Role of activin-A in cigarette smoke-induced inflammation and COPD

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ABSTRACT

Objective: Activin-A is a pleiotropic cytokine belonging to the TGF-β superfamily and has been implicated in asthma and pulmonary fibrosis. However, the role of activin-A and its endogenous inhibitor, follistatin, in the pathogenesis of Chronic Obstructive Pulmonary Disease (COPD) is unknown.

Methods: We first quantified activin-A and follistatin in lungs of air- or CS-exposed mice and in lungs of patients with COPD by immunohistochemistry, ELISA and qRT-PCR. We subsequently studied the effect of CS on primary human bronchial epithelial cells (HBECs) in vitro. Next, activin-A signalling was antagonized in vivo by administration of follistatin in mice exposed to air or CS for 4 weeks.

Results: Protein levels of activin-A were increased in the airway epithelium of patients with COPD compared with never-smokers and smokers. CS-exposed HBECs expressed higher levels of activin-A and lower levels of follistatin. Both mRNA and protein levels of activin-A were increased in lungs of CS-exposed mice, whereas follistatin levels were reduced upon CS exposure. Importantly, administration of follistatin attenuated the CS-induced increase of inflammatory cells and mediators in the bronchoalveolar lavage fluid in mice.

Conclusions: These results suggest that an imbalance between activin-A and follistatin contributes to the pathogenesis of CS-induced inflammation and COPD.

ABSTRACT WORD COUNT: 198

KEYWORDS: activin-A, cigarette smoke, COPD
INTRODUCTION

Chronic Obstructive Pulmonary Disease (COPD) is a chronic respiratory disease with obstruction of the small airways (obstructive bronchiolitis) and destruction of lung parenchyma (emphysema). The major risk factor for COPD is tobacco smoking, but air pollution and occupational exposure to dusts and chemicals are also important contributors together with genetic susceptibility [1]. COPD is a leading cause of morbidity and mortality worldwide [2]. Besides smoking cessation, current treatment of COPD is mainly symptomatic. It is believed that a complex interaction between a dysregulated immune response, oxidative stress, imbalanced proteolytic activity and increased apoptosis leads to the pathology seen in patients with COPD [3-5]. To date, the precise molecular mechanisms underlying the pathogenesis of COPD are not fully understood.

Members of the transforming growth factor (TGF)-β superfamily, which include TGF-βs, activins and bone morphogenetic proteins (BMPs) are pleiotropic cytokines regulating fundamental physiological processes and have been linked to numerous diseases [6]. Since TGF-β has a key role in immune regulation and tissue remodelling in COPD, other members of the TGF-β superfamily may also be involved in the pathogenesis of COPD [7-9]. Activin-A is a homodimeric protein comprising 2 inhibin βA subunits (INHBA) and signals via a constitutively active activin type II receptor (Act-RIIA and Act-RIIB) that recruits and phosphorylates an activin type I receptor (activin-receptor-like kinases (ALK) -4 and/or -7). The receptor complex propagates the signal by phosphorylating the same intracellular Smads (Smad2 and Smad3) as TGF-β. Phosphorylated Smad2/3 subsequently translocate to the nucleus in association with Smad4 to initiate gene transcription [10,11]. Regulation of activin-A signalling is achieved by the endogenous inhibitor, follistatin that binds activin-A with high
affinity and blocks the interaction between activin-A and the type II receptor [12]. Follistatin can also bind and inhibit several BMPs (BMP-2, -4, -5, -6, -7, -8) and TGF-β3, although the binding affinity of follistatin is much higher for activin-A than for BMPs [13,14].

Several groups have demonstrated activation of the activin-A signalling pathway in an experimental model of allergic asthma and in patients with asthma [15-18]. Similarly, patients with acute respiratory distress syndrome or pulmonary fibrosis have higher levels of activin-A in bronchoalveolar lavage (BAL) fluid and lung tissue, respectively, compared to control patients [19,20].

Since activin-A is an important inflammatory mediator in several respiratory diseases, we hypothesized that activin-A plays a significant role in the pathogenesis of COPD. To elucidate this, we investigated the expression of activin-A and follistatin in lungs of never-smokers and smokers with and without COPD and in primary human bronchial epithelial cell (HBEC) cultures exposed to cigarette smoke (CS). Finally, we used a CS-induced mouse model of COPD to examine the in vivo functional role of activin-A by administering its endogenous inhibitor follistatin.
MATERIALS AND METHODS

Detailed description of materials and methods can be found in the online data supplement.

Human study populations

Lung resection specimens were obtained from 100 patients, of which 89 from surgery for solitary pulmonary tumours (Ghent University Hospital, Ghent, Belgium) and 11 from explant lungs of end-stage COPD patients undergoing lung transplantation (University Hospital Gasthuisberg, Leuven, Belgium). Lung tissue of 64 (out of 100) patients, including 11 never-smokers, 24 smokers without airflow limitation and 29 patients with COPD GOLD II was used for mRNA expression analysis (Table 1). Lung tissue of 71 (out of 100) patients, including 10 never-smokers, 26 smokers without airflow limitation and 35 patients with COPD (21 GOLD II, 4 GOLD III and 10 GOLD IV) was used for immunohistochemical analysis (Table 2). Lung tissue of 35 (out of 100) patients was used for both mRNA and immunohistochemical analysis (Figure E1).

Written informed consent was obtained from all subjects. This study was approved by the medical ethical committees of the Ghent University Hospital and the University Hospital Gasthuisberg Leuven.

Activin-A immunohistochemistry and quantification

Paraffin-embedded sections of human lung tissue were subjected to activin-A staining using anti-activin-A antibody as previously described [21]. Immunohistochemical staining with anti-activin-A (R&D Systems, Abingdon, UK) was performed on paraffin sections of the left lung of mice. The area with positive activin-A staining was quantified using KS400 software (Zeiss) and was normalized to the length of the basement membrane.
**Phospho-Smad2 immunohistochemistry**

Paraffin sections were incubated overnight with primary phospho-Smad2 (Ser465/467) Ab (Cell Signaling Technology, Danvers, USA) or isotype rabbit IgG (Abcam, Cambridge, UK). Next, the slides were incubated with PowerVision poly-horseradish peroxidase-anti-rabbit (Immunovision Technologies, Burlingame, USA) and stained with 3,3’-diaminobenzidine substrate (Dako, Glostrup, Denmark). Finally, sections were rinsed in demineralised water, counterstained with Mayer’s hematoxylin (Sigma-Aldrich, St. Louis, USA) and mounted in DPX (Klinipath, Duiven, The Netherlands).

**Culture of human bronchial epithelial cells**

Primary human bronchial epithelial cells (HBECs) were obtained by enzymatic digestion from lung resection specimens obtained from anonymous donors during surgery for lung cancer, as described previously [22]. Cells from passage 2 were cultured at an air-liquid interface in medium containing a high concentration of retinoic acid to induce mucociliary differentiation, as described previously [23].

After the 14-day air-liquid interface culture period, HBECs were exposed to whole volatile smoke or air as a negative control, using a modification of the system reported by Beisswenger and coworkers [24]. In other experiments, HBECs were stimulated both on the apical and basal side with recombinant human activin-A (R&D Systems) or with recombinant human TGF-β1 (R&D Systems) as positive control.

**Cigarette smoke exposure**

C57BL/6 mice (Charles River Laboratories) were exposed to CS, as described previously [25].
Administration of follistatin

C57BL/6 mice were injected i.p. with 1 μg of recombinant mouse follistatin-288 (R&D Systems) or PBS (controls) [26]. Mice were injected 3 times a week in a subacute (4 weeks) CS experiment, 30 minutes before air or CS exposure.

Quantitative RT-PCR, ELISA, CBA and flow cytometry

Information is provided in the online data supplement.

Statistical analysis

Statistical analysis was performed with Sigma Stat software (SPSS 19.0, Chicago, IL, USA), using Kruskal-Wallis, Mann-Whitney U, Wilcoxon Signed Rank, Fisher’s exact test and Spearman correlation analysis. Characteristics of the study population are expressed as median and interquartile range. Pearson correlation and linear regression analysis were performed on log-transformed data. Values from the in vitro study with HBECs are reported relatively to the control group. Reported values are expressed as mean ± SEM. Differences at p-values < 0.05 were considered to be significant (*P<0.05, **P<0.01 and *** P<0.001).
RESULTS

Activin-A and follistatin mRNA expression in lungs of patients with COPD

To characterize the expression of activin-A and follistatin in human lung tissue, we extracted mRNA from total lung tissue of a study population containing 11 never-smokers, 24 smokers without airflow limitation and 29 patients with COPD, GOLD II. The characteristics of the different study groups are summarized in Table 1. Activin-A is a homodimer of inhibin βA subunits. By measuring the mRNA expression of the inhibin βA subunit (INHBA), we observed higher mRNA levels of activin-A in lung tissue of current smokers both with and without COPD, compared to never-smokers. Moreover, current smokers had significantly higher activin-A mRNA expression, compared to ex-smokers (Figure 1A). The mRNA expression of follistatin (Figure 1B) was similar between the different study groups. Taken together, the ratio of activin-A to follistatin is increased in current smokers both with and without COPD in comparison with never-smokers. (Figure 1C).

Increased activin-A signalling in airway epithelium of patients with COPD

In order to localize pulmonary activin-A signalling, we performed immunohistochemical staining for activin-A and phospho-Smad2 on lung tissue sections of never-smokers, smokers and patients with COPD GOLD II-IV. The demographic, clinical and lung functional characteristics of the study subjects are presented in Table 2. Figure 2 shows representative lung sections stained for activin-A in never-smokers (Figure 2A), smokers (Figure 2B), patients with COPD GOLD II (Figure 2C) and patients with COPD GOLD IV (Figure 2D and 2F). We observed positive staining for activin-A in bronchiolar epithelial cells with a minor staining of smooth muscle cells and alveolar macrophages in the lungs of never-smokers (Figure 2A). Using imaging analysis software, we quantified the activin-A positive
staining in the airway epithelium. Protein levels of activin-A in the airway epithelium were significantly increased in patients with COPD GOLD II and GOLD III-IV, compared to never-smokers and smokers without airflow limitation (Figure 2G). The expression of activin-A in the airway epithelium is significantly associated with disease severity, expressed as post-bronchodilator FEV₁ (% predicted) and FEV₁/FVC (%) (Table E1, Figure E2). After adjustment for various confounders, the association with FEV₁ remained significant (Table E2). After semi-quantitative scoring, we also demonstrated higher scores for activin-A in the airway smooth muscle cell layer and alveolar macrophages of patients with COPD compared to never smokers (Figure E3).

We evaluated the phosphorylation status and nuclear accumulation of Smad2 as a hallmark for activated activin-A signalling. Almost no positive nuclear staining was present in the airway epithelial cells of never smokers and smokers without COPD (Figure 2H-I), whereas, nuclei of airway epithelial cells of patients with COPD stained strongly (Figure 2J-M). Importantly, there was a significant, positive correlation between the semi-quantitative levels of p-Smad2 and activin-A in the airway epithelium (Figure E4).

**Cigarette smoke exposure increases expression of activin-A in human bronchial epithelial cells**

To confirm the results seen in patients with COPD, primary human bronchial epithelial cells (HBECs), grown *in vitro* at air-liquid interface, were exposed to mainstream CS. HBECs and basal media were harvested 3, 6 and 24 hours after CS exposure. RT-PCR analysis on mRNA extracted from HBECs revealed a significant increase in expression of activin-A 3, 6 and 24 hours after CS exposure, compared to air-exposed cells (Figure 3A). The follistatin mRNA expression was slightly lower in CS-exposed HBECs (Figure 3B). This resulted in a significantly increased ratio of activin-A to follistatin in CS-exposed HBECs compared to air-
exposed HBECs (Figure 3C). Accordingly, the release of activin-A in the basal medium from CS-exposed HBEC cultures was augmented, especially 24 hours after CS exposure (Figure 3D), whereas the release of follistatin was decreased after CS exposure (Figure 3E). In conclusion, the ratio of activin-A to follistatin was significantly increased in basal medium of CS-exposed HBECs 24 hours after CS exposure (Figure 3F). These data show that expression and release of activin-A in response to CS is enhanced in human HBECs.

Increased pulmonary expression of activin-A upon CS exposure in mice

As previously shown by our group [25], exposure of C57BL/6 mice to CS for 4 weeks or 24 weeks lead to inflammation in the bronchoalveolar lavage compartment and lung tissue, characterized by increased numbers of macrophages, neutrophils, dendritic cells and B and T lymphocytes (Figure E6 and E7). The effect of CS on the pulmonary expression of activin-A and follistatin was determined in total lung tissue by quantitative RT-PCR and immunohistochemistry and in BAL fluid supernatant by ELISA. Activin-A mRNA expression in lung tissue was significantly increased upon chronic CS exposure, compared to air-exposed littermates (Figure 4A), while CS significantly downregulated follistatin mRNA expression (Figure 4B). Importantly, immunohistochemistry on lung tissue revealed activin-A staining in airway epithelium (Figure 4H), which was increased upon chronic (24 weeks) CS exposure (Figure 4D), similar to our observations in patients with COPD. Activin-A protein levels were increased significantly in BAL fluid at all timepoints, with an especially marked increase after 24 weeks CS exposure (Figure 4E). In contrast, the protein levels of follistatin in BAL fluid were similar between air- and CS-exposed mice (Figure 4F). Taken together, the ratio of activin-A to follistatin was increased after CS exposure at all timepoints, both on mRNA and protein level (Figure 4C and 4G).
To determine if activin-A levels persist after smoke cessation, we analyzed protein levels of activin-A and numbers of inflammatory cells in BAL fluid of C57BL/6 mice on day 1, 14 and 56 following subacute exposure to air or CS (Figure 5). CS-induced inflammation (monocytes, dendritic cells, CD4\(^+\) T lymphocytes) was mostly resolved on day 14 post-exposure, except for neutrophils and CD8\(^+\) T lymphocytes (Figure 5B-D). Inflammation in the BAL fluid was completely cleared 56 days after exposure (Figure 5). Consistent with the inflammation data, there was a notable persistence of activin-A in the BAL fluid on day 14, whereas by day 56, activin-A levels were restored to normal (Figure 5A).

**CS-induced inflammation in BAL is attenuated upon neutralization of activin-A by follistatin**

To elucidate whether activin-A is involved in the pulmonary inflammation upon CS exposure, we exposed C57BL/6 mice to CS for 4 weeks (subacute exposure) and injected the mice i.p. 3 times a week with 1 μg follistatin or PBS. In PBS treated mice, exposure to CS significantly increased the total number of BAL cells and absolute numbers of monocytes, neutrophils and CD4\(^+\) and CD8\(^+\) T lymphocytes in BAL fluid (Figure 6A-F). In contrast, follistatin treatment significantly attenuated the accumulation of monocytes and CD8\(^+\) T lymphocytes in CS-exposed mice (Figure 6B, 6D and 6F).

**Follistatin affects cytokine and chemokine expression upon CS exposure**

Because we observed a decreased inflammatory cell recruitment in BAL of follistatin treated mice upon 4 weeks CS exposure, we measured cytokines IL-6, TNF\(\alpha\) and IL-10 and chemokines MCP-1 and KC in BAL fluid by cytometric bead array. In PBS treated mice, CS exposure significantly increased the protein levels of IL-6, TNF\(\alpha\), MCP-1 and KC. In follistatin treated mice, the increase of IL-6, MCP-1 and KC was significantly attenuated,
compared to PBS treated mice (Figure 7A and 7D-E). Follistatin treated mice showed a trend towards an attenuation of the CS-induced increase of TNFα in BAL fluid (P=0.061; Figure 7B). In contrast, IL-10 protein levels did not increase upon CS exposure in PBS-treated mice, while follistatin treatment significantly increased IL-10 protein expression upon CS (Figure 7C).

**Follistatin reduces CS-induced pulmonary activin-A/TGF-β signalling in mice**

We determined activin-A and TGF-β1 protein levels in BAL fluid of PBS and follistatin treated mice by ELISA. In follistatin treated mice, the CS-induced increase of activin-A and TGF-β1 was significantly attenuated, compared to PBS treated mice (Figure 8A-B). In contrast, the mRNA expression of activin-A was not influenced by the follistatin treatment (Figure 8D). Using ELISA, we also quantified the phosphorylation state of Smad2 as a marker of active signalling. CS exposure induced a significant increase of p-Smad2 in the lung homogenates of PBS-treated mice, which was strongly suppressed in follistatin-treated mice (Figure 8C). These results indicate that follistatin was effective as an antagonist for activin-A.
DISCUSSION

In this study, we demonstrate a marked role for activin-A in CS-induced inflammation and COPD using a combination of \textit{ex vivo} studies on human subjects, \textit{in vitro} studies on CS-exposed primary human bronchial epithelial cell (HBEC) cultures and \textit{in vivo} studies using CS-exposed mice. We show for the first time increased levels of activin-A in the lungs of CS-exposed mice and patients with COPD. Moreover, we can mimic this \textit{in vitro} by exposing HBEC cultures to CS. Neutralizing activin-A with follistatin, its natural inhibitor, in a mouse model of subacute CS exposure, attenuates inflammation in the bronchoalveolar lavage fluid.

We demonstrated a higher activin-A mRNA expression in the lungs of current smokers irrespective of airflow limitation, suggesting a CS-driven effect, rather than an effect of the disease itself. In addition, at the protein level, epithelial activin-A was solely augmented in patients with COPD and not in smokers without airflow limitation, implicating a disease effect. The discrepancy between mRNA and protein levels may be explained by epigenetic mechanisms and/or differences in translational control that contribute to the translation of mRNA into protein. Also in the CS-driven murine model of COPD, we revealed increased levels of activin-A in BAL fluid and lung tissue. Importantly, activin-A levels, together with neutrophil and CD8\(^+\) T lymphocyte counts, were still elevated 14 days after final CS exposure, suggesting that activin-A release is not merely due to an acute CS effect. After CS cessation, neutrophil counts and activin-A levels dropped more rapidly than CD8\(^+\) T lymphocytes. The persistence of CD8\(^+\) T lymphocytes in the airways can be explained by the recruitment of effector T cells which are generated in the draining lymph nodes after stimulation with airway-derived DCs.
Since the increase in activin-A in patients with COPD and CS-exposed mice is not accompanied by an up-regulation of follistatin, there is a relative excess of activin-A, unopposed by follistatin. Similarly, there is evidence from several studies that activin-A is increased in pulmonary diseases such as pulmonary fibrosis and asthma, but also in other non-pulmonary inflammatory disorders such as inflammatory bowel disease and rheumatoid arthritis \cite{16,20,27,28}. Accordingly, in airway epithelium of patients with COPD phosphorylation of Smad2, a downstream mediator of activin-A, was elevated. However, we should keep in mind that Smad2 can also be activated by other ligands of the TGF-\(\beta\) superfamily and that other non-canonical pathways (e.g. NF-\(\kappa\)B, ...) can propagate the activin-A signal \cite{29}.

To support the finding that epithelial cells produce activin-A upon CS exposure, we exposed primary human bronchial epithelial cells (HBECs) \textit{in vitro} to CS. \textit{In vitro}, it has been shown that CS can down-regulate the TGF-\(\beta\) pathway in bronchial epithelial cells \cite{30}. However, we demonstrated that mRNA levels of activin-A increase upon CS exposure while follistatin expression is hardly influenced. Furthermore, CS exposure stimulates the secretion of activin-A and inhibits the secretion of follistatin, resulting in a relative excess of activin-A. Interestingly, activin-A is also increased in cultured airway epithelial cells challenged with human rhinovirus, which is frequently associated with COPD exacerbations \cite{31}.

In accordance with the data in patients with COPD, activin-A was increased in the lungs and BAL fluid of mice exposed to CS. To elucidate whether activin-A is an innocent bystander or plays an active role in pulmonary inflammation upon CS, we blocked activin-A by administering follistatin in CS-exposed mice. We demonstrated a significant attenuation of the CS-induced increase of CD8\(^+\) T lymphocytes in the BAL fluid, which is the predominant
T cell population in patients with COPD [32]. Strikingly, the secretion of several chemokines (MCP-1 and KC) and cytokines (IL-6 and TNFα) was reduced in follistatin treated CS-exposed mice compared to the PBS treated group. Interestingly, the secretion of IL-10, an anti-inflammatory cytokine is increased upon follistatin treatment in CS-exposed mice. CS exposure induced increased regulatory T cell numbers in the lung and in the draining lymph nodes, however this was not affected by follistatin treatment (data not shown) [33].

This is the first study exploring the potential of blocking activin signalling to inhibit inflammation in a CS-driven murine model. Our in vivo data are in line with experimental data in animal models of other respiratory diseases, showing that the pro-inflammatory effects of activin-A can be blocked in an in vivo setting. Exogenous follistatin reduced the number of macrophages and neutrophils in BAL fluid of a bleomycin-induced rat model of pulmonary fibrosis [34]. Furthermore, neutralization of endogenous activin-A with ActRIIB-Fc protein reduced the acute lung injury-like pathology in LPS-instilled mice, diminishing neutrophilic inflammation and IL-6 protein levels in BAL [35]. Several reports studied blockade of the activin-A pathway as a treatment for allergic asthma, which resulted in conflicting outcomes. In one study, intranasal administration of follistatin reduced ovalbumin-induced Th2 immune responses in mediastinal lymph nodes of mice, while another study showed that systemic depletion of activin-A by a neutralizing antibody exacerbated ovalbumin-induced asthmatic disease [15,36]. These last papers suggest that the route of administration of follistatin may influence the outcome. In our study set-up – systemic administration of follistatin by intraperitoneal injection –, it is not possible to differentiate between a local or extrapulmonary effect.
An intriguing aspect of activin-A is that - like TGF-β – it has both pro- and anti-inflammatory activities depending on the type of tissue and injury [37]. A pro-inflammatory activity is suggested by the capability of activin-A to skew the macrophage polarization towards an M1 phenotype [38]. In contrast, activin-A is known to stimulate the development of FoxP3+ regulatory T cells [39]. The current hypothesis is that activin-A exerts a pro-inflammatory effect early on in the course of inflammation, but once the inflammatory response has been established, activin-A may stimulate anti-inflammatory effects [40]. Overall, our data suggest that activin-A promotes the pulmonary inflammation after CS. Accordingly, when we stimulated HBECs with increasing doses of activin-A for 24 or 48 hours, a dose-dependent, but not significant increase of IL-1β, TGF-β1 and IL-8 mRNA expression was observed, together with an enhanced secretion of IL-8 (Figure E5).

One of the strengths of our study is its translational character. Observations made ex vivo in patients with COPD were confirmed in vitro in HBEC cultures and in vivo in CS-exposed mice. A limitation is that only pulmonary inflammation was investigated which is only one aspect of the complex pathogenesis of COPD. Accumulating data suggest that activin-A is also involved in airway remodelling. In vitro results indicate that activin-A can stimulate proliferation of human lung fibroblasts and smooth muscle cells [41,42]. Moreover blocking activin-A inhibited collagen deposition and thickening of the subepithelial smooth muscle layer in two different murine models of allergic asthma [43,44]. Similarly, it would be helpful to study the relation between activin-A and peribronchial fibrosis in patients with COPD. In the murine model of COPD it is practically not feasible to study the effect of activin-A silencing on airway remodelling using a pharmacological approach (follistatin or a neutralizing antibody), since it is only after 24 weeks of CS exposure that airway wall remodelling appears [25]. Interestingly, we demonstrated that follistatin treatment suppresses
protein levels of the pro-fibrotic cytokine TGF-β1 in BAL supernatant of CS-exposed mice. Since follistatin does not antagonize TGF-β1, the attenuated TGF-β1 levels are likely the result of reduced activin-A based stimulation of TGF-β1 secretion [16]. Importantly, TGF-β1 can also stimulate activin-A expression, inducing a positive feedback loop [16]. In Figure 8E we schematically show the effect of CS exposure on activin-A signalling in the lungs.

In conclusion, these data demonstrate that activin-A is increased in the airway epithelium of patients with COPD and in the lungs of CS-exposed mice. Also, in vitro we confirmed that exposing HBEC cultures to CS modulates the delicate balance between activin-A and follistatin. Moreover, our in vivo studies in a CS-driven murine model indicate that activin-A contributes to the pathogenesis of CS-induced pulmonary inflammation.
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FIGURE LEGENDS

FIGURE 1. Pulmonary mRNA expression of INHBA (activin-A) and follistatin in human subjects. mRNA levels of activin-A (INHBA: inhibin βA subunit) (A) and follistatin (B) in total lung of never-smokers (N = 11), current-smokers (N = 12), ex-smokers (N = 12), current-smokers with COPD (N = 17) and ex-smokers with COPD (N = 12), as measured by qRT-PCR. In (C) the ratio of activin-A to follistatin mRNA expression is shown. mRNA levels were corrected using a calculated normalization factor based on mRNA expression of three reference genes (GAPDH, PPIA, HPRT-1). Data are expressed as box-and-whisker plots (*P<0.05 and **P<0.01).
FIGURE 1.
FIGURE 2. Pulmonary activin-A and phospho-Smad2 expression in human lungs by immunohistochemistry. Representative photomicrographs for activin-A of human lung sections of a never-smoker (A), smoker without airflow limitation (B), patient with COPD GOLD II (C) and patient with COPD GOLD IV (D). Isotype control is presented in (E). (F) is a detailed picture from (D). Quantification of activin-A protein expression in airway epithelium of never-smokers (N = 10), smokers (N = 26) and patients with COPD GOLD II (N = 21) and COPD GOLD III-IV (N = 14) is shown in (G). Results are expressed as the epithelial area positive for activin-A normalized to the length of the basement membrane (Pbm). Data are expressed as box-and-whisker plots (*P<0.05, **P<0.01). Representative photomicrographs for p-Smad2 of human lung sections of a never-smoker (H), smoker without airflow limitation (I), patient with COPD GOLD II (J) and patient with COPD GOLD IV (K). Isotype control is presented in (L). (M) is a detailed picture from (K). Original magnification 200x. Scale bar = 100 μm.
FIGURE 2.
FIGURE 3. Expression of activin-A and follistatin in air-liquid interface human bronchial epithelial cell (HBEC) cultures upon exposure to air or cigarette smoke. Basal media and cells were harvested 3, 6 and 24 hours after exposure. mRNA from HBECs was isolated and analyzed for the expression of INHBA (inhibin βA subunit of activin-A) (A), FST (follistatin) (B) by RT-PCR. In (C) the ratio of activin-A to follistatin mRNA expression is shown. mRNA levels were corrected using a calculated normalization factor based on mRNA expression of two reference genes (RPL13A, ATP5B). Protein levels of activin-A (D) and follistatin (E) in the basal medium were analyzed by ELISA. In (F) the ratio of activin-A to follistatin protein is shown. Data are reported relatively to the air-exposed group. Results are depicted as mean ± SEM of 5 different experiments each performed in duplicate and using cells from different donors (*P<0.05).
FIGURE 3.
FIGURE 4. Expression of activin-A and follistatin in C57BL/6 mice upon acute (3 days), subacute (4 weeks) and chronic (24 weeks) exposure to air or cigarette smoke. mRNA levels of Inhba (inhibin βA subunit of activin-A) (A) and follistatin (B) in total lung, as measured by qRT-PCR. In (C) the ratio of activin-A to follistatin mRNA expression is shown. mRNA levels were corrected using a calculated normalization factor based on mRNA expression of three reference genes (GAPDH, TFRC, HPRT-1). Quantification of activin-A protein expression in airway epithelium of mice upon exposure to air or CS is shown in (D). Results are expressed as the epithelial area positive for activin-A normalized to the length of the basement membrane (Pbm). (H) Representative images of activin-A-stained lung sections of air- and CS-exposed mice. Original magnification 200x. Scale bar = 100 μm. Protein levels of activin-A (E) and follistatin (F) in bronchoalveolar lavage (BAL) fluid measured by ELISA. In (G) the ratio of activin-A to follistatin protein is shown. Data are expressed as mean ± SEM (N = 8-10 animals/group; *P<0.05, **P<0.01 and *** P<0.001).
**FIGURE 4.**
FIGURE 5. Pulmonary expression of activin-A and pulmonary inflammation in C57BL/6 mice on day 1, 14 and 56 after subacute exposure to air or cigarette smoke. Protein levels of activin-A (A) in bronchoalveolar lavage (BAL) fluid measured by ELISA. (B-D) Cell differentiation in bronchoalveolar lavage (BAL) fluid. Neutrophils (B), lymphocytes (C) were counted on cytospins and CD8$^+$ T lymphocytes (D) were enumerated by flow cytometry. Data are expressed as mean ± SEM (N = 8 animals/group; *P<0.05, **P<0.01).
FIGURE 5.
FIGURE 6. Effect of CS exposure and neutralization of activin-A with follistatin on the total number of bronchoalveolar lavage (BAL) cells and cell subsets in BAL fluid (A-F). Total BAL cells (A), monocytes (B), neutrophils (C), lymphocytes (D), CD4⁺ T lymphocytes (E) and CD8⁺ T lymphocytes (F) in C57BL/6 mice upon 4 weeks exposure to air or CS, injected intraperitoneally 3 times a week with follistatin or PBS. All cell types were enumerated by cytopsin counts, except for the CD4⁺ and CD8⁺ T lymphocytes which were determined by flow cytometry. Data are expressed as mean ± SEM (N = 8 animals/group; *P<0.05, **P<0.01).
FIGURE 6.
FIGURE 7. Inflammatory cytokines and chemokines in the bronchoalveolar lavage (BAL) fluid of C57BL/6 mice upon 4 weeks exposure to air or CS, injected i.p. 3 times a week with follistatin or PBS. IL-6 (A), TNFα (B), IL-10 (C) MCP-1 (D) and KC (E) protein levels, measured by cytometric bead array. Data are expressed as mean ± SEM (N = 8 animals/group; *P<0.05, **P<0.01 and *** P<0.001).
FIGURE 7.
FIGURE 8. Activin-A/TGF-β signalling in the bronchoalveolar lavage (BAL) fluid (A-B) and lungs (C) of C57BL/6 mice upon 4 weeks exposure to air or CS, injected with follistatin or PBS. Activin-A (A), TGF-β1 (B) and phosphorylated-Smad2 (C—expressed as raw optical density values at 450 nm) levels were quantified by ELISA. Activin-A (Inhba: inhibin βA subunit) mRNA levels (D) were measured using qRT-PCR. Data are expressed as mean ± SEM (N = 8 animals/group; *P<0.05 and **P<0.01). In (E) a schematic overview of the effect of cigarette smoke (CS) exposure on pulmonary activin-A signalling is shown. CS induces activin-A expression in airway epithelial cells, but attenuates its endogenous antagonist follistatin. Activin-A binds to type II receptors on the surface of target cells, which oligomerize with type I receptors. The activated type I receptor phosphorylates intracellular protein Smad2, which forms a complex with Smad4 and translocates to the nucleus to initiate gene expression of target genes such as TGF-β1, which signals through the same Smad proteins. TGF-β1 in turn can induce the expression of activin-A, creating a positive feedback loop mechanism. TGF-β1: transforming growth factor-β1, I: activin type I receptors, II: activin type II receptors, P: phosphorylated form.
FIGURE 8.
TABLE 1

Characteristics of study subjects for lung mRNA analysis (by qRT-PCR)

<table>
<thead>
<tr>
<th></th>
<th>Never-smokers</th>
<th>Smokers</th>
<th>COPD II</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Number</strong></td>
<td>11</td>
<td>24</td>
<td>29</td>
</tr>
<tr>
<td><strong>Age (years)</strong></td>
<td>60 (47-70)</td>
<td>64 (55-71)</td>
<td>65 (59-69)</td>
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<tr>
<td><strong>Gender (m/f)</strong></td>
<td>3/8</td>
<td>18/6</td>
<td>29/0</td>
</tr>
<tr>
<td><strong>Current-smoker/Ex-smoker</strong></td>
<td>NA</td>
<td>12/12</td>
<td>17/12</td>
</tr>
<tr>
<td><strong>Pack-years</strong></td>
<td>NA</td>
<td>28 (14-49)</td>
<td>45 (40-60)</td>
</tr>
<tr>
<td><strong>FEV₁ (% predicted)</strong></td>
<td>104 (92-119)</td>
<td>96 (92-113)</td>
<td>69 (63-74)</td>
</tr>
<tr>
<td><strong>FEV₁/FVC (%)</strong></td>
<td>77 (75-80)</td>
<td>76 (73-78)</td>
<td>56 (54-60)</td>
</tr>
<tr>
<td><strong>ICS (yes/no)</strong></td>
<td>0/11</td>
<td>1/23</td>
<td>12/17</td>
</tr>
</tbody>
</table>

Footnote

m (male); f (female); FEV₁ (forced expiratory volume in 1 second); FVC (forced vital capacity); ICS (inhaled corticosteroids); NA (not applicable)

Data are presented as median (IQR)

Mann-Whitney U test: † P<0.001 vs never-smokers, * P<0.01 vs smokers, § P<0.001 vs smokers

Fisher’s exact test: ° P<0.01 and # P<0.001
TABLE 2

Characteristics of study subjects for immunohistochemical study

<table>
<thead>
<tr>
<th></th>
<th>Never-smokers</th>
<th>Smokers</th>
<th>COPD II</th>
<th>COPD III-IV</th>
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<tr>
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<td>21</td>
<td>14</td>
</tr>
<tr>
<td>Age (years)</td>
<td>59 (51-70)</td>
<td>57 (51-65)</td>
<td>66 (60-70)</td>
<td>52 (50-59)</td>
</tr>
<tr>
<td>Gender (m/f)</td>
<td>2/8 #</td>
<td>20/6 #</td>
<td>20/1 #</td>
<td>10/4 #</td>
</tr>
<tr>
<td>Current-smoker/Ex-smoker</td>
<td>NA</td>
<td>13/13 °</td>
<td>12/9 °</td>
<td>3/11 °</td>
</tr>
<tr>
<td>Pack-years</td>
<td>NA</td>
<td>24 (15-36) †</td>
<td>50 (40-61) †*</td>
<td>30 (25-39) †~</td>
</tr>
<tr>
<td>FEV₁ (% predicted)</td>
<td>103 (93-118)</td>
<td>102 (90-113)</td>
<td>68 (59-74) †§</td>
<td>25 (24-42) †§¶</td>
</tr>
<tr>
<td>FEV₁/FVC (%)</td>
<td>76 (75-82)</td>
<td>76 (72-80)</td>
<td>56 (53-60) †§</td>
<td>36 (32-42) †§¶</td>
</tr>
<tr>
<td>ICS (yes/no)</td>
<td>0/10 #</td>
<td>0/26 #</td>
<td>7/14 #</td>
<td>13/1 #</td>
</tr>
</tbody>
</table>

Footnote

m (male); f (female); FEV₁ (forced expiratory volume in 1 second); FVC (forced vital capacity); ICS (inhaled corticosteroids); NA (not applicable)

Data are presented as median (IQR)

Mann-Whitney U test: † P<0.001 vs never-smokers, * P<0.01 vs smokers, § P<0.001 vs smokers, ~ P<0.05 vs GOLD II, ◊ P<0.01 vs GOLD II and ¶ P<0.001 vs GOLD II

Fisher’s exact test: ◊ P<0.01 and # P<0.001