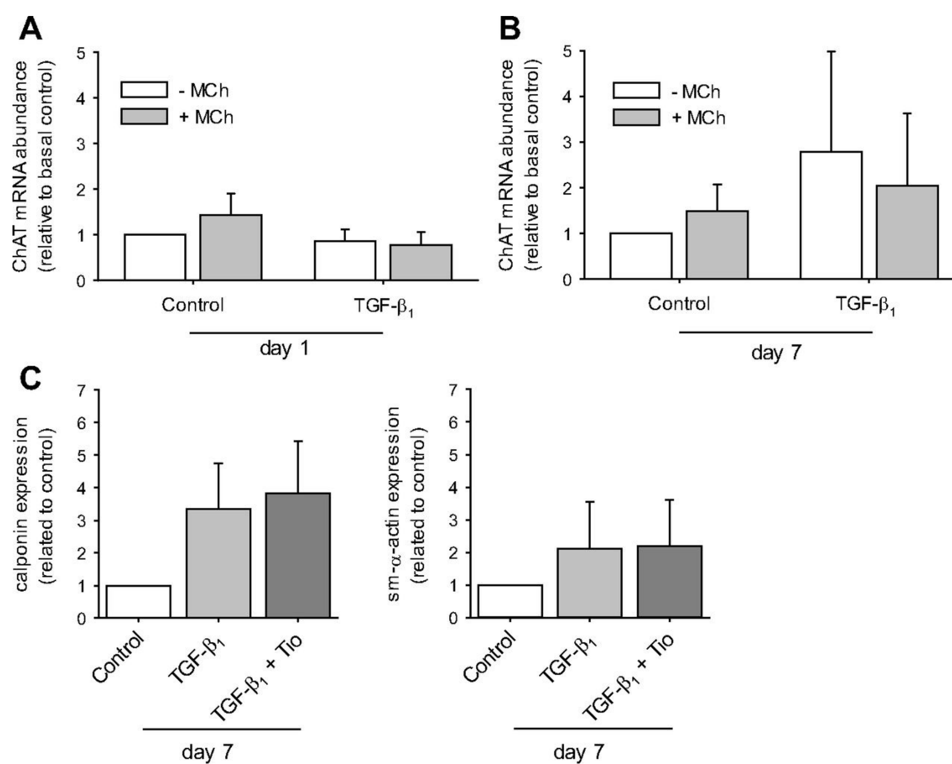

Figure 3.3. Autocrine ACh release is not involved in the induction of contractile proteins by TGF-β_1. Human ASM cells were grown to confluence. After 24 hours of serum deprivation, cells were treated with TGF-β_1 (2 ng/mL) in the absence or presence of MCh (10 µM). mRNA levels of ChAT were measured after 1 day (A) or 7 days (B) of stimulation. Further, cells were treated with TGF-β_1 (2 ng/mL) in the absence or presence of tiotropium bromide (10 nM) for 7 days. Cells lysates were analysed for the presence of calponin and sm-α-actin (C). Data shown are the means ± SE of 3-6 experiments.

3.3.3 Effect of muscarinic receptor stimulation on mRNA expression of contractile proteins induced by TGF-β_1

To establish whether the accumulation of contractile proteins was due to an increase in gene expression and/or increased translation, we investigated the mRNA expression of calponin and sm-α-actin after 1 and 7 days of treatment with TGF-β_1 and MCh as described above. After 1 day of stimulation with TGF-β_1 alone,

a significant increase in calponin (Figure 3.4A) and sm-α-actin (Figure 3.4B) mRNA expression was measured. Stimulation with MCh by itself had no effect on the expression of calponin and sm-α-actin mRNA (Figures 3.4A and 3.4B). Moreover, calponin expression induced by TGF-β_1 was not affected by MCh treatment (Figure 3.4A). Surprisingly, a significant decrease in sm-α-actin mRNA expression was observed after treatment with the combination of TGF-β_1 and MCh as compared with TGF-β_1 treatment alone (Figure 3.4B). On day 7, all treatments had no effect on the mRNA expression of either calponin or sm-α-actin (Figures 3.4C and 3.4D). Collectively, these data suggest that the increase in TGF-β_1-induced contractile protein expression by MCh observed after 7 days of treatment was not due to amplification of TGF-β_1-induced mRNA levels of these proteins.

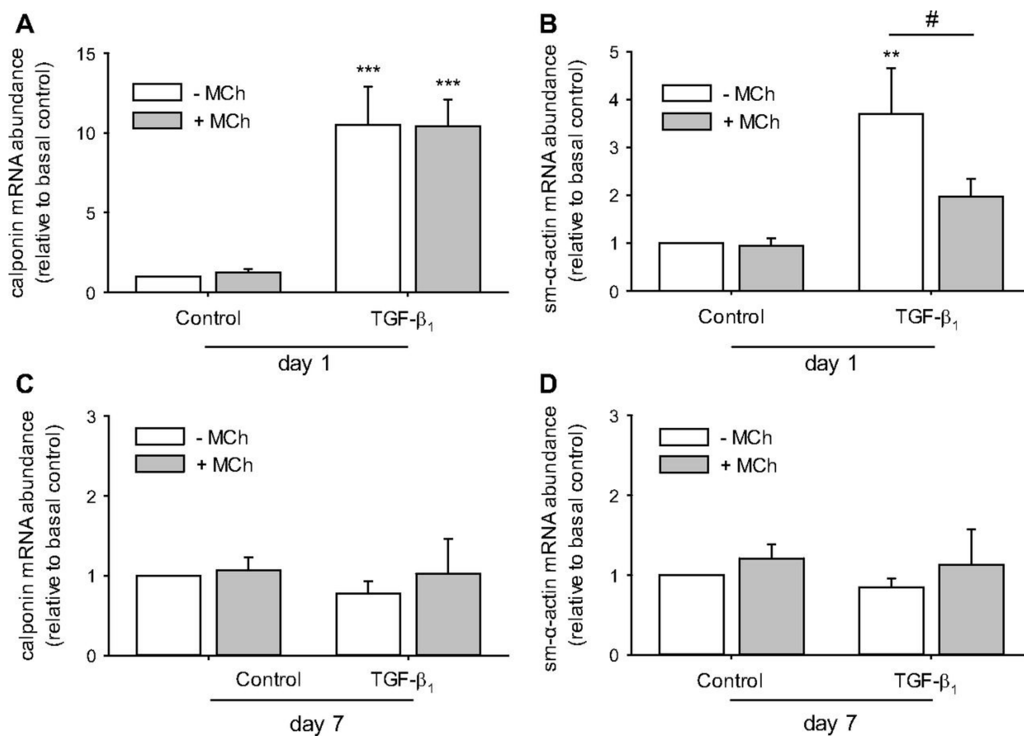

Figure 3.4. Muscarinic receptor stimulation does not augment mRNA expression of calponin and sm-α-actin induced by TGF-β_1. Human ASM cells were stimulated with TGF-β_1 (2 ng/mL), in the absence or presence of methacholine (MCh; 10 μM) for 1 or 7 days. mRNA levels of calponin (A, C) or sm-α-actin (B, D) were measured. Data shown are the means ± SE of 6-7 independent experiments. **$p \leq 0.01$, ***$p \leq 0.001$ compared to basal; $^{\#}p \leq 0.05$ compared to the absence of methacholine.

3.3.4 Effect of muscarinic receptor stimulation on translational processes activated by TGF-β_1

Next, we aimed to investigate the role of translational processes in the functional interaction between muscarinic receptor activation and TGF-β_1. We analysed the expression and the phosphorylation status of 4E-BP1 and GSK-3, proteins which are involved in the translation machinery of smooth muscle contractile phenotype marker proteins, and which have also been shown to play a critical role in TGF-β_1 induced contractile protein accumulation (13, 28).
The PI3K pathway can induces the phosphorylation of p4E-BP1 and GSK-3β to permit the expression of contractile protein. To confirm the involvement of the PI3K signalling in contractile protein accumulation induced by TGF-β_1, we inhibited the PI3K pathway with LY294002 (10µM). Treatment with LY294002 reduced the expression of calponin induced by TGF-β_1 and MCh (Figure 3.5A).

It has previously been demonstrated that TGF-β_1 is able to induce the phosphorylation of 4E-BP1 which contributes to TGF-β_1-induced sm-α-actin expression (29). Therefore, we investigated the potential involvement of 4E-BP1 phosphorylation in the TGF-β_1 response and the additive effect observed with MCh. To this aim, we stimulated ASM cells for 2 hours and 24 hours with TGF-β_1 in the absence or presence of MCh and analysed the phosphorylation of 4E-BP1. No significant increase in 4E-BP1 phosphorylation was observed after 2 or 24 hours stimulation of ASM cells with TGF-β_1 or MCh alone (Figure 3.5B). Interestingly, however, a significant effect was observed with the combination of both stimuli, at 24 hours after stimulation. To ensure that the PI3K pathway was involved in the phosphorylation of 4E-BP1 by TGF-β_1 and MCh, ASM cells were pre-treated with LY294002, which led to a reduction in the phosphorylation of p4E-BP1 induced by TGF-β_1 and MCh (Figure 3.5B).
Recently, it has been demonstrated that inhibition of GSK-3β is sufficient for the induction of ASM hypertrophy and increase in contractile protein expression (17, 29). GSK-3β is active in its unphosphorylated form and inhibits protein translation by phosphorylating eukaryotic initiation factor-2B (eIF-2B). Therefore, we investigated whether the phosphorylation of GSK-3 (ser9/ser21) was induced by TGF-β_1 and MCh. ASM cells were stimulated for 2 and 24 hours with TGF-β_1, MCh and their combination. For all treatments, only the phosphorylated β-isoform of GSK-3 was detected. Interestingly, after 2 hours of stimulation, TGF-β_1 and MCh alone were ineffective, whereas their combination induced a significant increase

in GSK-3β phosphorylation (Figure 3.5C). No significant effects were observed after 24 hours of stimulation. To reensure that the PI3K pathway was also involved in the phosphorylation of GSK-3β by TGF-β_1 and MCh, ASM cells were pre-treated with LY294002, which led to an inhibition of the phosphorylation of GSK-3β induced by TGF-β_1 and MCh (Figure 3.5C).

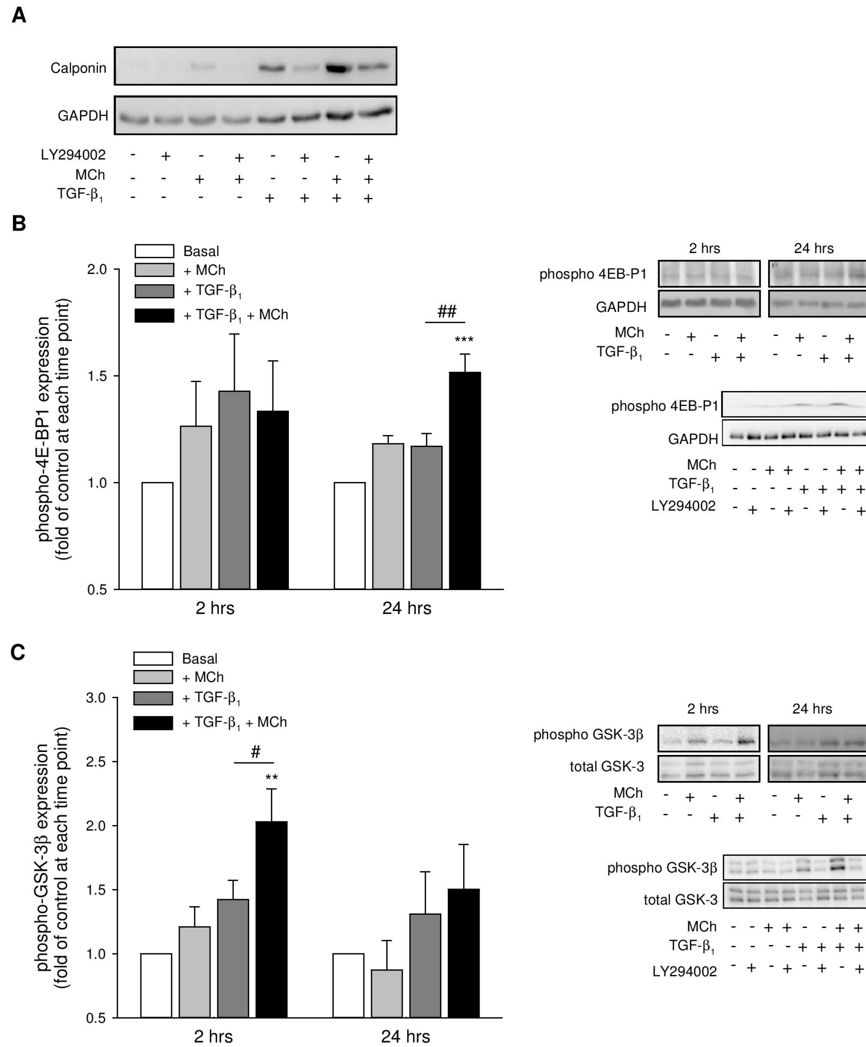

Figure 3.5. Muscarinic receptor stimulation augments TGF-β_1-induced phosphorylation of GSK-3β and 4E-BP1. To investigate the potential involvement of the PI3K pathway in contractile protein accumulation induced by TGF-β_1 and MCh, ASM cells were pre-incubated with LY294002 (10 μM) for 30 min, followed by 7 days stimulation with MCh (10

μM) and/or TGF-β_1 (2ng/mL) (A). Cells lysates were analysed for the presence of calponin. Data shown of 1 experiment. Human ASM cells were stimulated with TGF-β_1 (2 ng/mL), in the absence or presence of methacholine (MCh; 10 μM) for 2 and 24 hours. Cells lysates were analysed for the presence of phosphorylated 4E-BP1 (B) or phosphorylated GSK-3β (C). Representative blots are shown next to the graphs. Data shown are the means ± SE of 4-7 independent experiments. $^{**}p \leq 0.01$, $^{***}p \leq 0.001$ compared to basal; $^{\#}p \leq 0.05$, $^{\#\#}p \leq 0.01$ compared to the absence of methacholine. Additionally, to confirm the that PI3K is involved in the phosphorylation of 4E-BP1 and GSK-3β induced by TGF-β_1 and MCh, ASM cells were pre-incubated with LY294002 (10 μM) for 30 min, followed by 24 hrs (for phosphorylation of 4E-BP1 (B)) or 2 hrs (for phosphorylation of GSK-3β (C)) stimulation with MCh (10 μM) and/or TGF-β_1 (2ng/mL). Cells lysates were analysed for the phosphorylation of 4E-BP1 (B) or GSK-3β (C). Data shown of 1 experiment.

3.4 Discussion

In the present study, we demonstrate that a cross-talk between TGF-β receptors and muscarinic receptors regulate the expression of contractile phenotype marker proteins and the formation of sm-α-actin stress fibres in human ASM cells. We show that this cross-talk is independent of transcriptional regulation and of the autocrine release of ACh, but requires translational mechanisms, such as the phosphorylation of GSK-3β and 4E-BP1. These studies provide a potential mechanistic explanation for inhibition of allergen-induced contractile protein expression and ASM contractility by anticholinergic treatment in animal models of asthma (13). Furthermore, these studies are the first to demonstrate functional cross-talk between G-protein coupled receptors and TGF-β in ASM.

ASM remodelling in asthma is characterized by phenotype alterations leading to maturation of the muscle cells (19). The multifunctional cytokine TGF-β plays an important role in ASM remodelling in asthma (12). Patients with asthma as well as with COPD, diseases associated with airway remodelling, have increased expression of TGF-β in lung tissue (21, 30). In addition, its role in wound healing, it is well known that TGF-β induces the maturation of ASM cells. Goldsmith et al., reported that TGF-β induces a time-dependent increase in sm-α-actin expression in primary human bronchial smooth muscle cells (13). In agreement with this finding, we demonstrate that TGF-β induces the expression of the contractile phenotype protein markers sm-α-actin and calponin in human ASM cells. Interestingly, muscarinic receptor stimulation enhanced the TGF-β_1-induced

expression of these proteins, suggesting that muscarinic receptors facilitate ASM remodelling processes induced by TGF-β_1.

In support of a role for muscarinic receptors in ASM remodelling, an *in vivo* study from our group showed that tiotropium bromide reduces airway remodelling in repeatedly ovalbumin challenged guinea pigs. The administration of tiotropium bromide before each allergen challenge resulted in a reduction of ASM hyperplasia and of the increased contractility and contractile protein expression (6). *In vitro*, muscarinic receptor stimulation augmented the mitogenic responses of human ASM in response to EGF and PDGF (23, 25). Here, we show that muscarinic receptor stimulation also enhances the expression of contractile proteins in response of TGF-β_1 in human ASM cells, thus providing a plausible explanation for the observed effects of tiotropium *in vivo*. In line with this contention, in muscarinic M_3 receptor transfected canine ASM cells, carbachol has been reported to induce increased promoter activity of the sm-MHC and SM22 genes (31). Additionally, the exposure to carbachol in strained human ASM cells leads to an augmentation in the expression of myosin light-chain kinase (32). Furthermore, muscarinic receptor stimulation augments the IL-6 and IL-8 secretion by human ASM cells in response to various stimuli, including cigarette smoke extract and PDGF (**chapter 2**). Collectively, these findings and our current work are in agreement with a strong regulatory role for muscarinic receptors in ASM remodelling. The cholinergic system has also been implicated in fibrosis and fibroblast proliferation (33, 34), suggesting that anticholinergics may have beneficial effects on multiple pathological tissue remodelling processes in obstructive airways diseases.

Previous studies indicated that ASM expresses mRNA for ChAT, the synthesizing enzyme for ACh (35). Also, ChAT expression and autocrine ACh release by fibroblasts and epithelial cells was proposed to regulate cell proliferation and cytokine release by these cells (3, 36). Our studies show that although ChAT mRNA is expressed, the expression levels are in fact quite low. Furthermore, the expression of ChAT was unchanged in response to TGF-β_1, and antagonism of muscarinic receptors using tiotropium had no effect on TGF-β_1 induced contractile protein accumulation. This implies that an autocrine loop involving ACh release is not involved in the acquisition of contractile protein expression induced by TGF-β_1 and suggests that the above mentioned regulatory effects of muscarinic receptors on ASM require ACh release by for example, airway neurons and airway epithelium.

The signalling mechanisms that underpin these remodelling processes have thus so far not fully been established. The expression of contractile and contraction regulatory proteins, such as sm-α-actin, calponin, SM22 and desmin, which mark the maturation of ASM cells are under the control of transcriptional and translational processes. Smooth muscle-specific gene transcription is induced by the stimulation of the RhoA/Rho-kinase pathway and the Smad2/3 pathway leading to the nuclear translocation and transcriptional activation of the transcription factor serum response factor (SRF) and its co-activators myocardin and megakaryocytic acute leukaemia (MAL) (13-16, 37). The translation of the smooth muscle-specific genes is, however, dependent on the PI3K/Akt signalling pathway (13, 38-41). Thus, TGF-β_1-induced contractile protein expression is paralleled by the phosphorylation of GSK-3β and 4E-BP1, both downstream targets of PI3K/Akt signalling (13, 42), and the phosphorylation of 4E-BP1 is required for TGF-β-induced contractile protein expression (41). We and others have reported that muscarinic receptor stimulation facilitates PDGF-induced proliferation of human ASM cells through the cooperative activation of the PI3K pathway, leading to the synergistic phosphorylation of Akt, p70S6K and GSK-3β (22-25). Interestingly, our current results show that the increased expression of contractile proteins induced by TGF-β_1 and muscarinic receptor stimulation are also mediated by translational processes, including the phosphorylation of GSK-3β and 4E-BP1, but not by transcriptional processes, as mRNA expression of these smooth muscle specific genes was not affected. This suggests that muscarinic receptor stimulation exerts its action through cooperative activation of the PI3K/Akt/GSK-3-signaling pathway by TGF-β_1, inducing the accumulation of contractile proteins, and not through the regulation of RhoA/Rho-kinase/SRF pathway. In this context, and in the context of our earlier work (23), it is therefore of interest that in a mouse model of asthma increased phosphorylation of GSK-3β has been reported, which correlated with ASM hyperplasia, hypertrophy and expression of contractile proteins, including as sm-α-actin (42).

To our knowledge, this is the first study reporting cross-talk between a GPCR and TGF-β in ASM remodelling. Panettieri *et al*. showed that the expression of extracellular matrix proteins induced by TGF-β_1 was not influenced by CysLT1 receptor stimulation in human ASM cells (43), indicating that this cross-talk may be receptor dependent. In neural progenitor cells, Morishita et al. reported the ability of a GPCR and TGF-β to enhance the activity of the sm-α-actin promoter;

however, these activities were independent of each other (44). Collectively, our findings suggest that GPCRs and TGF-β are able to cooperatively induce airway remodelling processes, although clearly it cannot be assumed that this occurs for all GPCR classes. These findings are nonetheless of interest to future studies, as many remodelling processes, also outside the airways, involve cooperative regulation by GPCR ligands and growth factors (24).

In the Understanding Potential Long-term Impacts on Function with Tiotropium (UPLIFT) study, COPD patients treated with tiotropium bromide during a 4 year period had a better quality of life and lung function, whereas also the frequency of exacerbation was reduced. The rate of decline in FEV_1 (forced expiratory volume in 1 second) remained, however, unchanged during the trial period (45). In a post-hoc analysis of this study, a pre-specified subgroup of COPD patients, GOLD stage II patients treated only with tiotropium bromide, had a lower risk of exacerbations, but also a reduction in the rate of decline in post-bronchodilator FEV_1 over the 4 year period compared to placebo (46). In addition, a subgroup of young patients with COPD showed a significant reduction in the decline in post-bronchodilator $FEV_{1)}$ when treated with tiotropium on top of usual care (47). This may suggest that tiotropium has disease modifying properties in younger patients with COPD and in GOLD stage II patients. In support, regardless of the smoking status of the patient, the long-term benefits of treatment with tiotropium sustained (48). Moreover, exacerbations accelerate disease progression. Recently, tiotropium has also been proven to improve the lung function in patients with severe uncontrolled asthma (49). The mechanisms behind these effects are still unknown. However, muscarinic receptor induced airway remodelling, as demonstrated in the current and in other studies, could be involved. Indeed, a recent trial showed that repeated inhalations with the muscarinic receptor agonist methacholine induces airway remodelling in asthma patients, including the expression of TGF-β and collagen I in bronchial biopsies (50). This substantiates the hypothesis that cholinergic activation leads to remodelling via cross-talk with TGF-β in these patients. These effects were suggested by the authors to result from mechanical forces resulting from the bronchoconstriction itself. However, functional cross-talk of MCh and TGF-β on structural cells, resulting in remodelling cannot be ruled out.

In conclusion, our results indicate that the stimulation of muscarinic receptors enhances the expression of contractile phenotype marker proteins induced by TGF-β_1. These findings provide a plausible mechanistic explanation for our earlier observations that demonstrate protective effects of tiotropium on ASM remodelling in repeatedly allergen challenged guinea pigs. This implies a role for G protein coupled receptors in TGF-β_1-induced remodelling in the pathogenesis of chronic airway diseases like asthma and COPD.

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4 Crosstalk between TGF-β_1 and muscarinic M_2 receptor augments airway smooth muscle proliferation

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Abstract

Transforming growth factor-β_1 (TGF-β_1) is a central mediator in tissue remodelling processes, including fibrosis and airway smooth muscle (ASM) hyperplasia as observed in asthma. The mechanisms underlying this response, however, are unclear as TGF-β_1 has only weak mitogenic effects on ASM cells. In this study we hypothesized that the mitogenic effect of TGF-β_1 on ASM is indirect and requires prolonged exposure to allow extracellular matrix (ECM) deposition. To address this hypothesis, we investigated the effects of acute and prolonged treatment with TGF-β_1, alone and in combination with the muscarinic receptor agonist methacholine on human ASM cell proliferation. Acutely, TGF-β_1 had no mitogenic effect; however, prolonged treatment (7 days) with TGF-β_1 increased ASM cell proliferation and potentiated the PDGF-induced mitogenic response. Muscarinic receptor stimulation with methacholine synergistically enhanced the effect of TGF-β_1. Interestingly, the integrin-blocking peptide RGDS (Arg-Gly-Asp-Ser) as well as integrin α5β1 function-blocking antibodies inhibited the effects of TGF-β_1 and its combination with methacholine on cell proliferation. Accordingly, prolonged treatment with TGF-β_1 increased fibronectin expression, which was also synergistically enhanced by methacholine. The synergistic effects of methacholine on TGF-β_1-induced proliferation were reduced by the long-acting muscarinic receptor antagonist tiotropium and the M_2 receptor subtype selective antagonist gallamine, but not the M_3 selective antagonist DAU5884. In line with these findings, the irreversible G_i-protein inhibitor pertussis toxin also prevented the potentiation of TGF-β_1-induced proliferation by methacholine. We conclude that prolonged exposure to TGF-β_1 enhances ASM cell proliferation, which is mediated by ECM-integrin interactions and can be enhanced by muscarinic M_2 receptor stimulation.

4.1 Introduction

The pleiotropic growth factor transforming growth factor-β (TGF-β) is widely synthesized throughout the body and regulates a variety of cellular responses contributing to morphogenesis, embryonic development, inflammation and wound healing (1). Moreover, abnormal TGF-β regulation and expression in disease may result in fibrosis, characterized by the excessive deposition of

extracellular matrix (ECM) proteins (2). Traditionally, (myo)fibroblasts are perceived as the major source of ECM proteins; however, smooth muscle cells have also been identified as a rich source of these extracellular components (3, 4). Exposure of airway smooth muscle (ASM) cells to TGF-β increases the expression of various ECM proteins, including collagens, proteoglycans and fibronectin (3, 5, 6). In addition to their function as a scaffold, ECM proteins also modulate a variety of cellular functions, including migration, differentiation and proliferation. For example, ASM cells cultured on fibronectin or collagen type I matrices show enhanced mitogenic responses to growth factors such as platelet-derived growth factor (PDGF) (7-12). Interaction of these cells with the ECM occurs mainly through integrins, a group of heterodimeric transmembrane glycoproteins. Studies on the role of integrins in smooth muscle cell proliferation have indicated an important role for these ECM receptors; e.g. both angiotensin II- and PDGF-induced proliferation of vascular smooth muscle cells requires signalling through α5β1 integrins (7, 13). In addition, proliferation of human ASM cells can be mediated by integrins as well, in particular by the α5β1 integrins (9, 14, 15).
Various studies have addressed the mitogenic properties of TGF-β on smooth muscle proliferation. *In vivo* studies demonstrated that overexpression of TGF-β_1 in mice increases ASM mass, whereas allergen-induced increases in ASM mass in a murine model of asthma could be prevented by anti-TGF-β_1 antibodies (16-18). *In vitro* studies, however, have shown that TGF-β_1 has variable mitogenic properties on ASM cells (19-23).
In addition to receptor tyrosine kinases, serine threonine kinase receptors and integrins, G-protein coupled receptors (GPCR), including muscarinic receptors, have been associated with cell proliferation (24, 25). In bovine and human ASM cells, muscarinic M_3 receptor stimulation enhanced the mitogenic responses to PDGF and EGF (26, 27). ASM cell proliferation, leading to ASM thickening, is an important contributor to airway remodelling as observed in airway diseases (28, 29). *In vivo* animal models of asthma showed that muscarinic receptor stimulation also contributes to allergen-induced increase in ASM mass (30-32). Moreover, airway fibrosis could be prevented by treatment with the muscarinic receptor antagonist tiotropium bromide (32, 33). Interestingly, Matthiesen *et al*. and Haag *et al*. found that muscarinic M_2 receptor stimulation increases proliferation and collagen deposition by human lung fibroblasts *in vitro* (34, 35). Overall, these data suggest that muscarinic receptor stimulation plays a role in airway fibrosis and

ASM remodelling, which may involve both muscarinic M_2 and muscarinic M_3 receptors.

Collectively, a role for TGF-β and G-protein coupled receptors like muscarinic receptors in the induction of smooth muscle cell proliferation via autocrine production of ECM proteins and subsequent activation of integrins may be envisaged. In the present study, we hypothesized that exposure of human ASM cells to TGF-β_1 enhances the deposition of ECM proteins, resulting in the subsequent induction of proliferation of these cells via activation of integrins. Furthermore, we hypothesized that these processes may be reinforced by muscarinic receptor stimulation. Our results for the first time demonstrate that prolonged exposure to TGF-β_1 is followed by ASM cell proliferation, which occurs via deposition of ECM proteins such as collagen type I and fibronectin and subsequent activation of the α5β1 integrin. Combined treatment with the muscarinic receptor agonist methacholine synergistically enhanced fibronectin expression and ASM cell proliferation, via a mechanism involving the muscarinic M_2 receptor.

4.2 Materials and methods

4.2.1 Materials

Active, recombinant human TGF-β_1 was purchased at R&D systems (Abingdon, UK). Dulbecco's Modified Eagle's Medium (DMEM), foetal bovine serum (FBS), streptomycin, penicillin and amphotericin B were from Gibco BRL Life Technologies (Paisley, UK). Methacholine chloride was from ICN Biomedicals (Zoetermeer, The Netherlands). Horseradish peroxidase (HRP)-conjugated rabbit anti-mouse antibodies, HRP-conjugated rabbit anti-goat antibodies, human platelet-derived growth factor (PDGF)-AB, fibronectin (human plasma), gallamine and pertussis toxin (PTX) were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). Anti-GAPDH and anti-fibronectin (C-20) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). From Southern Biotech (ITK Diagnostics BV, Uithoorn), the anti-collagen I antibody was purchased. RGDS (H-Arg-Gly-Asp-Ser-OH) and GRADSP (H-Gly-Arg-Ala-Asp-Ser-Pro-OH) were purchased at Calbiochem (Nottingham, UK). Monomeric collagen type I (calf skin) was obtained from Fluka (Buchs, Switzerland). [*methyl*-^{3}H]-thymidine (0.25 µCi/mL) was purchased at Amersham (Buckingshamshire, UK). Tiotropium bromide and

DAU5884 were provided by Boehringer Ingelheim (Ingelheim, Germany). All other chemicals were of analytical grade.

4.2.2 Airway smooth muscle cell culture

Human bronchial smooth muscle cell lines, immortalized by stable expression of human telomerase reverse transcriptase (hTERT), were used for all experiments. hTERT airway smooth muscle cells were generated from primary cultured human bronchial smooth muscle cells as described previously (**chapter 2**). All procedures were approved by the Human Research Ethics Board of the University of Manitoba. Cells were grown to confluence on uncoated culture dishes using DMEM supplemented with 10% foetal bovine serum and antibiotics (50 U/mL streptomycin, 50 µg/mL penicillin and 1.5 µg/mL amphotericin B). Cultures were maintained in a humidified incubator at 37°C, gassed with 95% O_2 and 5% CO_2, Culture medium was replaced every 2-3 days.

4.2.3 Preparation of whole cell lysates

Cells were plated in 6-well plates and grown until confluence. After serum deprivation for 24 hours in DMEM supplemented with antibiotics, the cells were stimulated with TGF-β_1 (2 ng/mL) alone or in combination with the muscarinic receptor agonist methacholine (10 µM) for 7 days. Culture medium was refreshed after 4 days of stimulation. To obtain whole cell lysates, stimulated cells were washed once with ice-cold phosphate-buffered saline (PBS, composition: 140 mM NaCl, 2.6 mM KCl, 1.4 mM KH_2PO_4, 8.1 mM Na_2HPO_4, pH 7.4), followed by lysis using cold SDS-lysis buffer (composition: 62.5 mM Tris-HCl, 2% SDS, 1 mM NaF, 1 mM Na_3VO_4, 10 µg/mL aprotinin, 10 µg/mL leupeptin, 10 µg/mL pepstatin A, pH 8.0).

4.2.4 Western Blotting

Equal amounts of protein were separated on SDS polyacrylamide gels and transferred onto nitrocellulose. To avoid non-specific binding, membranes were blocked with blocking buffer (composition: 50 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20, 5% non-fat dried milk powder) for 1 hour at room temperature. Subsequently, the membranes were incubated with the primary antibodies diluted in blocking buffer for 1 hour at room temperature. After washing the membranes 3 times with 0.1% Tris Buffered Saline Tween-20 (0.1% TBS-T composition: 50 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20) for 10 min,

membranes were incubated with HRP-labelled secondary antibodies for 1 hour at room temperature, followed by an additional 3 washes with 0.1% TBS-T. By using enhanced chemiluminescence reagents, bands were recorded in the G:BOX iChemi gel documentation system equipped with GeneSnap image acquisition software (Syngene; Cambridge; UK). Band intensities were quantified by densitometry using GeneTools analysis software (Syngene; Cambridge; UK). All bands were normalized to GAPDH.

4.2.5 Coating of culture plates with extracellular matrix proteins

Monomeric collagen I was reconstituted in hydrochloric acid (10 mM) at 5 mg/mL. ECM-coated culture plates were prepared by covering 24-well culture plates with dilutions of collagen I (50 µg/mL, diluted in PBS) or fibronectin (10 µg/mL, diluted in PBS). ECM proteins were absorbed overnight and air-dried at room temperature. Unoccupied protein-binding sites were blocked using sterile 0.1% BSA in PBS for 30 min. Subsequently, plates were washed twice with plain DMEM and were dried before further use.

4.2.6 [m*ethyl*-^{3}H]-thymidine incorporation

Two different protocols were used to investigate DNA synthesis in ASM cells; an acute treatment and a prolonged treatment protocol (Figure 5.1A).

For the acute treatment protocol, ASM cells (20 000 cells/well) were plated on uncoated or ECM-coated 24-well plates and allowed to attach overnight. Subsequently, cells were serum deprived for 72 hours in DMEM supplemented with antibiotics and 1% ITS (5 µg/mL insulin, 5 µg/mL transferrin and 5 ng/mL selenium). After serum-deprivation, cells were stimulated with TGF-β_1 (2 ng/mL), PDGF-AB (10 ng/mL) or methacholine (10 µM) for 28 hours, the last 24 hours in the presence of [*methyl*-^{3}H]-thymidine.

For the prolonged treatment, ASM cells (20 000 cells/well) were plated on uncoated 24-well plates and allowed to attach overnight. Subsequently, cells were serum deprived for 24 hours in DMEM supplemented with antibiotics. After serum deprivation, cells were treated with TGF-β_1, alone or in combination with methacholine (10 µM) for 7 days. Subsequently, cells were washed and stimulated in the absence or presence of PDGF-AB (10 ng/mL) for 28 hours, the last 24 hours in the presence of [*methyl*-^{3}H]-thymidine.

After stimulation, cells were washed twice with PBS at room temperature and incubated on ice with ice-cold trichloroacetic acid (5%) for 30 min. The acid-

insoluble fraction was dissolved in 1 mL NaOH (1 M). Incorporated [*methyl*-^{3}H]-thymidine was quantified by liquid-scintillation counting using a Beckman LS1701 β-counter.

To investigate the involvement of integrins in TGF-β1-induced cell proliferation, cells were incubated 30 min before and during stimulation with the integrin-blocking peptide RGDS (100 μM) or its negative control GRADSP (100 μM); or with integrin function-blocking monoclonal anti-α5 (10 mg/mL, clone P1D6; Chemicon) and anti-β1 (10 mg/mL, clone 6S6; Chemicon, Chemicon, Chandler's Ford, UK) antibodies or mouse IgG control antibodies (10 mg/ml; Chemicon). Furthermore, to investigate the muscarinic receptor subtype involved, cells were incubated 30 min before and during stimulation with selective muscarinic receptor antagonists (gallamine (10 μM), DAU5884 (100 nM), tiotropium bromide (10 nM)). G_i proteins were inactivated by overnight incubation with pertussis toxin (PTX, 100 ng/mL) for 16 hours before stimulation. Newly synthesized G_i proteins were inhibited by incubation with PTX (50 ng/mL) during stimulation.

4.2.7 RNA isolation and real-time quantitative RT-PCR

Cells were plated in 6-well plates and grown until confluence. After serum deprivation for 24 hours in DMEM supplemented with antibiotics, the cells were stimulated with TGF-$β_1$ (2 ng/mL), alone or in combination with methacholine (10 μM) for 1 or 7 days. Culture medium was refreshed after 4 days of stimulation. Total cellular RNA was isolated using the Nucleospin RNA II kit (Machery-Nagel, Bioke, Leiden, The Netherlands). RNA concentration was determined by Nanodrop ND1000 (Thermo Scientific, Wilmington, MA). Total RNA was reverse transcribed using the Promega cDNA synthesis kit. Real-time quantitative PCR for fibronectin, collagen I α1, integrin β1 and integrin α5 was performed using an Illumina Eco Personal qPCR System (Westburg, Leusden, The Netherlands) using the specific primers listed in Table 4.1. Cycle parameters were: denaturation at 94°C for 30 seconds, annealing at 58°C for 30 seconds and extension at 72°C for 30 seconds for 40 cycles. The abundance of the target gene was normalized to the endogenous reference 18S rRNA (designated as ΔCq). Relative differences were determined by using the equation $2^{-(\Delta\Delta Cq)}$.

Table 4.1. Primer sequences of fibronectin, collagen I α1, integrin β1, integrin α5 and 18S rRNA used for real-time quantitative PCR.

Substance	Sequences	
Fibronectin	Forward	5'- TCGAGGAGGAAATTCCAATG -3'
	Reverse	5'- ACACACGTGCACCTCATCAT -3'
Collagen Iα1	Forward	5'- AGCCAGCAGATCGAGAACAT-3'
	Reverse	5'- TCTTGTCCTTGGGGTTCTTG-3'
Integrin α5	Forward	5'- GGAACTCAGATCCAGGACA -3'
	Reverse	5'- CATGTCTGGCCCAAAGAACT-3'
Integrin β1	Forward	5'- CCCTTGCACAAGTGAACAGA -3'
	Reverse	5'- TCTTGTCCTTGGGGTTCTTG -3'
18S rRNA	Forward	5’-CGCCGCTAGAGGTGAAATTC-3’
	Reverse	5’-TTGGCAAATGCTTTCGCTC-3’

4.3 Results

4.3.1 Mitogenic properties of TGF-β_1 in human ASM cells

To elucidate the mitogenic effects of TGF-β_1 on ASM cell proliferation, human ASM cells were treated with TGF-β_1 (2 ng/mL) for 1 or 7 days (Figure 4.1A). DNA synthesis was determined using a [^{3}H]-thymidine incorporation assay. After 1 day of treatment, TGF-β_1 did not enhance DNA synthesis in these cells, in contrast to the mitogen PDGF (10 ng/mL) (Figure 4.1B). However, prolonged treatment (7 days) with TGF-β_1 significantly ($p< 0.05$) increased DNA synthesis. After 7 days, PDGF still induced DNA synthesis, which was significantly enhanced in cells pre-treated with TGF-β_1 ($p< 0.001$, Figure 4.1C). A mitochondrial reduction assay and cell number determination confirmed these findings, as we observed a time-dependent increase in cell number when cells were stimulated with TGF-β_1 for up to 7 days (Table 4.2). Collectively, these results indicate that the TGF-β_1-induced proliferation of human ASM cells is delayed in its onset and requires prolonged TGF-β_1 treatment, which can be enhanced by PDGF.

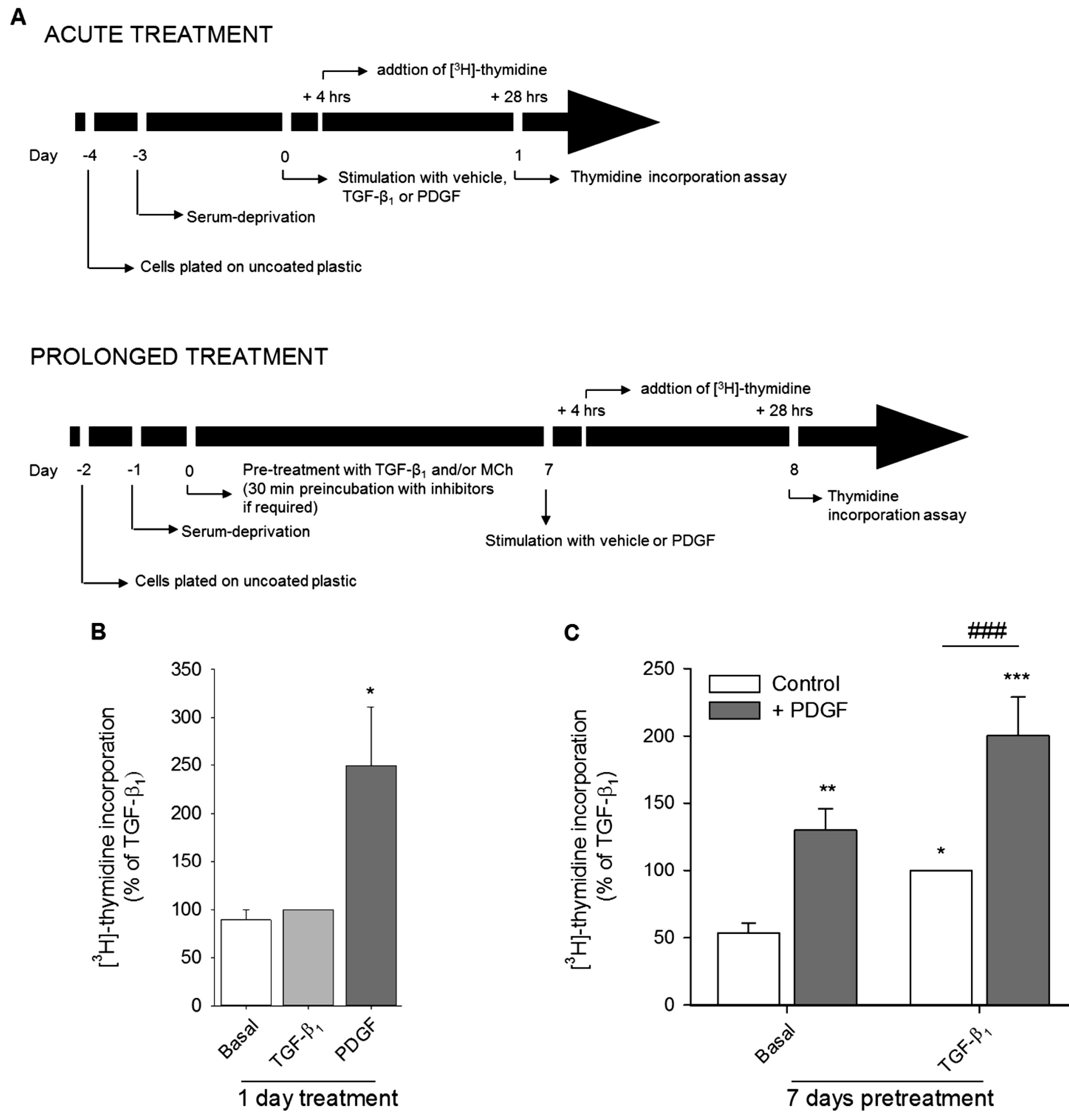

Figure 4.1. Mitogenic properties of TGF-β$_1$ in human ASM cells. Effects of TGF-β$_1$ on DNA synthesis in human ASM cells. Two different protocols were used to investigate the effects of TGF-β$_1$ on ASM proliferation. In the acute treatment protocol, ASM cells were plated and allowed to attach overnight before serum deprivation in the presence of 1% ITS for 3 days. Subsequently, cells were treated with serum-free medium, TGF-β$_1$ or PDGF-AB for 28 hours, the last 24 hours in the presence of [methyl-^{3}H]-thymidine. In the prolonged treatment protocol, ASM cells were plated and allowed to attach overnight after which

serum deprivation occurred for 1 day. Subsequently, cells were treated with serum-free medium or TGF-β_1 for 7 days. Cells were then washed and incubated with serum-free medium or PDGF-AB for 28 hours, the last 24 hours in the presence of [methyl-^{3}H]-thymidine. After both protocols thymidine incorporation was determined as described in the Materials and Methods section. (B) Effect of acute treatment with TGF-β_1 on ASM proliferation. Human ASM cells were treated with serum-free medium (basal), TGF-β_1 (2 ng/mL) or PDGF-AB (10 ng/mL), according to the acute DNA synthesis treatment protocol (Figure 4.1A). Data represent means ± s.e.m. of 7 experiments, each performed in triplicate. *p< 0.05 compared to basal (one-way ANOVA, post-hoc Newman-Keuls). (C) Effect of prolonged treatment with TGF-β_1 on ASM proliferation. Human ASM cells were treated with serum-free medium (basal) or TGF-β_1 (2 ng/mL) for 7 days after which cells were stimulated with serum-free medium (control) or PDGF-AB (10 ng/mL), according the prolonged treatment DNA synthesis protocol (Figure 4.1C). Data represent means ± s.e.m. of 9 experiments, each performed in triplicate. *p< 0.05, **p< 0.01, ***p< 0.001 compared to basal control. $^{###}$p< 0.001 compared to TGF-β_1 control (one-way ANOVA, post-hoc Newman-Keuls).

4.3.2 Mitogenic properties of TGF-β_1 are dependent on extracellular matrix protein production and integrins

The delay in onset of the mitogenic response of TGF-β_1 on human ASM cells could involve ECM production, therefore, we stimulated human ASM cells with TGF-β_1 and determined the expression of collagen type I and fibronectin by Western analysis. TGF-β_1 significantly (p< 0.01) increased the expression of collagen I and fibronectin protein after 7 days treatment (Figure 4.2A). In line, an increase of collagen I α1 and fibronectin mRNA was observed, both after 1 and after 7 days of TGF-β_1 stimulation (Figure 4.2A-B), whereas after 1 day of stimulation a significant increase in protein expression was observed for fibronectin, but not for collagen I (Figure 4.2B). ECM proteins such as fibronectin and collagen have been reported to increase basal ASM cell proliferation (8, 9). In addition, they may interact with growth factors like PDGF to enhance their mitogenic properties (9, 11). To investigate whether the enhanced proliferation was (partly) due to synergism between the produced ECM and TGF-β_1 itself, human ASM cells were plated on uncoated, collagen I- or fibronectin-coated plates and stimulated with TGF-β_1. As observed previously (8, 9), both collagen I and fibronectin increased ASM cell proliferation (p< 0.01). However, TGF-β_1 had no further effects on the proliferation induced by these ECM proteins (Figure 4.2C), suggesting that the mitogenic properties of TGF-β_1 might be indirect and could involve autocrine effects of collagen I and/or fibronectin.

ECM proteins may enhance ASM cell proliferation through binding of integrin receptors (9, 15). Integrins are heterodimeric transmembrane glycoproteins, which interact with specific sequences in ECM proteins. The α5β1 integrin has been shown to be importantly involved in collagen I- and fibronectin-induced smooth muscle cell proliferation, which can be inhibited by the integrin blocking peptide Arg-Gly-Asp-Ser (RGDS) (9, 13-15, 39). Using the RGDS peptide (100 µM), we investigated whether the TGF-β_1-induced proliferation could be due to the production of collagen I and fibronectin and their subsequent interaction with integrins on the ASM cell. We observed that treatment with RGDS reduced DNA synthesis induced by prolonged TGF-β_1 treatment (Figure 4.2D), whereas its negative control Gly-Arg-Ala-Asp-Ser-Pro (GRADSP, 100 µM) had no effect. Similarly, function-blocking anti-α5 and anti-β1 antibodies also fully blocked the TGF-β_1-induced proliferation (Figure 4.2E), indicating a key role for the major fibronectin binding α5β1 integrin in these effects. To investigate whether the TGF-β_1-induced ASM cell proliferation could also involve an increase in α5β1 integrin expression, we assessed the mRNA expression of the integrin α5 and β1 subunits. No effects of TGF-β_1 were observed on the mRNA expression of the α5 subunit. However, an increase in mRNA levels of the integrin subunit β1 was observed after 7 days of treatment with TGF-β_1 ($p < 0.001$), whereas a trend towards an increased mRNA expression was observed after 1 day ($p < 0.07$, Figure 4.2F). Collectively, these results indicate that TGF-β_1 enhances ASM proliferation primarily through the autocrine effects of ECM proteins such as collagen I and fibronectin on the α5β1 integrin, which could possibly be reinforced by enhanced expression of the integrin β1 subunit.

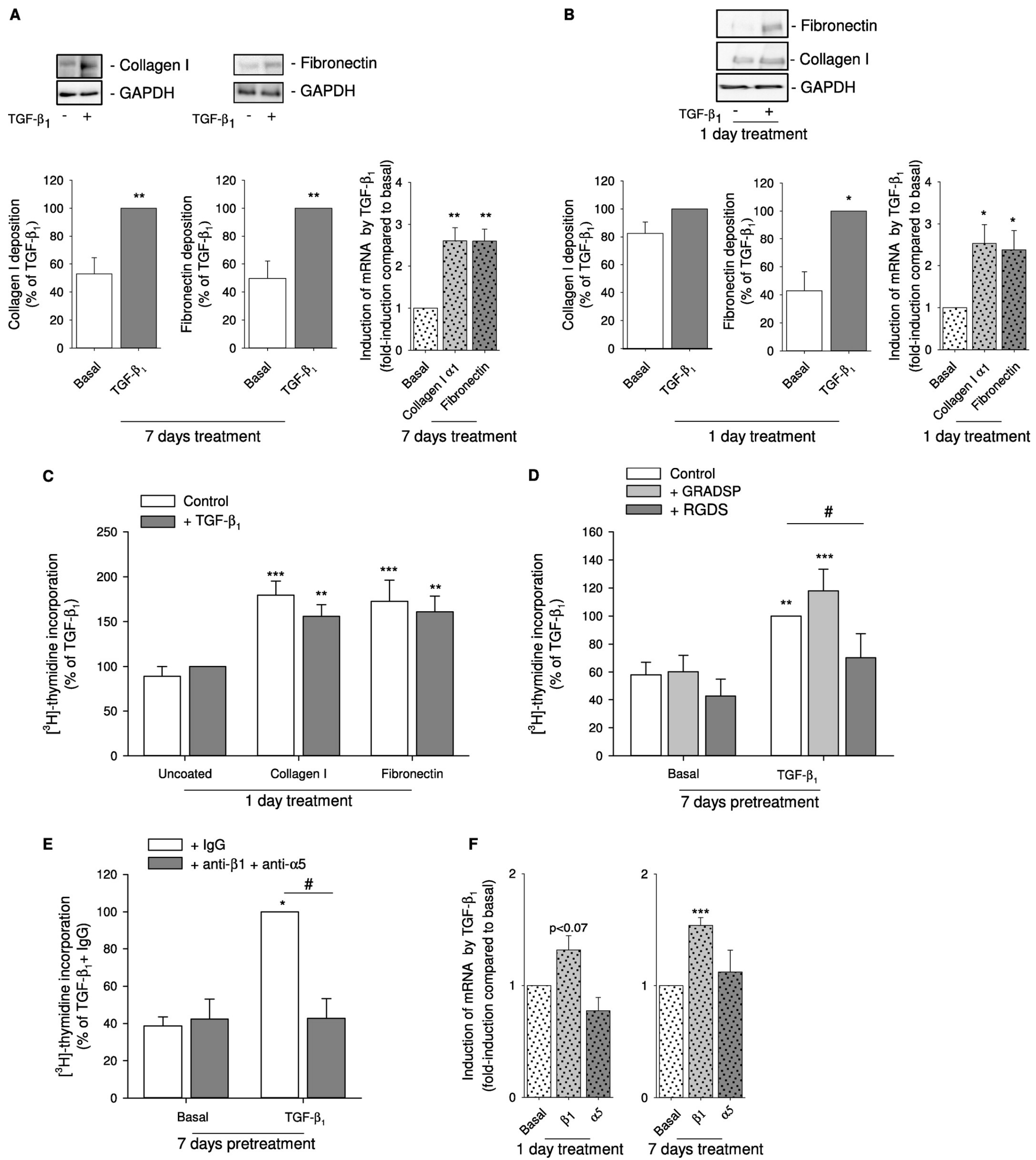

Figure 4.2. Involvement of extracellular matrix proteins in the mitogenic properties of TGF-β$_1$. (A) Human ASM cells were treated with serum-free medium (basal) or TGF-β$_1$ (2 ng/mL) for 7 days. Protein expression and mRNA levels of collagen I and fibronectin was

determined by Western analysis. Representative blots of collagen I, fibronectin and GAPDH are shown. Data represent means ± s.e.m. of 6-8 experiments. **$p < 0.01$ compared to basal (Student's t-test). (B) Protein expression of collagen I and fibronectin after 1 day of stimulation and mRNA levels of collagen I α1 and fibronectin in response to TGF-β_1 after stimulation for 1. Data represent means ± s.e.m. of 3-6 experiments. *$p < 0.05$ compared to basal (Student's t-test). (C) Human ASM cells were plated on uncoated plastic surfaces or on collagen type I- or fibronectin-coated matrices. Subsequently, cells were treated with serum-free medium (control) or TGF-β_1 (2 ng/mL), according to the acute treatment protocol (see Figure 4.1A). Data represent means ± s.e.m. of 7 experiments, each performed in triplicate. *$p < 0.05$, **$p < 0.01$ compared to uncoated control (one-way ANOVA, post-hoc Newman-Keuls). (D) Human ASM cells were treated with serum-free medium (basal) or TGF-β_1 (2 ng/ml) for 7 days, in the absence or presence of the integrin blocking peptide RGDS or its negative control GRADSP (both 100 µM), according to the prolonged treatment protocol (see Figure 4.1A). Data represent means ± s.e.m. of 8 experiments, each performed in triplicate. *$p < 0.05$, ** $p < 0.01$, ***$p < 0.001$ compared to basal control. $^{\#}p < 0.05$ compared to TGF-β_1 control (one-way ANOVA, post-hoc Newman-Keuls). (E) Human ASM cells were treated with serum-free medium (basal) or TGF-β_1 (2 ng/ml) for 7 days, in the absence or presence of the integrin function-blocking monoclonal anti-α5 (10 mg/mL) and anti-β1 (10 mg/mL) antibodies or mouse IgG control antibodies (10 mg/mL), according to the prolonged treatment protocol. Data represent means ± s.e.m. of 6-7 experiments, each performed in duplicate (one-way ANOVA, post-hoc Newman-Keuls) *$p < 0.05$ compared to basal control. $^{\#}p < 0.05$ compared to TGF-β_1 control. (F) mRNA levels of integrin subtypes β1 and α5 relative to basal expression of these subtypes after stimulation with TGF-β_1 for 1 and 7 days. Data represent means ± s.e.m. of 6 experiments (one-way ANOVA, post-hoc Newman-Keuls). ***$p < 0.001$ compared to basal.

4.3.3 Muscarinic receptor stimulation enhances TGF-β_1-induced ASM cell proliferation

To assess the functional interaction between TGF-β_1 and muscarinic receptor stimulation at the level of cell proliferation, ASM cells were pre-treated with TGF-β_1 (2 ng/mL), methacholine (10 µM) or their combination for 7 days. As described above, TGF-β_1 treatment significantly ($p < 0.001$) increased DNA synthesis after 7 days of treatment. Interestingly, this increase was significantly augmented by co-treatment with the muscarinic receptor agonist methacholine ($p < 0.001$), whereas no effect of methacholine was observed in the absence of TGF-β_1 (Figure 4.3A). Similar results were observed for cell number (Table 4.2).

Table 4.2. Proliferative responses of human ASM cells in the absence of TGF-β_1 (2 ng/mL) and/or methacholine (10 µM) after 7 days of incubation.

	Control		Methacholine	
	Basal	+ TGF-β_1	Basal	+ TGF-β_1
[^{3}H]-thymidine-incorporation	58 ± 9	100 ± 0***	60 ± 11	149 ± 10***$^{,###}$
Alamar Blue conversion	72 ± 1	100 ± 0***	74 ± 1	115 ± 6***$^{,##}$
Cell number	64 ± 6	100 ± 0**	70 ± 7	132 ± 6***$^{,##}$

Data are expressed as percentage of TGF-β_1-induced responses. Data represent means ± s.e.m. of 4-9 experiments performed I triplicate ([^{3}H]-thymidine-incorporation) or duplicate (Alamar Blue conversion and cell number). **p< 0.01, ***p< 0.001 compared to control. $^{##}$p< 0.01, $^{###}$p< 0.001 compared to TGF-β_1 control.

To investigate whether muscarinic receptor stimulation could amplify the mitogenic properties of the TGF-β_1-induced ECM proteins, ASM cells were plated on collagen I- or fibronectin-coated beds and treated with methacholine (10 µM) for 1 day. Stimulation with methacholine did not enhance the mitogenic effects of the ECM proteins (Figure 4.3B), indicating that the interaction between TGF-β_1 and muscarinic receptor stimulation was not due to a direct interaction between muscarinic receptors and collagen type I or fibronectin.

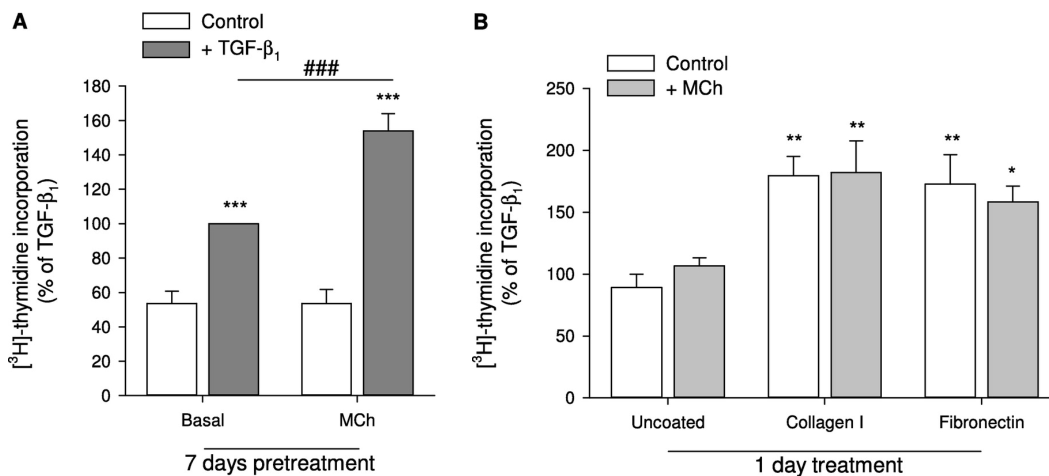

Figure 4.3. Muscarinic receptor stimulation enhances mitogenic properties of TGF-β_1. (A) Human ASM cells were treated with serum-free medium (basal), TGF-β_1 (2 ng/mL), methacholine (MCh, 10 µM) or their combination for 7 days, according to the prolonged treatment protocol (see Figure 4.1A). Data represent means ± s.e.m. of 9 experiments, each performed in triplicate. ***p< 0.001 compared to basal control. $^{###}$p< 0.001

compared to TGF-β_1 control (one-way ANOVA, post-hoc Newman-Keuls). (B) Human ASM cells were plated on uncoated plastic surfaces or on collagen I- or fibronectin-coated matrices and serum deprived for 3 days. Subsequently, cells were treated with serum-free medium or methacholine (10 μM), according to the acute treatment protocol. Data represent means ± s.e.m. of 7 experiments, each performed in triplicate (one-way ANOVA, post-hoc Newman-Keuls). *$p < 0.05$, **$p < 0.01$ compared to uncoated control.

Therefore, we investigated whether muscarinic receptor stimulation enhanced ECM deposition induced by TGF-β_1. ASM cells were treated with TGF-β_1 (2 ng/mL), methacholine (10 μM) or their combination for 7 days. Subsequently collagen I and fibronectin expression were measured by Western analysis. Interestingly, although TGF-β_1 clearly increased the expression of both collagen I and fibronectin, methacholine selectively increased the TGF-β_1-induced protein expression of fibronectin, while methacholine was ineffective by itself (Figure 4.4A). This suggests that the enhanced deposition of fibronectin might be involved in the functional interaction between TGF-β_1 and muscarinic receptor stimulation. To further substantiate this hypothesis, ASM cells were treated with or without RGDS while stimulated with TGF-β_1, methacholine or their combination for 7 days. RGDS blocked the enhanced mitogenic response induced by the co-stimulation with TGF-β_1 and methacholine (Figure 4.4B), whereas no effects were found for GRADSP (data not shown). Furthermore, function-blocking anti-α5 and anti-β1 antibodies significantly reduced the enhanced DNA synthesis induced by TGF-β_1 and methacholine (Figure 4.4C). In line with an increased activation of the α5β1 integrin, no effects of methacholine were observed on the mRNA expression of α5 and β1 integrins either, indicating that the synergistic effect of methacholine on TGF-β_1-induced proliferation cannot be explained by increased integrin abundance (Figure 4.4D). Collectively, these data indicate that the synergistically enhanced deposition of fibronectin and α5β1 activation in response to muscarinic receptor and TGF-β_1 stimulation importantly contributes to their functional interaction on ASM proliferation.

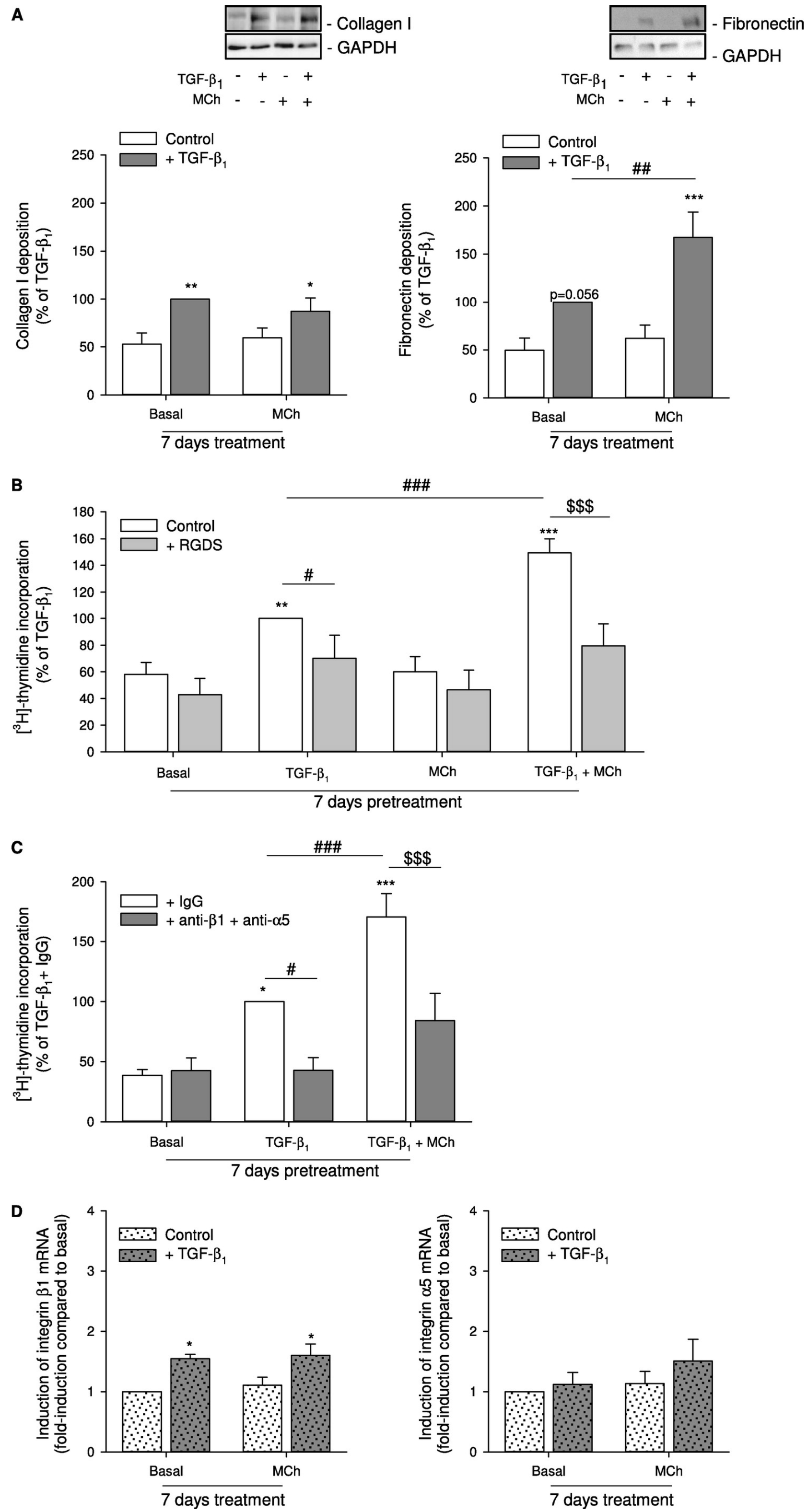
A
- Collagen I
- GAPDH
TGF-β1
MCh
- Fibronectin
- GAPDH
Control
+ TGF-β1
Collagen I deposition (% of TGF-β1)
Fibronectin deposition (% of TGF-β1)
p=0.056
Basal
MCh
7 days treatment
B
Control
+ RGDS
[3H]-thymidine incorporation (% of TGF-β1)
Basal
TGF-β1
MCh
TGF-β1 + MCh
7 days pretreatment
C
+ IgG
+ anti-β1 + anti-α5
[3H]-thymidine incorporation (% of TGF-β1+ IgG)
Basal
TGF-β1
TGF-β1 + MCh
7 days pretreatment
D
Control
+ TGF-β1
Induction of integrin β1 mRNA (fold-induction compared to basal)
Induction of integrin α5 mRNA (fold-induction compared to basal)
Basal
MCh
7 days treatment

Figure 4.4. Involvement of extracellular matrix proteins in the enhanced mitogenic response induced by muscarinic receptor stimulation in the presence of TGF-β_1. (A) Human ASM cells were treated for 7 days with serum-free medium (basal), TGF-β_1 (2 ng/mL), methacholine (MCh, 10 µM) or their combination. Protein expression of collagen I and fibronectin was determined by Western analysis. Representative blots of collagen I, fibronectin and GAPDH are shown. Data represent means ± s.e.m. of 7-8 experiments (one-way ANOVA, post-hoc Newman-Keuls). (B) Human ASM cells were treated with serum-free medium (basal), TGF-β_1 (2 ng/ml), methacholine (10 µM) or their combination for 7 days, in the absence or presence of the integrin blocking peptide RGDS (100 µM), according to the prolonged treatment protocol. Data represent means ± s.e.m. of 8 experiments, each performed in triplicate (one-way ANOVA, post-hoc Newman-Keuls). (C) Human ASM cells were treated with serum-free medium (basal), TGF-β_1 (2 ng/ml), or TGF-β_1 (2ng/mL) + methacholine (10 µM) for 7 days, in the absence or presence of the integrin function-blocking monoclonal anti-α5 (10 mg/mL) and anti-β1 (10 mg/mL) antibodies or mouse IgG control antibodies (10 mg/mL), according to the prolonged treatment protocol. Data represent means ± s.e.m. of 6-7 experiments, each performed in duplicate (one-way ANOVA, post-hoc Newman-Keuls). (D) mRNA levels of integrins subtypes β1 and α5 after stimulation with TGF-β_1 and methacholine for 1 and 7 days. Data represent means ± s.e.m. of 6 experiments (one-way ANOVA, post-hoc Newman-Keuls).*p< 0.05, **p< 0.01, ***p< 0.001 compared to basal control. $^{\#}$p< 0.05, $^{\#\#}$p< 0.01, $^{\#\#\#}$p< 0.001 compared to TGF-β_1 control. $^{\$\$\$}$p< 0.001 compared to TGF-β_1 + methacholine.

4.3.4 Muscarinic M_2 receptors are responsible for the cross-talk with TGF-β_1

We have previously reported that the enhancement of PDGF-induced ASM cell proliferation by methacholine is mediated by G_q-coupled muscarinic M_3 receptors (26). Distinct signalling pathways are involved in the activation of receptor tyrosine kinases by growth factors and serine/threonine kinase receptors by TGF-β_1 (38, 39). This could implicate that their cross-talk with muscarinic receptors may require different muscarinic receptor subtypes. Therefore, we aimed to investigate the muscarinic receptor subtype involved in the cross-talk between the serine/threonine kinase TGF-β receptor and muscarinic receptor stimulation. To this aim, we stimulated ASM cells with TGF-β_1 (2 ng/ml), methacholine (10 µM) and their combination in absence and presence of the subtype-selective muscarinic receptor antagonists DAU5884 and gallamine for 7 days. The clinically used, non-subtype-selective long-acting muscarinic receptor antagonist tiotropium bromide was also used. Concentrations of the selective antagonists were chosen based on the work of Roffel and coworkers, such that approximately 99% of the M_2 receptors and approximately 8 % of the M_3 receptors were occupied with the muscarinic M_2 receptor selective antagonist gallamine and less than 30% of the M_2 receptors and more than 99% of the M_3 receptors with the

muscarinic M_3 receptor selective antagonist DAU5884 (40-43). DNA synthesis was assessed using a $[^3H]$-thymidine incorporation assay. We observed that both the muscarinic receptor antagonist tiotropium bromide and the muscarinic M_2 receptor antagonist gallamine significantly ($p < 0.01$) reduced the DNA synthesis induced by co-stimulation with TGF-β_1 and methacholine, to levels observed after stimulation with TGF-β_1 alone (Figure 4.5A). No effects were observed for the muscarinic M_3 receptor antagonist DAU5884. In addition, no effects of the antagonists were observed on basal or TGF-β_1-induced proliferation. This indicates that the interaction between TGF-β_1 and muscarinic receptor stimulation was mediated by the muscarinic M_2 receptor.

Since muscarinic M_2 receptors couple to G_i proteins, the contribution of the muscarinic M_2 receptors to TGF-β_1 and methacholine-induced ASM cell proliferation was further assessed using the irreversible G_i protein inhibitor pertussis toxin (PTX). ASM cells were treated with PTX (100 ng/ml) for 16 hours, after which the cells were stimulated with TGF-β_1 (2 ng/mL), methacholine (10 μM) and their combination, in absence and presence of PTX (50 ng/mL) for 7 days. In line with a role for the muscarinic M_2 receptor, we observed that the synergistic effect of methacholine on the TGF-β_1-induced response could be attenuated by PTX treatment, whereas no effects of PTX were observed on any of the other treatments (Figure 4.5B). Collectively, these results demonstrate that cross-talk between G_i coupled muscarinic M_2 receptors and TGF-β_1 enhances ASM cell proliferation.

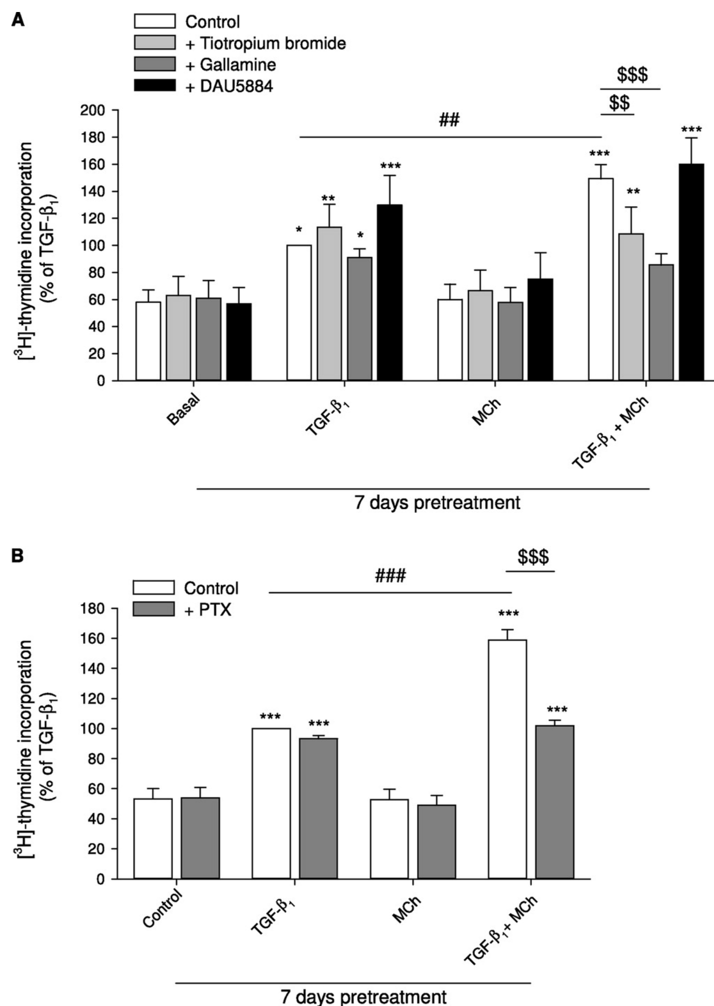

Figure 4.5. Muscarinic M_2 receptors enhance mitogenic properties of methacholine in the presence of TGF-β_1. (A) Human ASM cells were treated with serum-free medium (basal), TGF-β_1 (2 ng/mL), methacholine (MCh, 10 μM) or their combination in absence and presence of the non-selective muscarinic receptor antagonist tiotropium bromide (10 nM), the M_2-selective muscarinic receptor antagonist gallamine (10 μM) or the M_3-selective muscarinic receptor antagonist DAU5884 (100 nM) for 7 days, according to the prolonged treatment protocol. Data represent means ± s.e.m. of 8 experiments, each performed in triplicate (one-way ANOVA, post-hoc Newman-Keuls). (B) Human ASM cells were treated with serum-free medium (basal), TGF-β_1 (2 ng/mL), methacholine (10 μM) or their combination in presence or absence of PTX (100 ng/mL for 16 hours, followed by 50 ng/mL during the entire treatment period) for 7 days, according to the prolonged treatment protocol. Data represent means ± s.e.m. of 8 experiments, each performed in triplicate (one-way ANOVA, post-hoc Newman-Keuls). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to basal control. $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ compared to TGF-β_1 control. $^{\$\$}p < 0.01$, $^{\$\$\$}p < 0.001$ compared to TGF-β_1 + methacholine.

4.4 Discussion

In the current study, we demonstrate that prolonged treatment with TGF-β_1 is required to induce ASM cell proliferation via the enhanced deposition of ECM proteins and the subsequent autocrine activation of the α5β1 integrin. Our data show that TGF-β_1 enhances fibronectin and collagen I expression and that these ECM proteins subsequently interact with RGD-binding integrins, including the α5β1 integrin, to induce ASM cell proliferation. Moreover, TGF-β_1-induced proliferation was synergistically enhanced by muscarinic receptor stimulation, which required increased protein expression of fibronectin, but not collagen type I. Furthermore, we demonstrate that muscarinic M_2 receptors, but not muscarinic M_3 receptors, contribute to this enhancement of TGF-β_1-induced ASM cell proliferation.

The cytokine TGF-β is a ubiquitously expressed and multifunctional cytokine that regulates various processes in the human body, including morphogenesis, inflammation, cell growth, wound healing and fibrosis (1). Studies investigating the effects of TGF-β on smooth muscle indicate that TGF-β is importantly involved in differentiation and maturation. In ASM cells, TGF-β increases the expression of smooth muscle α-actin, calponin and smooth muscle myosin (**chapter 3**, 44, 45). Moreover, maturating ASM cells release higher concentrations of TGF-β, which in turn enhance ASM maturation in an autocrine fashion (46). In addition to its role in the maturation of smooth muscle cells, TGF-β also enhances fibrogenic responses. *In vitro* studies have demonstrated that TGF-β_1 enhances deposition of collagen type I, fibronectin and versican by smooth muscle cells as well (3, 4, 47). TGF-β also contributes to smooth muscle hyperplasia and/or hypertrophy; *in vivo* administration of an anti-TGF-β antibody reduces allergen-induced increases in ASM mass in a mouse model of asthma, which was associated with a decreased ECM deposition in the airway wall (47). Conversely, overexpression of TGF-β in mice increased smooth muscle mass and ECM deposition in the lung (17, 48). Previous *in vitro* studies have indicated that TGF-β could stimulate proliferation of ASM cells directly (19-23). In the current study, we also demonstrated that TGF-β_1 increased ASM proliferation, which required prolonged treatment and was associated with an enhanced production of ECM proteins fibronectin and collagen I. This increase in proliferation after prolonged exposure experiments could not be due to enhanced expression of endogenous PDGF (49) as the cells were washed

before [^{3}H]-thymidine incorporation was assessed. In line with previous studies showing that fibronectin and collagen I enhance both basal and growth factor-induced smooth muscle proliferation, we found that ASM proliferation was increased when cells were grown on fibronectin or collagen type I. Proliferation of ASM cells on these matrices requires multiple β1-integrins, including the α5β1 integrins (9, 15). These α5β1 integrins are also found to be important in the proliferation of vascular smooth muscle cells in response to angiotensin II and PDGF (7, 13). Proliferation in response to these stimuli could be inhibited by the use of integrin-blocking peptides containing the RGD motif, but also by anti-α5 and anti-β1 function-blocking antibodies (9, 13, 37). In the current study, we found that the RGD-binding integrin α5β1 is also importantly involved in the induction of ASM proliferation following prolonged TGF-β_1 treatment. These findings may not only be important *in vitro*, but also *in vivo* as RGD-binding integrins plays a key role in ASM remodelling in a guinea pig model of allergic asthma (9). In line with previous findings (37), we also found an increase in mRNA expression of integrin subtype β1 following TGF-β_1 stimulation. However, mRNA expression of the integrin subtype α5 remained unchanged. Taken together, our findings indicate an important role for TGF-β_1 and autocrine ECM signalling in smooth muscle remodelling, leading to smooth muscle proliferation.

Muscarinic receptors, belonging to the family of G protein-coupled receptors, are associated with contraction of smooth muscle cells in the airways. In addition, proliferation of ASM cells in response to peptide growth factors PDGF and EGF is enhanced by muscarinic receptor stimulation, with an important role for the muscarinic M_3 receptor (26, 27). The functional interactions between the TGF-β receptor serine/threonine kinase and GPCRs, however, are still not well characterized. Currently, we demonstrate that muscarinic receptor stimulation enhanced the mitogenic properties of TGF-β_1 and augmented the deposition of the ECM protein fibronectin, but not collagen I. As reported previously (26, 52), muscarinic receptor stimulation had no mitogenic properties by itself. Similarly, when grown on ECM beds of collagen I and fibronectin, methacholine did not enhance ASM cell proliferation. Using pharmacological antagonists at selective concentrations, we demonstrate that the interaction between TGF-β_1 and methacholine was dependent on the muscarinic G_i-coupled M_2 receptor. This was confirmed using the irreversible Gi protein inhibitor pertussis toxin. Also in human

lung fibroblasts, activation of the muscarinic M_2 receptors induces cell proliferation and collagen deposition (34, 35, 51).

The intracellular signalling pathways involved in the increased ECM deposition in response to muscarinic M_2 receptor and TGF-β receptor stimulation are still largely unknown. However, we have recently demonstrated that combined muscarinic receptor and TGF-β_1 stimulation enhanced the phosphorylation of 4E-BP1 and GSK-3β resulting in the increased expression of contractile proteins, including sm-α-actin and calponin in human ASM cells (**chapter 3**). Furthermore, as fibronectin expression after TGF-β_1 stimulation has been shown to be under the regulation of the GSK3β target β-catenin (3), these observations suggest an important role for the GSK-3 signalling axis in the increased fibronectin expression after TGF-β_1 and methacholine stimulation.

Taken together, these findings suggest that in addition to muscarinic M_3 receptors, also muscarinic M_2 receptors may importantly contribute to remodelling processes, leading to airway fibrosis and ASM remodelling.

ASM remodelling is considered to be a major factor contributing to lung function decline and airway hyperresponsiveness in asthma and chronic obstructive pulmonary disease (COPD) (52, 53). Airway hyperresponsiveness is characterized by increased responsiveness of the airways to pharmacological, chemical and physical stimuli such as methacholine, smoke and cold air (54-56). Acute, variable airway hyperresponsiveness is considered to be dependent on airway inflammation, whereas chronic persistent airway hyperresponsiveness is considered to reflect airway remodelling. The mechanisms underlying ASM remodelling in airway diseases are still incompletely understood, however, inflammation appears to play an important role. In addition, recent studies indicate that also bronchoconstriction may promote airway remodelling, as bronchoconstriction in response to methacholine challenge increases the expression of TGF-β and the deposition of subepithelial collagen in patients with asthma, without increasing inflammation (57). Not only ASM changes, but also ECM changes may contribute to airway responsiveness, as a significant correlation has been found between airway responsiveness and the deposition of collagen and elastin in the airway wall (58, 59). These findings indicate that remodelling characteristics, including the deposition of ECM by ASM cells, may contribute to airway hyperresponsiveness. Interestingly, our findings demonstrated that TGF-β_1 and methacholine induce proliferation of ASM cells via the deposition of the ECM protein fibronectin, which may contribute to allergen-induced airway

hyperresponsiveness and ASM remodelling in asthma (9). In line with these findings, increased ASM mass induced by repeated allergen challenges in guinea pig model of asthma is inhibited by the use of the muscarinic receptor antagonist tiotropium bromide, well-known for its bronchodilator effects (31). In a murine model of asthma, tiotropium bromide not only inhibited smooth muscle thickening but also the expression of TGF-β_1 in the bronchoalveolar lavage (BAL) fluid (32). This relationship between the cholinergic system and TGF-β_1 may in part be explained by the fact that cellular contraction is required for the release and activation of TGF-β from its inactive complex with latency-associated peptide by the αvβ5 integrin on the ASM cell membrane (60). In addition, our current data indicate that in addition to M_3 mediated effects, also M_2 mediated effects may contribute to ASM remodelling.

In summary, our study has demonstrated that TGF-β_1 enhances ASM proliferation by enhancing ECM deposition and the subsequent activation of RGD-binding integrins, in particular the α5β1 integrin. Muscarinic receptor agonists enhance TGF-β_1-induced ASM cell proliferation by the specific induction of fibronectin protein expression via the muscarinic M_2 receptor. Collectively, our data suggest that not only muscarinic M_3 receptors, but also muscarinic M_2 receptors play an important role in processes associated with airway remodelling.

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5 Bronchoconstriction induces TGF-β release and airway remodelling in guinea pig lung slices

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Abstract

Airway remodelling, including smooth muscle remodelling, is a primary cause of airflow limitation in asthma. Recent evidence links bronchoconstriction to airway remodelling in asthma. The mechanisms involved are poorly understood. A possible player is the multifunctional cytokine TGF-β, which plays an important role in airway remodelling. Guinea pig lung slices were used as an *in vitro* model to investigate mechanisms involved in bronchoconstriction-induced airway remodelling. To address this aim, mechanical effects of bronchoconstricting stimuli on contractile protein expression and TGF-β release were investigated. Lung slices were viable for at least 48 h. Both methacholine and TGF-β_1 augmented the expression of contractile proteins (sm-α-actin, sm-myosin, calponin) after 48 h. Confocal fluorescence microscopy showed that increased sm-myosin expression was enhanced in the peripheral airways and the central airways. Mechanistic studies demonstrated that methacholine-induced bronchoconstriction mediated the release of biologically active TGF-β, which caused the increased contractile protein expression, as inhibition of actin polymerization (latrunculin A) or TGF-β receptor kinase (SB431542) prevented the methacholine effects, whereas other bronchoconstricting agents (histamine and KCl) mimicked the effects of methacholine. Collectively, bronchoconstriction promotes the release of TGF-β, which induces airway smooth muscle remodelling. This study shows that lung slices are a useful *in vitro* model to study mechanisms involved in airway remodelling.

5.1 Introduction

Airway remodelling is an important pathological characteristic of chronic asthma (1). Airway remodelling encompasses all structural alterations of the airways, including remodelling of the airway smooth muscle layer which is one of the most striking pathological features of chronic asthma (2). Remodelling of the airway smooth muscle layer has been suggested to be a major cause of airflow obstruction in asthma (3, 4). The mechanisms underlying this pathology are, however, still unclear.

During bronchoconstriction, airways are subjected to mechanical forces, which promote gene expression and growth factor release in resident cells (5). As such,

mechanical forces could promote tissue remodelling. For example, compression of airway epithelial cells leads to features of remodelling, including upregulation of gene expression of TGF-β and protein expression of fibronectin (5-8). Grainge et al. demonstrated that bronchoconstriction induced by repeated methacholine challenges can induce features of airway remodelling in mild asthma patients (9), including an increase in epithelial TGF-β expression and an increase in subepithelial collagen deposition compared to saline-challenged controls. Moreover, we have previously demonstrated that treatment of sensitized guinea pigs with the anticholinergic bronchodilator drug tiotropium inhibits airway remodelling induced by repeated allergen challenge (10, 11). These findings have provided new insights into the causality of the relationship between persistent airflow obstruction and airway remodelling in asthma.

The multifunctional cytokine TGF-β plays an important role in airway remodelling (12). In the airways of asthmatics, this pro-fibrotic cytokine is highly expressed, particularly in epithelial cells and in eosinophils (12). In airway smooth muscle cells, TGF-β_1 can induce proliferation (12, 13, 14) as well as increased expression of contractile protein markers, such as sm-α-actin and calponin through both transcriptional and translational control (**chapter 3**, 13). Activation of serine/threonine kinase receptors by TGF-β_1 will provoke phosphorylation of Smad 2/3 (15), and promotes the nuclear localization of serum response factor, which cooperatively regulate the transcriptional activity for smooth muscle specific genes (16-18). Furthermore, TGF-β_1 can regulate cell proliferation of airway epithelial cells and fibroblasts, but also cell differentiation and the synthesis of extracellular matrix proteins in these cells (19). Although evidence exist that TGF-β is upregulated after mechanical compression of airway epithelial cells and is involved in tissue remodelling, a direct link between bronchoconstriction and TGF-β-induced airway remodelling has not been demonstrated yet.

Precision-cut lung slices have been proven a valuable in vitro tool in pharmacological research and drug development (20). Lung slices have various advantages compared to airway smooth muscle cell cultures, as all lung cell types are present and cell-cell contacts and cell-matrix interactions are preserved, which are important regulators in bronchoconstriction and airway remodelling (21). Additionally, the profound loss of contractile capacity of airway smooth

muscle cells in culture, associated with a loss of sm-myosin expression and contractile receptors, is avoided (22, 23). Moreover, a large number of lung slices can be prepared from the lungs of a single animal, which allows the direct comparison of experimental treatments with a control from the same animal (23). The use of precision-cut lung slices from guinea pigs has additional advantages. For example, Ressmeyer et al., showed that airway responsiveness to methacholine was almost identical for precision-cut lung slices from humans and guinea pigs (23). Moreover, in contrast to other small experimental animals, there are great anatomical and functional similarities between guinea pig and human airways including the presence of small airways (4).

Therefore, in the present study, precision-cut lung slices were used as an in vitro model to study mechanisms of bronchoconstriction-induced airway remodelling. Precision-cut lung slices from guinea pigs were stimulated with contractile agonists such as methacholine, histamine and potassium chloride, but also with the pleiotropic cytokine TGF-β_1. Contractile protein expression was studied in response to these stimuli as a marker of airway remodelling. Using specific inhibitors, the mechanisms inducing an increase in contractile protein expression by bronchoconstriction were studied and were found to be dependent on mechanically-induced release of endogenous TGF-β.

5.2 Materials and methods

5.2.1 Animals

Outbred, male, specified pathogen-free Dunkin Hartley guinea pigs (Harlan, Heathfield, UK) (740 ± 85g) were used. All protocols described in this study were approved by the University of Groningen Committee for Animal Experimentation, Groningen, The Netherlands. The animals were housed under a 12 hour light/dark cycle in a temperature- and humidity-controlled room with food and tap water ad libitum.

5.2.2 Precision-cut lung slices

Precision-cut lung slices were prepared as described in (23). In short, after euthanization by injection with pentobarbital (Euthasol 20%, Produlab Pharma, Raamsdonksveer, the Netherlands) the trachea was cannulated, and the animal

was ex-sanguinated via the aorta abdominalis. Lungs were filled through the cannula with a low melting-point agarose solution (1,5% final concentration (Gerbu Biotechnik GmbH, Wieblingen, Germany) in $CaCl_2$ (0.9mM), $MgSO_4$ (0.4 mM), KCl (2.7 mM), NaCl (58.2 mM), NaH_2PO_4 (0.6 mM), glucose (8.4 mM), $NaHCO_3$ (13 mM), Hepes (12.6 mM), sodium pyruvate (0.5 mM), glutamine (1 mM), MEM-amino acids mixture (1:50), and MEM-vitamins mixture (1:100), pH=7.2). Subsequently, lungs were placed on ice for at least 30 min, in order to solidify the agarose for slicing. Tissue cores were prepared from the lobes using a rotating sharpened metal tube (diameter 15 mm), followed by slicing the tissue in medium composed of $CaCl_2$ (1.8mM), $MgSO_4$ (0.8 mM), KCl (5.4 mM), NaCl (116.4 mM), NaH_2PO_4 (1.2 mM), glucose (16.7 mM), $NaHCO_3$ (26.1 mM), Hepes (25.2 mM), pH = 7.2, using a tissue slicer (Compresstome™ VF-300 microtome, Precisionary Instruments, San Jose CA, USA). Lung slices were cut at a thickness of 500 µm.

5.2.3 Culture medium

Before stimulation, lung slices were incubated individually in 24 well-plates in minimal essential medium composed of $CaCl_2$ (1.8mM), $MgSO_4$ (0.8 mM), KCl (5.4 mM), NaCl (116.4 mM), NaH_2PO_4 (1.2 mM), glucose (16.7 mM), $NaHCO_3$ (26.1 mM), Hepes (25.2 mM), sodium pyruvate (1mM), glutamine (2 mM), MEM-amino acids mixture (1:50), and MEM-vitamins mixture (1:100), pH=7.2, at 37°C in a humid atmosphere. In order to remove the agarose and cell debris from the tissue, medium was refreshed after 30 min, followed by 2 washes every hour. During the experiments, the lung slices were incubated in Dulbecco's Modification of Eagle's Medium (DMEM) supplemented with sodium pyruvate (1 mM), non-essential amino acid mixture (1:100), gentamycin (45 µg/mL), penicillin (100 U/mL), streptomycin (100 µg/mL) and amphotericin B (1.5 µg/mL) at 37°C-5% CO_2.

5.2.4 Lactate dehydrogenase release

To assess the viability of the lung slices, the amount of lactate dehydrogenase (LDH) released from the slices into the incubation medium relative to the total slice content was measured. Slices were incubated in 6 well-plates (2 slices/well) in 4 mL incubation medium for 1, 2, 3 or 4 days. Maximal LDH release was determined by lysing 2 slices with 1% Triton X-100 for 30 min at 37°C at the beginning of the experiment. Supernatants were stored -80 °C. LDH release in the supernatant was determined by routine clinical chemistry at the UMCG

(Groningen, The Netherlands) using the Roche/Hitachi Modular system (Roche, Mannheim, Germany).

5.2.5 Mitochondrial activity assay

Mitochondrial activity, as an additional measure of tissue viability, was assessed by conversion of Alamar blue into its reduced form, as described previously (24). Lung slices were incubated with Hanks' balanced salt solution, containing 10% Alamar blue solution (BioSource, Camarillo, CA), for at least 30 min at 37°C-5% CO_2.

5.2.6 Treatment of lung slices

Lung slices were cultured in 6-well plates, using 2 slices per well. The slices were stimulated with methacholine (10 µM), TGF-β_1 (0.1, 0.2, 1 and 2 ng/mL), KCl (60 mM) or histamine (1 µM) for 1 or 2 days continuously, as indicated. Where mentioned, lung slices were pre-incubated with the inhibitor of actin polymerization latrunculin A (0.3 µM) or the selective inhibitor of the TGF-β type I receptor activin receptor-like kinase ALK5, SB431542 (0.3 µM) for 30 min.

5.2.7 Stimulation of MRC-5 fibroblasts by conditioned media of stimulated lung slices

MRC-5 lung fibroblasts were cultured in Ham's F12 medium supplemented with 10% foetal bovine serum (FBS), L-glutamine (2 mM), streptomycin (100 µg/L) and penicillin (100 U/mL). For each experiment, cells were grown to confluence and subsequently culture medium was substituted with Ham's F12 medium supplemented with 0.5% FBS, L-glutamine and antibiotics for a period of 24 hours. Thereafter, cells were stimulated for 1 h with TGF-β_1 (2 ng/mL), methacholine (10 µM) or with conditioned media obtained from incubated lung slices. Conditioned media used were taken from control slices and from slices treated with TGF-β_1 (2 ng/mL) or MCh (10 µM), both in the presence and absence of the inhibitors latrunculin A (0.3 µM) or SB431542 (0.3 µM), for 48 h.

5.2.8 Immunofluorescence

Lung slices were stimulated in 6 well-plates, using 2 slices per well. Slices were stimulated with methacholine (10 µM) or TGF-β_1 (2 ng/mL) for 2 days continuously. After washing twice with cytoskeleton buffer (CB: MES (10 mM), NaCl (150 mM), EGTA (5 mM), $MgCl_2$ (5 mM), and glucose (5 mM) at pH=6.1), the

lung slices were fixed with CB containing 3% paraformaldehyde (PFA) for 15 min. The slices were subsequently incubated with CB buffer containing 3% PFA and 0.3% Triton-X-100 for 5 min, followed by an additional 2 washes with CB buffer. Lung slices were then blocked for 1 h in cyto-TBS (Tris-base (20 mM), NaCl (154 mM), EGTA (2.0 mM) and $MgCl_2$ (2.0 mM), pH=7.2), supplemented with BSA (1%) and normal donkey serum (2%). After that, lung slices were stained with mouse sm-myosin antibody overnight at 4°C. After washing 3 times with cyto-TBS containing 0.1% Tween-20 (cyto-TBS-T) for 10 min, incubation with the secondary antibody (Cy3-mouse, 1:50 in cyto-TBS-T) was performed during 3 h at room temperature. Lung slices were then washed 4 times for 15 min in cyto-TBS-T, followed by 4 washes with ultra-pure water. The slides were mounted with ProLong Gold anti-fade reagent (Invitrogen, Breda, The Netherlands). After staining, the slides were analysed using a Leica TCSSP2 confocal microscope. All conditions within one experiment were analysed in the same session using identical microscopic settings. Excitation wavelength was 543 nm.

5.2.9 Western Blotting

Lung slices or MRC-5 cells were washed once with ice-cold phosphate-buffered saline (PBS, composition: NaCl (140 mM), KCl (2.6 mM), KH_2PO_4 (1.4 mM), Na_2HPO_4 (8.1 mM), pH 7.4), followed by lysis using ice-cold SDS-lysis buffer (Tris-HCl (62.5 mM), SDS (2 %), NaF (1 mM), Na_3VO_4 (1 mM), aprotinin (10 µg/mL), leupeptin (10 µg/mL), pepstatin A (7 µg/mL) at pH 8.0). Equal amounts of protein, determined by Pierce protein determination according to the manufacturer's instructions, were separated on SDS polyacrylamide gels and transferred onto nitrocellulose, followed by standard immunoblotting techniques. All bands were normalized either to β-actin or to total Smad 2/3 expression.

5.2.10 Antibodies and reagents

Methacholine chloride was purchased from ICN Biomedicals (Zoetermeer, the Netherlands). Human recombinant TGF-β_1 was obtained from R&D systems (Abingdon, UK). Mouse anti-α smooth muscle actin (sm-α-actin) antibody, mouse anti-calponin antibody, mouse anti-β-actin (β-actin) antibody, horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG antibody, histamine were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). Mouse anti-myosin smooth muscle myosin (sm-myosin) was purchased from Neomarkers (Immunologics, Duiven, The Netherlands). Cy3-conjugated secondary antibody

was obtained from Jackson ImmunoResearch (West Grove PA, USA). The inhibitors latrunculin A and SB431542 were purchased from Tocris Biosciences (Bristol, UK). All other chemicals were of analytical grade.

5.2.11 Data analysis

Data are presented as mean values ± SEM. Statistical significance was determined by paired Student's *t*-test with two-tailed distribution or one-way ANOVA for paired observations, followed by a Newman-Keuls multiple comparisons test when appropriate. Data were considered statistically significant if $p < 0.05$.

5.3 Results

5.3.1 Lung slice viability

To determine the viability of cultured precision-cut lung slices, LDH release and mitochondrial activity were assessed after 1, 2, 3 and 4 days of incubation. We observed a significant increase in LDH release over time; however, LDH release was still below 15% of maximal content on day 2 (Figure 5.1A) as observed previously in Ressmeyer et al. (23). The time course of LDH release was closely paralleled by that of the mitochondrial activity assay, which demonstrated a significant reduction in activity starting on day 3 (Figure 5.1B). Based on these data, we used lung slices that were cultured for up to 2 days.

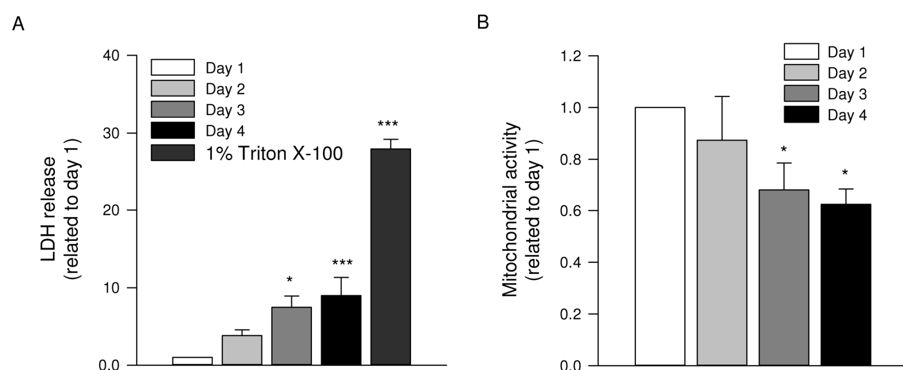


Figure 5.1. Lung slice viability. (A) LDH release from lung slices after 1, 2, 3 and 4 days of culture. Maximal LDH content of the slices was established by lysis with 1% Triton X-100. Data shown are the means ± SE of 3-7 independent experiments. (B) Mitochondrial activity in lung slices after 1, 2, 3 and 4 days of culture. Data shown are the means ± SE of 5 independent experiments. *: $p<0.05$ and ***: $p<0.001$ compared to basal (one-way ANOVA, posthoc Newman-Keuls).

5.3.2 Contractile protein expression in response to TGF-β1

TGF-β_1 is a potent cytokine that induces cellular biological processes leading to airway remodelling (12). Therefore, in order to investigate the usefulness of lung slices as an *in vitro* model to study airway remodelling, we studied the effect of TGF-β_1 treatment on contractile protein expression in lung slices. Lung slices were incubated in a dose- and time-dependent manner for 1 or 2 days in the presence or absence of TGF-β_1 (0.1, 0.2, 2 ng/mL) and analysed for the expression of sm-myosin, sm-α-actin and calponin. We observed that TGF-β_1 induced a time- and a concentration-dependent increase in the expression of sm-myosin, sm-α-actin and calponin (Figure 5.2A-B). The maximal response measured for sm-myosin was obtained by incubating the slices with 2 ng/mL TGF-β_1 for 2 days continuously (Figure 5.2B). This condition was chosen for further experiments with this cytokine.

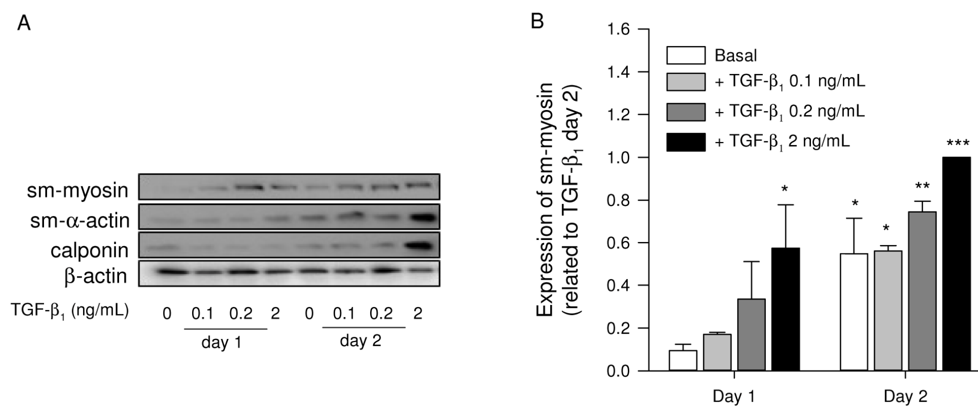


Figure 5.2. Concentration and time-dependent effects of TGF-β_1 on contractile protein expression. Lung slices were treated in a time- and concentration-manner with TGF-β_1 (0, 0.1, 0.2, 2 ng/mL) for 1 or 2 days, as indicated. Lung slice lysates were analysed for sm-myosin, sm-α-actin, or calponin, using β-actin as a loading control. (A) Representative blots of TGF-β_1-induced contractile protein expression. (B) Densitometric analysis of sm-myosin expression. Data shown are means ± SE of 3 independent experiments. *: $p<0.05$, **: $p<0.01$, and ***: $p<0.001$ compared to basal (one-way ANOVA, posthoc Newman-Keuls).

5.3.3 Contractile protein expression in response to methacholine-induced bronchoconstriction

We next determined whether bronchoconstriction also induced the expression of contractile proteins. Stimulation of lung slices with 10 µM methacholine for 2 days resulted in an increased expression of sm-myosin and calponin (1,69 ± 0.23- and 2.89 ± 0.59- fold induction, respectively). However, methacholine did not induce an increase in the expression of sm-α-actin (Figure 5.3A-D).

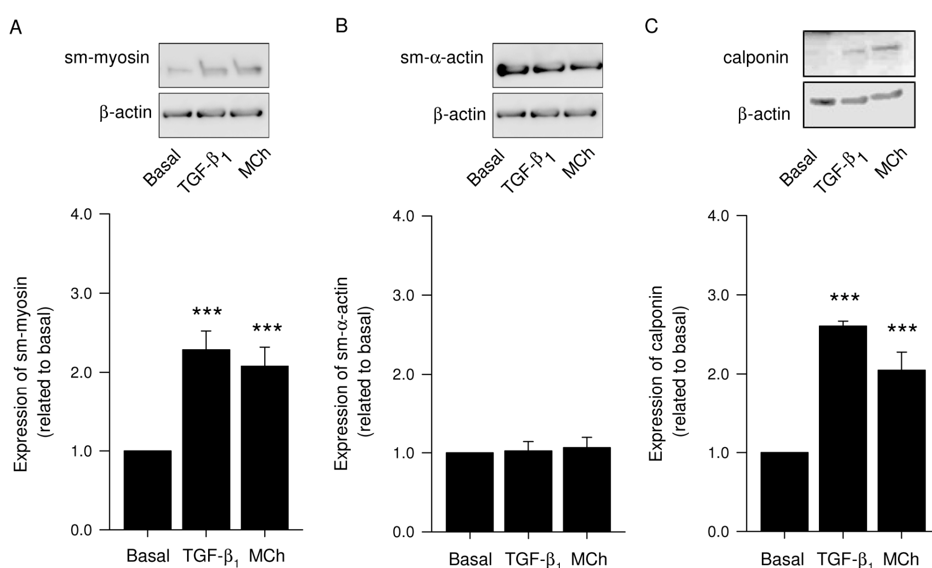
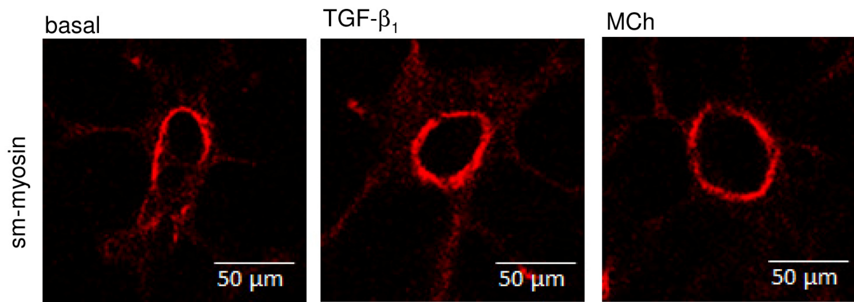


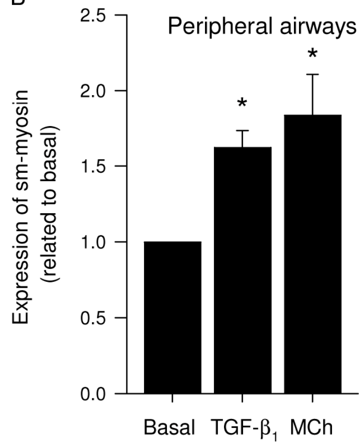
Figure 5.3. Methacholine stimulation induces contractile protein expression in lung slices. Lung slices were treated with TGF-β_1 (2 ng/mL), methacholine (MCh; 10 µM) or medium (basal) for 2 days. Lung slice lysates were then analysed for the presence of sm-myosin (B), sm-α-actin (C), or calponin (D) using β-actin as a loading control. Representative blots are shown in (A). Data shown in graphs are the means ± SE of 3-4 independent experiments. ***: p<0.001 compared to basal (one-way ANOVA, posthoc Newman-Keuls).

In view of these data, we further investigated the localization of increased contractile protein expression induced by TGF-β_1 and methacholine in the airways. Therefore, lung slices incubated with either TGF-β_1 (2 ng/mL) or methacholine (10 µM) were stained immuno-cytochemically for sm-myosin and visualized using confocal fluorescence microscopy. Staining intensity within the smooth muscle bundle was quantified (Figure 5.4A-C). To distinguish peripheral airways from central airways, the diameter of the airways was measured. Airways with a diameter under 100 µm were considered as peripheral airways and with a diameter above 400 µm as central airways. Increased expression of sm-myosin, but not of sm-α-actin (data not shown) in response to methacholine and TGF-β_1 as visualized by confocal fluorescence microscopy was found in peripheral airways with a diameter smaller than 100 µM (1.8 ± 0.3 and 1.6 ± 0.1 fold-induction for methacholine and TGF-β_1, respectively, Figure 5.4A-B), and in the central airways, with a diameter larger than 400 µM (1.6 ± 0.1 fold-induction for methacholine Figure 5.4C).

A



B



C

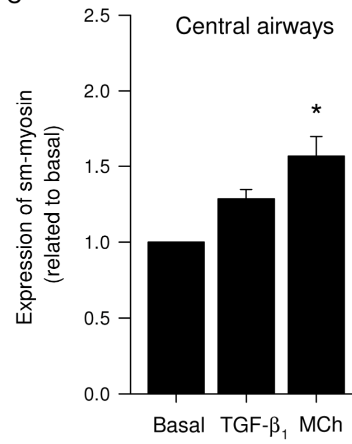


Figure 5.4. Localization of sm-myosin expression after TGF-β_1 and methacholine treatment. Lung slices were treated with TGF-β_1 (2 ng/mL), methacholine (MCh; 10 µM) or medium (basal) for 2 days. Lung slices were then fixed, stained for sm-myosin and analysed by confocal immunofluorescence microscopy. The images shown in (A) are taken from the peripheral airways (diameter smaller than 100 µm). Staining intensity within the muscle bundle was quantified and data shown from the peripheral airways (B) and central airways (diameter larger than 400 µm; C) are the means ± SE of 3 independent experiments. *: $p<0.05$ compared to basal (one-way ANOVA, posthoc Newman-Keuls).

5.3.4 Mechanisms of bronchoconstriction-induced airway remodelling

Bronchoconstriction induced by methacholine causes an increase in the release of epithelial TGF-β in asthma patients (11). Also, co-culture of airway smooth muscle cells and epithelial cells causes the release of biologically active TGF-β in response to contractile agonists such as lysophosphatidic acid and methacholine (26). Therefore, the increase in contractile protein expression we observed in response to bronchoconstriction may be due to the release of TGF-β by lung slices. To establish whether biologically active TGF-β is involved in the increased contractile protein expression in response to methacholine, biologically active TGF-β was determined in conditioned media from stimulated precision-cut lung slices, using Smad-3 phosphorylation in human MRC-5 fibroblasts as a bio-assay. Human MRC-5 fibroblasts were incubated for 1 hour with the conditioned media obtained from methacholine-stimulated lung slices, followed by analysis of phosphorylated Smad-3, which is specifically activated by TGF-β. The phosphorylation of Smad-3 was significantly increased by conditioned media from methacholine-stimulated lung slices compared to basal controls (Figure 5.5A). As controls, the direct phosphorylation of Smad-3 in response to TGF-β_1 and methacholine in human MRC-5 fibroblasts were investigated. TGF-β_1, but not methacholine, induced direct phosphorylation of Smad-3 in the MRC-5 cells (Figure 5.5A), confirming that the effect of methacholine was due to the release of biologically active TGF-β from the lung slices.

To investigate whether bronchoconstriction is involved in methacholine-induced contractile protein expression and release of TGF-β, we inhibited actin polymerization with latrunculin A. Latrunculin A prevented the increase in sm-myosin expression in response to methacholine and TGF-β_1 (Figure 5.5B). In addition, the release of bioactive TGF-β by methacholine was inhibited by latrunculin A, whereas latrunculin A had no direct effect on TGF-β_1-induced Smad-3 phosphorylation (Figure 5.5C). Moreover, inhibition of TGF-β type I receptor kinase with SB431542 prevented the increase in expression of sm-myosin induced by either TGF-β_1 or methacholine (Figure 5.5B). Collectively, these findings indicate that bronchoconstriction induced by methacholine leads to the release of TGF-β, which enhances the expression of contractile proteins.

To establish whether the increase in contractile protein expression was only seen with the contractile agonist methacholine, we stimulated lung slices also with the contractile agonists histamine (His, 1 μM) and potassium chloride (KCl, 60 mM) in the presence or absence of the inhibitors latrunculin A or SB431542. The

expression of sm-myosin was enhanced in response to the contractile agonists methacholine, histamine and KCl, and these responses were similarly inhibited by latrunculin A and SB431542 (Figure 5.5D). This suggests that bronchoconstriction leads to the release of TGF-β inducing the expression of the contractile protein sm-myosin irrespective of the contractile agonist used.

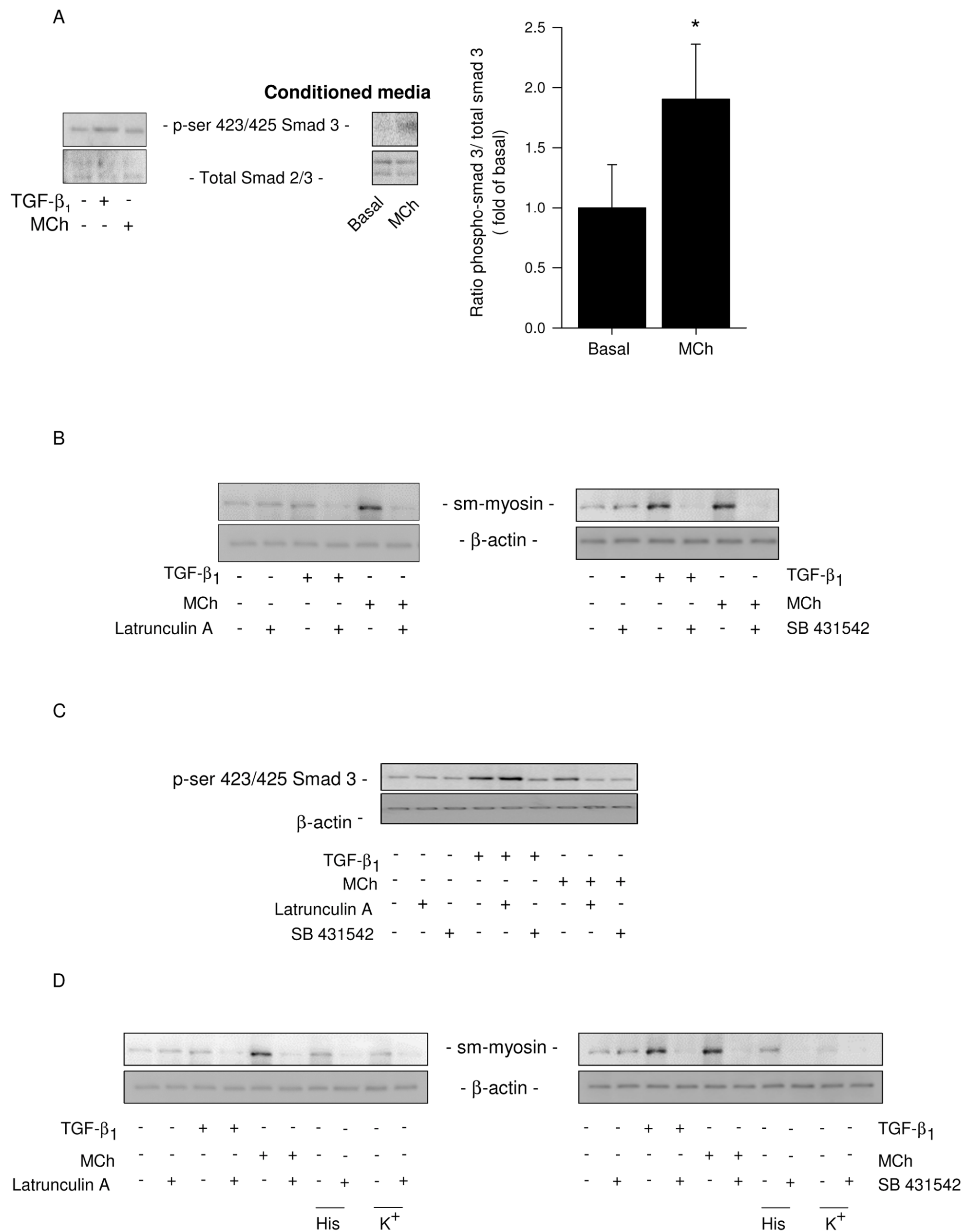


Figure 5.5. Bronchoconstriction induces the release of biologically active TGF-β leading to contractile protein expression. Human MRC-5 fibroblasts were stimulated for 1 hour with TGF-β_1 (2 ng/mL), methacholine (MCh; 10 µM) or medium (basal), or with conditioned media obtained from lung slice cultures treated for 2 days with and without 10 µM methacholine. MRC-5 cell lysates were analysed for phosphorylated (ser 423/425) and total Smad-3. Representative blots and quantified data of Smad-3 phosphorylation in

response to conditioned media are shown in (A). Data shown are the means ± SE of 4 independent experiments. [*]: $p<0.05$, compared to basal (paired Student's *t*-test with two-tailed distribution). (B) Lung slices were pre-treated with latrunculin A (0.3 µM), SB431542 (0.3 µM), or medium (basal) for 30 min, followed by 2 days of treatment with methacholine (MCh; 10 µM), TGF-β_1 (2ng/mL), or medium (basal). Lung slice lysates were analysed for the presence of sm-myosin, using ß-actin as a loading control. Blots shown are representative of 3 experiments. (C) Human MRC-5 fibroblasts were stimulated for 1 hour with conditioned media obtained from lung slice cultures after treatment with methacholine (MCh; 10 µM), TGF-β (2ng/mL) or medium (basal), in the absence and presence of latrunculin A (0.3 µM) or SB431542 (0.3 µM). MRC-5 cell lysates were analysed for phosphorylated (ser 423/425) and total Smad-3. Blots shown are representative of 3 experiments. (D) Lung slices were pre-incubated with latrunculin A (0.3 µM), SB431542 (0.3 µM), or medium (basal) for 30 min, followed by 2 days stimulation with methacholine (MCh; 10 µM), TGF-β_1 (2ng/mL), histamine (His, 1 µM), KCl (K^+, 60 mM) or medium (basal). Lung slice lysates were analysed for sm-myosin, using β-actin as a loading control. Blots shown are representative of 3 experiments.

5.4 Discussion

After validation of lung slices as an *in vitro* model for TGF-β induced airway smooth muscle remodelling, we studied the mechanisms involved in the induction of airway remodelling in response to bronchoconstriction. We show that bronchoconstriction induced by contractile agonists including methacholine, histamine and KCl, stimulates the release of TGF-β from lung tissue, which leads to an enhanced expression of contractile phenotype markers by the airway smooth muscle, similar to what is observed in patients with asthma (27, 28).
Airway remodelling is a multicellular process, in which structural cell-cell interactions and cell-matrix interactions play a major regulatory role (29). Therefore, lung slices appear to be a useful *in vitro* model to study multicellular remodelling processes as most cell-cell contacts, and the cell-matrix interactions are preserved in this model. This has already been established for other organ systems, such as liver slices, in which ethanol or CCl4 induced liver fibrosis can be adequately mimicked (30-32). Another advantage of lung slices in culture is that the loss of sm-myosin expression that is typical for cultured airway smooth muscle cells is not observed. The choice for guinea pig lung slices in the present study is based on the observations that airway responsiveness to methacholine in guinea pig lung slices is very similar to that in human tissue (4). Furthermore, guinea pigs

have great anatomical and functional similarities compared to human airways including the presence of small airways in contrast to other animals (24).

The use of lung slices also has some limitations. The major disadvantages of lung slices as an *in vitro* model include the lack of circulation and oedema formation (33), which interferes with the ability to clear soluble factors, including TGF-β. Also, relevant to the present study, tachyphylaxis to sustained bronchoconstriction could occur during 2 days of culture with bronchoconstricting agonists. However, as bronchoconstriction remained visible after 2 days of culture, and as other contractile agonists, including histamine and potassium chloride produced very similar results, we believe that even though tachyphylaxis may have occurred to some extent, its impact on the overall conclusion is small. Furthermore, the period during which lung slices can be maintained in culture is limited. Nonetheless, after 2 days of culture, we still measured low levels of LDH release and no decline in mitochondrial activity, indicating good viability. Very importantly, we observed an increase in contractile protein expression in response to TGF-β_1 as a marker of airway remodelling, which underscores the usefulness of this culture system. Overall, this model offers a great perspective for future experiments, particularly in studying research questions in which intact cell-cell and cell-matrix interactions are essential.

The pleiotropic cytokine TGF-β is an important growth factor involved in airway remodelling processes in asthma (12). Increased expression of TGF-β is found in lung tissue and bronchoalveolar lavage fluid of patients with asthma (33). TGF-β promotes important aspects of airway remodelling, including maturation of airway smooth muscle cells characterized by increased expression of contractile phenotype marker proteins (34). Indeed, we previously showed that TGF-β induces increased expression of the contractile phenotype markers sm-α-actin and calponin in human airway smooth muscle cells in a time-dependent manner, which was synergistically enhanced by muscarinic receptor stimulation (**chapter 3**, 13). Patients with chronic asthma show an increase in sm-α-actin and myosin light chain kinase staining in the airways (26, 27). In agreement with these data, we show that lung slices in culture exhibit a time- and concentration-dependent increase of contractile phenotype markers including sm-myosin, in response to TGF-β. This implies that TGF-β responses in lung slices are similar to those observed in airway smooth muscle cell systems.

Our studies provide important insights into the mechanisms behind the induction of contractile protein expression in response to bronchoconstricting agents. Our previous studies using cultured airway smooth muscle cells showed that muscarinic receptor stimulation had no effect on cell proliferation, cytokine production or contractile protein expression by its own but required functional interactions with growth factors (e.g. PDGF-AB, TGF-β), cytokines (e.g. TNF-α) or cigarette smoke extract to induce these cellular responses (35-37, **chapter 2 and 3**). Cooperative regulation of contractile protein expression by TGF-β and methacholine was found to be associated with enhanced GSK-3 and 4EBP-1 phosphorylation (**chapter 3**). The present study shows that in lung slices, in which cell-cell interactions and contractility are preserved, bronchoconstriction induced by methacholine is sufficient to promote the expression of contractile proteins, including sm-myosin and calponin, which is explained by the release of biologically active TGF-β which may functionally interact with methacholine to induce contractile expression. Other bronchoconstricting agents, including histamine and KCl were also sufficient to induce these effects. Notably, bronchoconstriction induced by methacholine did not enhance the expression of the contractile protein sm-α-actin. We previously demonstrated that sm-α-actin positive area in guinea pig airways is larger compared to sm-myosin positive area suggesting that actin positive, myosin negative cells exist within the smooth muscle bundle (10). Moreover, allergen-challenged guinea pigs show much greater induction of sm-myosin expression in comparison to sm-α-actin (10). Although we cannot directly compare the effect of allergen with methacholine, the similarity with our current data is remarkable and suggests that sm-α-actin is less susceptible to regulation than sm-myosin in guinea pig airways. The TGF-β response by itself was also affected by the actin polymerization inhibitor latrunculin A, suggesting a basal tone of the airways, leading to the release of TGF-β or a requirement of actin polymerization for the induction of smooth muscle specific gene expression, also in response to TGF-β (38-40). Taken together, this suggests that bronchoconstriction induces the release of biologically active TGF-β, leading to contractile protein expression.

In response to bronchoconstriction induced by methacholine or house dust mite, epithelial-TGF-β levels are increased in patients with asthma (9). In the airways biopsies of human lung, immunostaining revealed that TGF-β was mainly localized to the bronchial epithelial compartment, and to a lesser extent in smooth muscle

cells (41). Mechanical stimulation of bronchial epithelial cells induced the release of TGF-β, playing an important role in subepithelial processes observed in asthma (8). In response to static transmembrane pressures, rat epithelial cells also promote the gene expression of TGF-β, endothelin-1 and early growth response-1 (42). Additionally, mechanical stimulation induces an increase in fibronectin production in epithelial cells (5). This may suggest that the primary sources of TGF-β in the airways are the epithelial cells, which release this growth factor in response to mechanical stimulation during bronchoconstriction. Nonetheless, the airway smooth muscle may also play an important role as Tatler et al. demonstrated that airway smooth muscle cells can activate TGF-β through $\alpha_V\beta_5$ integrins in response to bronchoconstrictors *in vitro* (25). Moreover, blocking the $\alpha_V\beta_5$ integrins caused a reduction in the increased ASM layer *in vivo* with an ovalbumin-challenged mice model (25). Furthermore, in response to the contractile agonists lysophosphatidic acid and methacholine, airway smooth muscle cells from asthma patients released TGF-β to a greater extent than airway smooth muscle cells from healthy controls (25). Therefore, epithelial release of TGF-β and subsequent activation of the latent form into the biologically active form by $\alpha_V\beta_5$ integrins on airway smooth muscle is a plausible mechanism for the effects of bronchoconstriction observed in our study.

Our findings have important implications for the management of chronic asthma. As bronchoconstriction can promote airway remodelling, the beneficial effects of bronchodilator drugs may exceed their acute effects on lung function. *In vitro* and *in vivo* studies are supporting this hypothesis. In a murine model of asthma, levels of TGF-β were reduced in bronchoalveolar lavage fluid (BALF) by the anticholinergic drug tiotropium. Additionally, tiotropium inhibited the thickening of airway smooth muscle and airway fibrosis in this model (43). Tiotropium treatment also inhibited the increase in contractile protein expression and thickening of the airway smooth muscle layer, in response to allergen-challenge in guinea pigs (10, 11). In fact, anticholinergics have multiple anti-inflammatory and anti-remodelling properties in animal studies (for review, see **chapter 6**), however, the underlying mechanism are still not understood. β-agonists, which are widely prescribed as bronchodilator drugs to asthmatics, can also reduce, though only partially, TGF-β-induced contractile protein expression in human bronchial smooth muscle cells (44) and other remodelling processes (12).

In conclusion, using lung slices as an *in vitro* model, our findings demonstrate that bronchoconstriction can induce the release of TGF-β which promotes contractile protein expression. Therefore, our data suggest that bronchodilators may have beneficial effects on airway remodelling which should be followed up in future studies. Also our data suggest that the use of precision cut lung slices is a suitable model to study airway remodelling processes in response to bronchoconstriction.

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6 Regulation of airway inflammation and remodelling by muscarinic receptors

Perspectives on anticholinergic therapy in asthma and COPD

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Abstract

Acetylcholine is the primary parasympathetic neurotransmitter in the airways and an autocrine/paracrine secreted hormone from non-neuronal origins including inflammatory cells and airway structural cells. In addition to the well-known functions of acetylcholine in regulating bronchoconstriction and mucus secretion, it is increasingly evident that acetylcholine regulates inflammatory cell chemotaxis and activation, and also participates in signalling events leading to chronic airway wall remodelling that is associated with chronic obstructive airways diseases including asthma and COPD. As muscarinic receptors appear responsible for most of the pro-inflammatory and remodelling effects of acetylcholine, these findings have significant implications for anticholinergic therapy in asthma and COPD, which is selective for muscarinic receptors. Here, the regulatory role of acetylcholine in inflammation and remodelling in asthma and COPD will be discussed including the perspectives that these findings offer for anticholinergic therapy in these diseases.

6.1 Introduction

Acetylcholine is the primary parasympathetic neurotransmitter in the airways and a paracrine/autocrine hormone released from non-neuronal origins. The role of acetylcholine in the regulation of bronchomotor tone and mucus secretion from airway submucosal glands is well established (1). More recent findings suggest that acetylcholine, acting on muscarinic receptors, regulates additional functions in the airways, including inflammation and remodelling in obstructive airways diseases such as asthma and COPD (2-4). Based on these findings, we have previously questioned the traditional view on the role of acetylcholine, and suggested new possibilities for therapeutic targeting of muscarinic receptors in asthma and COPD (2). In this review, we will discuss the role of muscarinic receptors in obstructive airways disease further and update the discussion in view of these recent research papers and trials. In view of the selectivity of currently used anticholinergics for muscarinic receptors, we will not elaborate on the role of nicotinic receptors in this review. Nicotinic receptors are, however, expressed in the airways and mediate anti-inflammatory effects of acetylcholine. For excellent reviews on the anti-inflammatory role of nicotinic receptors, we would

like to refer to recently published reviews and to other reviews in this special issue (5-7).

6.2 Acetylcholine and muscarinic receptors in the airways

6.2.1 Biosynthesis, metabolism and mode of action of acetylcholine

Acetylcholine is synthesized from choline and acetyl-CoA mainly by the enzyme choline acetyltransferase (ChAT) (6). Airway neurons and non-neuronal cells such as airway epithelial cells express ChAT and release acetylcholine (8). Further, macrophages, mast cells, lymphocytes, granolucytes, fibroblasts and smooth muscle cells all have been suggested to express ChAT (6), although the release of acetylcholine from these cells has not yet been demonstrated directly. Acetylcholine can bind to and activate a family of G protein coupled muscarinic receptors, but also a family of nicotinic receptors, which are ligand gated cation channels (9). Most inflammatory and airway structural cells express muscarinic and/or nicotinic receptors (2). The individual receptor subtypes and subunits expressed by these cells have been reviewed extensively by Wessler and Kirkpatrick (6).

The mechanisms that regulate the metabolism of non-neuronal acetylcholine by airway epithelial cells are still not fully established, although recent studies have yielded important new insights. The uptake of choline is the rate-limiting step in the synthesis of acetylcholine. Choline uptake in airway epithelial cells is regulated by the high affinity choline transporter (CHT1) and by choline-specific transporter-like proteins (CTL) (10, 11). Organic cation transporter (OCT) subtypes 1 and 2 play a dominant role in the release of acetylcholine by airway epithelial cells (10, 11). Furthermore, the expression of the vesicular acetylcholine transporter (VAChT) by some epithelial cell types, including secretory cells, neuroendocrine cells and brush cells has been reported, suggesting that storage and release of acetylcholine via vesicles may mediate acetylcholine release by non-neuronal cell type (10, 11). The expression of muscarinic receptors, nicotinic receptors, synthesizing enzymes such as ChAT and the release of acetylcholine from non-neuronal cells is solid evidence for the existence of a non-neuronal cholinergic system in the airways next to the well-established neuronal cholinergic system.

6.2.2 Muscarinic receptor expression and function in the airways

Muscarinic receptors are the target for anticholinergic therapy in obstructive airways diseases as asthma and COPD and are the focus of this review. Muscarinic receptors are expressed by structural cells in the airways, predominantly airway smooth muscle, airway epithelium and airway fibroblasts. The parasympathetic neural network penetrates deep into the airway wall, and regulates bronchoconstriction, the release of mucus from submucosal glands, and to a lesser degree from goblet cells in the airway epithelium (2). The functional role of non-neuronal acetylcholine released from the airway epithelium is less well described, although recent studies suggest a role in airway smooth muscle contraction (12). It should be noted however that this finding is still controversial (13, 14). Additionally, acetylcholine, either neuronal or non-neuronal, may modulate airway inflammation and remodelling, as will be discussed further on.
The distribution of muscarinic receptor subtypes throughout the bronchial tree is mainly restricted to muscarinic M_1, M_2 and M_3 receptors (2). Muscarinic M_1 receptors are expressed by epithelial cells, where they play a modulatory role in electrolyte and water secretion, and in the ganglia, where they facilitate parasympathetic neurotransmission. Muscarinic M_2 receptors are expressed by neurons, where they function as autoreceptors, inhibiting the release of acetylcholine from both preganglionic nerves and from parasympathetic nerve terminals. Muscarinic M_2 autoreceptors are dysfunctional in allergic asthma due to eosinophil-derived release of major basic protein which acts as an allosteric antagonist of the M_2 receptor (15), augmenting acetylcholine release. Furthermore, M_2 receptors are widely expressed by airway mesenchymal cells such as fibroblasts and smooth muscle cells (2). Recent studies suggest that they may modulate cellular responses associated with airway remodelling (16). Also, a role in inhibition of G_s mediated airway smooth muscle relaxation has been proposed (1). Muscarinic M_3 receptors are probably the best characterized subtype and are the dominant receptor subtype in the regulation of mucus secretion from submucosal glands and airway smooth muscle contraction (2). As a result, muscarinic M_3 receptors are the primary target for anticholinergics, and M_3 subtype-selectivity has been advocated for by several research groups (17-22).

6.2.3 Muscarinic receptors as therapeutic targets for asthma and COPD

Anticholinergic therapy in COPD, and to a lesser extent asthma, is mainly aimed at inhibition of bronchoconstriction by inhibition of muscarinic receptors. Although

the term anticholinergic is most commonly used, all available anticholinergics used for the treatment of asthma and COPD are in fact specific antimuscarinics as they lack binding affinity at the nicotinic receptor. Clinically available anticholinergics are the short-acting ipratropium and the long-acting tiotropium. In addition to its longer duration of action, tiotropium has a considerably slower rate of dissociation from the M_1 and the M_3 receptor than from the M_2 receptor, making the drug 'kinetically selective' for M_1 and M_3 receptors (21). It is conceivable that this functional selectivity of tiotropium is beneficial, as smooth muscle contraction is primarily mediated by muscarinic M_3 receptors, whereas muscarinic M_2 receptor blockade facilitates acetylcholine release from parasympathetic nerves (2). However, direct evidence for a beneficial clinical effect of this functional M_3 selectivity of tiotropium is still lacking and the major difference between these drugs appears to be the duration of action.

The Understanding the Potential Long-term Impacts of on Function with Tiotropium (UPLIFT) trial has demonstrated that treatment with tiotropium provides a significant and sustained improvement in lung function and quality of life in COPD patients, and reduces exacerbations and hospitalizations (23). Currently available anticholinergics are the short-acting ipratropium and the long-acting tiotropium. These can be used either as monotherapy or in combination with β_2-agonists and provide significant improvement in FEV_1 in both asthma and COPD patients (24-27). The combination therapy with β_2-agonists is more effective than anticholinergic treatment alone; nonetheless, monotherapy is already markedly effective (104). The explanation for this relatively large effect of monotherapy may lie within the role that mediators of inflammation (e.g. thromboxane A_2, histamine) have in activating the airway cholinergic system. Airway inflammation has several ways to increase the output of neuronally released acetylcholine, as it results in exposure and activation of afferent C-fibres that facilitate ganglionic and central parasympathetic neurotransmission. Further, the release of acetylcholine can be facilitated directly via excitatory receptors for inflammatory mediators (e.g. prostaglandins, tachykinins) present on parasympathetic nerve terminals, and indirectly via inhibition of the M_2 autoreceptor through the release of eosinophil derived major basic protein that acts as an allosteric M_2 receptor antagonist (1, 2). As a result, the bronchoconstrictor response (and perhaps additional responses) induced by pro-inflammatory mediators such as thromboxane A_2 is for a large part mediated by neuronally released acetylcholine (28). Further, bronchoconstriction induced by

histamine after the early asthmatic response can be inhibited by ipratropium in a guinea pig model of asthma (29). This advocates for the use of anticholinergic therapy not only in COPD – where parasympathetic tone is the primary reversible component of airway obstruction (30) – but also in asthma. Indeed, recent clinical trials indicate significant improvements in lung function in asthma patients on top of usual care, and show that tiotropium therapy is non-inferior to β_2-agonist therapy when combined with corticosteroids in severe asthma patients (25, 26, 31). The additional observations that next to FEV_1 also exacerbation rate and lung function decline in subgroups of COPD patients are improved by treatment with tiotropium (20, 32) has prompted speculations on the possible beneficial effects of anticholinergics on airway inflammation and remodelling (33).

6.3 Airway inflammation

Asthma and COPD are both characterized by chronic airway inflammation, albeit that the patterns of inflammation are markedly different. Different subtypes of T cells are involved in asthma and COPD: in asthma there is an increase in T_H2 ($CD4^+$) cells, whereas in COPD $CD8^+$ T cells predominate. Furthermore, the inflammation that occurs in asthma can be described as eosinophilic, whereas that occurring in COPD is mainly neutrophilic. However, when disease severity increases these differences become less pronounced (34).

6.3.1 Inflammation and the non-neuronal cholinergic system

Increasing evidence suggests that acetylcholine contributes to airway inflammation. In 2004, Wessler et al. found that in patients with atopic dermatitis, a condition characterized by T_H2 type inflammation and often associated with bronchial asthma, expression of ChAT is increased in skin biopsies, with a consequent increase in acetylcholine (35). Further, Profita et al. (2011) demonstrated that cigarette smoke extract upregulated the non-neuronal cholinergic system in bronchial epithelial cells, by showing that expression of muscarinic M_2 and M_3 receptors and ChAT mRNA and protein were increased, whereas muscarinic M_1 receptor levels were not affected. Consequently, acetylcholine levels in cell extracts were significantly higher after stimulation with cigarette smoke extract. This increase could be reduced by tiotropium (36). In contrast, lungs of ovalbumin challenged rats and mice show a significant decrease

in ChAT and other components of the cholinergic system, including the functionally relevant choline transporter CHT1 (37). Future studies are clearly warranted within this area to better understand the complex mechanism of regulation of the cholinergic system by inflammation and the significance of this process in asthma and COPD.

6.3.2 Inflammatory cells

Acetylcholine has been shown to affect inflammatory cells involved in asthma and COPD directly, by inducing proliferation or cytokine release from these cells. Carbachol can induce the proliferation of macrophages from mice *in vitro* (38). Also T-cell proliferation can be observed ex vivo after treatment of rats with the muscarinic agonist oxotremorine, whereas atropine suppresses the proliferation of T-cells (39). These anti-inflammatory properties of atropine were also demonstrated in rats *in vivo*, were it suppressed the turpentine-induced infiltration of leukocytes (39). Moreover, bovine alveolar macrophages exhibit neutrophil, eosinophil and monocyte chemotactic activity in response to acetylcholine, which is likely explained by cholinergic induction of leukotriene B_4 (LTB_4) release (40). Recently, this was confirmed for primary human macrophages (41). Moreover, it was shown that acetylcholine-induced release of chemotactic activity from monocytes, macrophages and epithelial cells could be inhibited by tiotropium (41). It has also been shown that acetylcholine can induce the release of LTB_4 from sputum cells of COPD patients (42). These results are consistent with a study demonstrating that tiotropium and also acetylcholinesterase, the degrading enzyme of acetylcholine, inhibited alveolar macrophage mediated migration of neutrophils from COPD patients (43). Using the M_3-selective antagonist 4-DAMP it was shown that this effect is mediated via the muscarinic M_3 receptor (43). Further, although R,R-glycopyrrolate, a muscarinic receptor antagonist, did not inhibit LPS-induced TNF-α release by itself, it synergistically inhibited the rolipram and budesonide induced decrease in TNF-α release from human primary monocytes (44). All these findings support a broad role for acetylcholine acting on muscarinic receptors in the regulation of airway inflammatory cells (Figure 6.1).

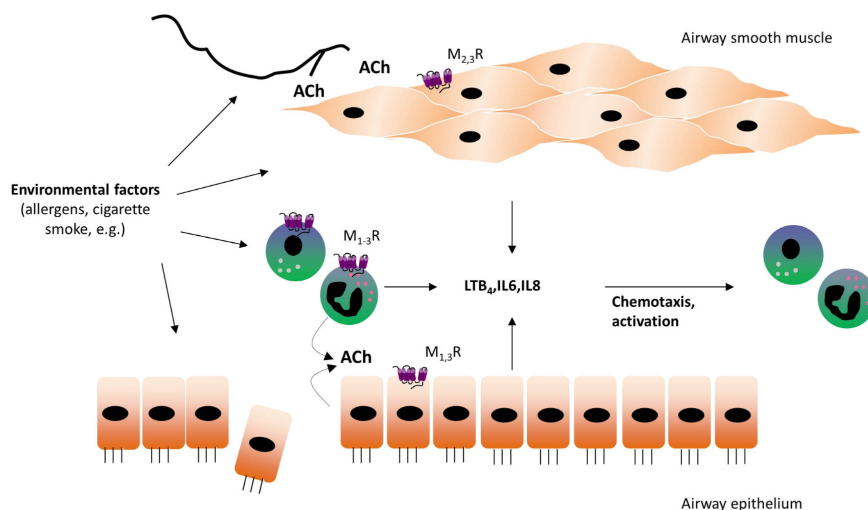

Figure 6.1. The regulatory role of acetylcholine in inflammatory cell chemotaxis and activation. Acetylcholine can be neuronally released or secreted as an autocrine or paracrine hormone from inflammatory cells and airway structural cells, most notably airway epithelial cells. In susceptible individuals, the release of acetylcholine may be enhanced in response to environmental factors such as cigarette smoke or allergens. As a consequence, pro-inflammatory cytokines including IL-6, IL-8 and LTB4 are produced, which attract and activate inflammatory cells, most notably neutrophils. Muscarinic M_3 receptors expressed on airway smooth muscle and muscarinic M1-3 receptors expressed by airway epithelial cells mediate the release of these factors via activation of ERK1/2 and NF-kB signaling pathways.

6.3.3 Epithelial cells

The expression of non-neuronal acetylcholine is relatively high in bronchial epithelial cells (8). Acetylcholine is known to induce eosinophil, monocyte and neutrophil chemotactic activity in bronchial epithelial cells (45, 46). The increase in epithelial neutrophil chemotactic activity by acetylcholine could be inhibited by tiotropium, indicating the involvement of muscarinic receptors in this response (47). The acetylcholine-induced neutrophil chemotactic activity from epithelial cells is partially dependent on IL-8 release, since it is inhibited by an anti-IL-8 monoclonal antibody (47). In line with this contention, the increase in IL-8 release in response to acetylcholine could be partially inhibited by tiotropium. In addition, acetylcholine induced LTB_4 release from bronchial epithelial cells in a tiotropium sensitive manner (36). Both IL-8 and LTB_4 release from bronchial epithelial cells is mediated via ERK1/2 and NF-κB signalling pathways and dependent on multiple

muscarinic receptor subtypes (M_1/M_2/M_3) (36, 47). Taken together, these studies implicate an important role for epithelial acetylcholine in airway inflammation, via the activation of muscarinic receptors (Figure 6.1).

Another potential mechanism by which tiotropium could inhibit inflammation induced by epithelial cells is by attenuating respiratory syncytial virus (RSV) replication in these cells (48). RSV is one of the major causes of acute lower respiratory tract infection and has been detected in patients with exacerbations of asthma and COPD (49). In an *in vitro* study, Iesato et al. demonstrated that the attenuation of virus replication by tiotropium was partially due to inhibition of RhoA activity. Moreover, tiotropium inhibited epithelial IL-6 and IL-8 production induced by RSV infection (48). *In vivo* studies are needed to investigate the importance of inhibition of infection-induced airway inflammation by tiotropium.

6.3.4 Airway smooth muscle cells

The airway smooth muscle is increasingly recognized for its role in modulating inflammation by secreting cytokines and chemokines (50), and it has been shown that muscarinic receptors on airway smooth muscle cells are involved in these responses. Stimulation of bovine airway smooth muscle strips with the muscarinic agonist carbachol induces pro-inflammatory gene expression, including IL-6, IL-8 and cyclo-oxygenase-2 (51). Furthermore, carbachol augmented the cyclic stretch-induced expression of these genes (51). Stimulation of airway smooth muscle cells with carbachol also induces the protein release of IL-6 and IL-8 via muscarinic M_3 receptors (52). Furthermore, methacholine strongly augmented cigarette smoke extract (CSE) induced IL-8 release (52). In line with findings in epithelial cells, IL-8 release induced by stimulation with methacholine and CSE in airway smooth muscle is ERK1/2 and NF-κB dependent (**chapter 2**).

6.3.5 *In vivo* studies

The regulatory role of muscarinic receptor signalling in inflammatory processes involved in asthma and COPD has been confirmed by *in vivo* studies, using animal models of these diseases.

Wollin and Pieper (2010) were the first to report anti-inflammatory properties of tiotropium in an animal model of cigarette smoke induced COPD. Total cell number and neutrophils in the bronchoalveolar lavage fluid (BALF) were concentration-dependently decreased after treatment with tiotropium. Furthermore, tiotropium inhibited the increase of several cytokines in the BALF,

including IL-6, KC, TNF-α and LTB_4 (53). Similar inhibitory effects of tiotropium on airway neutrophilia were observed in a guinea pig model of LPS-induced COPD (54). Moreover, neutrophilia was inhibited by ipratropium in a cadmium-induced rat model of pulmonary inflammation (55), by tiotropium in a HCl-induced rat model of gastro-oesophageal reflux (7) and by bilateral vagotomy or treatment with atropine in a diesel particle-induced rat model of pulmonary inflammation (56). Of interest, the latter study found that atropine was more effective in inhibiting pulmonary inflammation than bilateral vagotomy, suggesting a role for non-neuronal acetylcholine in this response (56).

These findings may also be relevant for asthma. Our group has shown that tiotropium also partially inhibits eosinophilia in a guinea pig model of asthma (57), which has been confirmed by Buels et al. (58). In line with these findings, infiltration of macrophages and eosinophils in the BALF was significantly inhibited by tiotropium treatment in a murine model of asthma. Furthermore, expression levels in BALF of IL-4, IL-5 and IL-13 were decreased by tiotropium treatment (59). In addition, aclidinium, a novel muscarinic receptor antagonist which is kinetically selective for the muscarinic M_3 receptor, inhibited infiltration of eosinophils in BALF in a mouse model of *Aspergillus fumigatus*-induced asthma (60). A recent study also suggested that M_3 receptors regulate these inflammatory responses, although the selectivity profile of the antagonist bencycloquidium that was used in this study precludes firm conclusions on the involvement of other receptor subtypes (61). Since both tiotropium and aclidinium are kinetically selective for the muscarinic M_3 receptor, this suggests predominant involvement of this receptor subtype in the observed anti-inflammatory effects in asthma and COPD models described above. This is supported by our own data on $M_3^{-/-}$ mice, in which neutrophilia and cytokine release in BALF were inhibited compared to wild-type mice after exposure to cigarette smoke (62).

Clearly, all these *in vivo* studies indicate a profound role for acetylcholine in inflammation in asthma and COPD, which is in accordance with results of *in vitro* studies that report pro-inflammatory effects of muscarinic receptors (Figure 6.1). The implication of these findings is that treatment with anticholinergics may have beneficial effects that exceed their bronchodilatory properties, a contention confirmed in several models of pulmonary inflammation. However, the exact mechanism responsible for the regulatory role of acetylcholine in inflammation is far from understood.

6.4 Airway remodelling

Airway inflammation in chronic airway diseases such as asthma and COPD is often associated with cellular and structural alterations in the airways, referred to as airway remodelling (63). Airway remodelling is considered a major component of irreversible airflow limitation in these diseases (64), is progressive, and correlates with disease severity (65, 66). Airway remodelling in asthma and COPD is characterized by mucus gland hypertrophy, goblet cell hyperplasia and pulmonary vascular remodelling (63). In addition, in asthma the basement membrane is thickened, there is subepithelial fibrosis, and there is considerable thickening of the airway smooth muscle bundle (64). In contrast, in COPD the fibrosis is mostly peribronchial, and although increased airway smooth muscle mass may occur, this appears restricted to severe stages of COPD (65). Airway structural alterations may accelerate decline of lung function (67).

6.4.1 Epithelial cells / mucus production

The airway epithelial layer is in continuous interaction with the external environment. To protect itself from exogenous stimuli, mucus is secreted under the control of the cholinergic system by muscarinic receptors (68). Mucus secretion can be increased by electrical field stimulation of the vagal nerve in bronchial preparations, predominantly via muscarinic M_3 receptors on the submucosal glands (68). In addition, electrolyte and water secretion are regulated by muscarinic M_1 and M_3 receptors (69, 70). Neuronal muscarinic M_2 autoreceptors appears to regulate the extent of the secretory response, by limiting neuronally released acetylcholine (70). In response to acetylcholine, glandular goblet cells also produce mucus (68).

Mucus hypersecretion is an important pathological feature of chronic airway diseases contributing to airway obstruction (68). MUC5A/C expression in airway epithelial cells and airway submucosal glands is directly correlated to airway obstruction in smokers (71) and in smokers, COPD patients and asthma patients; the expression of the MUC5A/C gene is augmented (32). Also, the expression of MUC5B and the insoluble MUC2 are increased, particularly in COPD. The ratio of mucus cells to serous cells in the submucosal glands is also increased in COPD patients (72). *In vitro* studies demonstrated that aclidinium suppressed carbachol-induced MUC5A/C overexpression in human bronchial tissue. Additionally, the increased expression of MUC5A/C by the co-stimulation of cigarette smoke

extract and carbachol could be attenuated by the use of aclidinium or atropine (73). Moreover, epidermal growth factor (EGF) stimulation enhanced the ACh-induced response on mucus cell activation in airway submucosal glands (74). *In vivo* studies confirm the role of acetylcholine in mucus hypersecretion and demonstrate that tiotropium reduces allergen-induced mucus gland hypertrophy and MUC5A/C-positive goblet cell number in guinea pigs (57). Further, it has been reported that tiotropium inhibits neutrophil elastase-induced goblet cell metaplasia in mice (75) and that treatment with tiotropium inhibited the increased MUC5A/C expression and mucus gland hypertrophy in a guinea pig model of COPD (54). This demonstrates the important role of acetylcholine in the regulation of mucus secretion, both *in vitro* and in animal models of asthma and COPD *in vivo* (Figure 6.2).

Acetylcholine may also regulate the proliferative and pro-fibrotic responses of airway epithelial cells. Bronchoconstriction induced by repeated challenges with methacholine induced epithelial cell proliferation and an increase in the expression of the profibrotic cytokine TGF-β by these cells in mild asthmatic subjects (76). In line with these findings, airway constriction induced by methacholine significantly increased the phosphorylation of the EGF receptor in airway epithelial cells (77). Moreover, in rat tracheal epithelial cells, acetylcholine induces proliferation mediated by muscarinic M_1 receptors (78) and autocrine release of acetylcholine is sufficient to induce monkey airway epithelial cell proliferation (8). Thus, the cholinergic system is able to regulate epithelial cell proliferation, either through the induction of mechanical strain or in an autocrine/paracrine manner, which is required for the repair of the airway epithelial layer.

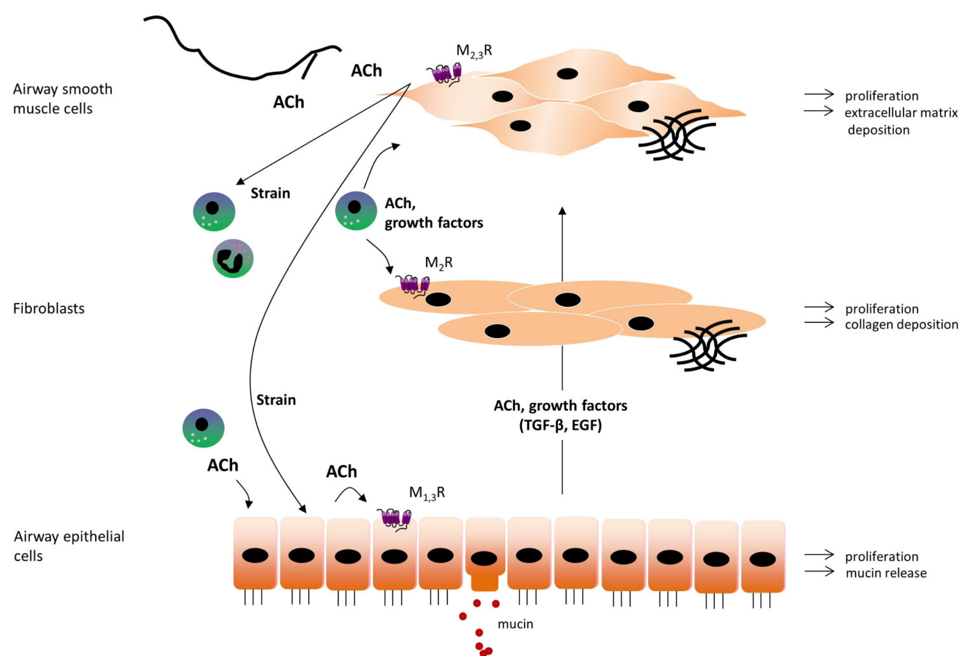

Figure 6.2. The regulatory role of acetylcholine in airway wall remodelling. Acetylcholine is neuronally released and secreted as an autocrine or paracrine hormone from airway structural cells and inflammatory cells. In the inflamed airway, inflammatory cells and airway epithelial cells also secrete growth factors that in concerted action with acetylcholine activate cell proliferation and matrix production by airway mesenchymal cells, including airway fibroblasts and airway smooth muscle cells. Furthermore, acetylcholine activates smooth muscle contraction leading to airway wall compression, which activates inflammatory cells and promotes remodelling responses by airway epithelial cells. Acetylcholine also directly promotes mucus production by and cell proliferation of airway epithelial cells.

6.4.2 Mesenchymal cells

Airway mesenchymal cells (e.g. fibroblasts, airway smooth muscle cells) contribute to airway remodelling by means of proliferation, contractile protein expression and the release of components such as mediators, extracellular matrix proteins and matrix metalloproteinases (MMPs) (79, 80). *In vitro* studies showed that the stimulation of muscarinic receptors on lung fibroblasts induces cell proliferation and the synthesis of collagen (16, 81) through the activation of the mitogen-activated protein kinase pathway (81, 82). This effect was mediated by the activation of muscarinic M_2 receptors (16). Interestingly, acetylcholine-induced cell proliferation is enhanced in human lung fibroblasts from COPD patients compared with healthy non-smokers and healthy smokers without COPD (83). The higher activation of cell proliferation in fibroblasts from COPD patients was due to enhanced ERK1/2 and NF-κB phosphorylation. Notably, the synthesizing enzyme ChAT was also increased in lung fibroblasts from healthy smokers and COPD patients (83).

MMPs play a key role in airway remodelling, inflammation and emphysema (84). In COPD patients, increased expression levels of MMP-1, MMP-2 and MMP-9 have been reported (85, 86). The activity of the MMPs can be inhibited by tissue inhibitor of matrix metalloproteinases (TIMPs) (84). Recently, it was demonstrated that tiotropium inhibited TGF-β-induced protein expression of both MMP-1 and MMP-2 in human lung fibroblasts, but had no effect on the TGF-β-induced TIMP-1 and TIMP-2 expression (87, 88). Therefore, these data suggest that treatment with tiotropium improves the balance between MMPs and TIMPs, inhibiting pro-fibrotic responses. As MMPs also play important roles in the infiltration of inflammatory cells, this effect could also contribute to the anti-inflammatory properties of anticholinergics.

Airway smooth muscle thickening is a characteristic pathological feature of asthma, and to a lesser extent of COPD. The induction of airway smooth muscle cell proliferation by growth factors, including PDGF and EGF, can be enhanced by the stimulation of muscarinic receptors (89-92). Specifically, $G_{\beta\gamma}$ subunits derived from G_q protein coupled receptors cooperate with receptor tyrosine kinases (e.g. the PDGF/EGF receptor) to induce synergistic activation of PI3K/Akt/p70S6K signalling leading to cell proliferation (89, 91, 92). Moreover, the activation of conventional PKC isoenzymes, likely via muscarinic M_3 receptor mediated $G_{\alpha q}$ stimulation, leads to GSK-3 inactivation, which potentiates both translational and transcriptional processes (90). These pathways are also involved in the acquisition

of contractile protein expression by TGF-β via transcriptional and translational processes (93-95) and can be activated by muscarinic receptor stimulation (96). Indeed, the expression of myosin light-chain kinase was augmented by carbachol in human airway smooth muscle cells exposed to cyclical mechanical strain (97). Additionally, we recently described that muscarinic receptor stimulation enhanced the TGF-β_1-induced contractile protein expression in human airway smooth muscle cells (**chapter 3**). Collectively, these findings suggest an important role of muscarinic receptor stimulation in the proliferation and maturation of mesenchymal cells (Figure 6.2).

6.4.3 *In vivo* studies

Inhibitory effects of anticholinergics on airway mesenchymal cell remodelling have indeed been reported in animal models of asthma and COPD. Treatment with tiotropium significantly inhibited airway smooth muscle remodelling in a guinea-pig model of chronic asthma using repeated challenges with ovalbumin (98). This was associated with the inhibition of increased contractile protein expression and of airway smooth muscle thickening. In a murine model of asthma, it was shown that tiotropium could also significantly inhibit smooth muscle thickening and the expression of TGF-β_1 in BALF (59). Similar effects have been described for the muscarinic M_3 receptor selective antagonist bencycloquidium bromide (61). Furthermore, bencycloquidium bromide reduced mucus production, globet cell metaplasia and collagen deposition and inhibited the upregulation of MMP-9, but not of TIMP-1 mRNA (61). Treatment with tiotropium also inhibited the increased peribronchial collagen deposition in a guinea pig model of COPD (54). Similarly, in a chronic gastro-oesophageal reflux model, tiotropium treatment prevented the increase in airway fibrosis (7). Taken together, these *in vivo* studies confirm *in vitro* studies showing that anticholinergics have anti-remodelling properties in asthma and COPD (Figure 6.2).

6.5 Clinical implications

The above mentioned *in vitro* and *in vivo* studies indicate significant pro-inflammatory and remodelling effects for acetylcholine via muscarinic receptors, suggesting that anticholinergics may have anti-inflammatory and anti-remodelling properties in asthma and COPD patients. This hypothesis still needs to be proven

in clinical studies, however. In the UPLIFT study, COPD patients treated with tiotropium during a 4 year period showed an improved quality of life and lung function, and a reduction in the frequency of exacerbations. Although tiotropium did not reduce FEV_1 decline in the overall study population (23), in pre-specified post-hoc studies, GOLD stage II and young COPD patients with rapid lung function decline had a significant improvement in the accelerated post-bronchodilator FEV_1 decline (20, 99). No notable reduction in exacerbation frequency was reported for ipratropium (100, 101). This suggests a beneficial role for tiotropium as a long-acting anticholinergic or a possible role for muscarinic M_3 receptor subtype selectivity, as tiotropium is kinetically selective for muscarinic M_3 receptors compared with ipratropium. Moreover, it also indicates anti-inflammatory effects of tiotropium, since patients who have more exacerbations demonstrate increased levels of inflammatory markers at stable state (102). However, Powrie et al. (2007) were not able to demonstrate a reduction in sputum IL-6 or IL-8 levels in patients treated with tiotropium during one year, even though the number of exacerbations was significantly decreased (103). A possible explanation for this discrepancy proposed by the authors is that the reduction in amount of sputum after tiotropium treatment might result in an increase in cytokine concentrations. Measurement of cytokine concentrations in sputum might therefore not be the optimal method. Also, Perng et al. (2009) did not find a decrease in sputum IL-8 levels after tiotropium treatment (104). However, the treatment group in their study was small and patients only received tiotropium for 12 weeks. Further studies are therefore needed to elucidate the mechanisms by which tiotropium reduces exacerbations and FEV_1 decline in subgroups of COPD patients and whether this is based on the anti-inflammatory effects of tiotropium discussed in this paper or by other effects, including a reduction in dyspnea or mucus hypersecretion. Likewise, further studies on the beneficial effects of anticholinergics in asthma patients are warranted. In patients with severe, uncontrolled asthma it has recently been shown that treatment with tiotropium improves lung function (25). Furthermore, a recent clinical trial showed that repeated inhalations with the muscarinic receptor agonist methacholine induces airway remodelling in asthma patients, including the expression of TGF-β and collagen I in bronchial biopsies (76). Therefore, although a rationale for beneficial effects of anticholinergics beyond the well-described bronchodilator properties in asthma and COPD certainly exists, it is evident that this still needs to be confirmed in clinical studies.

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7 General discussion and summary

Acetylcholine, the primary parasympathetic neurotransmitter in the airways, plays an important role in the regulation of bronchoconstriction and mucus production (1). Anticholinergics, by acting on muscarinic receptors inhibit the increased cholinergic tone in asthma and chronic obstructive pulmonary disease (COPD) patients. Both diseases are also characterised by inflammation and structural changes of the airways, defined as airway remodelling. Remodelling of the airway smooth muscle layer has been reported to contribute to the pathophysiology of both asthma and COPD.
Recent evidence suggests that the cholinergic system contributes to inflammation and remodelling of the airways, particularly at the level of airway smooth muscle. However, the mechanisms involved in these processes have only partially been solved. This thesis aims to explore the potential role of the cholinergic system in airway smooth muscle responses involved in airway inflammation and remodelling, as well as intracellular mechanisms underlying these processes. Novel muscarinic receptor-mediated mechanisms involved in the pro-inflammatory and remodelling responses of the airway smooth muscle are described in **chapters 2, 3, 4 and 5**. **Chapter 6** gives an overview of recent developments in the regulation of airway inflammation and remodelling by muscarinic receptors.

7.1 Role of muscarinic receptors in airway inflammation

The role of muscarinic receptors in the regulation of airway inflammation has been increasingly recognised during the last decade. Thus, various subtypes of muscarinic receptors are expressed on a variety of inflammatory cells (1, 2). Moreover, ChAT expression has been detected in immune cells, including eosinophils, neutrophils, lymphocytes, macrophages, and mast cells (3). Structural cells, including airway smooth muscle cells and epithelial cells, also express elements of the cholinergic system (2). Non-immune cells have been reported to regulate airway inflammation by secreting cytokines and chemokines (4). The airway smooth muscle plays an important role herein, by secreting a wide variety of immunomodulatory mediators, including IL-8, RANTES, IL-1β and TGF-β, in response to various pro-inflammatory stimuli (5). Recent work from our department has demonstrated that airway smooth muscle cells release pro-

inflammatory cytokines, including IL-8 and IL-6, in response to cigarette smoke extract (6). Muscarinic M_3 receptor activation synergistically enhanced this cytokine release and could also induce the production of IL-8 by itself, albeit to a minor extent ((6) and **chapter 2**). Similarly, others have shown that gene expression of pro-inflammatory cytokines, including IL-6 and IL-8, is up-regulated by carbachol in airway smooth muscle cells (7). These findings were supported by *in vivo* studies, as in a mouse model of COPD tiotropium bromide significantly reduced the cigarette smoke-induced increase of several cytokines, including IL-6, in the BALF (8). Moreover, in a guinea pig model of LPS-induced COPD, neutrophilia was reduced by tiotropium bromide (9). However, the precise mechanisms by which muscarinic M_3 receptors enhance the release of pro-inflammatory cytokines such as IL-6 and IL-8 remained unclear. In **chapter 2**, we demonstrate that the activation of muscarinic receptors on human airway smooth muscle cells induces the secretion of pro-inflammatory cytokines IL-8 and IL-6, particularly in combination with TNF-α, PDGF-AB and cigarette smoke extract. The mechanism behind the synergism between cigarette smoke extract- and methacholine-induced IL-8 secretion involves signalling by PKC, NF-κB and ERK1/2. This mechanism could be of importance for COPD patients using anticholinergics.

7.2 Role of muscarinic receptors in airway remodelling

An important mediator of airway remodelling is TGF-β, a multifunctional cytokine, which, among others, is involved in inflammation-induced tissue repair by inducing mesenchymal cell growth and extracellular matrix production. In chronic inflammatory conditions, as in obstructive airway diseases, this may lead to fibrosis. The expression of TGF-β is upregulated in the airways of asthma and COPD patients. Indeed, various *in vivo* studies have demonstrated that overexpression of TGF-β_1 in mice increases airway smooth muscle mass and fibrosis (10, 11). In addition, allergen-induced increased airway smooth muscle mass was prevented by anti-TGF-β_1 antibodies (12). Nonetheless, *in vitro* studies on the mitogenic effect of TGF-β_1 on airway smooth muscle cells are not yet conclusive. It appears that TGF-β can regulate both proliferation and maturation of airway smooth muscle cells, depending on its concentration (13). In this thesis (**chapters 3, 4 and 5**), we investigated the effects of TGF-β, alone and in concert

with muscarinic receptor stimulation, on various processes involved in airway smooth muscle remodelling.

To determine the impact of TGF-β_1 and muscarinic receptors on human airway smooth muscle phenotype, we studied the effects of TGF-β_1, methacholine and their combination on contractile protein expression and cell proliferation (**chapters 3 and 4**). In **chapter 3**, we demonstrated that TGF-β_1 on its own increases the expression of contractile phenotype markers, including sm-α-actin, calponin and sm-myosin, in airway smooth muscle cells (**chapter 3**, (20-22)). Despite the fact that muscarinic receptor stimulation on its own did not induce the expression of contractile proteins, it synergistically enhanced the TGF-β_1-induced contractile protein expression in airway smooth muscle cells. Furthermore, **chapter 3** describes a mechanism by which the cooperative regulation of contractile protein expression by TGF-β_1 and muscarinic receptor stimulation is mediated, namely through enhancing the translational activity by phosphorylation of glycogen synthase kinase (GSK)-3 and eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) and not by upregulation of gene expression in human airway smooth muscle cells (Figure 7.1). To our knowledge, this is the first study reporting crosstalk between a G protein-coupled receptor and TGF-β in airway smooth muscle; however, it remains unclear whether this crosstalk is applicable to other G protein-coupled receptors. This is worthwhile to further elucidate as many remodelling processes, also outside the airways, involve cooperative regulation by G protein-coupled receptor ligands and growth factors (14).

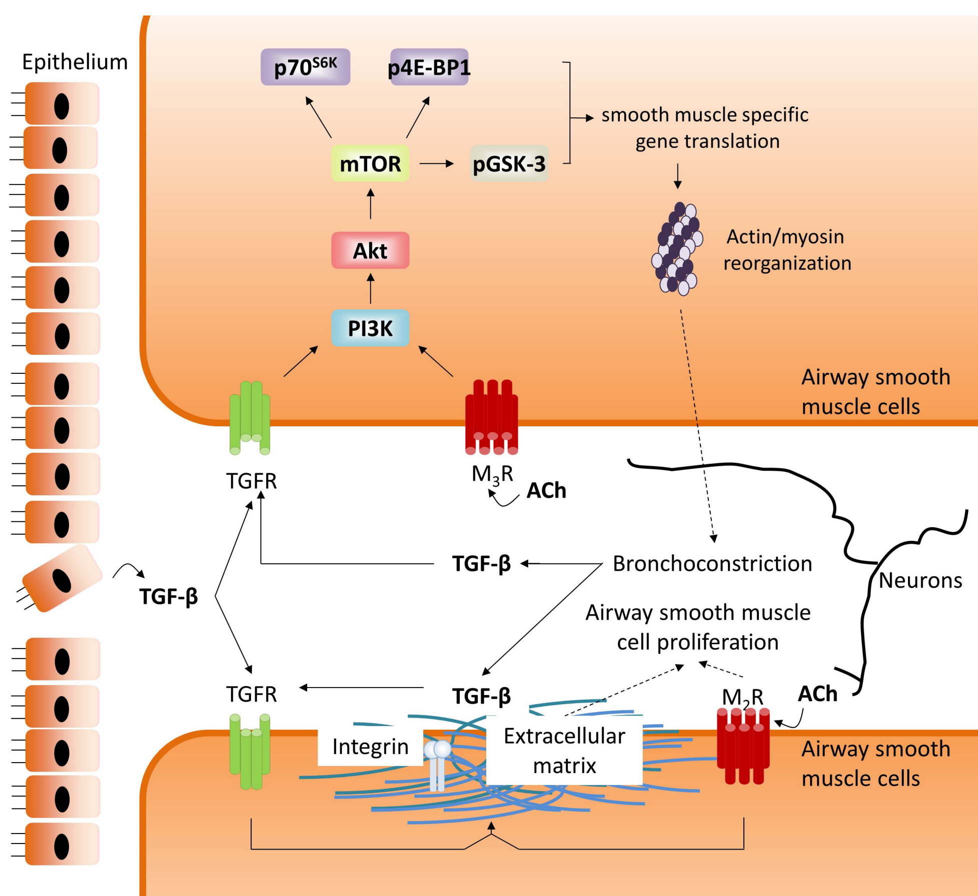

Figure 7.1. Functional interactions of muscarinic receptors and TGF-β in airway smooth muscle remodelling. The release of TGF-β by damaged epithelial cells, inflammatory cells and extracellular matrix proteins triggers airway smooth muscle phenotype switching by inducing airway smooth muscle cell proliferation and contractile protein expression. Muscarinic receptor activation on the airway smooth muscle cell enhances these processes. In particular, muscarinic receptors interact with TGF-β activation to allow phosphorylation of 4E-BP1 and GSK-3, leading to increased smooth muscle-specific gene translation in airway smooth muscle cells, followed by reorganisation of actin and myosin filaments to induce contraction. The interaction of muscarinic M_2 receptors with TGF-β promotes the deposition of extracellular matrix proteins, which triggers airway smooth muscle cell proliferation through integrins. The cooperative interactions of TGF-β and muscarinic receptors promoting airway smooth muscle remodelling may lead to airway hyperresponsiveness. TGFR: transforming growth factor receptor, TGF-β: transforming growth factor-β, ACh: acetylcholine, M_2R: muscarinic M_2 receptor, M_3R: muscarinic M_3 receptor, mTOR: mammalian target of rapamycin; PI3K: phosphatidyl inositide 3-kinase, GSK-3: glycogen synthase kinase 3; 4E-BP1: eukaryotic translation initiation factor 4E-binding protein 1.

Interestingly, in addition to its role in airway smooth muscle maturation, we also demonstrated that prolonged exposure (7 days) to TGF-β_1 induced airway smooth muscle cell proliferation, which was similarly significantly augmented by muscarinic receptor stimulation (**chapter 4**). Overall, this indicates a pivotal role for TGF-β_1 in airway smooth muscle phenotype switching, which is under important cholinergic control. Airway smooth muscle cells and extracellular matrix proteins communicate with each other through heterodimeric glycoproteins integrins (15). Integrins have been shown to be involved in airway smooth muscle cell proliferation; however, the potential interaction of muscarinic receptors with these processes has not been explored yet. **Chapter 4** demonstrates that TGF-β_1-induced airway smooth muscle cell proliferation is dependent on the production of the extracellular matrix proteins fibronectin and collagen I and their subsequent interaction with RGD-binding integrin α5β1 (Figure 7.1). Prolonged treatment with TGF-β_1 was required for the production of extracellular matrix proteins, which is necessary to induce airway smooth muscle cell proliferation. Also, muscarinic receptor activation enhanced TGF-β_1-induced cell proliferation and fibronectin deposition. Remarkably, this process was mediated through activation of muscarinic M_2 receptors and not M_3 receptors, as previously reported for the interaction between PDGF and muscarinic receptors in inducing airway smooth muscle cell proliferation (16, 17). This implies that in addition to muscarinic M_3 receptor-mediated effects, also muscarinic M_2 receptor mediated effects may contribute to airway smooth muscle remodelling. Furthermore, a role for GSK-3 signalling can be suggested, as fibronectin expression after TGF-β_1 stimulation has been shown to be under the regulation of the GSK-3β target β-catenin (18), whereas **chapter 3** demonstrates that crosstalk between muscarinic receptors and TGF-β_1 enhanced the phosphorylation of 4E-BP1 and GSK-3β resulting in increased expression of contractile proteins in the human airway smooth muscle cells. Overall, an important role for GSK-3 signalling in the crosstalk between muscarinic receptor and TGF-β_1 signalling in airway smooth muscle remodelling processes is suggested.

Recently, evidence for a role of methacholine-induced bronchoconstriction in airway remodelling was found in patients with asthma (19). Indeed, increased levels of collagen deposition, TGF-β expression and proliferating epithelial cells were measured in asthma patients, who were subjected to repeated methacholine challenges (19). In vitro, cyclical mechanical strain of airway smooth muscle enhanced the expression of contractile proteins in response to muscarinic

agonists (20). Moreover, contractile agonists, including methacholine, activated the release of TGF-β from airway smooth muscle cells through reorganization of the cytoskeleton and inside-out activation of $\alpha_v\beta_5$ integrins (25). This process was enhanced in asthmatic cells (21). In this thesis, we hypothesised that bronchoconstriction contributes to airway remodelling by inducing the release of TGF-β (Figure 7.1). This hypothesis was investigated in precision-cut lung slices. As demonstrated in airway smooth muscle cells ((22) and **chapter 3**) we observed that exogenous TGF-β also induced increased expression of contractile proteins in precision-cut lung slices in a time-and concentration-dependent manner (**chapter 5**). Moreover, we demonstrated that muscarinic receptor activation induced the release of endogenous biologically active TGF-β in these slices, resulting in an increase of contractile protein expression, including sm-myosin and sm-α-actin. This remodelling process was shown to be due to bronchoconstriction induced by the muscarinic agonist, as inhibition of actin polymerization (latrunculin A) prevented these effects. This was apparently in contrast to **chapter 3**, where we demonstrated that muscarinic receptor stimulation by itself did not induce increase contractile protein expression in cultured airway smooth muscle cells. Moreover, in precision cut lung slices, bronchoconstriction induced by other contractile agonists, including histamine and potassium chloride, increased the expression of contractile protein phenotype markers as well. The above mentioned findings are in agreement with the study of Grainge et al. (19), demonstrating that repeated inhalations with methacholine induce the release of TGF-β and airway remodelling in bronchial biopsies of patients with mild asthma (19). Based on our findings and those of Grainge et al., we propose that bronchoconstriction in asthma and COPD induces airway remodelling through the release of biologically active TGF-β. Our results in the precision-cut lung slices further extend the importance of the cooperative regulation of airway remodelling by muscarinic receptors and TGF-β. Overall, this would imply a beneficial effect of bronchodilators, including anticholinergics, on airway remodelling, which should be followed up in future studies. Furthermore, the use of precision-cut lung slices has proven to be a suitable model to study processes involved in airway remodelling induced by bronchoconstriction.

In conclusion, the findings presented in **chapters 2-5** point out the important functional role of muscarinic receptors in pro-inflammatory and remodelling processes in the airway smooth muscle cells and support a beneficial non-

bronchodilator role for anticholinergics to protect against these processes. A role for GSK-3/4E-BP1 signalling in airway smooth muscle remodelling processes induced by muscarinic receptor and TGF-β is proposed.

7.3 Clinical implications

The UPLIFT study (Understanding Potential Long-Term Impacts on Function with Tiotropium), a recent 4 year double-blind, randomised, placebo-controlled clinical trial investigating the effect of tiotropium on the progressive decline in FEV_1 in COPD patients, showed that tiotropium improved lung function, the risk of respiratory failure and quality of life, and reduced exacerbations and hospitalisations, but did not reduce the rate of decline of FEV_1 (23). However, in post-hoc studies, young COPD patients presenting a rapid lung function decline and GOLD stage II patients had a reduced rate of decline of FEV_1 when treated with tiotropium (24, 25), suggesting a potential disease-modifying effect of anticholinergics such as tiotropium in specific subpopulations of COPD patients. Recently, Kerstjens et al. demonstrated the potential of tiotropium in patients with severe and uncontrolled asthma, by showing a significantly improved lung function and reduced exacerbation frequency in these patients (26, 27). These effects might be explained by beneficial effects on airway inflammation and remodelling; however, further clinical studies are needed to substantiate this hypothesis. In support, repeated inhalations of methacholine induced airway remodelling in patients with mild asthma, by increasing the expression of TGF-β and collagen I (19).

7.4 Future perspectives

In this thesis, we show for the first time that muscarinic M_2 receptors are involved in airway smooth muscle phenotype switching, by promoting cell proliferation through the deposition of extracellular matrix proteins. In addition, muscarinic M_2 receptors mediate both proliferation and collagen synthesis in fibroblasts (28,29). These findings raise the question as to whether the kinetic selectivity towards the M_3 receptor of particularly the clinically used long-acting anticholinergics is

beneficial. Therefore, it will be important to study the distinct roles of M_1, M_2, and M_3 receptors in airway remodelling and inflammation.

Several approaches can be used to determine the involvement of the muscarinic receptor subtype(s) in inflammatory and remodelling processes. First, *in vitro* studies using selective antagonists in cultured cells are useful to identify the specific role of muscarinic receptor subtypes in specific cellular functions. However, many cell types are present in the airways and their response is highly dependent on intercellular communication and interaction with the environment. In this regard a more informative approach would be *in vivo* studies in animal models of asthma and COPD, using different experimental subtype-selective muscarinic receptor antagonists or muscarinic receptor subtype specific knockout mice. Alternatively, precision cut lung slices as described in **chapter 5** can be used, which may also be applied to the muscarinic receptor subtype knockout mice. Cellular infiltration from the bloodstream is lacking in these slices; however, responses of infiltrated cells can be studied when the animals are first subjected to challenges. Using this model, fewer animals would be required, as several conditions can be investigated in one animal. By understanding the effects of specific muscarinic receptor subtypes on airway remodelling, inflammation and mucus production, including the intracellular mechanisms involved, an improvement in the treatment of asthma and COPD may be acquired.

Further, findings described in this thesis demonstrate the ability of muscarinic receptors in airway smooth muscle to synergize with TGF-β-induced responses. Although TGF-β has anti-inflammatory properties, TGF-β is highly expressed in the airways of asthma and COPD patients and show as well pro-inflammatory and pro-remodelling effects (30, 31), therapy against TGF-β could therefore be envisaged. To investigate the cooperative effects of TGF-β and muscarinic receptor activation in the airways, *in vivo* models could be used. For example, the effect of an anticholinergic in transgenic mice overexpressing TGF-β could be studied. Alternatively, muscarinic receptor subtype specific knockout mice (M_1/M_2/M_3) could be treated with TGF-β and anti-TGF-β to acquire more information on the cooperative interaction of muscarinic receptors and TGF-β on airway remodelling and inflammation. The development of potent specific inhibitors of TGF-β signalling, possibly in combination with selective anticholinergics might be a promising drug therapy for asthma and COPD patients to halt and reverse both airway remodelling, inflammation and mucus production. However, decreased TGF-β signalling has also been reported to mediate parenchymal tissue

destruction in emphysema (31). SD-208, a TGF-β inhibitor has been developed; however, there may be long term concerns about the inhibition of TGF-β, like maintaining sufficient levels of regulatory T lymphocytes (32). Overall, this stresses the importance of further elucidating TGF-β signalling in chronic airway diseases.

7.5 Main conclusions

In conclusion, the studies described in this thesis revealed that:

- Muscarinic receptor stimulation augments the pro-inflammatory response of airway smooth muscle in response to cigarette smoke extract, TNF-α and PDGF, as measured by IL-8 and IL-6 release (**chapter 2**).
- The augmentation of IL-8 release is mediated by activation of PKC, followed by activation of the NF-κB- and ERK1/2-dependent pathways. This response is mediated by muscarinic M_3 receptors (**chapter 2**).
- TGF-β mediates phenotype switching, i.e. proliferation and maturation, of airway smooth muscle cells (**chapters 3, 4 and 5**).
- Muscarinic receptor stimulation enhances TGF-β-induced contractile protein expression in airway smooth muscle cells (**chapters 3 and 5**).
- The enhanced contractile protein expression by muscarinic receptor stimulation is mediated by activation of the PI3K pathway, as indicated by the phosphorylation of 4E-BP1 and GSK-3 (**chapter 3**).
- Prolonged exposure to TGF-β is required to induce airway smooth muscle cell proliferation by this cytokine. This involves enhanced deposition of extracellular matrix proteins, which subsequently induce proliferation via interaction with RGD-binding ($\alpha_5\beta_1$) integrins (**chapter 4**).
- Muscarinic M_2 receptors, but not muscarinic M_3 receptors, enhance TGF-β-induced airway smooth muscle cell proliferation by deposition of fibronectin, but not of collagen I (**chapter 4**).
- Precision cut lung slices are a suitable model to study airway remodelling processes *in vitro* (**chapter 5**).

- Bronchoconstriction induces the release of endogenous biologically active TGF-β, which contributes to airway remodelling by promoting contractile protein expression (**chapter 5**).

Beyond their role in the contraction of airway smooth muscle cells, muscarinic receptors are also important in inflammatory and remodelling responses by these cells, involving complex intracellular signalling mechanisms.

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Nederlandse samenvatting

Chronische aandoeningen van de luchtwegen, zoals astma en chronisch obstructief longlijden (COPD) vormen een groot wereldwijd gezondheidsprobleem. De wereldwijde incidentie van zowel astma als COPD stijgt en naar verwachting zal COPD in 2020 epidemische proporties aannemen. Zowel astma als COPD worden gekenmerkt door beperking van de luchtstroom en chronische aanhoudende luchtwegontsteking. Acetylcholine is de primaire parasympathische neurotransmitter in de luchtwegen en speelt een belangrijke rol in luchtwegconstrictie en mucusproductie door activering van muscarinereceptoren in de luchtwegen. Astma- en COPD-patiënten kunnen behandeld worden met anticholinergica (deze blokkeren de werking van acetylcholine op de muscarinereceptoren) om de verhoogde cholinerge tonus (ten gevolge van overmatige afgifte van acetylcholine) te blokkeren. Naast een verhoogde cholinerge tonus spelen ontsteking en structurele veranderingen van de luchtwegen (luchtweg-remodelling) een belangrijke rol bij astma en COPD. Remodelling van de zg. gladde spierlaag rond de luchtwegen bijvoorbeeld draagt sterk bij aan de pathofysiologie van deze ziektes. Hierbij kunnen luchtweg-gladde spiercellen fenotypische veranderingen ondergaan, waarbij een omkeerbare overgang van een contractiel naar een proliferatief fenotype mogelijk is.

Recentelijk is aangetoond dat acetylcholine ook kan bijdragen aan ontsteking en luchtweg-remodelling, in het bijzonder van de luchtweg-gladde spierlaag. Het is echter nog onbekend welke mechanismen hieraan ten grondslag liggen.

In dit proefschrift wordt de potentiële rol van acetylcholine bij ontsteking en luchtweg-remodelling in de luchtweg-gladde spierlaag onderzocht. In **hoofdstuk 2, 3, 4 en 5** worden nieuwe mechanismen besproken die betrokken zijn bij deze processen. **Hoofdstuk 6** geeft een overzicht van de recente ontwikkelingen op het gebied van luchtwegontsteking en -remodelling en de betrokkenheid van de muscarinereceptor hierbij.

Rol van muscarinereceptoren bij ontsteking van de luchtwegen

De bijdrage van muscarinereceptoren aan luchtwegontsteking wordt steeds duidelijker. Niet alleen is duidelijk geworden dat verschillende subtypes van muscarinereceptoren tot expressie worden gebracht in ontstekingscellen, maar ook is gevonden dat ChAT (het enzym dat acetylcholine synthetiseert) in ontstekingscellen, zoals eosinofielen, neutrofielen, lymfocyten, macrofagen en mestcellen, tot expressie wordt gebracht. In structurele cellen, zoals luchtweg-gladde spiercellen en epitheelcellen komen ook verschillende onderdelen van dit cholinerge systeem tot expressie. Ook is aangetoond dat deze laatstgenoemde celtypes kunnen bijdragen aan luchtwegontsteking d.m.v. de aanmaak en afgifte van specifieke cytokinen en chemokinen. Luchtweg-gladde spiercellen bijvoorbeeld reguleren de productie en afgifte van immunomodulerende mediatoren, zoals interleukine (IL)-8, IL-1β en transforming growth factor (TGF)-β, na activatie door pro-inflammatoire stimuli. Dit proefschrift toont aan dat luchtweg-gladde spiercellen ook pro-inflammatoire cytokinen, zoals IL-6 en IL-8, vrijzetten na activatie met sigarettenrook. Activatie van muscarine M_3 receptoren versterkt deze respons, en kan ook zelf in geringe mate de IL-8 productie induceren (**hoofdstuk 2**). Deze *in vitro* bevindingen worden ondersteund door verschillende *in vivo* studies die aangeven dat het anticholinergicum tiotropium bromide de expressie van verschillende cytokinen, waaronder IL-6, significant remt in de bronchoalveolaire lavagevloeistof van muizen die blootgesteld zijn aan sigarettenrook. Tevens verminderde tiotropium bromide de neutrofiele luchtwegontsteking na herhaalde lipopolysaccharide-blootstelling in een caviamodel voor COPD. De precieze mechanismen waarmee muscarine M_3 receptoren het vrijzetten van cytokinen zoals IL-6 en IL-8 bevorderen waren echter nog onbekend. De resultaten in **hoofdstuk 2** tonen aan dat de activatie van muscarinereceptoren op humane luchtweg-gladde spiercellen de afgifte van de cytokinen IL-8 en IL-6 bevordert, in het bijzonder in combinatie met TNF-α, platelet derived growth factor (PDGF)-AB en sigarettenrookextract. Het mechanisme achter deze synergistische effecten berust op signaaltransductie via de eiwitten PKC, NF-κB en ERK1/2. Dit mechanisme kan van belang zijn voor patiënten met COPD die anticholinergica gebruiken, omdat onsteking een belangrijk onderdeel is van de ziekte.

Rol van muscarinereceptoren bij luchtweg-remodelling

Een belangrijke groeifactor voor luchtweg-remodelling is TGF-β. Deze factor is betrokken bij meerdere processen, waaronder het herstel van weefselschade die door ontsteking is ontstaan. Bij weefselschade zal de groeifactor TGF-β celgroei (proliferatie) induceren, waaronder die van gladde spiercellen, maar ook de aanmaak van extracellulaire matrixeiwitten bevorderen. Bij chronische ontstekingsziektes, zoals COPD en astma, leiden deze mechanismen tot fibrose (overmatig bindweefsel). De expressie van TGF-β is verhoogd in de luchtwegen van astma- en COPD-patiënten. *In vivo* studies hebben aangetoond dat overexpressie van TGF-β_1, een isovorm van TGF-β, in muizen leidt tot verdikking van de luchtweg-gladde spierlaag en tot fibrose. Het toedienen van anti-TGF-β_1 antilichamen (antilichamen die de werking van TGF-β_1 blokkeren) voorkomt de verdikking van de luchtweg-gladde spierlaag na blootstelling van muizen aan allergenen. Desondanks zijn de resultaten met betrekking tot de mitogene eigenschappen van TGF-β_1 in luchtweg-gladde spiercellen *in vitro* niet eenduidig. Het blijkt dat TGF-β_1 *in vitro* zowel proliferatie (celgroei) als maturatie (verhoogde expressie van contractiele eiwitten) van de luchtweg-gladde spiercel kan induceren, afhankelijk van de concentratie. In dit proefschrift (**hoofdstuk 3, 4, en 5**) hebben we de invloed van TGF-β_1 op luchtweg-remodelling en de interactie met muscarinereceptoractivatie verder bestudeerd.

Om de invloed van TGF-β_1 en muscarinereceptoractivatie op het humane luchtweg-gladde spiercel fenotype te bepalen, hebben we de effecten van TGF-β_1, methacholine en hun combinatie op de expressie van contractiele eiwitten (**hoofdstuk 3**) en celproliferatie (**hoofdstuk 4**) onderzocht. De resultaten beschreven in **hoofdstuk 3** tonen aan dat TGF-β_1 de expressie van contractiele eiwitten zoals sm-α-actin, calponin en sm-myosin kan induceren in luchtweg-gladde spiercellen. Hoewel muscarinereceptoractivatie op zichzelf de expressie van contractiele eiwitten niet bevorderde, versterkte het significant de effecten van TGF-β_1 hierop. Muscarinereceptoractivatie draagt derhalve bij aan de maturatie van de luchtweg-gladde spiercellen. Het synergistisch effect van TGF-β_1 en muscarinereceptoractivatie wordt veroorzaakt door een verhoging van de fosforylering van glycogen synthase kinase (GSK)-3 en eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1), en beinvloedt hiermee wel de eiwit-, maar niet de genexpressie van de contractiele eiwitten. Dit is de eerste studie die crosstalk van een G-eiwit gekoppelde receptor (in dit geval de

muscarinereceptor) en TGF-β in luchtweg-gladde spiercellen heeft aangetoond. Verdere studies zijn nodig om te onderzoeken of deze crosstalk voorkomt bij andere klassen G-eiwit gekoppelde receptoren. Het is zeker de moeite waard om dit verder te onderzoeken, omdat veel remodellingsprocessen, ook buiten de luchtwegen, gekenmerkt worden door de betrokkenheid van zowel G-eiwit gekoppelde receptorliganden als van groeifactoren, zoals TGF-β.

Hoofdstuk 4 laat zien dat langdurige blootstelling van luchtweg-gladde spiercellen aan TGF-β_1 ook proliferatie kan induceren. Deze respons wordt eveneens significant versterkt door muscarinereceptoractivatie. Het mechanisme waarop de inductie van luchtweg-gladde spiercelproliferatie door TGF-β_1 en muscarinereceptoractivatie wordt bevorderd, is nader onderzocht in dit hoofdstuk. Luchtweg-gladde spiercellen en extracellulaire matrix (ECM) eiwitten (eiwitten die toegenomen zijn bij fibrose) communiceren met elkaar d.m.v. integrinen, receptoren voor ECM eiwitten op de gladde spiercel. Eerder is aangetoond dat sommige integrinen betrokken zijn bij luchtweg-gladde spiercelproliferatie, maar de mogelijke interactie met muscarinereceptoren was nog niet eerder bestudeerd. **Hoofdstuk 4** toont aan dat de door TGF-β geïnduceerde luchtweg-gladde spiercelproliferatie afhankelijk is van de productie van ECM eiwitten, zoals fibronectine en collageen I, en van hun interactie met α5β1 integrinen. Deze integrinen binden aan een specifieke aminozuursequentie (RGD) op de genoemde ECM eiwitten. Langdurige blootstelling aan TGF-β_1 is noodzakelijk om voldoende ECM eiwit te produceren voor de inductie van luchtweg-gladde spiercelproliferatie. Daarnaast verhoogt muscarinereceptor-activatie de door TGF-β_1 geïnduceerde celproliferatie en fibronectine productie, maar niet de productie van collageen I. Dit proces wordt gemedieerd door muscarine M_2 receptoren en niet door M_3 receptoren, zoals eerder was aangetoond voor de interactie met de groeifactor PDGF bij de inductie van luchtweg-gladde spiercelproliferatie. **Hoofdstuk 4** is de eerste studie die aantoont dat muscarine M_2 receptoren ook betrokken zijn bij remodellingsprocessen van luchtweg-gladde spiercellen.

Bij astmapatiënten is recent aangetoond dat bronchoconstrictie ook een rol kan spelen in luchtwegremodelling. In luchtwegbiopten van patiënten met astma die herhaald blootgesteld werden aan methacholine, werd een verhoogde collageendepositie, TGF-β-expressie en epitheelcelproliferatie aangetoond. Contractiele agonisten zoals methacholine kunnen reorganisatie van het cytoskelet en activatie van αvβ5 integrinen veroorzaken, wat kan leiden tot afgifte

van TGF-β door de luchtweg-gladde spiercellen. Dit proces bleek versterkt in luchtweg-gladde spiercellen van astmapatiënten. In dit proefschrift hebben we de hypothese onderzocht dat bronchoconstrictie bijdraagt aan luchtweg-remodelling door de TGF-β vrijzetting te bevorderen (**hoofdstuk 5**). Deze hypothese hebben we onderzocht door gebruik te maken van zogenaamde *precision-cut lung slices* (met precisie gesneden dunne longplakjes). Met behulp van deze methode hebben we aangetoond dat methacholine op een tijds- en concentratie-afhankelijke manier de expressie van contractiele eiwitten bevordert. Daarnaast tonen de resultaten van **hoofdstuk 5** aan dat muscarinereceptoractivatie de vrijzetting van endogeen, biologisch actief, TGF-β induceert in deze slices en dat dit de oorzaak is van de toename in contractiele eiwitexpressie. Dit effect werd geremd door het gebruik van latrunculin A ("verlamt" de spier door remming van de actinepolymerisatie nodig voor contractie) en een TGF-β receptorblokker. In **hoofdstuk 5** werd tevens een verhoogde expressie van contractiele eiwitten in *precision-cut lung slices* gevonden met andere contractiele stimuli, zoals histamine en kaliumchloride. Gebaseerd op onze bevindingen en de eerder genoemde klinische studie, concluderen wij dat bronchoconstrictie leidt tot luchtwegremodelling via de vrijzetting van TGF-β. Onze bevindingen in de *precision-cut lung slices* ondersteunen derhalve het belang van de coöperatieve regulatie van luchtwegremodelling door muscarinereceptoractivatie en TGF-β. Daarnaast wijst het op potentieel gunstige effecten van bronchusverwijders, zoals anticholinergica, op de luchtwegremodelling. Dit zal verder moeten worden onderzocht. Het gebruik van *precision-cut lung slices* is een zeer bruikbare methode gebleken om bronchoconstrictie-geïnduceerde luchtweg remodelling *in vitro* te bestuderen.

Samenvattend wijzen de bevindingen in **hoofdstuk 2-5** op het belang van muscarinereceptoren bij ontstekings- en remodellingsprocessen in luchtweg-gladde spiercellen en de mogelijk gunstige effecten van anticholinergica op deze processen. Dit proefschrift beschrijft ook een nieuwe rol voor GSK-3/4E-BP1 signalering in remodellingsprocessen in de luchtweg-gladde spier, gemedieerd door muscarinereceptoractivatie en TGF-β.

Toekomstperspectieven

In dit proefschrift wordt voor de eerste keer bewezen dat muscarine M_2 receptoren betrokken zijn bij fenotype veranderingen van luchtweg-gladde spiercellen, met name door het bevorderen van celproliferatie via de afzetting van ECM eiwitten. In fibroblasten zijn soortgelijke bevindingen gedaan, waar muscarine M_2 receptoren een rol hebben bij zowel proliferatie als collageensynthese. Deze bevindingen trekken het veronderstelde gunstige effect van selectieve muscarine M_3 receptorantagonisten in twijfel. Daarom is het belangrijk om de specifieke rol van muscarine M_1, M_2 en M_3 receptoren in luchtwegremodelling en -ontsteking verder in kaart te brengen.

Er zijn verschillende benaderingen om de rol van de muscarinereceptorsubtype(s) bij ontstekings- en remodellingsprocessen te bepalen. *In vitro* studies met selectieve antagonisten in gekweekte cellen kunnen bruikbaar zijn om de specifieke rol van muscarinereceptorsubtypes bij specifieke celfuncties te identificeren. Er zijn echter veel verschillende celtypes aanwezig in de luchtwegen en hun respons is sterk afhankelijk van intercellulaire communicatie en interactie met de omgeving. In dit verband zullen *in vivo* studies met diermodellen voor astma en COPD een meer informatieve benadering vormen, waarin verschillende experimentele subtype-selectieve muscarinereceptorantagonisten of muscarinereceptorsubtype-specifieke knockout-muizen onderzocht kunnen worden. Een ander alternatief is het gebruik van *precision-cut lung slices* zoals beschreven in **hoofdstuk 5**, wat ook kan worden toegepast op de muscarinereceptorsubtype knockout-muizen. Alhoewel de circulatie van bloedcellen ontbreekt in deze slices, kan de respons van geïnfiltreerde cellen wel onderzocht worden wanneer de dieren eerst blootgesteld worden aan bijvoorbeeld allergeen. Met dit model zouden minder dieren nodig zijn, omdat verschillende omstandigheden van één dier onderzocht kunnen worden. Het begrijpen van de rol van specifieke muscarinereceptorsubtypen op luchtwegremodelling, -ontsteking en slijmproductie, en de betrokken intracellulaire signaaltransductiemechanismen, kan leiden tot een verbetering in de behandeling van astma en COPD.

De bevindingen beschreven in dit proefschrift tonen het vermogen aan van muscarinereceptoren om de effecten van TGF-β op de luchtweg-gladde spieren te bevorderen. Hoewel TGF-β ontstekingsremmende eigenschappen heeft, komt TGF-β sterk tot expressie in de luchtwegen van astma- en COPD-patiënten en

heeft het ook ontstekingsbevorderende en remodellingsbevorderende effecten. Therapie gericht tegen TGF-β zou daarom overwogen moeten worden. Om de coöperatieve effecten van TGF-β en muscarinereceptoractivering in de luchtwegen te onderzoeken, kunnen *in vivo* modellen gebruikt worden. Zo zou bijvoorbeeld het effect van een anticholinergicum op transgene muizen die TGF-β tot overexpressie brengen bestudeerd kunnen worden. Een alternatief is om muscarinereceptorsubtype-specifieke knockout-muizen (M_1/M_2/M_3) te behandelen met TGF-β en anti-TGF-β. Dit zou meer informatie op kunnen leveren over de coöperatieve interactie van muscarinereceptoren en TGF-β bij luchtwegremodelling en ontstekingsprocessen. De ontwikkeling van specifieke inhibitoren van TGF-β signalering, eventueel in combinatie met een selectieve anticholinergica, zou een veelbelovende therapie voor astma- en COPD-patiënten kunnen opleveren om de luchtwegremodelling, -ontsteking en slijmproductie te verminderen. Hierbij moet echter opgemerkt worden dat juist verminderde TGF-β signalering betrokken is bij de parenchymale weefselafbraak bij emfyseem. Bovendien is met de TGF-β-remmer SD-208 aangetoond dat langdurige remming van TGF-β kan leiden tot afname van regulatoire T-lymfocyten. Daarom is verdere opheldering van de rol van TGF-β bij chronische luchtwegaandoeningen, zoals astma en COPD, noodzakelijk.

Conclusies

De belangrijkste conclusies van dit proefschrift zijn:

- Activatie van muscarinereceptoren versterkt de door sigarettenrook, TNF-α en PDGF geïnduceerde vrijzetting van IL-6 en IL-8 door luchtweg-gladde spiercellen (**hoofdstuk 2**).
- De bovengenoemde toename in IL-8 afgifte wordt gemedieerd door PKC-activatie, gevolgd door de activatie van NF-κB en ERK1/2-afhankelijke signaaltransductieroutes. De cholinerge respons wordt gemedieerd door muscarine M_3 receptoren (**hoofdstuk 2**).
- TGF-β kan zowel proliferatie als maturatie van luchtweg-gladde spiercellen induceren (**hoofdstuk 3, 4 en 5**).
- Activatie van muscarinereceptoren verhoogt de door TGF-β geïnduceerde contractiele eiwitexpressie in luchtweg-gladde spiercellen (**hoofdstuk 3 en 5**).

- De door muscarinereceptoractivatie verhoogde contractiele eiwitexpressie wordt gemedieerd door fosforylatie van 4E-BP1 en GSK-3 (**hoofdstuk 3**).
- Langdurige blootstelling aan TGF-β is noodzakelijk om proliferatie te induceren in luchtweg-gladde spiercellen. Hiebij speelt een verhoogde depositie van ECM eiwitten door TGF-ß een cruciale rol. De proliferatie wordt veroorzaakt door interactie tussen de ECM eiwitten en de luchtweg-gladde spiercel via de RGD-bindende (α5β1) integrinen (**hoofdstuk 4**).
- Muscarine M_2 receptoren versterken de door TGF-β geïnduceerde luchtweg-gladde spiercelproliferatie door bevordering van de depositie van fibronectine, maar niet van collageen I (**hoofstuk 4**).
- *Precision-cut lung slices* zijn een uitstekend model om luchtwegremodelling *in vitro* te bestuderen (**hoofdstuk 5**).
- Bronchoconstrictie induceert de afgifte van endogeen en biologisch actief TGF-β. Dit draagt bij aan luchtwegremodelling, waaronder de expressie van contractiele eiwitten (**hoofdstuk 5**).

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Curriculum Vitae

The author of this thesis was born in Groningen, The Netherlands, on the 11th of September 1984. After finishing her pre-university education (Baccalauréat Scientifique, specialty Biology, Évry, France) in 2002 with high honours, she moved to Groningen, the Netherlands and studied Biology at the University of Groningen. She obtained her Bachelor degrees Molecular Biology and Medical Biology (BSc.) in 2005 and her Master's degree Medical Pharmaceutical Sciences (MSc.) in 2008. During her Master, she performed an internship on the regulation of death receptors mediated-apoptosis in colorectal cancer cell lines at the Department of Medical Oncology, University Medical Center Groningen (Groningen, the Netherlands) under the supervision of dr. S. de Jong and B. Pennarun, followed by an internship on the diversity of T cell receptors within an immune-dominant CD8 CTL response to HSV-2 at the University of Washington, Seattle, USA under the supervision of D. Koelle, MD and dr. S. Welling-Wester. After graduation, she initiated her PhD-study at the Department of Molecular Pharmacology, where she worked on a research project funded by Boehringer Ingelheim Pharma GmbH & Co.KG, Germany, entitled: 'Muscarinic receptors as master switches of airway smooth muscle phenotype and function', the results of which are presented in this thesis. In the forthcoming year, she is active as a lecturer at the same department.

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List of abbreviations

ACh	Acetylcholine
AHR	Airway hyperresponsiveness
Akt	Protein kinase B
ANOVA	Analysis of variance
AP-1	Activator protein-1
ASM	Airway smooth muscle
BAL(F)	Bronchoalveolar lavage (fluid)
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
ChAT	Choline acetyltransferase
CHT1	Choline transporter
CTL	Choline-specific transporter-like
Coll	Collagen
COPD	Chronic obstructive pulmonary disease
COX-2	Cyclooxygenase-2
CSE	Cigarette smoke extract
CTGF	Connective tissue growth factor
DMEM	Dulbecco's modified eagle medium
ECM	Extracellular matrix
EGF	Epidermal growth factor
eIF4E	Eukaryotic initiation factor 4E
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular signal-regulated kinase
FBS	Foetal bovine serum
FEV_1	Forced expiratory volume in 1 second
FN	Fibronectin
GM-CSF	Granulocyte macrophage colony-stimulating factor
GOLD	Global Initiative for Obstructive Lung Disease
GPCR	G protein coupled receptor
GRADSP	Glycine-Arginine-Alanine-Aspartic acid-Serine-Proline
GRGDSP	Glycine-Arginine-Glycine-Aspartic acid-Serine-Proline
GSK-3	Glycogen synthase kinase 3
hASMc	Human airway smooth muscle cells

HBSS	Hank's buffered salt solution
HRP	Horseradish peroxidase
hTERT	Human telomerase reverse transcriptase
Ig	Immunoglobulin
IκBα	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
IKK	IκB kinase
IL	Interleukin
ITS	Insulin, transferrin and selenium
KC	Keratinocyte chemoattractant
LTB_4	Leukotriene B_4
LPS	Lipopolysaccharide
MAPK	Mitogen activated protein kinase
MCh	Methacholine
MEK	Mitogen-activated protein kinase kinase
MMP	Matrix metalloproteinase
(m)RNA	(messenger) Ribonucleic acid
mTOR	Mammalian target of rapamycin
MUC5	Mucin 5
NF-κB	Nuclear factor kappa B
OCT	Organic cation transporter
P70S6K	p70 ribosomal S6 kinase
PBS	Phosphate-buffered saline
PDGF	Platelet-derived growth factor
PI3-kinase	Phosphatidyl inositol 3-kinase
PKC	Protein kinase C
PMA	phorbol 12-myristate 13-acetate
RANTES	Regulated upon Activation, Normal T-cell Expressed and Secreted
RGD	Arginine-Glycine-Aspartic acid
RGDS	Arginine-Glycine-Aspartic acid-Serine
RhoA	Ras homolog gene family, member A
RSV	Respiratory syncytial virus
RT	Room temperature
SDS/PAGE	Sodium dodecyl sulfate/polycrylamide gel electrophoresis
SM22	Transgelin
sm-α-actin	*S*mooth muscle α-actin

sm-MHC	Smooth-muscle-specific myosin heavy chain
SRF	Serum response factor
TBST	Tris-buffered saline with tween
TGF-β	Transforming growth factor-β
T_H cells	T helper cells
TIMP	Tissue Inhibitor of MMPs
TNF-α	Tumor necrosis factor-α
UPLIFT	Understanding the Potential Long-term Impacts on Function with Tiotropium
VAChT	Vesicular acetylcholine transporter
VEGF	Vascular endothelial growth factor