Differential effects of fluticasone on extracellular matrix production by airway and parenchymal fibroblasts in severe COPD

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Chronic obstructive pulmonary disease (COPD) is a progressive lung disease that is predominantly caused by longstanding smoking. Its progression can generally not be halted by currently available therapies. COPD is characterized by abnormal repair in the lung resulting in airway obstruction associated with emphysema and loss of lung tissue (emphysema) (9). The molecular basis and inflammation in the small airways and lung parenchyma in COPD is needed and clinically relevant with respect to its implications for improved and better targeted treatment.

Next to smoking cessation, which is crucial in preventing worsening of disease, bronchodilators and inhaled steroids are currently standard treatment options for patients with COPD (6). None of these treatments can cure COPD, but inhaled steroids either alone or in combination with bronchodilators have been shown to improve health status and respiratory symptoms next to reducing frequent exacerbations (1, 2, 18, 21). Some recent studies have demonstrated that inhaled steroids can also reduce the decline in lung function at least in a subset of COPD patients (3, 13).

It is yet unclear whether and to what extent inhaled steroids can affect the abnormal repair process in the airways and lung parenchyma in COPD. We investigated the effects of fluticasone on transforming growth factor (TGF)-β-induced changes in mothers against decapentaplegic homolog (Smad) signaling and extracellular matrix (ECM) production in airway and parenchymal lung fibroblasts from patients with severe COPD. We showed that TGF-β-induced ECM production by pulmonary fibroblasts, but not activation of the Smad pathway, was sensitive to the effects of fluticasone. Fluticasone induced decorin production by airway fibroblasts and partly reversed the negative effects of TGF-β treatment. Fluticasone inhibited biglycan production in both airway and parenchymal fibroblasts and procollagen 1 production only in parenchymal fibroblasts, thereby restoring the basal difference in procollagen 1 production between airway and parenchymal fibroblasts. Our findings suggest that the effects of steroids on the airway compartment may be beneficial for patients with severe COPD, i.e., restoration of decorin loss around the airways, whereas the effects of steroids on the parenchyma may be detrimental, since the tissue repair response, i.e., biglycan and procollagen production, is inhibited. More research is needed to further disentangle these differential effects of steroid treatment on the different lung compartments and its impact on tissue repair and remodeling in COPD.

Airway fibrosis can take place in one organ, the lung, are intriguing, especially so since they are caused by one common etiological factor (smoking). The presence and degree of airway fibrosis and emphysema varies between patients and may explain the heterogeneity in the response to treatment in COPD patients. Therefore, further insight in the underlying molecular mechanisms of tissue breakdown, repair, and fibrosis in COPD is needed.

Chronic obstructive pulmonary disease; tissue repair and remodeling

CHRONIC OBSTRUCTIVE PULMONARY disease (COPD) is a progressive lung disease that is predominantly caused by longstanding smoking. Its progression can generally not be halted by currently available therapies. COPD is characterized by persistent airway obstruction. This airway obstruction is, apart from changes in large airways like smooth muscle and goblet cell hyperplasia, predominantly caused by peripheral airway fibrosis and inflammation in the small airways and lung parenchyma, and loss of lung tissue (emphysema) (9). The molecular mechanisms by which tissue breakdown and repair as well as

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METHODS

Subjects. Primary lung fibroblasts were freshly isolated from lung tissue of patients with severe stage IV COPD, undergoing lung transplant surgery. All patients were female, had quit smoking for at least one year, and were using inhaled and/or oral steroids at the time of surgery. Emphysema was confirmed by routine histological review of lung tissue sections by an experienced pulmonary pathologist. The study protocol was consistent with the Research Code of the University Medical Center Groningen (http://www.rug.nl/umcg/onderzoek/researchcode/index) and national ethical and professional guidelines (http://www.federa.org).

Isolation, culture, and stimulation of primary fibroblasts. Fibroblast cultures were established by means of an explant technique as described previously (15, 16). For our airway fibroblast cell cultures, we start at the main stem bronchus and dissect airway tissue ranging from the third to fifth generation. For parenchymal fibroblasts, lung tissue is taken from peripheral areas of the lung with exclusion of visible blood vessels and airways. There was no mycoplasma contamination in the fibroblast cultures, as confirmed by a mycoplasma detection kit (Roche Diagnostics, Almere, The Netherlands). Isolated cells were characterized as fibroblasts by morphological appearance and expression pattern of specific proteins as described previously (16). Fibroblasts were cultured in complete culture medium [Ham’s F-12, 10% FBS, penicillin, streptomycin, and glutamin (all from Lonza, Breda, the Netherlands)]. Experiments were performed on paired sets (n = 6) of airway and parenchymal fibroblasts of passage 5/6. After reaching confluence, fibroblasts were cultured for 24 h on 0.5% FBS culture medium before the stimulations started. Cells were washed with Ham’s F-12 culture medium (Lonza) without FBS and incubated with the appropriate stimulus, diluted in 0.5% FBS culture medium. Fluticasone (GSK, London, UK) was used in a concentration of 10^{-8} M, TGF-β (R&D Systems, Abingdon, UK) was used in a concentration of 100 U/ml (= 3.1 ng/ml), and cigarette smoke extract (CSE) was used in a concentration of 2.5%. CSE was prepared according to a standardized protocol by bubbling the puff of four 3R4F reference cigarettes (Kentucky University research cigarettes) through 50 ml of Ham’s F-12 medium (100% CSE). The smoke extract medium was sterilized with a 22-μm filter. After 1, 24, and 72 h of stimulation, cells were harvested, and the supernatants were collected. For RNA isolation, the cells were washed two times with Hank’s balanced salt solution (Lonza) and lysed using the lysis buffer of the RNeasy mini kit (Qiagen, Hilden, Germany). For protein assessment, the cells were lysed in a lysis buffer containing 20 mmol/l Tris (pH 7.4), 150 mmol/l NaCl, 1 mmol/l EDTA, 1% Triton, 1 mmol/l β-glycerophosphate, 1 mmol/l Na3VO4, 1 μg/ml leupeptin, and 1 mmol/l phenylmethylsulfonyl fluoride.

Optimal concentrations for the different stimuli and optimal time points for mRNA and protein measurements of the different outcome parameters were determined in several test experiments (data not shown). Based on these findings, we decided to assess Smad3 phosphorylation at 1 h, mRNA levels at 24 h, and protein levels in cell lysates and supernatants at 72 h.

RNA isolation and real-time PCR. Fibroblast total RNA was isolated using the RNeasy mini kit (Qiagen). RNA quantity and quality (OD 260/280) were determined by the Nanodrop D1000 (Nanodrop Technologies, Wilmington, DE). Genomic DNA contamination was removed using a Qiagen RNaase-free DNase set. Fifteen hundred nanograms of mRNA were transcribed into cDNA by reverse transcriptase II (Invitrogen, Breda, the Netherlands). Real-time PCR was performed on an ABI7900HT Sequence using “Assays on Demand” from Applied Biosystems (Foster City, CA), according to the manufacturers’ instructions. An input of 3 ng cDNA was used per sample. Expression of the following genes was analyzed in the 24-h samples: mothers against decapentaplegic homolog-3 (SMAD3), SMAD7, biglycan, collagen type I α2, and decorin. Data were analyzed by the 2^{ΔΔCt} method, as described by Livak and Schmittgen (14). In brief, concentration in time (Ct) values of the genes of interest were corrected for Ct values from a housekeeping gene, resulting in a Δ−Ct value. The 2^{ΔΔCt} was calculated and used for our analysis. Several housekeeping genes were tested for the influence of the experimental procedure on the expression. Ribosomal protein S9 was chosen as the optimal housekeeping gene.

Protein analysis. Total protein concentrations were measured by the Bio-Rad (Veenendaal, The Netherlands) protein assay. The amount of decorin, procollagen I, and phospho-Smad3 was determined by enzyme-linked immunosorbent assay (ELISA). The amount of Smad3, Smad7, and biglycan was measured by Western blot. The R&D Systems human Duoset ELISA was used to measure decorin concentration in cell lysates after 72 h stimulation. Procollagen I was determined by the Takara-Bio ELISA kit (Lonza) in lysates after 72 h stimulation. Phospho-Smad3 was detected in the lysates using the ELISA assay published by Kapoun et al. (10) after 1 h stimulation. In brief, 96-well plates were coated with anti-Smad2/3 monoclonal antibody (BD Bioscience, Breda, The Netherlands) overnight at 4°C. Nonspecific binding sites were blocked with 0.3% BSA/FBS for 2 h at room temperature. After washing the 96-well plates with 0.5% Tween 20/PBS, we added cell lysates (10 g of total protein), incubated them overnight at 4°C. After washing the plates, we added a polyclonal anti-phospho-Smad3 antibody (Cell Signaling, Leiden, Netherlands) diluted in 2% BSA-0.05% Tween 20-PBS, incubated for 2 h at room temperature on a shaker. Wells were washed and incubated with goat-anti-rabbit IgG-HP (Dako, Heverlee, Belgium) for 1 h. Staining was visualized using tetramethylbenzidine (Sigma Aldrich, Zwijndrecht, Netherlands), and plates were read at an optical density of 450 nm. MRC5 cells (fetal lung fibroblasts, ATCC, CCL-171) that were stimulated with TGF-β for 1 h were used for the standard curve. All values were calculated relative to this standard curve.

The amount of Smad3, Smad7, and biglycan was detected in the 72-h samples using Western blot. Ten micrometers of total protein were run on 10% denaturing polyacrylamide gels and transferred to nitrocellulose blots. The blots were blocked with 5% skim milk (Smad3 and -7) or 5% FBS/PBS (biglycan) for 1 h and incubated overnight with primary antibodies against Smad3 (rabbit polyclonal; Zymed, Invitrogen), Smad7 (monoclonal; Abnova, Heidelberg, Germany), and biglycan (goat polyclonal; R&D Systems). After being rinsed, the blots were incubated with peroxidase-labeled secondary antibodies for 1 h. Staining was visualized using Supersignal chemiluminescent substrate (Pierce, Etten-Leur, Netherlands), and densities of the bands were determined by densitometry using Quantity One software from Bio-Rad. β-Actin (rabbit-polyclonal; Abcam) was used as loading control, and Smad3, Smad7, and biglycan values were expressed as a ratio compared with β-actin.

Statistical analysis. Differences between all groups, including different stimulations and airway and parenchymal samples, were analyzed using the Kruskall-Wallace test. When significant, differences in basal expression levels between airway and parenchymal samples were analyzed using the nonparametric Mann-Whitney U-test. Differences between basal expression and the different stimulations, e.g., basal vs. TGF or basal + fluticasone, within the airway and parenchymal samples were analyzed using the nonparametric Wilcoxon paired-samples test. P < 0.05 was considered significant.

RESULTS

TGF-β-induced changes in Smad gene and protein expression are not sensitive to fluticasone in both airway and parenchymal fibroblasts. At basal conditions, Smad3 mRNA levels were lower in airway fibroblasts than parenchymal fibroblasts (Fig. 1A). Fluticasone did not affect Smad3 mRNA and protein levels in airway fibroblasts, but it significantly reduced both Smad3 mRNA and protein levels in parenchymal fibroblasts at basal conditions (Fig. 1A).
Fig. 1. Mothers against decapentaplegic homolog (Smad) gene and protein expression in airway and parenchymal fibroblasts. Smad3 (A) and Smad7 (B) mRNA (24 h) and protein (72 h) expression and phosphorylated Smad3 protein (1 h) expression (C) is depicted in airway (left) and parenchymal (right) fibroblasts from severe chronic obstructive pulmonary disease (COPD) patients at basal conditions and after transforming growth factor-β (TGF-β) and cigarette smoke extract (CSE) stimulation with and without fluticasone. Each fibroblast cell line derived from a single COPD patient is indicated with a different symbol. mRNA data are expressed as $2^{-ΔΔCt}$ values, which are raw values corrected for housekeeping gene expression. Smad3 and Smad7 protein levels are expressed as density values relative to β-actin expression, and representative Western blot images are shown below the graph for 1 fibroblast cell line. Phosphorylated Smad3 protein levels are expressed as relative enzyme-linked immunosorbent assay (ELISA) units. $^*$Significant difference ($P < 0.05$, Mann-Whitney U-test) in basal expression levels between airway and parenchymal fibroblasts. $^\#$Significant difference ($P < 0.05$, Wilcoxon paired-samples test) in stimulated cells compared with basal expression levels. *Significant effect of fluticasone ($P < 0.05$, Wilcoxon paired-samples test).
TGF-β reduced Smad3 mRNA, and not protein expression, in both airway and parenchymal fibroblast. These effects were not affected by fluticasone treatment.

CSE increased Smad3 mRNA levels in airway fibroblasts, and this effect was inhibited by fluticasone treatment. A similar inhibitory effect of fluticasone was present on Smad3 protein levels in CSE-treated airway and parenchymal (trend) fibroblasts.

TGF-β clearly increased Smad7 mRNA, and not protein expression, in both airway and parenchymal fibroblasts (Fig. 1B). These effects were not affected by fluticasone. CSE had no effect on Smad7 mRNA and protein expression.

CSE increased Smad3 mRNA levels in airway fibroblasts, and this effect was inhibited by fluticasone treatment. A similar inhibitory effect of fluticasone was present on Smad3 protein levels in CSE-treated airway and parenchymal (trend) fibroblasts.

Fluticasone reduces biglycan protein levels in airway and parenchymal fibroblasts and partly inhibits the effect of TGF-β in airway fibroblasts. At basal conditions, procollagen 1 levels were lower in airway than parenchymal fibroblasts (Fig. 4). Fluticasone inhibited procollagen 1 protein production in pa-

Fluticasone reduces biglycan mRNA levels in airway fibroblasts, whereas it significantly induced Smad3 phosphorylation in parenchymal fibroblasts, and this effect was not affected by fluticasone (Fig. 1C). Fluticasone decreased Smad3 phosphorylation in airway fibroblasts treated with CSE. CSE treatment itself decreased Smad3 phosphorylation in parenchymal fibroblasts.

Fluticasone induces decorin mRNA and protein expression in airway and parenchymal fibroblasts and restores the negative effect of TGF-β treatment. TGF-β reduced decorin mRNA levels in airway fibroblasts and decorin protein levels in both airway and parenchymal fibroblasts (Fig. 2). Under (almost) all conditions, fluticasone increased decorin mRNA and protein levels in airway and parenchymal fibroblasts, and thus to a considerable extent restored the negative effects of TGF-β. Clearly, fluticasone effects on decorin protein levels were most pronounced in airway fibroblast. The effects of fluticasone in parenchymal fibroblasts were only of borderline significance at basal conditions and after TGF-β treatment. Fluticasone did not further increase the CSE-induced increase in decorin protein levels in parenchymal fibroblasts.

Fluticasone reduces biglycan mRNA and protein levels in both airway and parenchymal fibroblasts (Fig. 3). Fluticasone had no effect on TGF-β-induced biglycan protein levels in airway fibroblasts, whereas it significantly reduced TGF-β-induced biglycan protein levels in parenchymal fibroblasts. At basal conditions and in combination with CSE treatment, fluticasone reduced biglycan protein levels in both airway and parenchymal fibroblasts.

Fluticasone reduces procollagen 1 protein levels in parenchymal fibroblasts and only partly inhibits the effect of TGF-β in airway fibroblasts. At basal conditions, procollagen 1 levels were lower in airway than parenchymal fibroblasts (Fig. 4). Fluticasone inhibited procollagen 1 protein production in pa-

Fig. 2. Decorin (DCN) gene and protein expression in airway and parenchymal fibroblasts. Decorin mRNA (24 h) and protein (72 h) expression in cell lysates is depicted in airway (left) and parenchymal (right) fibroblasts from severe COPD patients at basal conditions and after TGF-β and CSE stimulation with and without fluticasone. Each fibroblast cell line derived from a single COPD patient is indicated with a different symbol. mRNA data are expressed as 2ΔCt values, which are raw values corrected for housekeeping gene expression. Protein levels are expressed in μg/ml as assessed in the ELISA. *Significant difference (P < 0.05, Wilcoxon paired-samples test) in stimulated cells compared with basal expression levels. **Significant effect of fluticasone (P < 0.05, Wilcoxon paired-samples test).
renchymal fibroblasts under basal conditions and thus restored this difference.

TGF-β increased collagen 1α2 mRNA expression in airway and parenchymal fibroblasts as well as procollagen 1 protein levels in airway fibroblasts (Fig. 4). Fluticasone had only minimal effects on TGF-β-induced increases in collagen 1α2 mRNA and procollagen 1 protein levels in airway fibroblasts, whereas it significantly reduced procollagen 1 protein levels in parenchymal fibroblasts under all conditions.

**DISCUSSION**

To find new and more individualized treatment strategies for patients with COPD, better insight is needed into the abnormal smoke-induced tissue remodeling and repair process in COPD lungs. This is particularly true for the differences between the airway and parenchymal lung compartments given their putative role in progression of the disease. We hypothesized that different lung fibroblast phenotypes exist and that steroids differentially affect fibroblasts derived from airways and lung parenchyma.

Our findings demonstrated that airway and parenchymal fibroblasts from patients with severe COPD differ in ECM production and response to TGF-β and that fluticasone differentially affects airway and parenchymal fibroblasts.

At basal conditions, airway fibroblasts produced significantly lower levels of Smad3 mRNA and procollagen-1 protein than parenchymal fibroblasts. Particularly after TGF-β stimulation, we found some essential differences between airway and parenchymal fibroblasts. In airway fibroblasts, TGF-β significantly increased procollagen 1 production without affecting Smad3 phosphorylation, whereas in parenchymal fibroblasts TGF-β did not induce procollagen production, while Smad3 phosphorylation was clearly increased. These observations may suggest a less-controlled collagen production in the...
airways of COPD patients, which could favor airway remodeling together with a hampered Smad pathway-induced procollagen production in the parenchyma, which fits with a lack of repair. Our findings regarding these phenotypic differences between pulmonary fibroblasts from different lung compartments in severe COPD are in line with previous studies in asthma patients (12, 24), healthy donors and cancer patients (17), and COPD (8). The observation that Smad3 mRNA levels and procollagen 1 protein levels were both higher in parenchymal than airway fibroblasts at baseline could be explained as a reflection of an ongoing attempt to repair parenchymal damage.

A next question was whether an inhaled corticosteroid affects the TGF-β/Smad pathway and ECM production in airway and parenchymal fibroblasts. Fluticasone did not affect TGF-β-induced Smad pathway activation, as demonstrated by increased Smad7 mRNA expression and Smad3 phosphorylation, whereas it clearly affected ECM production by pulmonary fibroblasts. Fluticasone increased decorin mRNA and protein production by airway fibroblasts, and the negative effect of TGF-β treatment on decorin production was restored to a considerable extent. Additionally, fluticasone inhibited biglycan protein production in both airway and parenchymal fibroblasts, albeit that TGF-β-induced increases in biglycan production in airway fibroblasts were not inhibited by fluticasone. Interestingly, fluticasone inhibited procollagen 1 production only in parenchymal fibroblasts, thereby restoring the basal difference in procollagen 1 production between airway and parenchymal fibroblasts.

Our findings regarding the effects of fluticasone on ECM protein production in airway and parenchymal fibroblasts are of particular interest, since the presence of these matrix proteins is the end result of remodeling and repair processes in the lung. We have previously shown that decorin expression is decreased around the airways of COPD patients (20, 23). Our current observation that decorin production is stimulated by fluticasone in airway fibroblasts indicates that steroids may still be capable of restoring decorin loss around the airways in patients with very severe COPD. On the other hand, fluticasone inhibited the production of biglycan and procollagen in parenchymal fibroblasts from these severe COPD patients, indicating that steroids may inhibit the repair response particularly of fibroblasts that are located in emphysematous areas of the lung that may need repair.

When comparing our findings with other studies investigating steroid effects on ECM proteins in human lung fibroblasts, we found some overlapping but also different findings. Goulet et al. showed that fluticasone reduced total ECM and collagen

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**Fig. 4.** Collagen gene and protein expression in airway and parenchymal fibroblasts. Collagen 1α2 mRNA (COL1A2) expression (24 h) and procollagen-1 protein expression (72 h) in cell lysates is depicted in airway (left) and parenchymal (right) fibroblasts from severe COPD patients at basal conditions and after TGF-β and CSE stimulation with and without fluticasone. Each fibroblast cell line derived from a single COPD patient is indicated by a different symbol. mRNA data are expressed as 2^(-ΔΔCt) values, which are raw values corrected for housekeeping gene expression. Protein levels are expressed in ng/ml as assessed in the ELISA. $Significant difference (P < 0.05, Mann-Whitney U-test) in basal expression levels between airway and parenchymal fibroblasts. #Significant difference (P < 0.05, Wilcoxon paired-samples test) in stimulated cells compared with basal expression levels. *Significant effect of fluticasone (P < 0.05, Wilcoxon paired-samples test).
deposition at basal conditions in healthy peripheral lung fibroblasts (7). This is similar to what we found at basal conditions in our parenchymal fibroblasts from severe COPD patients. However, in the presence of FCS, which was used to mimic inflammatory conditions, Goulet et al. showed that ECM and collagen production was not reduced but increased by fluticasone, which is clearly different from the decrease we found in severe COPD. This discrepancy could signify that parenchymal fibroblasts from healthy donors, as used by Goulet et al., still have the capability to repair lung tissue by increasing their ECM production in inflammatory conditions, whereas fibroblasts from our severe COPD patients do not have this capability.

Our findings regarding the effects of fluticasone on decorin and biglycan production are in line with recent findings in asthma (19). Todorova et al. showed that budesonide could not reverse TGF-β-induced increases in biglycan production by airway fibroblasts from asthmatic subjects. Furthermore, they demonstrated a reversal of the TGF-β-induced decrease in decorin production by budesonide, also similar to our observations in severe COPD.

Our study provides a first clue about differences between fibroblasts from different lung compartments in severe COPD and the differential effects of steroid treatment in this respect. In summary, there appears to be uncontrolled collagen production in airway fibroblasts, whereas there is a diminished repair capacity by parenchymal fibroblasts in patients with severe COPD. Our findings regarding the effects of steroids on the different compartments indicate that steroids may have a beneficial effect on the airway compartment in severe COPD patients, i.e., restoration of decorin loss around the airways, whereas the effects of steroids may be detrimental on the parenchymal compartment, since the tissue repair response is (further) inhibited. The net balance between these effects has yet to be established. Clearly, this is the first step toward better understanding of heterogeneity of COPD and interindividual differential effects of inhaled steroids in COPD. Our data suggest that established treatments and novel treatments yet to be discovered should be investigated as to their effects on repair capacity of cells in central and peripheral lung compartments separately.

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AUTHOR CONTRIBUTIONS
Author contributions: C.A.B. and M.R.J. analyzed data; C.A.B., W.T., and D.S.P. interpreted results of experiments; C.A.B. prepared figures; C.A.B. drafted manuscript; C.A.B., W.T., M.R.J., and D.S.P. edited and revised manuscript; C.A.B., W.T., M.R.J., B.R., J.A.N., and D.S.P. approved final version of manuscript; W.T., M.R.J., and D.S.P. conception and design of research; M.R.J., B.R., and J.A.N. performed experiments.

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