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Original article

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Losartan reduces cigarette smoke-induced airway inflammation and mucus hypersecretion

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Summary: Inflammation and mucus hypersecretion, associated with cigarette smoke-induced chronic airway diseases, is ameliorated by losartan in human airway epithelial cells *in vitro* and in the upper airways of human subjects in a small clinical trial.

Abstract

The aim was to determine whether losartan reduces cigarette smoke (CS)-induced airway inflammation and mucus hypersecretion in an *in vitro* model and a small clinical trial.

Primary human bronchial epithelial cells (HBECs) were differentiated at the air-liquid interface (ALI) and exposed to CS. Expression of transforming growth factor beta 1 (TGF-β1) and the mucin MUC5AC, and expression or activity of matrix metalloproteinase-9 (MMP-9) was measured after CS exposure. Parameters of mucociliary clearance were evaluated by measuring airway surface liquid (ASL) volumes, mucus concentrations, and conductance of cystic fibrosis transmembrane conductance regulator (CFTR) and large conductance, Ca^{2+}-activated and voltage-dependent potassium (BK) channels. Nasal cells were collected from study participants and expression of *MUC5AC*, *TGF-β1*, and *MMP-9* mRNAs was measured before and after losartan treatment.

In vitro, CS exposure of HBECs caused a significant increase in mRNA expression of *MUC5AC* and *TGF-β1* and MMP-9 activity and decreased CFTR and BK channel activities, thereby reducing ASL volumes and increasing mucus concentrations. Treatment of HBECs with losartan rescued CS-induced CFTR and BK dysfunction and caused a significant decrease in *MUC5AC* expression and mucus concentrations, partially by inhibiting TGF-β signalling. In a prospective clinical study, cigarette smokers showed significantly reduced mRNA expression levels of *MUC5AC*, *TGF-β1*, and *MMP-9* in the upper airways after two months of losartan treatment.

Our findings suggest that losartan may be an effective therapy to reduce inflammation and mucus hypersecretion in CS-induced chronic airway diseases.

Key Words: CFTR, BK channel, mucus, airway inflammation

Introduction

The consequences of cigarette smoking are well documented, with smoking-related diseases causing over six million deaths per year worldwide [1]. Chronic obstructive pulmonary diseases (COPD) include chronic bronchitis and mostly result from long-term smoking. Chronic bronchitis, even without obstruction, is characterized by persistent airway inflammation and mucus hypersecretion [2], which both decrease mucociliary clearance and accelerate disease progression. Unfortunately, no effective therapies exist, but their goal would be to decrease mucus hyperconcentration by controlling inflammation and facilitating the removal of mucus from the airways. One of the primary gel-forming mucins in the airways, MUC5AC [3], is induced by cigarette smoke (CS) *in vivo* and in primary human airway epithelial cells *in vitro* [4]. In fact, recent reports have found increased levels of MUC5AC in the sputum of smokers when compared to non-smokers [5], with MUC5AC expression levels correlating with COPD progression [6]. Transforming growth factor-beta 1 (TGF-β1) and matrix metalloproteinase-9 (MMP-9) are also known to be major players in the pathogenesis of COPD [7-9] as well as other chronic airway diseases such as cystic fibrosis (CF) [10-12]. They have been proposed to serve as relevant biomarkers in COPD [13, 14].

Losartan is a Food and Drug Administration (FDA) approved angiotensin II receptor type 1 (AGTR1) blocker (ARB), widely used to treat hypertension. However, losartan also exhibits anti-inflammatory properties, possibly independent of its ARB activity [7, 12, 15, 16]. We showed that losartan can increase levels of peroxisome proliferator-activated receptor gamma (PPAR-γ) and thereby rescue TGF-β1-induced inflammation and mucociliary dysfunction in relevant CF models *in vitro* and *in vivo* [12]. Induction of PPAR-γ was also shown to prevent and reverse CS-induced emphysema in mouse models [7, 17]. A large clinical trial is underway to assess losartan's effects on emphysema progression (LEEP, NCT02696564). However, this trial does not focus on airway disease. Thus, we examined how CS impacts the levels of

MUC5AC and inflammation markers (TGF-β1 and MMP-9) in airway epithelia *in vitro* and *in vivo*. We further determined whether losartan acts as an effective anti-inflammatory therapy to reduce airway inflammation and thereby mucin concentrations both *in vitro* and in a small clinical trial (NCT02416102).

Methods

Lungs

Lung tissue was obtained from organ donors whose lungs were rejected for transplant and recovered for research by the Life Alliance Organ Recovery Agency at the University of Miami (Miami, FL, USA), LifeCenter Northwest (Seattle, WA, USA), and the Midwest Transplant Network (Kansas City, KS, USA). A ring of the trachea or main bronchi was cut and fixed in 10% formalin at 4°C until embedded in paraffin for sectioning and tissue staining. Cells were taken from airways of non-smokers and smokers. The diagnosis of COPD was made by clinical criteria before the death of the patient and taken verbatim from the chart. No lung function was available confirming obstructive disease. Thus, the diagnosis was only accepted for these COPD cells if the donor had a significant smoking history and there were macro-pathological signs of emphysema. All COPD subjects here were still actively smoking. Airways were dissected and the tissue exposed to protease overnight as described [18-20]. Cells were harvested the following day and frozen in liquid nitrogen (considered P0 HBECs). The University of Kansas deemed the use of these materials non-human subjects research.

Cell culture and losartan treatments

Culturing of HBECs at the air-liquid interface (ALI) was performed as described (see also online supplementary methods) [18-20]. *In vitro* losartan (#61188, Millipore Sigma, St. Louis, MO, USA) treatment in the media at 10 μM was started the day P1 HBECs went to an ALI and maintained throughout differentiation, except for cells from smokers (including COPD) that were treated for 24h

with losartan before CS exposure. LY2157299 (#S2230, Selleckchem, Houston, TX, USA) at 10 μM or EXP3179 (#18855, Cayman Chemicals, Ann Arbor, MI, USA) at 5 μM was added to the basolateral media of ALI cultures 24 h before CS exposure.

Immunofluorescence staining

Immunofluorescence staining of tissue sections was performed as described [21]. Tissue sections were incubated with anti-MUC5AC antibody (#MA1-38223, ThermoFisher Scientific, Waltham, MA, USA) at 0.4 μg/mL overnight at 4°C and with Hoechst (#H3569, ThermoFisher Scientific) at 2 μg/mL for 10 min. For staining of P1 HBECs, ALI cultures on Transwell inserts were fixed with a solution of 50%/50% methanol/acetone for 2 min at -20°C followed by three washes with PBS. A 3% BSA solution was used to block for 1 h before incubation with primary antibodies. P1 HBECs exposed to CS or room air were washed and fixed 48 h after exposure following the same immunostaining protocol. All slides were imaged with a Nikon C2+ confocal microscope (Nikon Instruments, Tokyo, Japan).

Quantitative PCR

P1 HBECs exposed to CS or room air were lysed 24 h after exposure and total RNA was isolated using the E.Z.N.A.® Total RNA Kit (Omega Bio-tek, Norcross, GA, USA). qPCR was performed as described [8, 22] using TaqMan Gene Expression Assays (ThermoFisher Scientific) for *MMP9* (Hs00234579_m1), *MUC5AC* (Hs01365601_m1) and *TGF-β1* (Hs00998133_m1), and normalized to reference gene *GAPDH*.

MMP-9 activity assay

MMP-9 activity was measured in 200 μL apical PBS washes collected 24 h after room air or CS exposure using the Human Active MMP-9 Fluorokine E Kit (#F9M00, R&D Systems, Minneapolis, MN, USA), following manufacturer's instructions for non-activated samples.

Ussing chamber

Cystic fibrosis transmembrane conductance regulator (CFTR) and large conductance, Ca^{2+}-activated and voltage-dependent K^+ channel (BK) activities were recorded in Ussing chambers as previously described (see also online supplementary methods) [23, 24]. CFTR and BK currents from P1 HBECs on Snapwell filters were measured at 4 h after the cells were exposed to CS or room air.

Airway surface liquid (ASL) volume measurements

ASL volume estimation was performed by meniscus scanning as previously published [8, 25]. P1 HBECs exposed to CS or room air were scanned 1 h and 4 h after exposure and the ΔASL was plotted.

Ciliary beat frequency (CBF)

CBF was recorded 4 h after exposure to CS or room air using a high-speed camera and analysed using the individual Region-of-Interest (ROI) method of SAVA software [26, 27]. Ciliary beating was recorded 1-2 mm away from the centre of the insert for 2 seconds and four ROIs were plotted.

Mucus concentration measurements

The percent solids of mucin-containing fluid on top of cultures was measured according to published methods of mucus wet and dry weights using a UMX2 ultra-microbalance (Mettler Toledo, Columbus, OH, USA) [28, 29]. P1 HBECs exposed to CS or room air were tested 24 h after exposure.

Cigarette smoke (CS) exposure

CS exposure of P1 HBECs was done as described [8, 21, 30]. Briefly, P1 HBECs were exposed to 24 puffs of a Kentucky research cigarette (3R4F) with a volume of 35 mL delivered every 60 seconds using the Vitrocell VC10 smoking robot (Vitrocell, Waldkirch, Germany) following ISO standard 3308. As controls, P1 HBECs were exposed to room air. Nicotine

deposition onto the surface of HBECs after CS exposures was validated by mass spectrometry (LS-MS/MS, Florida International University, FL, USA) and showed depositions of approximately 100-120 μM of nicotine onto ALI cultures comparable to *in vivo* deposition of 1-2 cigarettes [31].

Human subjects and study approval

The clinical study was approved by the University of Miami Human Subject Research Office and informed consent was obtained from each participant. Clinicaltrials.gov registration can be found under NCT02416102.

The study enrolled a total of 31 participants: 16 healthy never-smokers (<100 cigarettes in a lifetime) and 15 current smokers with a smoking history of >10 pack-years and no signs of COPD by PFT with DLCO, FEV1/FVC, FEV1, and FVC values from both groups in the normal range. From those 31 patients, 14 (7 smokers and 7 non-smokers) completed the study with laboratory test results and only 5 subjects in each group had complete nasal sample data sets. The subjects were aged 35 to 70 years and not taking any angiotensin receptor blockers (ARBs) prior to enrolment. Participants received 50 mg losartan for 4 weeks and then 100 mg losartan for another 4 weeks. The exclusion criteria are described in online supplementary methods.

Nasal cell collection

Nasal cells were collected from study participants using sterile cytology brushes (Medical Packaging Corporation, Camarillo, CA, USA) as described in online supplementary methods.

Statistical analyses

Data are shown as dot plots / bar graph combinations with means ± S.E.M. Differences between two groups were compared by parametric or non-parametric tests as indicated in the figure captions depending on whether the data passed Shapiro-Wilk normality testing. p values

for significance were accepted at $p < 0.05$. All analyses were performed using Prism (GraphPad Software, San Diego, CA, USA).

Results

Expression of *MUC5AC, TGF-β1* and *MMP-9* is elevated in lung tissues from smokers

We analysed mRNA expression levels of the mucin *MUC5AC* and the inflammation markers *TGF-β1* and *MMP-9* in freshly isolated HBECs from lungs of age-matched non-smoking and smoking donors without COPD (demographics of donors can be found in online supplementary table S1). We refer to these as passage zero (P0) cells (never expanded or cultured). mRNA expression levels of *MUC5AC, TGF-β1*, and *MMP-9* were significantly increased in P0 HBECs of smokers compared to non-smokers (figure 1A). Immunofluorescence staining of tracheal/bronchial tissue sections from the same donors showed that smokers displayed an increased percentage of MUC5AC-positive cells in the epithelium compared to non-smokers (figure 1B,C). These results are largely consistent with previous reports showing increased absolute concentrations of both MUC5AC and TGF-β1 in smokers without COPD compared to non-smokers [6, 32]. When HBECs were expanded and fully re-differentiated at the ALI, now referred to as P1 HBECs or ALI cultures, differences in mRNA expression levels of *MUC5AC, TGF-β1*, and *MMP-9*, as well as the percentage of MUC5AC-positive cells between non-smokers and smokers were no longer apparent (figure 2A-C). Thus, P1 HBECs lose some of their *in vivo* characteristics during re-differentiation.

Cigarette smoke exposure induces inflammation and mucociliary dysfunction in P1 HBECs of non-smokers

Chronic bronchitis, defined as productive cough, indicates a failure of normal mucociliary function, usually indicating a reduction in ASL volume. ASL homeostasis depends on the proper activities of ion channels such as CFTR and Ca^{2+}-activated chloride channels (CaCC)

as well as the apically expressed BK channel [33]. Function of the BK α subunit, KCNMA1, depends on Leucine Rich Repeat Containing protein 26 (LRRC26), a γ subunit necessary to open BK in non-excitable tissues [24, 34]. CFTR and BK activities, ASL volumes, and mucus concentrations (% solids) in P1 HBECs were not significantly different between non-smokers and smokers (figure 2D-G). To recreate features of inflammation and mucus overproduction observed in P0 HBECs from smokers, we exposed P1 ALI cultures from non-smoking subjects to CS (24 puffs of a 3R4F Kentucky research cigarette, at one puff every minute with 8 s exhaust time via the Vitrocell VC-10) [8]. As a control, P1 HBECs were exposed to room air. CS significantly increased mRNA expressions of *MUC5AC* and *TGF-β1* (figure 3A) and activity of MMP-9 (figure 3B) 24 h after exposure. Parameters of mucociliary function were also affected: CS-exposed ALI cultures showed a significant decrease in activities of CFTR and BK (correlating with mRNA expression levels of *CFTR* and *LRRC26*, respectively) compared to air control (figure 3C,D). In addition, CS caused a concomitant loss of ASL volume (figure 3E), and a significant increase in MUC5AC-positive cells and mucus concentration (figure 3F,G). The increase in mucus solids from approximately 2% to 4% is consistent with previously published data for smokers *in vivo* and another study demonstrating the relationship of mucus concentrations and mucociliary clearance *in vitro* [28, 29].

Losartan reduces cigarette smoke-induced mucus hypersecretion and mucociliary dysfunction *in vitro*

Next, we tested whether losartan, an FDA approved drug with a good safety track record and known anti-inflammatory properties [7, 15, 16], reduced CS-induced airway inflammation and thereby mucus concentration to levels compatible with normal mucociliary clearance. Fully re-differentiated P1 HBECs from non-smokers were treated with 10 μM losartan throughout differentiation before exposing them to 24 puffs of CS through the VC-10 robot. We observed an improvement in ASL volumes (figure 4A), ciliary beat frequency (CBF; figure

4A), and a decrease in the percentage of MUC5AC-positive cells (figure 4B) and mucus concentrations (figure 4B).

TGF-β1 is likely a driver of cigarette smoke-induced inflammation and mucociliary dysfunction as pre-treatment of ALI cultures with LY2157299 (Galunisertib), a selective TGF-β receptor 1 (TGFBR1) inhibitor, improved ASL volumes upon TGF-β1 and smoke exposure (figure 4C).TGF-β1- and CS-induced decreases in ASL volume were also rescued by EXP3179, the losartan metabolite with anti-inflammatory but no ARB activities, suggesting losartan's effects are independent of AGTR1 signalling (figure 4D).

In support of the TGF-β pathway being responsible, LY2157299 decreased smoke-induced TGF-β1 and MMP-9 mRNA expression (figure 5A,B). LY2157299 had no effect on expression of TGF-β2, IL-13, MMP-2 and MMP-9 mRNA expression up[on smoke exposure (figure 5A,B), while TGF-β1-induced MMP-2 and MMP-12 mRNA expressions were suppressed by LY2157299 (figure 5B).

Treatment with losartan also significantly reduced *MUC5AC* and *TGF-β1* mRNA expression as well as MMP-9 activity (figure 6A,B). Finally, losartan rescued CS-induced decreases in CFTR and BK function as well as mRNA expressions of *CFTR* and the functionally most relevant γ subunit of BK, *LRRC26* (figure 6C-F). Interestingly, losartan also increased *CFTR* and *LRRC26* mRNA in air controls (figure 6D,F).

In analogy to the data obtained with airway cells from non-smokers, cells from smokers with or without COPD also showed improvements in parameters of mucociliary function upon smoke exposure in the presence of losartan (figure 7). There were significant improvements in 1) CFTR and BK currents as well as *CFTR* and *LRRC26* mRNA expression (figure 7A-D), 2) CBF (figure 7E), 3) and a reduction in MMP-9 activity (figure 7F).

While a previous study found that losartan could ameliorate CS-induced parenchymal changes in mice [7], we provide the first evidence that losartan can restore important parameters of CS-induced mucociliary dysfunction using primary HBECs *in vitro*.

Losartan reduces expression levels of *MUC5AC*, *TGF-β1* and *MMP-9* in the upper airways of smokers in a clinical study

These results set the stage for a small prospective clinical study to determine whether losartan could reduce inflammation in the upper airways of currently healthy smokers. A schematic of the study can be found in figure 8A with subject demographics in figure 8B. Consistent with P0 HBECs, human nasal epithelial cells (HNECs) from smokers exhibited at baseline significantly elevated expression levels of *MUC5AC, TGF-β1*, and *MMP-9* mRNA compared to non-smokers (figure 8C). Losartan was administered for 4 weeks at 50 mg daily and for an additional 4 weeks at 100 mg daily before the same parameters were re-examined. mRNA levels were compared before (baseline) and after losartan treatment (week 1 and week 8) and are presented here as relative values of baselines. HNECs from smokers receiving losartan displayed significant decreases in *MUC5AC, TGF-β1,* and *MMP-9* mRNA expression levels after 8 weeks of treatment with losartan (figure 8D), while expression levels in non-smokers were unaffected (online supplementary figure S1). Thus, losartan effectively reduced the levels of important markers of airway inflammation as well as *MUC5AC* expression in the upper airways of smokers.

Discussion

Airway inflammation and mucin hyperconcentration are hallmarks of cigarette smoking-related chronic airway diseases. Mucin concentrations are elevated in chronic bronchitis and absolute concentrations of MUC5AC are increased in smokers, even without COPD [6]. CS induces the expression of MUC5AC in airway epithelial cells *in vitro* [4], and increases levels of TGF-β1 protein and mRNA have been reported in small airway epithelial cells of smokers with and without COPD [32], Levels of MMP9 were also significantly higher in sputum from

smokers compared to non-smokers [5]. Since inflammation can persist after smoking cessation, safe and non-toxic therapeutics that ameliorate airway inflammation and mucus hypersecretion would therefore provide a sound approach to treat smoking-related respiratory diseases.

Here, we therefore tested losartan *in vitro* and *in vivo*. We found that mRNA expressions of *MUC5AC, TGF-β1*, and *MMP-9* are indeed elevated in lung tissue from smokers compared to non-smokers (figure 1). Although these differences were apparent in HBECs that were never expanded, key features of CS-induced inflammation and mucus hyperconcentration were lost once HBECs were expanded and re-differentiated at the ALI (P1 HBECs). Loss of features in P1 HBECs present in the native airways could make it difficult to study airway diseases using cells. However, P1 HBECs were shown to be suitable surrogates for changes that occur in the airway epithelium after exposure to CS, airway pollutants, and other irritants [35]. Here, we exposed P1 HBECs from non-smokers to CS generated to confirm that changes observed between smokers and non-smokers *in vivo* can be consistently reproduced *in vitro*. We found that *in vitro* CS exposure of P1 HBECs from non-smokers increased mucus concentrations from ~2% to ~4% solids (figure 3G), values consistent with previously published *in vivo* data [28] and a study demonstrating the relation of mucus concentrations and mucociliary clearance *in vitro* [29]. In fact, the values we obtained in CS-exposed P1 HBECs were more consistent with mucin concentrations found in chronic bronchitis subjects *in vivo* rather than non-symptomatic smokers. These results suggest that *in vitro* exposure to CS induces relevant effects in P1 HBECs from non-smokers and that the criteria of how many cigarettes or puffs to use should be carefully evaluated when designing studies.

CS exposure had detrimental effects on mucociliary clearance *in vitro*, consistent with *in vivo* data demonstrating a negative correlation between mucus concentration and mucociliary clearance in subjects with chronic bronchitis [29]. Exposure of P1 HBECs to CS impaired ion transport through both CFTR and BK and further led to a loss of ASL volume (figure 3). These effects are likely mediated

through CS-induced increases in TGF-β1 expression as TGF-β1 signalling has been previously shown to reduce CFTR and BK activities through regulation of *CFTR* and *LRRC26* mRNA expression, respectively [24, 36, 37]. Furthermore, we show here that the TGFBR1 inhibitor LY2157299 can ameliorate CS-induced ASL volume loss and reduce expression of *MMP-9* mRNA (figure 5). There is evidence that losartan can inhibit TGF-β1 signalling and we recently showed that losartan can rescue TGF-β1-induced mucociliary dysfunction in CF airways *in vitro* and in a large animal model of CF-like airway disease [12]. Indeed, we found that treatment of P1 HBECs with losartan during re-differentiation could effectively decrease the expression of TGF-β1 and MMP-9 as well as MUC5AC upon CS exposure compared to non-losartan-treated and CS-exposed control P1 HBECs. Furthermore, CS-induced mucociliary dysfunction was reversed by losartan as shown by rescue of CFTR and BK channels functions, increasing ASL volume availability and reducing mucus concentration. These effects were also seen using EXP-3179, the losartan metabolite without angiotensin receptor blocking ability, indicating that these effects were not related to the ARB property of losartan.

We also showed that the results obtained in P1 cells from non-smokers could be repeated when using cells from smokers with or without COPD (figure 7). Thus, losartan was effective even when cells were *in vivo* chronically exposed to smoke and possibly underwent epigenetic changes.

These *in vitro* findings set the stage for a small clinical study to determine whether losartan could reduce inflammation and mucus hypersecretion in the upper airways of smokers. In nasal cells of participants, we measured significant increases in *TGF-β1*, *MMP9* and *MUC5AC* mRNA expressions in smokers compared to non-smokers, consistent with elevation of these markers observed in subjects with chronic bronchitis [6, 32]. Despite the small number of subjects completing the study and the pre-/post-treatment design (suboptimal to a placebo control), we observed a significant decrease in the expression of *TGF-β1*, *MMP9* and *MUC5AC* mRNAs in those who remained on losartan for the full eight weeks of treatment (figure 8). Although numerous studies using HNECs as a surrogate for HBECs have been described [38], their usefulness for studying airways diseases continues to be debated [39].

However, our data reveal that the relative increases in mRNA expression of *TGF-β1*, *MMP-9*, and *MUC5AC* in airway cells of smokers versus non-smokers is comparable between HNECs and P0 HBECs. More importantly, losartan successfully reduced the expression of these markers in both HNECs derived from smoking subjects and CS-exposed P1 HBECs, suggesting HNECs can serve as a surrogate for HBECs in *in vitro* models of CS-induced airway disease.

These studies provide support for losartan as a potential therapeutic to combat inflammation and mucus hyperconcentration in smoking-related chronic airway diseases. Losartan may also be effective in treating other airway diseases, where MUC5AC tethering impairs mucociliary transport[29, 40] or where mucus hypersecretion contributes to the pathogenesis of the disease [41].

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Author contributions: Conceived and designed the study: N. Baumlin and M. Salathe. Executed experiments and analysed the data: N. Baumlin, J.S. Dennis, M. Yoshida, A. Kis, M.D. Kim, M. Salathe. Clinical study recruitment and collection and interpretation of data: N. Baumlin, C. Aguiar, A. Schmid, E. Mendes, M. Salathe. Wrote the manuscript: N. Baumlin, M.D. Kim, and M. Salathe. Discussed the results and commented on the manuscript: all authors.

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Captions

FIGURE 1. Freshly isolated human bronchial epithelial cells (P0 HBECs) from non-smokers and smokers. (**A**) Quantitative mRNA expression of *MUC5AC*, *TGF-β1*, and *MMP-9* of P0 HBECs from non-smokers and smokers. Data shown as relative to GAPDH and non-smokers. n≥6 donors for each group. (**B**) Representative confocal images of immunofluorescent staining of bronchial tissue from a non-smoker (upper panels) and a smoker (lower panels). MUC5AC in red; nuclei stained with Hoechst in blue. Scale bar represents 50 μm. (**C**) Quantification of MUC5AC-positive cells relative to nuclei. n=7 from ≥3 donors for each group. * $p < 0.05$, Student's t-test after passing Shapiro-Wilk normality test.

FIGURE 2. Fully re-differentiated human bronchial epithelial cells (P1 HBECs) from non-smokers and smokers. (**A**) Quantitative mRNA expression of *MUC5AC*, *TGF-β1*, and *MMP-9* of fully re-differentiated P1 HBECs from non-smokers and smokers. Data shown as relative to GAPDH and non-smokers. n≥4 donors for each group. (**B**) Representative images of immunofluorescent staining of P1 HBECs from a non-smoker (upper panels) and a smoker (lower panels). MUC5AC is stained in red, nuclei were stained with Hoechst and represented in blue. Scale bar represents 50 μm. (**C**) Quantification of MUC5AC-positive cells relative to nuclei. n=8 from ≥3 donors. (**D**) CFTR currents measured by short circuit current changes upon $CFTR_{inh}172$ (10 μM) application after 10 μM forskolin stimulation 4 h after exposure to room air or CS (represented as ΔI_{sc} upon $CFTR_{inh}172$; thus, decreases indicate enhanced CFTR function). n≥9 donors for each group. (**E**) BK currents measured upon ATP stimulation and represented as ΔI_{sc}. n≥11 donors for each group (decreases indicate enhanced BK function). (**F**) ASL volumes measured 4-6 weeks after establishment of ALI and represented as baselines. n=12 donors for each group. (**G**) Mucus concentration depicted as % mucus solids measured 4-6 weeks after establishment of ALI and represented as baselines. n≥4 donors for each group.

None of the comparisons were significant by Student's t-test after passing Shapiro-Wilk normality test.

FIGURE 3. Fully re-differentiated human bronchial epithelial cells (P1 HBECs) from non-smokers exposed to cigarette smoke (CS). (**A**) Quantitative mRNA expression of *MUC5AC* and *TGF-β1* in P1 HBECs 24 h after exposure to room air or CS (24 puffs). Data shown as relative to GAPDH and air control. n=8 donors for each group. (**B**) MMP-9 activity assay from PBS washes of P1 HBECs from non-smokers 24 h after exposure to room air or CS (24 puffs). Data shown as relative to air control. n=8 from 4 donors for each group. (**C**) *Left panel:* representative CFTR trace measured by short circuit current changes upon $CFTR_{inh}172$ (10 μM) application after 10 μM forskolin stimulation 4 h after exposure to room air or CS (represented as ΔI_{sc} upon $CFTR_{inh}172$; thus, decreases indicate enhanced CFTR function). n=5 donors for each group. *Middle panel:* quantification of CFTR currents upon $CFTR_{inh}172$. *Right panel: CFTR* mRNA expression. Data shown as relative to GAPDH and air control. n=18 from 6 donors for each group. (**D**) *Left panel:* representative BK trace and quantification of currents measured upon ATP stimulation 4 h after exposure to room air or CS (represented as ΔI_{sc} with decreases indicating better BK function). n=6 donors for each group. *Right panel: LRRC26* mRNA expression (γ subunit of BK critical for BK function). Data shown as relative to GAPDH and air control. n=18 from 6 donors for each group. (**E**) ASL volumes represented as Δ volume between 1 and 4 h after air or CS exposure. n=11 donors for each group. (**F**) Quantification of MUC5AC-positive cells relative to nuclei 24 h after air or CS exposure. n=9 from 3 donors for each group. (**G**) Mucus concentration depicted as % mucus solids measured 24 h after air or CS exposure. n=5 donors for each group. * $p < 0.05$, Student's t-test after passing Shapiro-Wilk normality test for all except for the BK data (Mann-Whitney).

FIGURE 4. Fully re-differentiated human bronchial epithelial cells (P1 HBECs) from non-smokers exposed to cigarette smoke (CS) or TGF-β1 and treated with losartan, LY2157299, and EXP3179. (**A**)

Left: ASL volumes represented as Δ volume between 1 and 4 h after CS ± losartan exposure. n=13 donors for each group. *Right:* Ciliary beat frequency measured 4 h after exposure to room air, CS ± losartan. n=12 from 3 donors in each group. (**B**) *Left:* Quantification of MUC5AC-positive cells relative to nuclei 24 h after CS ± losartan exposure. n=8 from 3 donors for each group. *Right:* Mucus concentration depicted as % mucus solids measured 24 h after CS ± losartan. n=5 donors for each group. (**C**) *Left:* P1 HBECs from non-smokers were treated with TGF-β1 (10 ng/mL) in the presence or absence of the TGFBR1 inhibitor LY2157299 (10 μM) for 24 h. Control contained appropriate concentrations of DMSO. LY2157299 prevents TGF-β1-induced ASL volume loss. n=3 donors. *Right:* P1 HBECs from non-smokers were pre-treated with 10 μM LY2157299 before exposure to CS (24 puffs). LY2157299 ameliorated CS-induced ASL volume loss. (**D**) *Left:* P1 HBECs from non-smokers were treated with TGF-β1 (10 ng/mL) in the presence or absence of EXP3179 (5 μM) for 24 h. EXP3179 prevents TGF-β1-induced ASL volume loss. n=7 donors. *Right:* P1 HBECs from non-smokers were pre-treated with 5 μM EXP3179 before exposure to CS (24 puffs). EXP3179 ameliorated CS-induced ASL volume loss. n=3 donors. * $p < 0.05$ either compared to all groups by one-way ANOVA followed by Holm-Sidak post hoc test or Student's t-test; both after passing Shapiro-Wilk normality test.

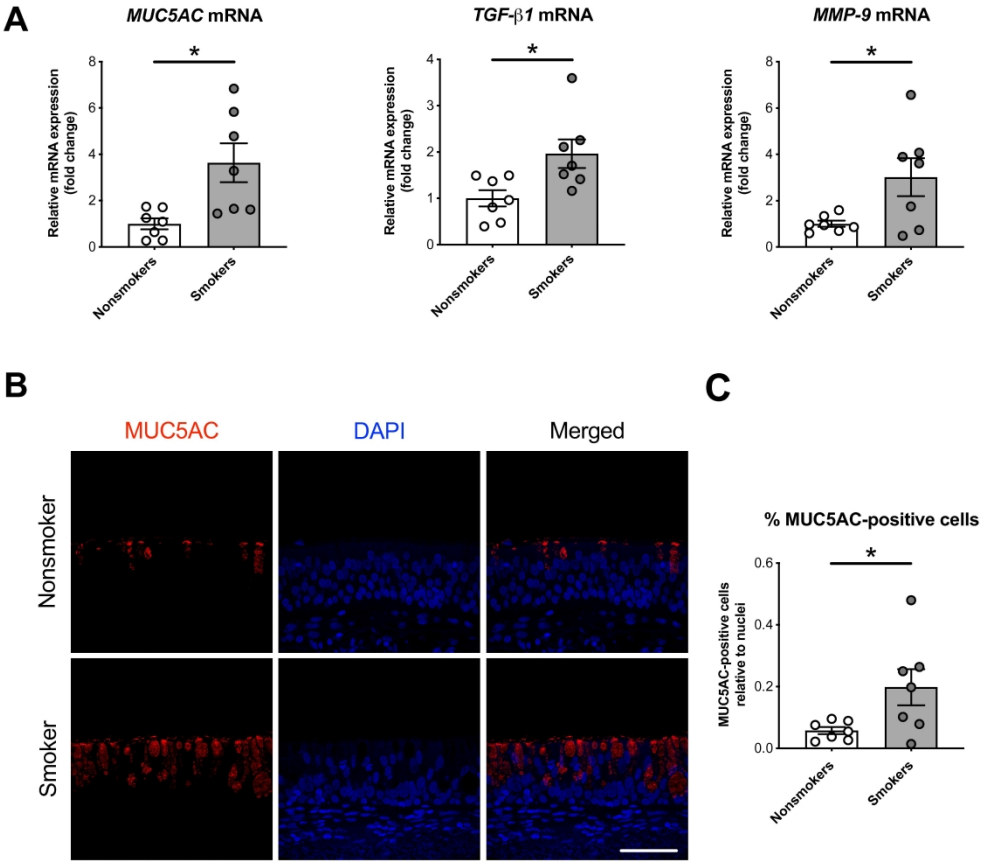
FIGURE 5. Fully re-differentiated human bronchial epithelial cells (P1 HBECs) from non-smokers exposed to cigarette smoke or TGF-β1 and treated with losartan or LY2157299. (**A**) Expression of TGF-β1 mRNA but not of TGF-β2 mRNA is increased by smoke (two left panels). IL-13 mRNA is not significantly upregulated by smoke and is not changed by LY-2157299 (10 μM). Note the low expression (right panel). (**B**) Of MMP-2, MMP-9, and MMP-12, only MMP-9 mRNA is upregulated by smoke in a TGF-β1-dependent fashion. LY2157299 also reduced *MMP-9* mRNA expression upon smoke exposure. $n \geq 4$ from ≥3 donors. (**A, B**) * $p < 0.05$ compared to all groups and # $p < 0.05$ compared to smoke only by one-way ANOVA followed by Holm-Sidak post hoc test; all after passing Shapiro-Wilk normality test. All data shown are mean ± SE. One-way ANOVA followed by Holm-Sidak after passing Shapiro-Wilk normality test or Kruskal Wallis.

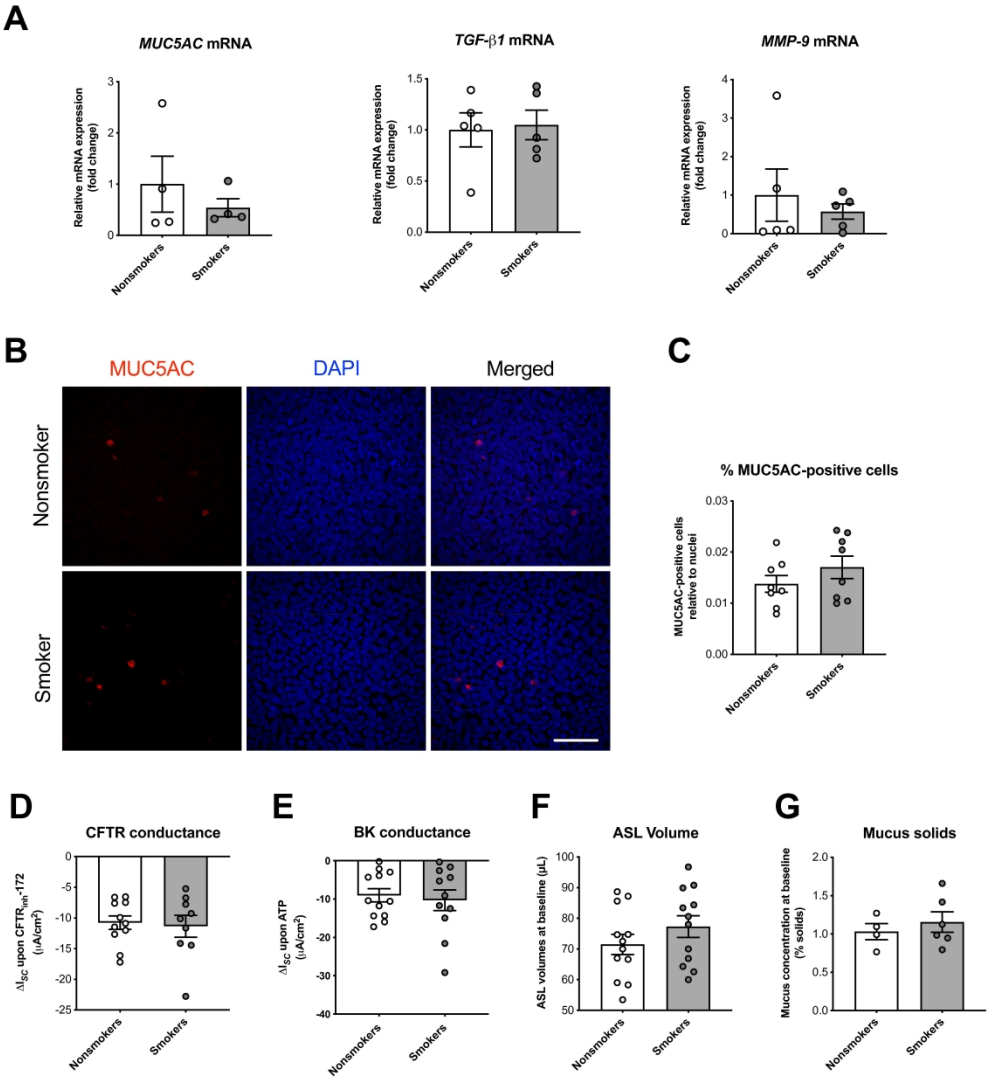
FIGURE 6. Fully re-differentiated human bronchial epithelial cells (P1 HBECs) from non-smokers exposed to cigarette smoke (CS) ± losartan. (**A**) Quantitative mRNA expression of *MUC5AC* and *TGF-β1* in P1 HBECs 24 h after exposure to CS (24 puffs) without and with losartan treatment (10 μM throughout differentiation). Data shown as relative to GAPDH and smoke. n=5 donors for each group. (**B**) MMP-9 activity assay from PBS washes of P1 HBECs from non-smokers 24 h after exposure to CS (24 puffs) without and with losartan treatment. Data shown as relative to smoke. n=6 from 2 donors for each group. (**C**) *Left panel:* CFTR currents represented by short circuit current changes upon $CFTR_{inh}172$ (10 μM) application after 10 μM forskolin stimulation 4 h after exposure to CS ± losartan (represented as ΔI_{sc} upon $CFTR_{inh}172$). n=5 for each group. *Right panel: CFTR* mRNA expression. Data shown as relative to GAPDH and smoke. n=6 donors for each group. (**D**) *CFTR* mRNA expression in air control. Data shown as relative to GAPDH and air. n=3 donors for each group. (**E**) Left panel: BK currents measured upon ATP stimulation 4h after exposure to CS ± losartan. n=5 donors for each group. *Right panel: LRRC26* mRNA expression. Data shown as relative to GAPDH and smoke. n=6 donors for each group. (**F**) *LRRC26* mRNA expression in air control. Data shown as relative to GAPDH and air. n=3 donors for each group. * $p < 0.05$; all Student's t-test after passing Shapiro-Wilk normality test.

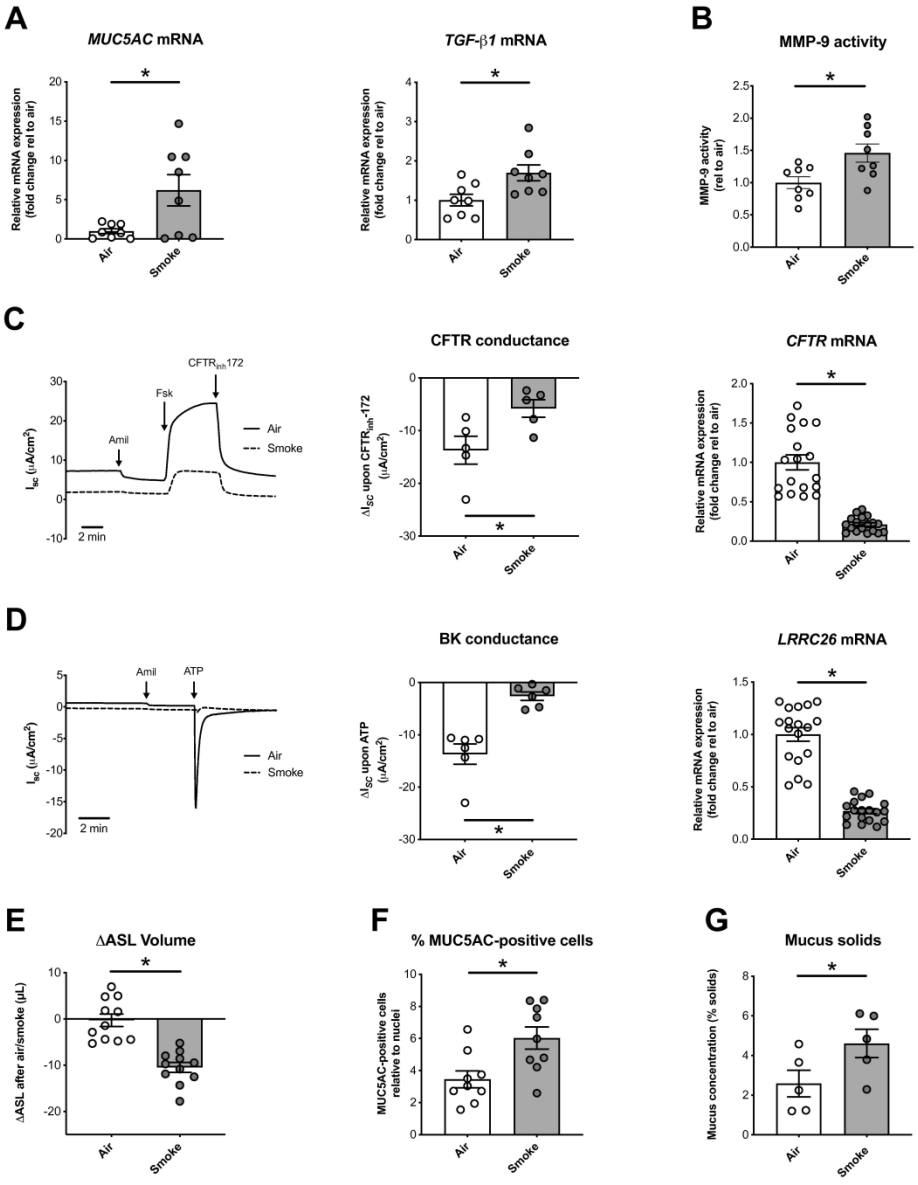
FIGURE 7. Fully re-differentiated human bronchial epithelial cells (P1 HBECs) from active smokers with and without COPD exposed to cigarette smoke (CS) and treated with losartan. (**A**) CFTR conductance expressed as a ratio of CS exposure over air exposure of short circuit current changes upon $CFTR_{inh}$-172 (10 μM) application after 10 μM forskolin stimulation (ΔI_{sc} smoke/air). Note reverse y axis to depict similar as in figure 3 (more downward = positive = improved conductance). n=8 from 7 donors. * $p < 0.05$ using t-test. (**B**) *CFTR* mRNA expression. Data shown as relative to GAPDH and smoke. n=18 from 6 donors for each group. n=7 from 7 donors. * $p < 0.05$ using t-test. (**C**) BK currents expressed as a ratio of CS exposure over air exposure of short circuit current changes upon ATP stimulation (ΔI_{sc} smoke/air). Note reverse y axis to depict similar as in figure 3 (more downward = positive = improved conductance). n=5 from 5 donors. * $p < 0.05$ using t-test. (**D**) *LRRC26* mRNA

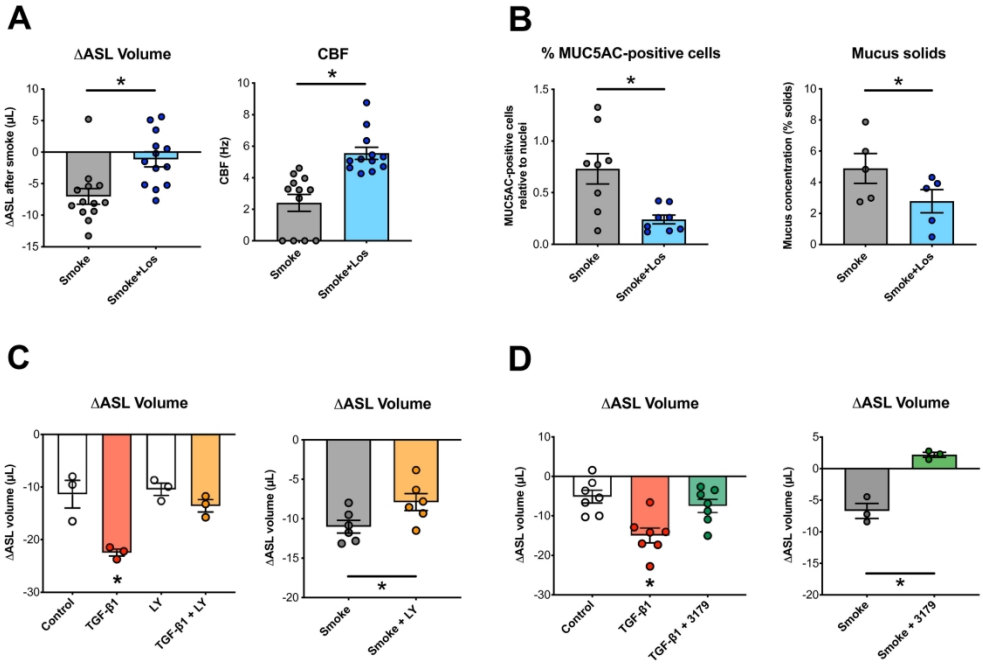
expression. Data shown as relative to GAPDH and smoke. n=7 from 7 donors. * $p < 0.05$ using t-test. (**E**) Ciliary beat frequency after exposure to CS ± losartan. n=26 from 7 donors. * $p < 0.05$ using Mann Whitney. (**F**) MMP-9 activity assay from apical PBS washes obtained 24 h after exposure to CS ± losartan. Data shown as relative to smoke. n=12 from 7 donors. * $p < 0.05$ using t-test.

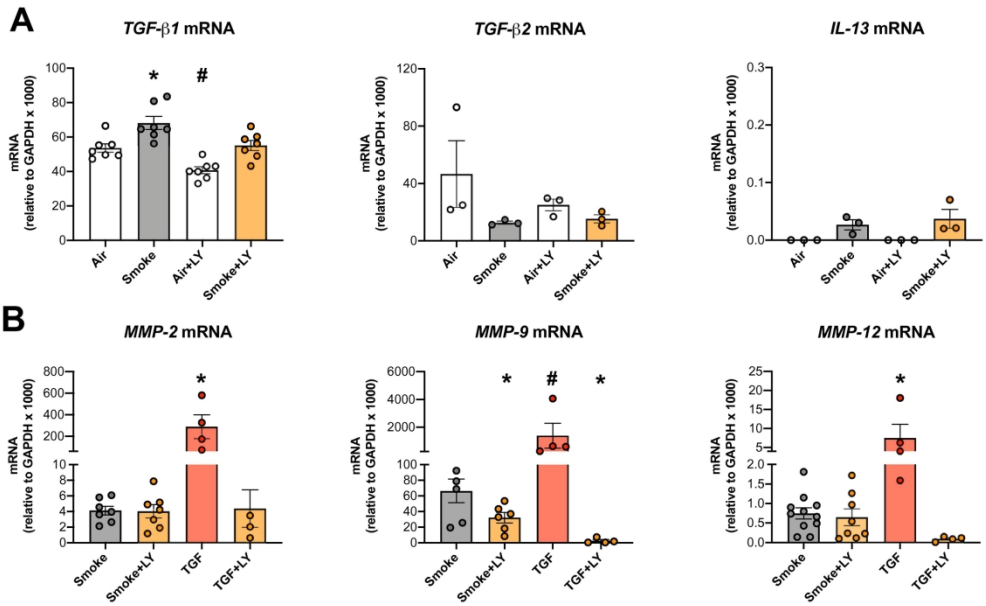
FIGURE 8. Clinical trial with oral losartan and analysis of human nasal epithelial cells (HNECs) from non-smokers and smokers. (**A**) Study schematic (clinicaltrials.gov registration NCT02416102). (**B**) Table with participants' demographics. ns: not significant. (**C**) Quantitative mRNA expression of *MUC5AC, TGF-β1*, and *MMP-9* of HNECs from non-smokers and smokers at baseline. Data shown as relative to GAPDH and non-smokers. n=5 subjects for each group. (**D**) Quantitative mRNA expression of *MUC5AC, TGF-β1*, and *MMP-9* of nasal cells from smokers, before losartan treatment and 2 months after (50 mg daily for 4 weeks and 100 mg daily for an additional 4 weeks). Data shown as relative to baseline prior to losartan administration. n=5 subjects from each group for each measurement. * $p < 0.05$, all Student's t-test after passing Shapiro-Wilk normality test.

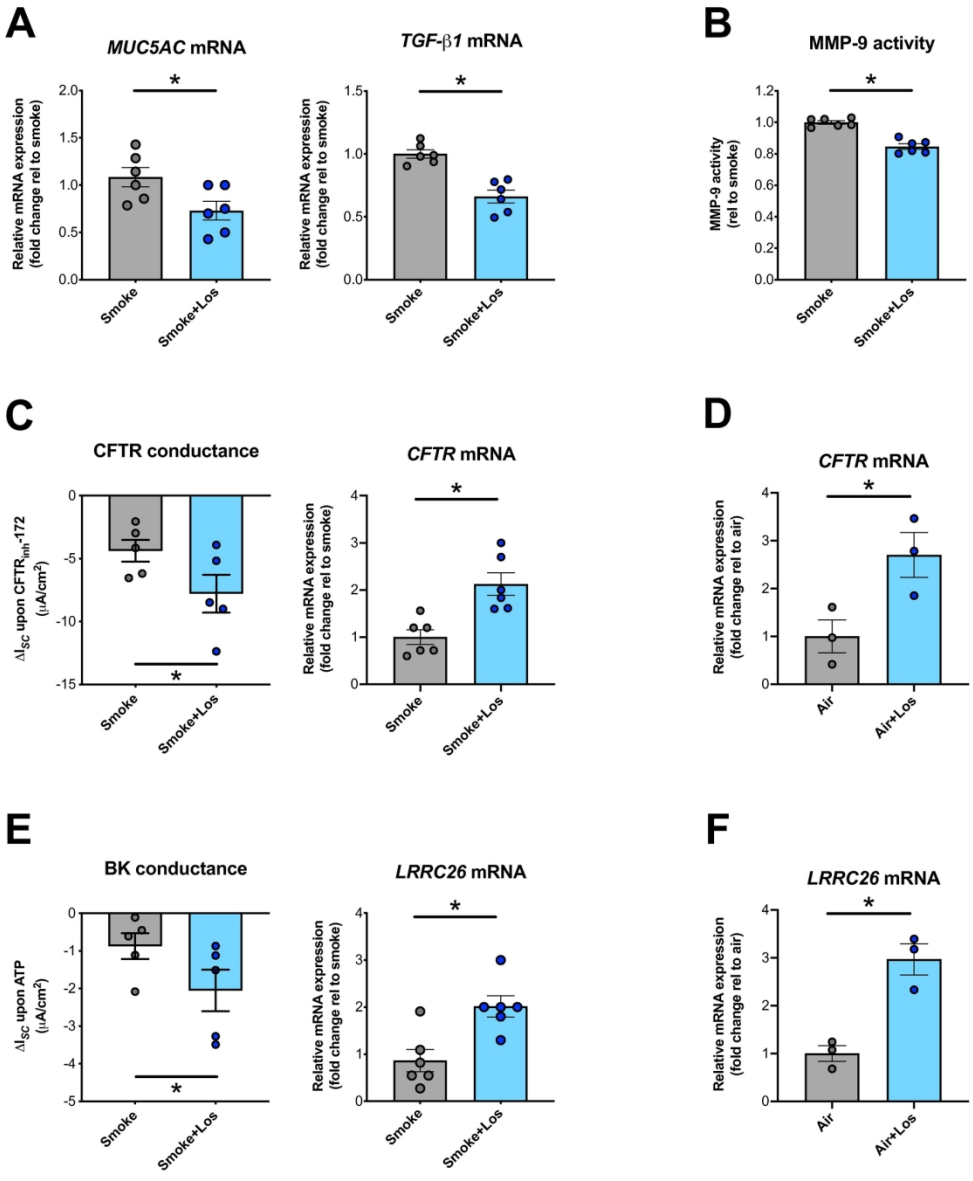

A
MUC5AC mRNA
TGF-β1 mRNA
MMP-9 mRNA
Relative mRNA expression (fold change)
Nonsmokers
Smokers
B
MUC5AC
DAPI
Merged
Nonsmoker
Smoker
C
% MUC5AC-positive cells
MUC5AC-positive cells relative to nuclei

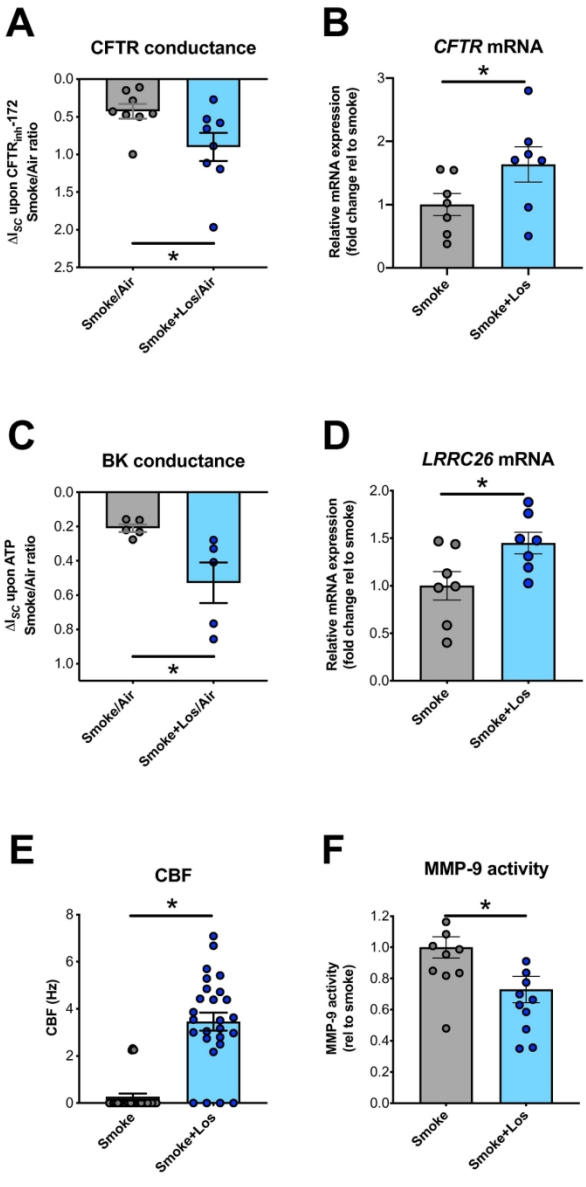

A
MUC5AC mRNA
TGF-β1 mRNA
MMP-9 mRNA
Relative mRNA expression (fold change)
Nonsmokers
Smokers
B
MUC5AC
DAPI
Merged
Nonsmoker
Smoker
C
% MUC5AC-positive cells
MUC5AC-positive cells relative to nuclei
D
CFTR conductance
E
BK conductance
F
ASL Volume
ASL volumes at baseline (µL)
G
Mucus solids
Mucus concentration at baseline (% solids)


A
MUC5AC mRNA
Relative mRNA expression (fold change rel to air)
Air
Smoke
TGF-β1 mRNA
Relative mRNA expression (fold change rel to air)
B
MMP-9 activity
MMP-9 activity (rel to air)
C
CFTR conductance
CFTRinh172
Fsk
Amil
Air
Smoke
2 min
ΔISC upon CFTRinh-172 (μA/cm²)
CFTR mRNA
Relative mRNA expression (fold change rel to air)
D
BK conductance
Amil
ATP
ΔISC upon ATP (μA/cm²)
LRRC26 mRNA
E
ΔASL Volume
ΔASL after air/smoke (μL)
F
% MUC5AC-positive cells
MUC5AC-positive cells relative to nuclei
G
Mucus solids
Mucus concentration (% solids)

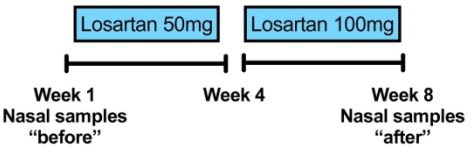

A
ΔASL Volume
ΔASL after smoke (μL)
Smoke
Smoke+Los
CBF
CBF (Hz)
Smoke
Smoke+Los
B
% MUC5AC-positive cells
MUC5AC-positive cells relative to nuclei
Smoke
Smoke+Los
Mucus solids
Mucus concentration (% solids)
Smoke
Smoke+Los
C
ΔASL Volume
ΔASL volume (μL)
Control
TGF-β1
LY
TGF-β1 + LY
ΔASL Volume
ΔASL volume (μL)
Smoke
Smoke + LY
D
ΔASL Volume
ΔASL volume (μL)
Control
TGF-β1
TGF-β1 + 3179
ΔASL Volume
ΔASL volume (μL)
Smoke
Smoke + 3179


A
TGF-β1 mRNA
TGF-β2 mRNA
IL-13 mRNA
mRNA (relative to GAPDH x 1000)
Air
Smoke
Air+LY
Smoke+LY
B
MMP-2 mRNA
MMP-9 mRNA
MMP-12 mRNA
Smoke
Smoke+LY
TGF
TGF+LY


A
MUC5AC mRNA
Relative mRNA expression (fold change rel to smoke)
2.0
1.5
1.0
0.5
0.0
*
Smoke
Smoke+Los
TGF-β1 mRNA
Relative mRNA expression (fold change rel to smoke)
1.5
1.0
0.5
0.0
*
Smoke
Smoke+Los
B
MMP-9 activity
MMP-9 activity (rel to smoke)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
Smoke
Smoke+Los
C
CFTR conductance
ΔISC upon CFTRinh-172 (μA/cm2)
0
-5
-10
-15
*
*
Smoke
Smoke+Los
CFTR mRNA
Relative mRNA expression (fold change rel to smoke)
4
3
2
1
0
*
Smoke
Smoke+Los
D
CFTR mRNA
Relative mRNA expression (fold change rel to air)
4
3
2
1
0
*
Air
Air+Los
E
BK conductance
ΔISC upon ATP (μA/cm2)
0
-1
-2
-3
-4
-5
*
Smoke
Smoke+Los
LRRC26 mRNA
Relative mRNA expression (fold change rel to smoke)
4
3
2
1
0
*
Smoke
Smoke+Los
F
LRRC26 mRNA
Relative mRNA expression (fold change rel to air)
4
3
2
1
0
*
Air
Air+Los


A
CFTR conductance
ΔISC upon CFTRinh-172
Smoke/Air ratio
0.0
0.5
1.0
1.5
2.0
2.5
*
Smoke/Air
Smoke+Los/Air
B
CFTR mRNA
Relative mRNA expression
(fold change rel to smoke)
3
2
1
0
*
Smoke
Smoke+Los
C
BK conductance
ΔISC upon ATP
Smoke/Air ratio
0.0
0.2
0.4
0.6
0.8
1.0
*
Smoke/Air
Smoke+Los/Air
D
LRRC26 mRNA
Relative mRNA expression
(fold change rel to smoke)
2.0
1.5
1.0
0.5
0.0
*
Smoke
Smoke+Los
E
CBF
CBF (Hz)
8
6
4
2
0
*
Smoke
Smoke+Los
F
MMP-9 activity
MMP-9 activity
(rel to smoke)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
Smoke
Smoke+Los

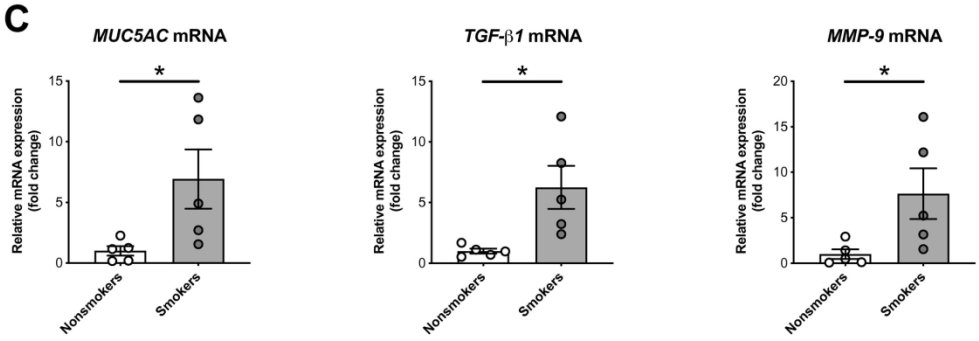
A



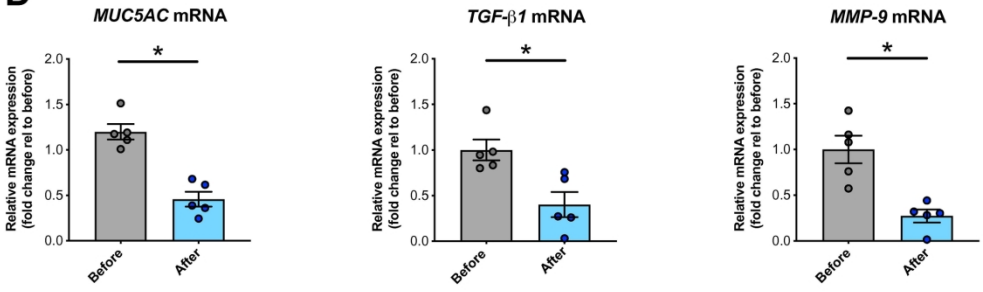
B

	Nonsmokers	Smokers	
n	5	5	
Age	53.8 ± 5.3	52 ± 3	ns
Female	5 (100%)	2 (40%)	
Male	0 (0%)	3 (60%)	
BMI	27.6 ± 0.81	32.0 ± 2.17	ns
FEV1/FVC	80.8 ± 0.02	78.0 ± 0.02	ns
FEV1 (L)	2.55 ± 0.23	2.55 ± 0.24	ns
FVC (L)	3.26 ± 0.2	2.87 ± 0.3	ns

C



D



Supplementary data

Losartan reduces cigarette smoke-induced airway inflammation and mucus hypersecretion

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Supplementary methods

Lungs

Lung tissue was obtained from organ donors whose lungs were rejected for transplant and recovered for research by the Life Alliance Organ Recovery Agency at the University of Miami (Miami, FL, USA), LifeCenter Northwest (Seattle, WA, USA), and the Midwest Transplant Network (Kansas City, KS, USA). A ring of the trachea or main bronchi was cut and fixed in 10% formalin at 4°C until embedded in paraffin for sectioning and tissue staining. Airways were dissected and the tissue exposed to protease overnight as previously described.[1 2] Cells were harvested the following day and frozen in liquid nitrogen. These cells are considered P0 HBECs.

Cell culture and drug treatments

Culturing of HBECs at the air-liquid interface (ALI) was performed as described.[1 2] Briefly, P0 HBECs were thawed at 37°C, resuspended in warm bronchial epithelial cell growth medium (BEGM) and spun at 360 x g for 5 min at room temperature. The supernatant was discarded and the cell pellet resuspended in BEGM with Amphotericin B. The density of viable cells plated in a 10 cm dish was approximately 1-2 x 10^6 cells. Once the cells reached confluence, they were trypsinized and plated on Transwell and/or Snapwell inserts at a minimum of 150,000 cells/cm^2 in ALI media.[1] HBECs were maintained submerged for 4-6 days before exposing them to air and allowing them to re-differentiate for a minimum of 28 days before experiments were performed. Fully re-differentiated HBECs are referred to as P1 HBECs. *In vitro* losartan (#61188, MilliporeSigma, St. Louis, MO, USA) treatment at 10 μM was started the day P1 HBECs went to an ALI. Losartan was maintained in the media throughout differentiation. LY2157299 (#S2230, Selleckchem, Houston, TX, USA) at 10 μM or EXP3179 (#18855, Cayman Chemicals, Ann

Arbor, MI, USA) at 5 μM was added to the basolateral media of ALI cultures 24 h before CS exposure.

Immunofluorescence staining

Immunofluorescence staining of tissue sections was performed as previously described.[3] Slides were first incubated overnight at 60°C. The following day, a deparaffinization step of 2 x 5 min in xylene was performed, followed by several hydration steps of 2 x 3 min in 100% ethanol, 2 x 1 min in 95% ethanol, and finally, 2 x 1 min in 80% ethanol before rinsing in distilled water. Antigen retrieval was achieved using 10 mM trisodium citrate (#S1804, MilliporeSigma) with 0.05% Tween 20 in distilled water with pH adjusted to 6.0 for 30 min in a water bath at 98°C. The slides were allowed to cool down before a 10 min rinsing step in phosphate buffered saline (PBS) with 0.05% Tween 20. All the following steps were performed on a shaker. 3% bovine serum albumin (BSA; #BP1600-100, ThermoFisher Scientific, Waltham, MA, USA) in PBS was used for 1 h at room temperature (RT) to block for nonspecific bindings. Anti-MUC5AC primary antibody (#MA1-38223, ThermoFisher Scientific) at 0.4 μg/mL in 3% BSA was incubated overnight at 4°C. After two washing steps of 10 min with PBS with 0.05% Tween 20, secondary antibody in 3% BSA was applied for 1 h at RT. Two more washing steps of 10 min were performed before adding 2 μg/mL Hoechst (#H3569, ThermoFisher Scientific) for 10 min in PBS with 0.05% Tween 20. One final washing step of 10 min was performed before adding Fluoro-Gel with Tris Buffer (#17985-11, Electron Microscopy Sciences, Hatfield, PA, USA). For staining of P1 HBECs, Transwell inserts were fixed with a solution of 50% methanol and 50% acetone for 2 min at -20°C followed by 3 washes of PBS. A solution of 3% BSA was used to block for one hour at RT on a shaker. Immunostaining with primary and secondary antibodies were performed as described

above. P1 HBECs exposed to CS or room air were washed and fixed 48 h after exposure following the same immunostaining protocol. All slides were imaged with a Nikon C2+ confocal microscope (Nikon Instruments, Tokyo, Japan).

Quantitative PCR

P1 HBECs exposed to CS or room air were lysed 24 h after exposure and total RNA was isolated using the E.Z.N.A.® Total RNA Kit (Omega Bio-tek, Norcross, GA, USA). qPCR was performed as described[4 5] using TaqMan Gene Expression Assays (ThermoFisher Scientific) for *MMP9* (Hs00234579_m1), *MUC5AC* (Hs01365601_m1) and *TGF-β1* (Hs00998133_m1), and normalized to reference gene *GAPDH*.

MMP-9 activity assay

MMP-9 activity was measured in 200 µL PBS washes collected 24 h after room air or CS exposure using Human Active MMP-9 Fluorokine E Kit (#F9M00, R&D Systems, Minneapolis, MN, USA) and following manufacturer's instructions for non-activated samples.

Ussing chamber

Cystic fibrosis transmembrane conductance regulator (CFTR) and large conductance, Ca^{2+}-activated and voltage-dependent K^+ channel (BK) activities were recorded in Ussing chambers as previously described.[6 7] Briefly, ENaC currents were blocked by amiloride (10 µM; MilliporeSigma) before CFTR currents were stimulated by forskolin (10 µM; MilliporeSigma) and inhibited by $CFTR_{inh}$-172 (10 µM; MilliporeSigma). Prior to measuring BK currents, the basolateral membranes were permeabilized with amphotericin B (20 µM; MilliporeSigma),

Nigericin (10 μM; Tocris Bioscience, Minneapolis, MN, USA), and Valinomycin (10 μM; Tocris Bioscience). The cells were exposed to a K^+ gradient of 140 mM at the permeabilized basolateral side and 5 mM at the apical side. After ENaC currents were blocked by amiloride (10 μM), BK currents were stimulated by ATP (10 μM; Tocris Bioscience). The transepithelial membrane potential was clamped at 0 mV (model VCC MC8, Physiologic Instruments, San Diego, CA, USA), using Ag/AgCl electrodes in agar bridges. Signals were digitized and recorded using the Acquire and Analyze revision II module according to the manufacturer's instructions. The experiments were performed at 37°C using heated water jackets and bubbled with a gas mixture of 95% O_2 / 5% CO_2 for CFTR current or air for BK current. The CFTR and BK currents from P1 HBECs on Snapwells were measured at 4 h after the cells were exposed to CS or room air.

Airway surface liquid (ASL) volume measurements

ASL volume estimation was performed by meniscus scanning as previously published.[5 8] P1 HBECs exposed to CS or room air were scanned 1 h and 4 h after exposure and the delta was plotted.

Ciliary beat frequency (CBF)

CBF was recorded 4 h after exposure to CS or room air using a high-speed camera and analyzed using the individual Region-of-Interest (ROI) method of SAVA software.[9 10] Ciliary beating was recorded 1-2 mm away from the center of the insert for 2 seconds and four ROIs were plotted.

Mucus concentration measurements

The percent of solids on top of cultures was measured according to published methods of mucus wet and dry weights using an ultra-microbalance (UMX2 Mettler Toledo) capable of detecting accurately to 100 ng.[11 12] Briefly, mucus was lifted off the cultures using a 5-mm diameter Kimwipe paper mesh that was cut with a laser to assure equal size (Epilog Mini 30W laser). All weights were recorded with the mesh in an aluminum boat. The dry weight of the mesh was recorded first, then the mesh was applied onto the cultures and left on the surface for 10 min at 37°C in an incubator before being pulled off and immediately measured again for the wet weight. After an overnight incubation at 60°C (oven), the dry weight with only mucus left was recorded again. All measurements were corrected for elapsed time after pulling off the mesh from the cultures and when taken out of the oven until it was placed onto the scale. Percent mucus solids were calculated as follows: [(dry weight-mesh weight) / (wet weight-mesh weight)] x 100 – 0.9. P1 HBECs exposed to CS or room air were tested 24 h after exposure.

Cigarette smoke (CS) exposure

CS exposure of P1 HBECs was done as previously described.[3 5 13] Briefly, cells were washed with PBS and the media was replaced the day before the exposure. P1 HBECs were exposed to 24 puffs of a Kentucky research cigarette (3R4F) with a volume of 35 mL delivered every 60 seconds using the Vitrocell VC10 smoking robot (Vitrocell, Waldkirch, Germany) following ISO standard 3308. As controls, P1 HBECs were exposed to room air. Nicotine deposition onto the surface of HBECs after CS exposures was validated by mass spectrometry (LS-MS/MS, Florida International University, FL, USA) and showed depositions of approximative 100-120 μM of nicotine onto ALI cultures comparable to *in vivo* deposition of 1-2 cigarettes.[14]

Study approval

The study was approved by the University of Miami Human Subject Research Office and informed consent was obtained from each participant. Clinicaltrials.gov registry under NCT02416102.

Human subjects

The study enrolled a total of 31 participants: 16 healthy never-smokers and 15 current smokers with a smoking history of >10 pack/years and no signs of COPD by PFT with DLCO. FEV1/FVC, FEV1, and FVC values from both groups were in the normal range. Thus, none of the subjects had COPD by spirometric definitions. From those 31 patients, 14 (7 smokers and 7 nonsmokers) completed the study with laboratory test results and only 5 subjects in each group had complete nasal sample data sets. The subjects were aged 35 to 70 years and not taking any angiotensin receptor blockers (ARBs) prior to enrollment. None of the subjects were taking corticosteroids for 6 weeks prior to enrollment, were pregnant, had hypoxemia, untreated arterial hypertension, blood pressure lower than 100 mm Hg systolic or 70 mm Hg diastolic while standing. Furthermore, there was no significant difference in complete blood count (CBC), comprehensive metabolic panel (CMP) or c-reactive protein (CRP) measured in the blood samples, between week 1 and week 8. Participants received 50 mg losartan for 4 weeks and then 100 mg losartan for another 4 weeks.

Nasal cells collection

Nasal cells were collected using sterile cytology brushes (Medical Packaging Corporation, Camarillo, CA, USA). The brushes were introduced into the nasal cavity under direct visual guidance and were placed between the nasal septum and the inferior turbinate. No anesthesia was used. The cells were harvested by a few careful backward-forward and rotary movements before

twirling the brush into 5 mL of sterile PBS in a 15 mL tube to release the cells. The same procedure was repeated 3 times in each nostril. Immediately after the harvest, the tube was centrifuged at 360 x g for 5 min at 4°C. The supernatant was discarded and the remaining pellet was frozen at -80°C until qPCR assay was performed.

Statistical analyses

Data are shown as dot plots / bar graph combinations with means ± S.E.M. Differences between two groups were compared by parametric or non-parametric tests as indicated in the figure captions depending on whether the data passed Shapiro-Wilk normality testing. p values for significance were accepted at $p < 0.05$. All analyses were performed using Prism (GraphPad Software, San Diego, CA, USA).

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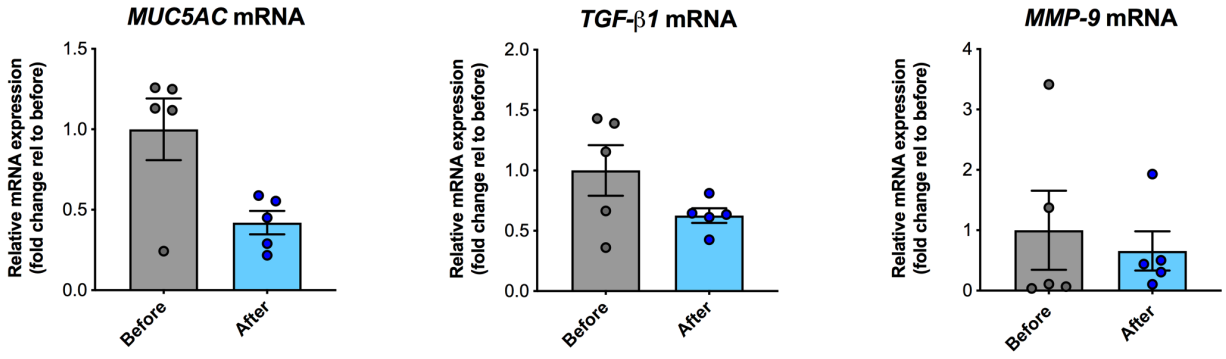
Supplementary table and figures

Table S1

	Nonsmokers	Smokers
n	10	7
Age	44.5 ± 2.3	43 ± 2.9
Female	4	2
Male	6	5
Caucasian	8	3
African-American	1	1
Hispanic	1	3
Pack-years	0	18.9 ± 2.1

Supplementary Table S1. Table with demographics of all lung donors used for Figs. 1-5.

Figure S1



Supplementary Figure S1. Human nasal epithelial cells (HNECs) of nonsmokers treated with losartan. Quantitative mRNA expression of *MUC5AC*, *TGF-β1*, and *MMP-9* of nasal cells from nonsmokers, before losartan treatment and 2 months after (50 mg daily for one month and 100 mg daily for an additional month). Data shown as relative to baseline prior to losartan administration. n=5 subjects for each group. None of the comparisons were significant by Student's t-test after passing Shapiro-Wilk normality test.