Muscarinic receptor stimulation augments TGF-β1-induced contractile protein expression by airway smooth muscle cells

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Acetylcholine (ACh) is the primary parasympathetic neurotransmitter in the airways. It can act as a paracrine or autocrine mediator to induce cell proliferation, airway fibroblasts, and airway smooth muscle cells (48). It can also regulate airway remodeling in animal models of asthma (49), chronic obstructive pulmonary disease (COPD), and, to a lesser extent, with gastroesophageal reflux disease (8). Acetylcholine (ACh) is often prescribed to patients with chronic obstructive pulmonary disease; receptor cross-talk between the cholinergic system and TGF-β1 in ASM is, however, still unknown. We hypothesized that muscarinic receptors contribute to ASM remodeling by enhancing TGF-β1 function. Therefore, in human ASM cells, we investigated the effect of muscarinic receptor stimulation on TGF-β1-induced expression of acetylcholine; airway remodeling; sm-α-actin; calponin; glycogen synthase kinase-3β; 4E-binding protein 1; asthma; chronic obstructive pulmonary disease; receptor cross-talk

ACETYLCHOLINE (ACh) is the primary parasympathetic neurotransmitter in the airways that is associated with the regulation of bronchoconstriction and mucus secretion (21). 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 ACh (either neuronal or nonneuronal) also regulates airway inflammation and remodeling, which might contribute to the therapeutic effectiveness of these drugs (21). ACh 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 (48). 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 patients with COPD, and therefore the increased release of ACh may promote airway inflammation and remodeling (41, 43).

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, 16, 38). In addition, in animal models of COPD, tiotropium reduced LPS-induced airway neutrophilia, collagen deposition, and muscularization of microvessels (40), inhibited neutrophil elastase-induced goblet cell metaplasia (1), and reduced cigarette smoke-induced pulmonary inflammation (50). Anti-inflammatory and antiremodeling properties of tiotropium have also been demonstrated in a mouse model of gastroesophageal reflux disease (8).

The multifunctional cytokine TGF-β1 plays an important role in remodeling of the airways in various chronic airway diseases. This profibrotic cytokine is highly expressed in many cell types of the airways of patients with these diseases (26). TGF-β1 has been reported to induce proliferation and maturation of airway smooth muscle (ASM) cells, depending on its concentration (15, 26). In ASM cells, TGF-β1 promotes the expression of contractile phenotype markers, including sm-α-actin, through both transcriptional and translational control (15). Transcriptional regulation requires signaling to RhoA and Smad2/3, which promotes the nuclear presence and transcriptional activity of serum response factor (SRF) at smooth muscle-specific genes (6, 7, 15, 25). In parallel, TGF-β1 signals to phosphatidylinositol 3-kinase (PI3K), which results in downstream phosphorylation of proteins that control protein translation, including p70S6K, glycogen synthase kinase-3β (GSK-3β), and 4E-binding protein 1 (4E-BP1), a cellular response that requires the presence of caveolae (2, 12, 15, 18). Furthermore, TGF-β1 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 (9, 29).

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 (4, 17, 28, 30). Functional crosstalk between the cholinergic system and TGF-β1 in ASM is, however, still unknown. We hypothesized that muscarinic receptors contribute to ASM remodeling by enhancing TGF-β1 function. Therefore, in human ASM cells, we investigated the effect of muscarinic receptor stimulation on TGF-β1-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.

MATERIALS AND METHODS

Antibodies and reagents. Methacholine chloride (MCh) was purchased from ICN Biomedicals (Zoetermeer, The Netherlands). Human recombinant TGF-β1 was obtained from R & D Systems (Abingdon, UK). Mouse anti-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-1β (IL-1β) were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). Phospho-GSK-3-α/β (Ser21/9) antibody and phospho-4E-BP1 antibody were obtained from Cell Signaling Technology (Beverly, MA). GADPH (Ser21/9) antibody and phospho-4E-BP1 (Thr37/46) antibody were purchased from Tocris Biosciences (Bristol, UK). Antibody and total GSK-3 antibody were purchased from Jackson ImmunoResearch (West Grove, PA). The Cy3-conjugated secondary antibody was obtained from Boehringer Ingelheim Pharma (Biberach an der Riss, Germany). All other chemicals were of analytical grade.

Cell culture. Human bronchial smooth muscle cell lines immortalized by stable expression of human telomerase reverse transcriptase (hTERT) were prepared as described previously (19). The primary cultured human bronchial smooth muscle cells used to generate hTERT ASM cells were prepared from macroscopically healthy segments of second- to fourth-generation main bronchi 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% fetal bovine serum, streptomycin 50 U/ml, penicillin 50 U/ml, and amphotericin B 1.5 μg/ml. Cultures were maintained in a humidified incubator at 37°C, 5% CO2, and media were changed every 2–3 days. The expression and function of muscarinic M2 and M3 receptors in these cells have previously been described (20).

Stimulation of human ASM cells. Cells were cultured in six-well plates and grown until confluence. After serum deprivation for 24 h 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 MCh (10 μM), TGF-β1 (2 ng/ml), or the combination of MCh (10 μM) and TGF-β1 (2 ng/ml) for 2 h, 1 day, 4 days, or 7 days in serum and ITS-free media. Where mentioned, cells were preincubated with LY294002 (10 μM) or tiotropium iodide (10 μM) for 30 min. Western blotting. To obtain whole cell lysates, stimulated cells were washed once with ice-cold PBS (composition in mM: 140 NaCl, 2.6 KCl, 1.4 KH2PO4, 8.1 Na2HPO4, pH 7.4), followed by lysis using ice-cold SDS-lysis buffer (62.5 mM Tris·HCl, 2% SDS, NaF 1 mM, Na3VO4 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 nonspecific binding, membranes were blocked with blocking buffer (Tris·HCl 50 mM, Tween-20 0.1%, nonfat dried milk powder 5%) for 1 h at room temperature. Afterward, the membranes were incubated with the specific primary antibody, all diluted in blocking buffer, for 1 h at room temperature. After the membranes were washed 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 labeled with HRP was performed for 1 h at room temperature, followed by an additional three washes with TBS-T 0.1%. Bands were subsequently visualized on film using enhanced chemiluminescence reagents and analyzed by densitometry (TotalLab; Nonlinear Dynamics, Newcastle, UK). Expression and phosphorylation of proteins was normalized to GAPDH expression or to total GSK-3 for phospho-GSK-3-α/β.

Immunofluorescence. Cells were cultured on Labtek II chamber slides and grown until confluence. After serum deprivation for 24 h in DMEM supplemented with antibiotics and 1% ITS, the cells were stimulated with the muscarinic receptor agonist MCh (10 μM), alone and in combination with TGF-β1 (2 ng/ml) for 1, 4, or 7 days. After the cells were washed 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 two washes with CB. Cells were then blocked for 1 h 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, the cells were stained with mouse sm-α-actin antibody overnight at 4°C. After three washes 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 for 3 h at room temperature. Cells were then washed four times for 15 min in cyto-TBS-T, and the nuclei were stained with Hoechst 33342 (dilution 1: 1,000 in cyto-TBS-T) (Invitrogen, Breda, The Netherlands) for 1 min. Before the slides were mounted with ProLong Gold anti-fade reagent (Invitrogen), cells were washed four times with ultrapure water. After the staining, the slides were analyzed using an Olympus AX70 microscope equipped with digital image capture system (ColorView Soft System with Olympus U CMAD2 lens).

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). 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 (Bio-Rad, 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 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-ΔΔCt method.

Data analysis. Data are presented as mean values ± SE. 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 ≤ 0.05.

RESULTS

Effect of muscarinic receptor stimulation on contractile protein expression induced by TGFB-β1. We first analyzed the effects of MCh (10 μM), alone and in combination with TGFB-β1 (2 ng/ml), on the expression of sm-α-actin, calponin, ChAT, and 18S rRNA used for real-time quantitative PCR

Table 1. Primer sequences of sm-α-actin, calponin, ChAT, and 18S rRNA used for real-time quantitative PCR

<table>
<thead>
<tr>
<th>Substance</th>
<th>Primer Sequences</th>
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<tbody>
<tr>
<td>sm-α-actin</td>
<td>Forward 5′-GACCCCTGAGATCACCCTTATAGACG-3′</td>
</tr>
<tr>
<td></td>
<td>Reverse 5′-GGGAAAGACAAAGGACTCCGT-3′</td>
</tr>
<tr>
<td>Calponin</td>
<td>Forward 5′-TCCTTTCAGACCAAGCACTACACAC-3′</td>
</tr>
<tr>
<td></td>
<td>Reverse 5′-GGTTCTCTTTGGCTTCCGCA-3′</td>
</tr>
<tr>
<td>ChAT</td>
<td>Forward 5′-TTTGGTGGAGACCGCTACGT-3′</td>
</tr>
<tr>
<td></td>
<td>Reverse 5′-CAAGAGCCTATAGGCGAGCA-3′</td>
</tr>
<tr>
<td>18S rRNA</td>
<td>Forward 5′-CCGGCGCTAGAGGTTGAAATTCC-3′</td>
</tr>
<tr>
<td></td>
<td>Reverse 5′-TGGCGAAAATGCTTCTTCCGCT-3′</td>
</tr>
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ChAT, choline acetyltransferase.
on contractile protein expression (calponin and sm-α-actin) by human ASM cells. TGF-β₁ alone induced a significant increase in calponin and sm-α-actin expression in these ASM cells after 7 days (Fig. 1, A–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-β₁-induced calponin and sm-α-actin expression were significantly increased by 7 days of costimulation with MCh, to a similar extent (1.4-fold; Fig. 1, A–C). The induction of sm-α-actin was synergistic, whereas for calponin the response was additive. Also, the effect of MCh was concentration dependent (Fig. 1D).

As we expected, alterations in the morphology of the cells due to the increased contractile phenotype marker protein expression were seen after immunocytochemistry treatment was performed. Interestingly, increased sm-α-actin stress fiber formation was observed after 7 days of treatment with TGF-β₁ (Fig. 2). Costimulation with MCh clearly amplified this effect, whereas the muscarinic receptor agonist had no effect by itself (Fig. 2). Collectively, these data indicate that human ASM

![Diagram A](image)

![Diagram B](image)

![Diagram C](image)

![Diagram D](image)

Fig. 1. A–C: muscarinic receptor stimulation augments contractile protein expression induced by TGF-β₁. Human airway smooth muscle (ASM) cells were stimulated with TGF-β₁ (2 ng/ml), in the absence or presence of methacholine (MCh; 10 μM) for 1, 4, or 7 days. Cell lysates were analyzed 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 with basal; ##P < 0.01 compared with the absence of MCh. D: dose response for MCh was performed using the concentration 10⁻⁷ M, 10⁻⁶ M, 10⁻⁵ M in absence or presence of TGF-β₁ (2 ng/ml) for 7 days. Cell lysates were analyzed for the presence of calponin or sm-α-actin. Data shown of 1 experiment.
cells acquired a contractile phenotype by TGF-β1 treatment, which could be enhanced by muscarinic receptor stimulation.

**Autocrine ACh secretion is not involved in TGF-β1-induced contractile protein expression.** To investigate whether human ASM cells express ChAT in response to TGF-β1, we measured mRNA levels of ChAT induced by TGF-β1 and/or MCh after 1 (Fig. 3A) and 7 days (Fig. 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-β1, MCh, or their combination. We next preincubated ASM cells with tiotropium bromide for 30 min, followed by stimulation with TGF-β1 for 7 days. TGF-β1 induced an increase in the expression of calponin and sm-α-actin; however, pretreatment 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-β1 stimulation is not dependent on the autocrine production of ACh by ASM.

**Effect of muscarinic receptor stimulation on mRNA expression of contractile proteins induced by TGF-β1.** 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-β1 and MCh as described above. After 1 day of stimulation with TGF-β1 alone, a significant increase in calponin (Fig. 4A) and sm-α-actin (Fig. 4B) mRNA expression was measured. Stimulation with MCh by itself had no effect on the expression of calponin and sm-α-actin mRNA (Fig. 4, A and B). Moreover, calponin expression induced by TGF-β1 was not affected by MCh treatment (Fig. 4A). Surprisingly, a significant decrease in sm-α-actin mRNA expression was observed after treatment with the combination of TGF-β1 and MCh compared with TGF-β1 treatment alone (Fig. 4B). On day 7, all treatments had no effect on the mRNA expression of either calponin or sm-α-actin (Fig. 4, C and D). Collectively, these data suggest that the increase in TGF-β1-induced contractile protein expression by MCh observed after 7 days of treatment was not due to amplification of TGF-β1-induced mRNA levels of these proteins.

**Effect of muscarinic receptor stimulation on translational processes activated by TGF-β1.** We next aimed to investigate the role of translational processes in the functional interaction...
between muscarinic receptor activation and TGF-β₁. We analyzed the expression and the phosphorylation status of 4E-BP1 and GSK-3, proteins that are involved in the translation machinery of smooth muscle contractile phenotype marker proteins and that also have been shown to play a critical role in TGF-β₁-induced contractile protein accumulation (14, 15).

The PI3K pathway can induce the phosphorylation of 4E-BP1 and GSK-3 to permit the expression of contractile protein. To confirm the involvement of the PI3K signaling in contractile protein accumulation induced by TGF-β₁, we inhibited the PI3K pathway with LY294002 (10 μM). Treatment with LY294002 reduced the expression of calponin induced by TGF-β₁ and MCh (Fig. 5A).

It has previously been demonstrated that TGF-β₁ is able to induce the phosphorylation of 4E-BP1, which contributes to TGF-β₁-induced sm-α-actin expression (11). Therefore, we investigated the potential involvement of 4E-BP1 phosphorylation in the TGF-β₁ response and the combined effect ob-

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**Fig. 3.** Autocrine acetylcholine (ACh) release is not involved in the induction of contractile proteins by TGF-β₁. Human ASM cells were grown to confluence. After 24 h of serum deprivation, cells were treated with TGF-β₁ (2 ng/ml) in the absence or presence of MCh (10 μM). mRNA levels of choline acetyltransferase (ChAT) were measured after 1 day (A) or 7 days (B) of stimulation. Furthermore, cells were treated with TGF-β₁ (2 ng/ml) in the absence or presence of tiotropium bromide (10 nM) for 7 days. Cell lysates were analyzed for the presence of calponin and sm-α-actin (C). Data shown are the means ± SE of 3–6 experiments.

**Fig. 4.** A–D: muscarinic receptor stimulation does not augment mRNA expression of calponin and sm-α-actin induced by TGF-β₁. Human ASM cells were stimulated with TGF-β₁ (2 ng/ml), in the absence or presence of 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 ≤ 0.01, ***p ≤ 0.001 compared with basal; #p ≤ 0.05 compared with the absence of MCh.
served with MCh. To this aim, we stimulated ASM cells for 2 h and 24 h with TGF-β₁ and MCh alone (Fig. 5B). Interestingly, however, a significant, additive effect was observed with the combination of both stimuli, at 24 h after stimulation. To ensure that the PI3K pathway was involved in the phosphorylation of 4E-BP1 by TGF-β₁ and MCh, ASM cells were pretreated with LY294002, which led to a
reduction in the phosphorylation of 4E-BP1 induced by both stimuli (Fig. 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 (3, 11). GSK-3β is active in its unphosphorylated form and inhibits protein translation by phosphorylating eukaryotic initiation factor-2B. Therefore, we investigated whether the phosphorylation of GSK-3 (ser9/ser21) was induced by TGF-β1 and MCh. ASM cells were stimulated for 2 and 24 h with TGF-β1, MCh, and their combination. For all treatments, only the phosphorylated β-isof orm of GSK-3 was detected. Interestingly, after 2 h of stimulation, TGF-β1 and MCh alone were ineffective, whereas their combination induced a significant, additive increase in GSK-3β phosphorylation (Fig. 5C). No significant effects were observed after 24 h of stimulation. To ensure that the PI3K pathway was also involved in the phosphorylation of GSK-3β by TGF-β1 and MCh, ASM cells were pretreated with LY294002, which led to an inhibition of the phosphorylation of GSK-3β induced by both stimuli (Fig. 5C).

DISCUSSION

In the present study, we demonstrate that a cross-talk between TGF-β receptors and muscarinic receptors regulates the expression of contractile phenotype marker proteins and the formation of sm-α-actin stress fibers 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 (23, 33), suggesting that anticholinergics may have beneficial effects on multiple pathological tissue remodeling processes in obstructive airways diseases.

Previous studies indicated that ASM expresses mRNA for ChAT, the synthesizing enzyme for ACh (49). Also, ChAT expression and autocrine ACh release by fibroblasts and epithelial cells were proposed to regulate cell proliferation and cytokine release by these cells (41, 47). 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-β1, and antagonism of muscarinic receptors using tiotropium had no effect on TGF-β1-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-β1 and suggests that the above-mentioned regulatory effects of muscarinic receptors on ASM require ACh release, for example, by airway neurons and away epithelium.

The signaling mechanisms that underpin these remodeling processes have thus 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 SRF and its coactivators myocardin and megakaryocytic acute leukemia (6, 7, 15, 25, 34). The translation of the smooth muscle-specific genes is, however, dependent on the PI3K/Akt signaling pathway (15, 24, 32, 42, 51). Thus TGF-β1-induced contractile protein expression is paralleled by the phosphorylation of GSK-3β and 4E-BP1, both downstream targets of PI3K/Akt signaling (2, 15), and the phosphorylation of 4E-BP1 is required for TGF-β1-induced contractile protein expression (51). 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 (4, 17, 28, 30). Interestingly, our current results show that the increased expression of contractile proteins induced by TGF-β1 and muscarinic receptor stimulation are also mediated by translational processes, including the phosphorylation of GSK-3β and 4E-BP1, but not by the PI3K/Akt signaling pathway.
by transcriptional processes, as mRNA expression of these smooth muscle-specific genes was not affected. Furthermore, total protein expression in these cells is increased by TGF-β1, which is enhanced by MCh treatment (data not shown). This suggests that muscarinic receptor stimulation exerts its action through cooperative activation of the PI3K/Akt/GSK-3-signaling pathway by TGF-β1, 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 (17), 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, hyper-trophy, and expression of contractile proteins, including sm-α-actin (2).

To our knowledge, this is the first study reporting cross-talk between a G protein-coupled receptor and TGF-β in ASM remodeling. Panettieri et al. (39) showed that the expression of extracellular matrix proteins induced by TGF-β1 was not influenced by CysLT1 receptor stimulation in human ASM cells, indicating that this cross-talk may be receptor dependent. In neural progenitor cells, Morishita et al. (36) reported the ability of a G protein-coupled receptor and TGF-β to enhance the activity of the sm-α-actin promoter; however, these activities were independent of each other. Collectively, our findings suggest that G protein-coupled receptors and TGF-β are able to cooperatively induce airway remodeling processes although clearly it cannot be assumed that this occurs for all G protein-coupled receptor classes. These findings are nonetheless of interest to future studies, as many remodeling processes, also outside the airways, involve cooperative regulation by G protein-coupled receptor ligands and growth factors (28).

In the Understanding Potential Long-term Impacts on Function with Tiotropium (UPLIFT) study, patients with COPD treated with tiotropium bromide during a 4-yr period had a better quality of life and lung function, and the frequency of exacerbation was also reduced. Recently, tiotropium has also been proven to improve the lung function in patients with severe uncontrolled asthma (27). The mechanisms behind these effects are still unknown. However, muscarinic receptor-induced airway remodeling, 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 MCh induces airway remodeling in patients with asthma, including the expression of TGF-β and collagen I in bronchial biopsies (22). This substantiates the hypothesis that cholinergic activation leads to remodeling via cross-talk with TGF-β in these patients. These effects were suggested by the authors as the result of mechanical forces resulting from the bronchoconstriction itself. However, functional cross-talk of MCh and TGF-β on structural cells, resulting in remodeling, 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-β1. These findings provide a plausible mechanistic explanation for our earlier observations that demonstrate protective effects of tiotropium on ASM remodeling in repeatedly allergen-challenged guinea pigs. This implies a role for G protein-coupled receptors in TGF-β1-induced remodeling in the pathogenesis of chronic airway diseases like asthma and COPD.

**DISCLOSURES**

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**AUTHOR CONTRIBUTIONS**


**REFERENCES**


