Losartan Rescues Inflammation-related Mucociliary Dysfunction in Relevant Models of Cystic Fibrosis

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Abstract

Rationale: Despite therapeutic progress in treating cystic fibrosis (CF) airway disease, airway inflammation with associated mucociliary dysfunction remains largely unaddressed. Inflammation reduces the activity of apically expressed large-conductance Ca^{2+} -activated and voltage-dependent K^{+} (BK) channels, critical for mucociliary function in the absence of CFTR (CF transmembrane conductance regulator).

Objectives: To test losartan as an antiinflammatory therapy in CF using CF human bronchial epithelial cells and an ovine model of CF-like airway disease.

Methods: Losartan's antiinflammatory effectiveness to rescue BK activity and thus mucociliary function was tested *in vitro* using primary, fully redifferentiated human airway epithelial cells homozygous for F508del and *in vivo* using a previously validated, now expanded pharmacologic sheep model of CF-like, inflammation-associated mucociliary dysfunction.

Measurements and Main Results: Nasal scrapings from patients with CF showed that neutrophilic inflammation correlated with reduced expression of LRRC26 (leucine rich repeat containing 26),

the γ subunit mandatory for BK function in the airways. TGF- β 1 (transforming growth factor β 1), downstream of neutrophil elastase, decreased mucociliary parameters *in vitro*. These were rescued by losartan at concentrations achieved by nebulization in the airway and oral application in the bloodstream: BK dysfunction recovered acutely and over time (the latter via an increase in LRRC26 expression), ciliary beat frequency and airway surface liquid volume improved, and mucus hyperconcentration and cellular inflammation decreased. These effects did not depend on angiotensin receptor blockade. Expanding on a validated and published nongenetic, CF-like sheep model, ewes inhaled CFTR_{inh}172 and neutrophil elastase for 3 days, which resulted in prolonged tracheal mucus velocity reduction, mucus hyperconcentration, and increased TGF- β 1. Nebulized losartan rescued both mucus transport and mucus hyperconcentration and reduced TGF- β 1.

Conclusions: Losartan effectively reversed CF- and inflammation-associated mucociliary dysfunction, independent of its angiotensin receptor blockade.

Keywords: transforming growth factor β ; angiotensin receptor antagonists; mucus; cilia; human neutrophil elastase

(Received in original form May 14, 2019; accepted in final form October 9, 2019)

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†Deceased.

This work was supported by the NIH (R01 HL133240 to M.S. and R01 GM105817 to F.T.H.), the Cystic Fibrosis Foundation (SALATH14G0 and SALATH16G0 to M.S.), and the Protein and Monoclonal Antibody Production Shared Resource at Baylor College of Medicine with funding from NIH Cancer Center Support Grant P30 CA125123.

Author Contributions: M.D.K., N.B., D.P., F.T.H., J.R.S., W.M.A., and M.S. contributed to the concept and/or design of the study. All authors contributed to the acquisition, critical analysis and interpretation of the data. M.S. drafted the manuscript. All authors critically revised it for intellectual content and approved the final version before submission.

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This article has a related editorial.

This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org.

Am J Respir Crit Care Med Vol 201, Iss 3, pp 313–324, Feb 1, 2020

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Originally Published in Press as DOI: 10.1164/rccm.201905-0990OC on October 15, 2019

Internet address: www.atsjournals.org

At a Glance Commentary

Scientific Knowledge on the

Subject: Despite progress in small molecule therapies, the life span of patients with cystic fibrosis (CF) continues to be shortened by progressive lung disease because of failure to maintain adequate mucociliary clearance. Airway inflammation contributes to mucociliary dysfunction and thus remains an important but unaddressed therapeutic target in CF.

What This Study Adds to the Field:

Angiotensin receptor blockers, including losartan, have antiinflammatory properties. Independent of its receptor blocking activity, losartan rescues CF-related and inflammation-induced mucociliary dysfunction (reduced ciliary beating, increased mucus concentration, abnormal ion conductance, and reduced airway surface liquid) in primary CF airway epithelial cells *in vitro* and in a sheep model of CF-associated slowing of mucus transport *in vivo*. The studies presented here set the stage for clinical trials in CF to test the efficacy of a safe medication as an antiinflammatory therapy.

Despite progress in small molecule therapies, the life span of patients with cystic fibrosis (CF) continues to be shortened by progressive lung disease. Adequate mucociliary clearance is maintained at near-normal levels in young patients and in CFTR (CF transmembrane conductance regulator)-null pigs at birth (1), likely because of an *in utero* adaption of alternative ion channels to compensate for CFTR dysfunction. Airway surface liquid (ASL) volume in human CF airway epithelial cells cultured at the air-liquid interface (ALI) is lower but sufficient to maintain mucociliary clearance (reviewed in Reference 2). The development of mucociliary dysfunction occurs via ASL volume depletion, increased mucus concentration, and mucus adhesion (3) as a direct consequence of airway inflammation, shown in this study as simulated *in vitro* and *in vivo*.

Development of nontoxic antiinflammatory therapy for patients with CF with established lung disease is an important goal. This is especially true because lung function continues to decline, after an initial rise, in modulator-treated patients, albeit at a slower rate.

Neutrophil elastase is found abundantly in CF airways and contributes to airway disease and mucociliary dysfunction (4, 5). Although elastase has several effects on the airway, it increases TGF- β 1 (transforming growth factor β 1) (6). In fact, TGF- β 1 is found to be elevated in CF airways, contributing to airway inflammation (7–10). Consequently, we explored antiinflammatory therapy *in vitro* using TGF- β 1 exposure of fully redifferentiated airway epithelial cells from F508del homozygous patients with CF. We also evaluated these effects *in vivo* using CFTR inhibitors and neutrophil elastase to create CF-like, inflammation-associated mucociliary dysfunction in sheep, expanding on a previously validated nongenetic model of CF (11).

From a biomarker perspective, an increase in the mucus concentration of airway secretions predicts mucociliary dysfunction, with >8% solids revealing mucociliary stasis in CF (12–14), consistent with airway dehydration. Not surprisingly, mucociliary clearance depends on balanced ion fluxes through multiple channels to maintain adequate airway hydration or ASL volume: Na⁺ absorption via epithelial Na⁺ channels, Cl[−] secretion via CFTR, and Ca²⁺-activated Cl[−] channels (in part TMEM16A), as well as K⁺ secretion via large-conductance Ca²⁺-activated and voltage-dependent K⁺ (BK) channels (15–20). Apical secretion of K⁺ provides a driving force for Cl[−], forming a so-called apical loop current that is critically important for ASL availability (21, 22). In CF airway epithelial cells, TGF- β 1 decreases BK activity by reducing availability of the BK γ subunit LRRC26 (leucine rich repeat containing 26), which is required for BK function in nonexcitatory cells (21). Thus, inflammation impairs the backup mechanism for K⁺ and alternative Cl[−] secretion to maintain ASL volume (21).

In this study we tested the antiinflammatory properties of losartan, used clinically as a type 1 angiotensin II receptor blocker (ARB). It is known to inhibit TGF- β signaling, independent of its receptor-blocking action (23–26). In fact, a

recent clinical trial demonstrated losartan's effectiveness in treating Marfan syndrome, in which TGF- β signaling plays a pathophysiologic role (27). Downstream of elastase, TGF- β 1 contributes to CF airway disease and inflammation and is found abundantly in CF airways (4, 5). Therefore, TGF- β 1 was used to simulate inflammation *in vitro*. Losartan was effective *in vitro* and *in vivo* for improving parameters of mucociliary function in inflamed CF airway epithelial cells and sheep airways.

Methods

Human Nasal Samples

Nasal cell scraping data were obtained from a previously published CF cohort (28) and analyzed for a correlation between LRRC26 and CD45, a leukocyte marker (*see online supplement*).

ALI Cultures, Ciliary Beat Frequency, ASL Volume, Ussing Chambers, and Mucus Concentrations

See online supplement.

Animals and Aerosols

Adult ewes were used. All procedures were approved by the Mount Sinai Medical Center animal research committee. Sheep were conscious and intubated during the course of the study. Methods for airway aerosol challenges and tracheal mucus velocity (TMV) have previously been published (29–31). Aerosols were generated by an Airline nebulizer with a dosimetry system. A piston respirator was used to deliver aerosols directly into the trachea during inspiration at a frequency of 20 breaths/min and a V_T of 500 ml. Stock solutions of human neutrophil elastase (HNE; Elastin Product Co.) were diluted on the experimental day in 3 ml of PBS to contain 1,190 mU (low dose) or 2,380 mU (high dose) of enzyme. CFTR_{inh}172 (1 or 10 mg) was dissolved in 0.3 ml DMSO and 2.7 ml ethanol. Also used was 7% hypertonic saline (3 ml). TGF- β 1 and losartan were dissolved in 1% β -cyclodextrin in water (25 μ g and 50 mg/3 ml total solution). The solvents did not influence TMV.

A baseline TMV measurement was obtained, followed by nebulization of specified agents. Subsequent TMV measures were obtained as indicated in the figures.

Statistics

In the graphs, distinct letters above the bars indicate unique group assignments that are significantly different from each other ($P < 0.05$) by the statistical analyses described. All data shown are mean $\pm$ SE. Significance was accepted at $P < 0.05$. All data were analyzed for normality using the Shapiro-Wilk test. If data passed normality testing, multiple groups were analyzed using one-way ANOVA, followed by the Holm-Sidak test. Two groups were compared with a paired or nonpaired t test, as appropriate.

If data did not pass normality testing, multiple groups were analyzed using the Friedman test (repeated measures) or the Kruskal-Wallis test (one-way) followed by the Dunn multiple-comparisons test. Two groups were compared with Wilcoxon or Mann-Whitney tests, as appropriate.

TMV in sheep was analyzed using two-way ANOVA.

Human Study Approval

All normal human bronchial epithelial cells and one set of CF bronchial epithelial (CFBE) cells were isolated from donors whose lungs were found unsuitable for transplantation. Although organ donations were done with appropriately consented donors by the organ procurement agencies, cell and tissue use is not considered human subject research because cells and tissue came from deceased individuals. However, most CFBE cells were obtained from explanted lungs, with appropriately consented patients undergoing lung transplantation. These protocols were approved by the University of Miami's institutional review board (IRB). Human nasal cell scraping was approved by the IRB at UNC Chapel Hill and the IRB of each participating institution where the study was conducted. For all patients, written informed consent was received from participants before inclusion in the study.

Results

Neutrophilic Inflammation Is Associated with LRRC26 Reduction in Nasal Cells from Patients with CF

To assess relevance of our study to human beings, we conducted a secondary analysis of RNA-sequencing data from nasal scrapings of a large cohort of patients with CF (28). There was an inverse relationship between

LRRC26 expression and CD45 presence in nasal scrapings. LRRC26 is the γ subunit required for BK channel function in airway cells. CD45 is a marker of leukocytes and thus indirectly of neutrophil elastase (Figure 1A).

Losartan Rescues TGF- β 1-mediated BK Channel Dysfunction In CF ALI Cultures

These experiments involved basolateral exposure of CFBE cells to TGF- β 1 (10 ng/ml) for 24 hours (Figure 1B). This was associated with reduced LRRC26 expression (Figure 1C), consistent with decreased BK channel activity (Figures 1D and 1E) without an effect on other channels (Figures E1A and E1B in the online supplement). TGF- β 1's effects on BK activity and LRRC26 mRNA were blocked by a TGF receptor blocker, 10 μ M LY2157299 (32), demonstrating a specific TGF- β 1 action (Figures E1C and E1D).

Losartan (10 μ M) ameliorated the effects of TGF- β 1 on BK activity by partially rescuing the expression and function of the BK channel's γ subunit LRRC26 (Figures 1C–1E). Because oral losartan intake results in peak blood levels of ~ 1 μ M, we repeated the experiments with 1 μ M losartan. This concentration was not effective when cells were exposed to 10 ng/ml TGF- β 1, but it rescued mucociliary parameters of cells exposed to 5 ng/ml TGF- β 1 (Figure 1F). The latter TGF- β 1 concentration is still higher than those measured in sheep mucus (*see below*).

Twenty-four hours of losartan exposure did not increase the association of LRRC26 with the pore-forming α subunit of BK (KCNMA1; Figure E2A). However, losartan had a short-term positive effect on BK activity: currents improved by a median of 40% (range, -49% to 381% ; $n = 12$) after 5 minutes of losartan exposure (Figures 1G and 1H).

Losartan Rescues TGF- β 1-mediated ASL Volume Decreases in CF ALI Cultures (Homozygous F508del)

As shown previously by us (21), TGF- β 1 reduced ciliary beat frequency (CBF) secondary to airway dehydration in CF cells (schematic in Figure 2A). ASL volumes (baseline, 57 ± 16 μ L; $n = 47$) and CBF (baseline, 9.3 ± 0.2 Hz; $n = 36$) decreased on 24-hour exposure to TGF- β 1 compared with control (Figures 2B–2E). This was prevented by LY2157299 (Figures E1E and

E1F). Losartan (1 and 10 μ M) partially reversed TGF- β 1-mediated airway dehydration and CBF decreases (Figures 2B–2E).

Losartan Reduces TGF- β 1-induced Mucus Hyperconcentration (Percentage of Solids) in CF Cultures

Mucus was collected with meshes from the surface of ALI cultures, and the percentage of solids was determined as described (33). Treatment of ALI-cultured CF cells with TGF- β 1 basolaterally (10 ng/ml) for 24 hours increased the mucus concentration to levels reported to cause transport stasis (14). This was prevented by losartan (10 μ M) at 24 hours (Figure 2F).

Losartan's Effect on BK Is Independent of Its ARB Ability

EXP3179 (5 μ M), a losartan metabolite without an ARB property (34, 35), rescued TGF- β 1-mediated reductions in BK activity, ASL volume, and CBF after 24 hours in CFBE cells (Figures 3A–3D). In addition, EXP3179 acutely potentiated BK activity in normal human bronchial epithelial cells (Figure 3E), naïve or pre-treated with TGF- β 1 for 24 hours (Figure 3F). Similar to losartan, EXP3179 did not increase association of LRRC26 with the pore-forming α subunit of BK (Figure E2B). Finally, losartan was converted in culture to EXP3179, further supporting the notion that losartan's effect does not involve ARB (Figures E3A and E3B).

Losartan Has Antiinflammatory Properties

TGF- β 1 for 24 hours increased COX-2 (cyclooxygenase 2) mRNA expression in homozygous F508del cells. Both losartan and EXP3179 attenuated the increased expression of COX-2, supporting their antiinflammatory properties in CFBE cells (Figures 4A and 4B). Furthermore, losartan significantly reversed TGF- β 1-induced increases in the basolateral secretion of IL-8 and IL-1 β (Figures 4C and 4D).

On a signaling level, losartan reduced elevated ERK (extracellular signal-regulated kinase) phosphorylation and increased levels of PPAR- γ (peroxisome proliferator-activated receptor γ) compared with TGF- β 1 alone at the 24-hour time point (Figures 4E and 4F).

Figure 1. Neutrophilic inflammation (elastase) reduces LRRC26 (leucine rich repeat containing 26) *in vivo* in patients with cystic fibrosis (CF) and TGF- β 1 (transforming growth factor β 1)-mediated LRRC26 reduction and large-conductance Ca^{2+} -activated and voltage-dependent K^{+} (BK) channel dysfunction are ameliorated by losartan in fully differentiated homozygous F508del cells. (A) Human data ensure relevance of neutrophil elastase to decrease LRRC26 mRNA: correlation between human nasal cell LRRC26 and CD45 (leukocyte marker) expression (RNA sequencing) from a cohort of 134 patients (28). The linear regression line indicates an inverse correlation (red; $P = 0.0002$ and $r^2 = 0.1$). (B) Schematic of experiments in B–F: fully differentiated, homozygous F508del CF bronchial epithelial cells on 12-mm Transwell inserts were exposed for 24 hours to basolateral TGF- β 1 and/or losartan. Expected outcomes are indicated with arrows. (C) Quantitative PCR data of LRRC26 mRNA expression. $n = 7$ from three different CF lungs. One-way ANOVA followed by Holm-Sidak after passing Shapiro-Wilk normality test. (D) BK activity in CF bronchial epithelial cells, represented by the outward short-circuit current (I_{sc}) evoked by 10 μM apical ATP in the presence of 10 μM apical amiloride, is reduced 24 hours after exposure to basolateral TGF- β 1 (10 ng/ml) but partially rescued by treatment with 10 μM losartan. Representative traces are shown. (E) Quantitative analysis of BK channel activity. $n = 10$ experiments from a total of five different CF lungs. Friedman followed by Dunn multiple-comparisons test. (F) Quantitative analysis of BK channel activity relative to control with lower TGF- β 1 and losartan concentrations. $n = 8$ experiments from a total of six different CF lungs. (G) Acute effects of losartan on BK activity: addition of losartan increased BK currents within 5 minutes by a median of 53% (range, 5–380%). $n = 8$ from five normal lungs. (H) Within 30 minutes, TGF- β 1-mediated reduction in BK activity was also acutely enhanced by losartan. $n = 6$ from six normal lungs. Distinct letters above bars indicate groups that are significantly different from each other ($P < 0.05$). All data shown are mean $\pm$ SE. Wilcoxon test was used in E–G. Los = losartan.

Expanded Nongenetic Ovine Model of CF Inflammation-related Mucociliary Dysfunction

We confirmed that ion channels are similar in ovine and human airway epithelia. Nonexpanded, short-term (3 d), polarized, ovine airway epithelial cell cultures revealed functional expression of apical BK channels, CFTR, and Ca^{2+} -activated Cl^{-} channels

(Figure E4). Furthermore, basolateral addition of TGF- β 1 (10 ng/ml) for 24 hours reduced apical potassium currents (Figure E4G) and ovine LRRC26 mRNA (Figure E4H).

For the *in vivo* assessment of TMV, ewes were challenged with an aerosol of the CFTR inhibitor CFTR_{inh}172. TMV decreased in a dose-dependent

manner (Figure 5A), and the effect was sustained for 24 hours when 25 μg TGF- β 1 was added to the aerosol challenge.

To simulate the combination of defective CFTR function and neutrophilic inflammation in CF, a combination challenge of CFTR_{inh}172 plus HNE was used. HNE at the higher of two doses

Figure 2. TGF- β 1 (transforming growth factor β 1)-mediated airway dehydration, ciliary beat frequency (CBF) decrease, and mucus hyperconcentration are ameliorated by losartan in fully differentiated homozygous F508del cells. (A) Schematic of experiments in B–F: cystic fibrosis (CF) bronchial epithelial cells on 12-mm Transwell inserts were exposed for 24 hours to basolateral TGF- β 1 and/or losartan. Expected outcomes are indicated with arrows. (B) Change in airway surface liquid (ASL) volume (Δ ASL volume) estimated by meniscus scanning in CF bronchial epithelial cells. $n = 10$ experiments from a total of four different CF lungs. (C) Lower TGF- β 1 and losartan concentrations result in the same ASL volume outcome. $n \geq 4$ from a total of six different CF lungs. (D) Same experiment as in B but measuring CBF. $n = 22$ measurements from 11 cultures from a total of three CF lungs. (E) Lower TGF- β 1 and losartan concentrations result in the same CBF outcome. $n \geq 9$ from a total of four different CF lungs. (F) Same experiment as in B measuring mucus solids as described in METHODS. $n \geq 5$ experiments from three CF lungs. Distinct letters above bars indicate groups that are significantly different from each other ($P < 0.05$). All data shown are mean $\pm$ SE. One-way ANOVA followed by Holm-Sidak was used after passing Shapiro-Wilk normality test, except for C and E, which were mixed-effect models followed by Holm-Sidak. BK = large-conductance Ca^{2+} -activated and voltage-dependent K^{+} ; Los = losartan.

extended TMV slowing to at least 24 hours when given with CFTR_{inh}172 (Figure 5C). HNE but not CFTR_{inh}172 alone increased TGF- β 1 in airway secretions (Figure 5D), supporting the importance of TGF- β 1. Finally, both aerosolized CFTR_{inh}172 and HNE increased mucus concentrations (Figure 5E).

Losartan and EXP3179 Reverse Ovine TMV Slowdown in Combined Challenge Models

Inhaled losartan reversed the CFTR_{inh}172 plus TGF- β 1-mediated slowing of TMV (Figure 6A).

To simulate more prolonged ovine airway inflammation that is closer to human CF

airway disease, we challenged sheep with CFTR_{inh}172 plus HNE on 3 consecutive days, which led to prolonged TMV reduction lasting at least 48 hours beyond the final challenge (Figure 6B). Inhaled CFTR_{inh}172 plus HNE also increased the concentration of TGF- β 1 in airway secretions (Figure 6C) and mucus concentrations to levels close to

Figure 3. Losartan's effect is not mediated by angiotensin II receptor blocker in fully differentiated homozygous F508del cells. (A) Schematic of experiments in B–F: cystic fibrosis (CF) bronchial epithelial cells on 12-mm Transwell inserts were exposed for 24 hours to basolateral TGF- β 1 (transforming growth factor β 1; 10 ng/ml) and/or EXP3179 (5 μ M). Expected outcomes are indicated with arrows. (B) Quantitative analysis of large-conductance Ca^{2+} -activated and voltage-dependent K^+ (BK) channel activity in CF bronchial epithelial cells. $n=8$ experiments from a total of five different CF lungs. (C) Change in airway surface liquid (ASL) volume (Δ ASL volume) estimated by meniscus scanning depicted as change in volume to control ($\Delta\Delta$ ASL volume). $n=7$ experiments from a total of three different CF lungs. (D) Same experiment as in B but measuring ciliary beat frequency. $n \geq 8$ measurements from three cultures from a total of three CF lungs. (E) Acute effects of EXP3179 (5 μ M) on BK activity: addition of EXP3179 increased BK currents within 30 minutes by a median of 37% (range, 12–700%). $n=6$ from six normal lungs. (F) Within 30 minutes, TGF- β 1-mediated (for 24 h) reduction in BK activity was acutely enhanced by 5 μ M EXP3179. $n=6$ from six normal lungs. Distinct letters above bars in B–F indicate groups that are significantly different from each other ($P < 0.05$). All data shown are mean $\pm$ SE. (B and D) One-way ANOVA followed by Holm-Sidak after passing Shapiro-Wilk normality test. (C, E, and F) Wilcoxon test. CBF = ciliary beat frequency; I_{sc} = short-circuit current.

human CF mucus (Figure 6D). Inhaled losartan partially affected TGF- β 1 but clearly decreased mucus concentration in ovine tracheal secretions (Figures 6C and 6D), possibly indicating that its effect is mostly on TGF- β 1 signaling and not production. Importantly, losartan partially reversed mucociliary dysfunction and did so in a sustained fashion in this model (Figure 6B).

EXP3179 had a similar effect but the duration was limited, possibly because of metabolism (Figure 6E).

Discussion

CF is the most common inherited disease in the white non-Hispanic population, causing

a shortened life span and affecting $\sim 30,000$ people in the United States, with annual healthcare costs of at least \$1.8 billion (36). The median age of predicted survival of these patients has improved to >40 years in the United States; however, patients still die from respiratory failure. Small molecules are being developed that rescue specific CFTR mutants. These agents have produced remarkable improvements in some patients, especially for the G551D mutation with ivacaftor (37). Almost 24 months of treatment with ivacaftor also decreased airway inflammation but did not eliminate it (38, 39). This is important because CF airway inflammation is associated with morbidity (reviewed in Reference 40) and, as shown in this study, with mucociliary dysfunction.

Several large independent clinical studies have demonstrated that TGF- β 1 polymorphisms in CF are associated with worse lung function (8, 41). Not only are BAL fluid levels of TGF- β 1 elevated in patients with CF, they also are associated with diminished lung function and recent hospitalization (42). TGF- β 1 is increased in media of CF cells (our unpublished results; Reference 43). Neutrophil elastase is also found abundantly in CF airways, contributing to airway disease (4, 5) and, as shown in this study, increasing TGF- β 1. *In vitro*, TGF- β 1 downregulates CFTR function in normal airway cells and impedes CFTR-restoring therapies in F508del homozygous cells (9, 10). This issue will not necessarily be resolved with new modulator therapy; when examining the response to triple therapy (two correctors and one potentiator), many patients respond well with respect to lung function and sweat chloride improvements (44). However, a noticeable number of patients still show little response even on this therapy (waterfall plots in online supplement), possibly because of the effects of TGF- β on CFTR mRNA levels (9, 10). TGF- β 1 also decreases airway surface hydration in CF cells by impairing the function of apical BK channels crucial for maintaining ASL volume (21) and in normal cells despite CFTR function (45). Consequently, losartan still may be an important additional therapy after US Food and Drug Administration (FDA) approval of triple therapy.

Although inflammation decreases airway hydration, leading to mucociliary dysfunction, a growing body of literature

Figure 4. Losartan reduces TGF- β 1 (transforming growth factor β 1)-mediated inflammation in fully differentiated homozygous F508del cells. (A and B) Losartan and EXP3179 reduced TGF- β 1-mediated mRNA upregulation of COX-2 (cyclooxygenase 2) 24 hours after addition of basolateral TGF- β 1. Each $n = 6$ from two or more different CF lungs. (C and D) Losartan reduced basolateral accumulation of IL-8 and IL-1 β protein, which are induced by TGF- β 1. (E) Losartan decreases ERK1/2 (extracellular signal-regulated kinase 1 or 2) phosphorylation 24 hours after exposure of cystic fibrosis (CF) cells to TGF- β 1. Plot is normalized to control, originally calculated by ERK1/2 minus phosphorylated ERK1/2. $n = 14$ from three different CF lungs. (F) Losartan rescues total PPAR- γ (peroxisome proliferator-activated receptor γ) that was reduced by TGF- β 1 exposure for 24 hours. $n = 12$ from three different CF lungs. Statistics: distinct letters above bars indicate unique group assignments that are significantly different from each other ($P < 0.05$). All data shown are mean $\pm$ SE. Friedman followed by Dunn multiple-comparisons test was used for A and B, one-way ANOVA followed by Holm-Sidak after passing Shapiro-Wilk normality test was used for C and D, and Kruskal-Wallis followed by Dunn multiple-comparisons test was used for E and F. Los = losartan; pERK1/2 = phosphorylated ERK1/2.

Figure 5. Expanding a validated, nongenetic ovine model of cystic fibrosis (CF)-related mucociliary dysfunction. (A) Ewes inhaled the CFTR (CF transmembrane conductance regulator) inhibitor CFTR_{inh}172 at two different concentrations. The 10-mg dose resulted in mucociliary dysfunction that lasted for the duration of the 8-hour experiment. A lower dose of CFTR_{inh} 172 (1 mg) had no effect. Nebulization of hypertonic saline (HTS) at 4 hours after 10 mg CFTR_{inh}172 (dashed arrow) temporarily rescued mucociliary dysfunction. All $n \geq 3$ experiments in three or more different sheep. (B) While adding 25 μ g TGF- β 1 (transforming growth factor β 1) to the 10-mg CFTR_{inh}172 challenge also resulted in sustained mucociliary dysfunction for 24 hours, sole TGF- β 1 inhalation caused only a 5-hour reduction in transport. All $n = 3$ experiments from three different sheep. (C) Human neutrophil elastase (HNE) was nebulized 2 hours after 10 mg CFTR_{inh}172. Low-dose HNE (1,190 mU) was insufficient to keep transport reduced at 24 hours, but high-dose HNE (2,380 mU) resulted in mucociliary dysfunction that was sustained for 24 hours. All $n \geq 3$ experiments from three or more different sheep. (D) Sheep mucus was collected by direct suctioning in the trachea at the indicated time after challenge. HNE inhalation alone caused a decrease in tracheal mucus velocity, as published previously by us (29; data not shown) and caused acutely increased TGF- β 1 levels. All $n \geq 4$ measurements in four different sheep. (E) Airway secretions were collected for mucus concentration measurements. Both CFTR_{inh}172 and high-dose HNE increased mucus solids. All $n \geq 6$ measurements from four different sheep. Statistics: light gray areas in A–C indicate statistically significant differences of time points compared with baseline, and dark gray areas indicate statistically significant differences of the same time points between different treatments using two-way ANOVA of repeated measures followed by Sidak multiple comparison test. On average, baseline tracheal mucus velocity was 9.8 ± 0.26 mm/min ($n = 15$ sheep). Letters above bars in D and E indicate group assignments of the statistical analysis using one-way ANOVA followed by Holm-Sidak after passing Shapiro-Wilk normality test. TMV = tracheal mucus velocity.

shows that *in vitro* improvements in ASL volumes are often, but not always, predictive of improved mucociliary clearance (37, 46) or lung function in

clinical studies (47–50). These facts suggest that effective and safe antiinflammatory therapy has the potential to improve mucociliary dysfunction in patients with

CF, even in the absence of small molecule therapy. However, currently used chronic antiinflammatory therapies such as high-dose ibuprofen and steroids produce

Figure 6. Losartan ameliorates CFTR (cystic fibrosis transmembrane conductance regulator) inhibitor CFTR_{inh}172 plus TGF-β1 (transforming growth factor β1)-mediated ovine mucociliary dysfunction and long-term CFTR_{inh}172 plus human neutrophil elastase (HNE)-induced ovine mucociliary dysfunction. (A) Losartan (50 mg inhaled) ameliorated CFTR_{inh}172 (10 mg) plus TGF-β1 (25 μg)-mediated reductions in tracheal mucus velocity (TMV) in sheep. All $n \geq 3$ experiments from three different sheep. (B) A 3-day challenge with CFTR_{inh}172 (10 mg) and HNE (2,380 mU) led to prolonged mucociliary dysfunction that lasted at least 48 hours beyond the last challenge. Inhaled losartan (50 mg) partially rescued TMV in a prolonged fashion. All $n = 4$ experiments from four different sheep. (C) TGF-β1 in ovine tracheal secretions increased 4 hours after inhalation of a combination of CFTR_{inh}172 (10 mg) plus HNE (2,380 mU). Losartan attenuated this effect, assessed 4 hours after the first measurements (or 8 hours after initial challenge with CFTR_{inh}172 plus HNE). All $n = 7$ from three different sheep. (D) Mucus concentrations (percentage of solids) increased significantly in ovine secretions 4 hours after inhalation of a combination of CFTR_{inh}172 (10 mg) plus HNE (2,380 mU). Losartan significantly attenuated this effect, measured 4 hours after the first measurements (or 8 hours after initial challenge with CFTR_{inh}172 plus HNE). All $n \geq 21$ from three different sheep. (E) A 3-day challenge with CFTR_{inh}172 (10 mg) and high-dose HNE (2,380 mU) was used. Inhaled EXP3179 (50 mg) temporarily rescued TMV. All $n = 4$ experiments from four different sheep. Light gray areas in A, D, and E indicate statistically significant differences of time points compared with baseline, and dark gray areas indicate statistically significant differences of the same time points between different treatments using two-way ANOVA of repeated measures followed by Sidak multiple comparison test. Average baseline TMV was 9.8 ± 0.26 mm/min. Letters above bars in B and C indicate group assignments of the statistical analysis using Kruskal-Wallis followed by Dunn multiple-comparisons test.

unwanted side effects that negate their effectiveness in adults. Furthermore, a 4-day steroid regimen in sheep failed to correct HNE-induced slowing of TMV (51). Additional medications failed in clinical trials (eg, GSK SB-656,933), and several others are still in development.

Evidence from the cardiovascular literature supports the antiinflammatory properties of losartan, which is well tolerated and FDA approved for oral intake. Losartan is mainly a prodrug with biological activity mediated by two metabolites. One, EXP3174, has 100-fold higher ARB potency than losartan (52). The other, EXP3179, is not an ARB (34, 35) but rather an antiinflammatory molecule. The exact mechanism by which losartan blocks TGF- β signaling is unknown, although as shown in this study, activation of PPAR- γ might be involved (53–55).

Our *in vitro* and *in vivo* experiments show that losartan improves airway hydration in inflammatory conditions in CF cells *in vitro* and that these are paralleled by improvements in TMVs measured in sheep *in vivo*. Losartan has a benign side-effect profile and an excellent safety track record, even in

nonhypertensive patients. Its use as an antiinflammatory agent in CF could provide a rapid and safe way of improving overall mucociliary clearance in CF airways, not only in patients with CF currently ineligible for CFTR recovery therapy but also in patients with CF treated with new small molecule therapies.

For *in vivo* studies, we used an expanded large-animal model of CF-like, inflammation-associated mucociliary dysfunction in sheep, using a nongenomic approach. Although there are multiple genetic animal models of CF (reviewed in Reference 56), these models do not easily lend themselves for multiple mucociliary function assessments with an array of interventions. The nongenetic ovine model recapitulates several important aspects of CF-related human airway disease: 1) mucociliary dysfunction is caused by a block of CFTR function (and pharmacologic CFTR inhibition was validated in ovine cells); 2) mucus concentration on pharmacologic challenge is increased similar to CF; 3) TGF- β -related inflammation is induced with CFTR inhibition plus neutrophil elastase, the latter commonly found in inflamed CF

airways; 4) mucociliary dysfunction is maintained beyond the immediate pharmacologic challenges; and 5) pharmacologic challenges recapitulate ion channel dysfunctions typically seen in CF. We acknowledge that this is a model, but ultimate proof of efficacy no matter what model is used will be settled in human studies.

This model system also lends itself to test multiple interventions measuring multiple outcomes including mucus velocity, mucus concentration, and airway inflammation. Although recent clinical trials, especially with epithelial Na⁺ channel inhibitors, did not seem to translate their effectiveness from the sheep model, there are two important considerations: in some of these trials, the effective sheep dosing was not applied to humans and the lower human dosing regimen failed (H. Danahay, Ph.D., oral communication, June 25, 2019), and the sheep model used was less rigorously inducing inflammation in the airways.

In the ovine model, losartan reversed low mucus transport rates rapidly and in a sustained manner. Initially, the rapid reversal was puzzling. However, we found that losartan potentiated BK activity, as

Figure 7. Schematic of the effects of neutrophil elastase, TGF- β 1 (transforming growth factor β 1), and losartan on mucociliary function in cystic fibrosis and how losartan can block these detrimental pathways to restore mucociliary function. Neutrophil elastase, abundantly present in cystic fibrosis airways, stimulates TGF- β 1 production (left). TGF- β 1 blocks PPAR- γ (peroxisome proliferator-activated receptor γ) signaling, which results in downregulation of LRRC26 (leucine rich repeat containing 26 or γ subunit necessary for large-conductance Ca²⁺-activated and voltage-dependent K⁺ [BK] function in nonexcitable cells) and thus dysfunction of the BK channel, necessary to maintain airway hydration and mucociliary clearance. Calcium-activated chloride channels cannot effectively secrete chloride because of the lack of potassium in the airway surface liquid. Losartan blocks TGF- β 1 signaling (right), restoring PPAR- γ and LRRC26, allowing BK channels to function with restoration of mucociliary clearance. Losartan also has an unknown but direct and positive effect on BK channels. ASL = airway surface liquid; CaCC = Ca²⁺-activated Cl[−] channel; CBF = ciliary beat frequency; CFTR = cystic fibrosis transmembrane conductance regulator.

previously described in smooth muscle (57). There are data implicating the close association of BK and stimulated AT1R (angiotensin II type 1 receptor) that is lost when receptors are blocked, resulting in a 40% increase in BK function (58). However, AT1R is not expressed in airway epithelia, and EXP3179 does not block the receptor. Consequently, another mechanism must be responsible. Further investigations into this area will be needed. We deduce that the rapid response in mucus clearance seems to rely on BK potentiation, whereas the more long-term response relies on replenishing cellular LRRC26. The importance of the latter effect is highlighted by the inverse correlation between LRRC26 expression in

human nasal cells from patients with CF and CD45 in nasal scrapings, indicating a loss of LRRC26 with inflammation. The failure of EXP3179 to reverse mucociliary dysfunction for more than 4 hours could be attributed to metabolism.

Conclusions

Losartan is an effective antiinflammatory molecule that improves multiple aspects of CF- and inflammation-related mucociliary dysfunction in human CF cells *in vitro* and in an ovine model of CF-like airway disease *in vivo* (Figure 7). Based on these findings, we started clinical trials to test the effect of oral losartan in patients with CF (NCT03206788 and NCT03435939). ■

Author disclosures are available with the text of this article at www.atsjournals.org.

Acknowledgment: The authors acknowledge Michael Myerburg for kindly providing the airway surface liquid volume-measurement, meniscus-scanning software; Brian Button for helping to establish the percentage mucus solid measurements; and Siddharth Rawal and Ashutosh Agarwal for their help using an Epilog Mini 30W laser to cut precise tissue meshes for lifting mucus off the cultures. The authors also thank LifeCenter Northwest (Washington), the Life Alliance Organ Recovery Agency (Florida), and the Midwest Transplant Network (Kansas) for their assistance with organ procurement and patients with cystic fibrosis who donated their lungs and nasal cells for research.

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