Reduced miR-146a Increases Prostaglandin E₂ in Chronic Obstructive Pulmonary Disease Fibroblasts

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Rationale: Persistent inflammation plays a major role in chronic obstructive pulmonary disease (COPD) pathogenesis, but its mechanisms are incompletely defined. Overproduction of the inflammatory mediator prostaglandin (PG) E₂ by COPD fibroblasts contributes to reduced repair function.

Objectives: The present study determined if fibroblasts from subjects with COPD overproduce PGE₂ after stimulation with the inflammatory cytokines IL-1 β and tumor necrosis factor- α , and further defined the mechanism for overproduction.

Methods: Fibroblasts were isolated from parenchymal tissue obtained from smokers with and without COPD undergoing lung surgery. PGE₂, cyclooxygenases (COX), and miR-146a in these cells were evaluated by *in vitro* studies.

Measurements and Main Results: After stimulation with inflammatory cytokines, COPD fibroblasts produced 2.7-fold more PGE₂ compared with controls with similar smoking history. The increase in PGE₂ depended on induction of COX-2, which increased to a greater degree in fibroblasts from subjects with COPD. Cytokines also induced microRNA miR-146a expression in both fibroblasts, but significantly less in COPD fibroblasts. miR-146a caused degradation of COX-2 mRNA; reduced expression prolonged COX-2 mRNA half-life in fibroblasts from subjects with COPD. Cytokine-stimulated PGE₂ production and miR-146a expression in cultured fibroblasts correlated with clinical severity assessed by expiratory airflow and diffusion capacity.

Conclusions: miR-146a seems to play a pathogenetic role in the abnormal inflammatory response in COPD. Increased half-life of inflammatory mRNAs is a mechanism of abnormal inflammation in this disease.

Keywords: chronic obstructive pulmonary disease; miR-146a; prostaglandin E₂; cyclooxygenase-2; fibroblasts

Chronic obstructive pulmonary disease (COPD), currently the fourth leading cause of death in the United States, will become the third leading cause by 2020 in the United States and globally (1). Cigarette smoking is the most important cause, and smoking cessation early in the course of the disease can slow

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Reduced expression of miR-146a is a pathogenetic mechanism that contributes to the abnormal inflammatory response in chronic obstructive pulmonary disease.

What This Study Adds to the Field

The reduced expression of miR-146a is a novel pathogenetic mechanism and suggests a new therapeutic target that can control the abnormal inflammatory response in chronic obstructive pulmonary disease.

the rate at which lung function is lost (2). An abnormal inflammatory response of the lung, which persists despite cessation of smoking (3–6), is characteristic of COPD. This inflammation is believed to play a major role in the pathogenesis and progression of COPD (1, 7). Although this inflammation is believed to be “abnormal” (1), there is little information on how inflammation in COPD differs from normal.

The tissue alterations that underlie COPD result not only from tissue damage, but also from the inability of repair responses to restore tissue structure (1, 7, 8). In this context, cigarette smoke directly inhibits repair processes (9–12). Inflammatory mediators also modulate repair. Prostaglandin (PG) E₂, an inflammatory mediator increased in the lungs of patients with COPD, is overproduced by COPD fibroblasts under basal culture conditions (13, 14). PGE₂ is also a potent inhibitor of lung fibroblast repair functions (15–19). COPD fibroblasts cultured *ex vivo* are deficient in a number of repair functions in part because of overproduction of PGE₂ by the cells under “baseline” culture conditions. However, the release of PGE₂ increases greatly in response to a variety of inflammatory stimuli including IL-1 β and tumor necrosis factor (TNF)- α , which are believed to play key roles in COPD. The current study, therefore, was designed to determine if COPD fibroblasts also overproduce PGE₂ in response to inflammatory mediators.

We obtained lung tissue from smokers who did and did not have concurrent COPD who were undergoing surgery, generally for suspected lung cancer. Fibroblasts were cultured from cancer-free areas of the lung. This allowed us to compare cells from patients with COPD to a similarly aged group with similar smoking history and clinical diagnosis.

MicroRNAs (miRNAs) are a family of small, approximately 19- to 25-nucleotide, noncoding RNAs. They are transcribed from specific genes and generally undergo two cleavage steps that result in mature miRNAs. The mature miRNAs cause post-transcriptional gene repression by increasing mRNA degradation or by inhibiting translation (20). In the current study, we demonstrated that fibroblasts from patients with COPD pro-

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duce increased amounts of PGE₂ in response to the cytokines and that this is caused by the underexpression of miR-146a that, in turn, leads to overexpression of cyclooxygenase (COX)-2 and production of PGE₂. Importantly, the level of expression of miR-146a correlated with both PGE₂ production and two measures of COPD clinical severity. This represents a novel mechanism accounting for the altered inflammatory response that characterizes COPD.

METHODS

Human Subject Samples

Primary lung fibroblasts from 14 subjects with mild to very severe COPD and 16 subjects without clinical or functional signs of COPD (controls) were included (Table 1). All subjects were undergoing surgery for lung tumor resection except for two of the subjects with COPD undergoing volume reduction surgery. Acquisition of samples was approved by the Human Studies Committee of the Medical Board of the State of Schleswig-Holstein, and all subjects provided written informed consent for research.

Human lung fibroblasts were cultured as described (14, 21) from normal-appearing areas of the pulmonary parenchyma in a region as far as possible from the tumor that was free of pleura or large airways.

Measurement of PGE₂

PGE₂ production from lung fibroblasts was determined by enzyme immunoassay (Cayman Chemical, Ann Arbor, MI) following the manufacturer's instructions.

Western Blot Analysis

Western blot analysis was performed as described (14).

COX Activity Assay

COX activity was determined using a commercially available kit (Cayman) following the manufacturer's instructions.

COX Silencing

Silencing of COX-1 and COX-2 by small interfering RNA (siRNA) was performed as described (22).

Real-Time Polymerase Chain Reaction

Total RNA was isolated from cells by using Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA) and real-time polymerase chain reaction (PCR) was performed as described (23).

mRNA Stability Assay

Measurement of mRNA stability was accomplished as described (24, 25).

miRNA Preparation and Microarray Assay

Cells were pretreated either in the presence or absence of IL-1 β and TNF- α . miRNAs were extracted and profiled as described (26).

Northern Blot Analysis

Northern blot analysis for the detection of miR-146a by DIG-labeled LNA probe (Exiqon, Vedbaek, Denmark) was performed as described with modifications (27).

Transfection of miRNA Mimic or Inhibitor

Cells were plated without antibiotic and antifungal agents and cultured. At 50% confluence, cells were treated with IL-1 β and TNF- α for 24 hours and then transfected with miR-146a mimic, inhibitor, or negative control miRNA (final concentration = 50 nM each; Dharmacon, Lafayette, CO). After 6-hour transfection, cells were further cultured for 24 hours before assay.

TABLE 1. CLINICAL FEATURES OF SUBJECTS

	Control	COPD	P Value
n of subjects	16	14	
Age (yr)	63.8 $\pm$ 2.2	60.2 $\pm$ 2.3	0.27
Gender (M/F)	13/3	8/6	0.27
Histology of lung tumor			
Adenocarcinoma	7	7	
Squamous cell carcinoma	6	4	
Large cell carcinoma	2	1	
Carcinoid	1	0	
No tumor*	0	2	
Height, cm	169.6 $\pm$ 2.0	173.3 $\pm$ 2.5	0.29
Weight, kg	71.1 $\pm$ 3.3	71.6 $\pm$ 4.1	0.57
Smoking (pack-years)	43.2 $\pm$ 7.6	43.7 $\pm$ 4.3	0.63
Current smoker†	6	6	0.82
GOLD stage (I/ II/ III/ IV)	0/ 0/ 0/ 0	1/ 6/ 5/ 2	
Pre-medications			
LAMA	0	7	
LABA	0	7	
ICS	0	8	
Oral steroids	0	5	
Theophylline	0	2	
Statins	4	3	
VC (% of predicted value)	103.9 $\pm$ 2.6	90.1 $\pm$ 4.8	0.02
FEV ₁ (% of predicted value)	93.5 $\pm$ 2.6	49.2 $\pm$ 4.6	<0.0001
DL _{CO} (% of predicted value)‡	86.6 $\pm$ 3.4	63.1 $\pm$ 5.2	0.003

Definition of Abbreviations: COPD = chronic obstructive pulmonary disease; DL_{CO} = diffusion capacity for carbon monoxide; GOLD = Global Initiative for Chronic Obstructive Lung Disease; ICS = inhaled corticosteroids; LABA = long acting β -agonist; LAMA = long-acting muscarinic antagonist.

Values are mean $\pm$ SEM, except for sex, histology of lung tumor, current smoker, GOLD stage, and premedications (number).

* These subjects were undergoing volume reduction surgery.

† Current smoker defined as someone who has smoked 100 cigarettes in his or her lifetime and who currently smokes cigarettes.

‡ DL_{CO} values are from 13 subjects of each group.

Luciferase Reporter Assay

A luciferase (LUC) reporter construct containing 3' untranslated region (UTR) of COX-2 mRNA (GeneCopoeia, Germantown, MD) was transformed following the manufacturer's instructions. LUC reporter assay was performed as described (28).

Statistical Analysis

Data are expressed as mean $\pm$ SEM. All data were analyzed using the GraphPad Prism 4 (GraphPad Software, San Diego, CA). Analysis of variance was performed with the use of the nonparametric Kruskal-Wallis test. When applicable, the Mann-Whitney *U* test was used for comparisons between groups. Correlation coefficients were calculated with the use of Spearman rank method. For the primary outcomes including PGE₂ production, COX expression, and miR-146a expression, all 30 subjects were evaluated. For the mechanistic studies, cells cultured from selected subjects were evaluated. In these cases, each separate experiment, which included internal replicates, counted as a test. A *P* value of less than 0.05 was considered significant.

Additional details of all methods are available in the online supplement.

RESULTS

Clinical Features of Subjects

The clinical features of subjects without COPD (control) and subjects with COPD are presented in Table 1. These two groups were closely matched for age and smoking status. Using the Global Initiative for Chronic Obstructive Lung Disease classification based on FEV₁, subjects with COPD were classified as stage I to IV (mild to very severe COPD). All controls lacked the criteria for COPD. Subjects with COPD showed the expected physiologic alterations including significantly lower FEV₁ and lower diffusion capacity for carbon monoxide

(DL_{CO}). Also as expected, subjects with COPD received more medications than did controls. All control subjects and 12 of 14 subjects with COPD had lung tumors. Two subjects with COPD were operated on for volume reduction.

Effect of IL-1 β and TNF- α on PGE₂ Production in COPD and Control Fibroblasts

We first sought to evaluate PGE₂ production by lung fibroblasts from COPD and control subjects in response to IL-1 β and TNF- α . These cytokines are known to stimulate PGE₂ release synergistically in lung fibroblasts (29). We first confirmed that IL-1 β stimulated PGE₂ production in control and COPD fibroblasts and that the combination of IL-1 β and TNF- α stimulated more than the individual cytokines (*see* Figure E1A). In the absence of IL-1 β and TNF- α (baseline condition), compared with control fibroblasts, COPD fibroblasts produced 1.6-fold more PGE₂ ($P = 0.07$) (Figure 1A). Exogenous stimulation with IL-1 β and TNF- α stimulated the production of PGE₂ from fibroblasts from both controls and subjects with COPD ($P < 0.0001$). After exogenous stimulation, COPD fibroblasts produced 2.7-fold more PGE₂ than did control fibroblasts ($P < 0.0001$) and the magnitude of the stimulation in the COPD fibroblasts was significantly greater, increasing approximately 63-fold compared with the 29-fold increase in control cells ($P = 0.02$).

We next evaluated the relationship between baseline and stimulated PGE₂ production and two physiologic measures that

characterize the severity of COPD and, in particular, emphysema: FEV₁ and DL_{CO} . The relationship between baseline PGE₂ levels and both FEV₁ and DL_{CO} did not achieve statistical significance (Figure 1B). In contrast, there were significant correlations between PGE₂ production after IL-1 β and TNF- α stimulation and these physiologic measures (Figure 1C).

To explore the mechanism for PGE₂ overproduction in COPD fibroblasts, we first defined the concentration dependence and time-course for PGE₂ production in response to IL-1 β and TNF- α stimulation in selected cell strains. Cytokines stimulated PGE₂ production from both COPD and control fibroblasts in a concentration-dependent manner with a similar 50% effective dose, although the amount of PGE₂ produced differed significantly (2.10 vs. 1.63 ng/ml) (*see* Figure E1B). The time-course of PGE₂ production was also similar and showed that PGE₂ levels from both COPD and control fibroblasts started to increase by 4 hours and reached a maximum around 24 hours after treatment (*see* Figure E1C).

Role of COX-2 in IL-1 β - and TNF- α -induced PGE₂ Production

Because IL-1 β and TNF- α induced PGE₂ has been reported to induce increased COX-2 expression, but not COX-1 (30, 31), we evaluated COX-1 and COX-2 protein and mRNA expression. In the absence of stimulation, both protein and mRNA expression of COX-1 but not COX-2 were clearly demonstrated (Figures 2A and 2B). After 24 hours of cytokine stimulation, COX-2 protein and mRNA expression in both COPD and

Figure 1. Effect of IL-1 β and tumor necrosis factor (TNF)- α on prostaglandin (PG) E₂ production and correlations between PGE₂ production and lung function in chronic obstructive pulmonary disease (COPD) and control fibroblasts. (A) PGE₂ production by 14 COPD and 16 control fibroblasts was measured over 24 hours. Each dot represents a separate subject. The bars show the mean values. Note that the vertical axis, which shows PGE₂ production, is a log scale. * $P < 0.0001$. (B) Correlation of baseline (without cytokine treatment) PGE₂ level with FEV₁ and diffusing capacity of carbon monoxide (DL_{CO}). (C) Correlation of IL-1 β /TNF- α stimulated PGE₂ level with FEV₁ and DL_{CO} . Subjects with COPD are indicated by the dark squares, and control subjects by the open squares. A solid line shows the regression line. FEV₁ values are from all 30 subjects and DL_{CO} were from 13 subjects (all available) of each group.

Figure 2. Effect of IL-1 β and tumor necrosis factor (TNF)- α on cyclooxygenase (COX) protein and mRNA expression in chronic obstructive pulmonary disease (COPD) and control fibroblasts. (A) COX-1 protein and mRNA expression. (B) COX-2 protein and mRNA expression. Fourteen COPD and 16 control fibroblasts were stimulated with or without cytokines (2 ng/ml each) for 24 hours. Protein expression assessed by Western blot analysis is expressed relative amount to β -actin. COX mRNA expression quantified by real-time polymerase chain reaction is normalized to the amount of rRNA and expressed as $2^{-\Delta\Delta C_T}$ values. Each dot represents a separate subject. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.0001$.

control fibroblasts increased to a great degree, whereas there was no significant change in COX-1 expression. Moreover, COPD fibroblasts showed higher protein and mRNA levels of COX-2 compared with controls after IL-1 β and TNF- α stimulation ($P = 0.005$ and 0.02 , respectively), whereas COX-1 expression in COPD fibroblasts did not differ from control.

We next confirmed the functional role of COX-2 in IL-1 β and TNF- α induced PGE₂ production. First, we assessed the COX enzyme activity. After stimulation with cytokines, there was an increase in total COX activity that was greater in COPD fibroblasts and was entirely accounted for by increased COX-2 activity (Figure 3A). We further demonstrated that pharmacologic inhibition with a COX-2 selective inhibitor, NS-398, completely blocked IL-1 β - and TNF- α -induced PGE₂ production. In contrast, use of a COX-1 selective inhibitor, SC-560, had no effect on cytokine-induced PGE₂ production in control fibroblasts, but inhibited PGE₂ release in COPD cells by about 50% (Figure 3B). Use of an siRNA to suppress COX-2 mRNA also resulted in complete inhibition of IL-1 β - and TNF- α -induced PGE₂ production, whereas a COX-1 targeting siRNA had no effect (Figure 3C).

To evaluate the mechanism for the increased expression of COX-2 mRNA and protein, we first evaluated the time-course of the protein and mRNA response in selected strains. After stimulation with cytokines, there was no consistent change in COX-1 protein expression (*see* Figure E2). In contrast, COX-2 protein expression increased in a time- dependent manner and reached a maximum after 24 hours; the increase was approximately 52-fold in the control and 82-fold in the COPD strains tested ($P = 0.04$). Over the next 24 hours, the levels decreased approximately 54% in the control and 18% in the subjects with COPD ($P = 0.02$). Similarly, after cytokine stimulation, there was a time- dependent increase in COX-2 mRNA in both COPD and control fibroblasts (Figure 3D). The increase in COPD cells was of greater magnitude. The peak mRNA level was observed after 8 hours for the control and after 12 hours in the COPD cells. Moreover, the mRNA levels seemed to be more persistent in COPD fibroblasts, decreasing by 57% in COPD fibroblasts after 48 hours compared with an 80%

decrease in the control fibroblasts ($P = 0.01$). Because this persistence might reflect a difference in mRNA stability, we next explicitly evaluated COX-2 mRNA stability in selected IL-1 β and TNF- α treated COPD and control fibroblasts. The half-life of COX-2 mRNA was significantly longer in COPD fibroblasts compared with controls (11.9 vs. 4.61 h; $P = 0.009$) (Figure 3E).

Because several miRNAs have been suggested to contribute to COX-2 gene regulation and could account for a difference in COX-2 mRNA stability (28, 32, 33), we suspected that altered miRNA expression may contribute to altered COX-2 regulation and PGE₂ overproduction in COPD fibroblasts after stimulation with IL-1 β and TNF- α . Therefore, we used a microarray-based approach to assess miRNA expression with and without cytokine stimulation using two selected strains each of COPD and control fibroblasts. Two potential miRNA candidates were underexpressed in COPD fibroblasts compared with control after cytokine stimulation, miR-146a and miR-138 (*see* Figure E3A), although neither had been previously described to regulate COX-2. In contrast, the miRNAs that were previously reported to regulate COX-2 were expressed to the same extent in COPD and control fibroblasts and were not altered in expression by cytokine stimulation. Moreover, miR-138 expression was not affected by cytokine stimulation through the current microarray analyses (*see* Figure E3B). Nevertheless, next, we evaluated candidate miRNA target sites in the COX-2 3' UTR by bioinformatic analysis using online databases (www.targetscan.org; www.mirbase.org). MiR-146a was found to have a homology with the COX-2 3' UTR (*see* Figure E3C), whereas miR-138 had no homology revealed. Based on the microarray and *in silico* assessments, we focused on miR-146a for further analysis.

We first quantified miR-146a expression both at baseline and after IL-1 β and TNF- α stimulation in all 30 strains of COPD and control fibroblasts by real-time PCR. Consistent with a previous report (34), IL-1 β and TNF- α induced the expression of miR-146a. Although there was overlap among the miR-146a expression levels between COPD and control fibroblasts, the magnitude of the induction was significantly less in the COPD

Figure 3. Role of cyclooxygenase (COX) in IL-1 β and tumor necrosis factor (TNF)- α induced prostaglandin (PG) E₂ production by chronic obstructive pulmonary disease (COPD) and control fibroblasts. (A) COX enzyme activity after IL-1 β /TNF- α stimulation. Total COX (open bars) and COX-2 (dark bars) activity was determined by COX activity assay. + $P < 0.05$ and ++ $P < 0.001$, compared with control group. (B) Effect of pharmacologic inhibitors of COX on IL-1 β /TNF- α induced PGE₂ production. Cells were treated with an inhibitor of COX-1 (SC-560: 10^{-7} M) or COX-2 (NS-398: 10^{-6} M) for 1 hour and then stimulated with cytokines (2 ng/ml each) for 24 hours. (C) Effect of COX silencing on IL-1 β /TNF- α induced PGE₂ production. To inhibit specific cyclooxygenases, cells were transfected with 100 nM of COX-1, COX-2, or nontarget siRNA for 6 hours after stimulation with cytokines. (D) IL-1 β /TNF- α induction of COX-2 mRNA level, time-course. COX-2 mRNA expression expressed as fold change compared with the time 0 level of control fibroblasts. Note that the vertical axis is a log scale. (E) Stability of COX-2 mRNA. Cells were stimulated with cytokines for 24 hours, after which stimuli were removed and actinomycin D (2 μ g/ml) was added. COX-2 mRNA is expressed as a percentage of mRNA level at the time of adding actinomycin D. Four strains each of COPD and control cells were evaluated on two separate occasions. Values are mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Comparison for D and E = COPD vs. control at indicated time point.

fibroblasts than in the control cells ($P = 0.008$) (Figure 4A). Importantly, the expression of miR-146a assessed showed significant negative correlation with PGE₂ production following cytokine stimulation (Figure 4B). Expression of miR-146a after cytokine stimulation was also significantly correlated with COPD severity assessed by FEV₁ and DLCO (Figures 4C and 4D). Because real-time PCR can detect both mature and precursor forms of miRNAs, we confirmed reduced expression of the mature form by Northern blot analysis. In all six strains of control and COPD fibroblasts assessed, IL-1 β and TNF- α induced the expression of both precursor and mature forms of miR-146a (see Figure E4A). Densitometric quantification confirmed reduced expression of mature miR-146a in COPD cells and reduced conversion of pre-miR-146a to mature miR-146a

in COPD fibroblasts compared with control cells (3.42 vs. 5.09; $P = 0.04$) (see Figure E4B).

Role for miR-146a in Modulation of COX-2 and PGE₂ Production in COPD Fibroblasts

To define a mechanistic role for miR-146a in modulation of COX-2 and PGE₂ production, we transfected COPD and control fibroblasts with miR-146a mimics or inhibitors. After stimulation with IL-1 β and TNF- α , an miR-146a mimic reduced COX-2 mRNA levels when transfected into both COPD and control fibroblasts and an miR-146a inhibitor had the opposite effect (Figure 5A). Moreover, we confirmed this mechanism of action by demonstrating that the miR-146a mimic reduced the prolonged COX-2 mRNA half-life present in cytokine-treated

Figure 4. miR-146a expression after IL-1 β and tumor necrosis factor (TNF)- α stimulation by chronic obstructive pulmonary disease (COPD) and control fibroblasts and *in vivo* correlation. (A) Effect of IL-1 β /TNF- α on miR-146a expression by real time polymerase chain reaction. Fourteen COPD and 16 control cell strains were stimulated with or without cytokines (2 ng/ml each) for 24 hours. miR-146a expression is normalized to the amount of rRNA and expressed as $2^{-\Delta\Delta C_T}$ values. Each dot represents a separate subject. * $P < 0.01$, ** $P < 0.0001$. (B) Correlation between miR-146a expression and prostaglandin (PG) E₂ production after IL-1 β /TNF- α stimulation. (C) Correlation between miR-146a expression after IL-1 β /TNF- α stimulation and FEV₁. (D) Correlation between miR-146a expression after IL-1 β /TNF- α stimulation and diffusing capacity of carbon monoxide (DLCO). Subjects with COPD are indicated by the dark squares, and control subjects by the open squares. A solid line shows the regression line. FEV₁ values are from all 30 subjects and diffusing capacity of carbon monoxide (all available) were from 13 subjects of each group.

COPD fibroblasts (Figure 5B) and that the miR-146a inhibitor enhanced the stability of COX-2 mRNA (Figure 5C). We then demonstrated binding of miR-146a to COX-2 3' UTR using a LUC reporter assay. The LUC activity significantly decreased only after the cotransfection with both the LUC reporter construct containing COX-2 mRNA 3' UTR and miR-146a mimic (Figure 5D), and this effect was clearly observed in both COPD and control cells. Finally, the functional role of miR-146a in COPD fibroblasts was further established by demonstrating significantly reduced COX-2 protein expression ($P = 0.02$) (Figure 5E) and PGE₂ production ($P = 0.0003$) (Figure 5F) after transfection of the miR-146a mimic into cytokine-treated COPD fibroblasts.

DISCUSSION

The present study demonstrates that, after stimulation with IL-1 β and TNF- α , fibroblasts from subjects with COPD produce more PGE₂ than do fibroblasts from control subjects. This increased production of PGE₂ was caused by an increase in the expression of COX-2 and was associated with increased expression of COX-2 mRNA. The increased COX-2 mRNA expression was caused, at least in part, by prolonged COX-2 mRNA half-life. IL-1 β and TNF- α stimulation also induced the expression of miR-146a in both COPD and control fibroblasts, but the induction was less in COPD fibroblasts. Because miR-146a has homology with the 3' UTR of COX-2 mRNA, this suggests a mechanism whereby reduced expression of miR-146a in COPD fibroblasts leads to reduced feedback degradation of

COX-2 mRNA and subsequent prolonged half-life and overexpression of COX-2 and consequent overproduction of PGE₂. This mechanism was confirmed by demonstrating that transfection with an miR-146a mimic reduced COX-2 mRNA stability, COX-2 protein expression, and PGE₂ production in COPD fibroblasts. Conversely, an miR-146a inhibitor prolonged the stability of COX-2 mRNA in control fibroblasts. The functional role of miR-146a in modulating COX-2 was further confirmed by a demonstration of binding of miR-146a to the 3' UTR of COX-2 mRNA. Finally, the statistically significant correlations between miR-146a expression and PGE₂ production after cytokine stimulation and functional measures of COPD severity suggests that miR-146a expression could play a key mechanistic role in the pathogenesis of COPD.

The COPD and control subjects from whom samples were obtained had similar smoking histories and were of similar ages. As a result, it is unlikely that cigarette smoking or age accounts for the differences observed. Similarly, the control subjects and all but two of the subjects with COPD were operated on for suspected lung cancer, suggesting that underlying diagnosis was not a reason for the differences observed. Although every effort was made to culture cells from tissue free of involvement with cancer, we cannot exclude the possibility that our observations are specific for the population of subjects with COPD and cancer. The subjects with COPD were taking more medications. Thus, we cannot exclude an effect of the COPD medications causing the differences observed. However, because these differences persisted with passage in culture, it is unlikely that the differences are caused by concurrent exposure to COPD-related medications.

Figure 5. miR-146a regulates cyclooxygenase (COX)-2 expression and prostaglandin (PG) E₂ production by a direct binding to 3' untranslated region (UTR) of COX-2 mRNA. (A) Effect of miR-146a mimic or inhibitor on COX-2 mRNA expression. COX-2 mRNA expression represented as a percentage of control (indicated as a black bar). (B) Effect of miR-146a mimic on COX-2 mRNA stability. (C) Effect of miR-146a inhibitor on COX-2 mRNA stability. COX-2 mRNA was assessed after 6 hours treatment of actinomycin D (2 μ g/ml) after IL-1 β and TNF- α stimulation and is expressed as a percentage of mRNA level at the time of adding actinomycin D. (D) Direct binding of miR-146a to COX-2 3'UTR-LUC construct. Cells were cotransfected with a COX-2 3' UTR-LUC construct, control vector, miR-146a mimic, miR-146a inhibitor, and control microRNA. Cell layers were harvested 48 hours after transfection, and luciferase (LUC) activities were determined by dual LUC assay. Data are normalized to *Renilla* LUC activity and expressed as a relative value to control (indicated as a black bar). (E) Modulation of COX-2 protein in cytokine-treated chronic obstructive pulmonary disease (COPD) fibroblasts by miR-146a mimic. The inset shows an example of the Western blot for COX-2 with and without exogenous miR-146a mimic for a single cell strain. Densitometric quantification of COX-2 is expressed relative to control after normalization to β -actin. The statistics were calculated from two strains of cells each evaluated on three separate occasions. (F) Effect of miR-146a mimic on PGE₂ production by cytokine-treated COPD fibroblasts. Two strains each of COPD or control cells were evaluated on three separate occasions in all experiments. Values are mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

In a prior study, our group demonstrated that fibroblasts from patients with COPD produce more PGE₂ than do fibroblasts from control subjects (14). The current study is consistent with this prior study, demonstrating a trend toward increased production of PGE₂ under baseline conditions. Importantly, the current study extends our prior results by assessing PGE₂ production after stimulation with the inflammatory cytokines, IL-1 β and TNF- α . PGE₂ is produced not only by cells under baseline conditions, but, perhaps more importantly, in response to stimulation by proinflammatory mediators. IL-1 β and TNF- α were used in the current study because they are prominent inflammatory mediators believed to play a pathogenic role in COPD, and they are known to stimulate potently, and potentially synergistically, the production of PGE₂ by fibroblasts (29). The current study, therefore, extends prior results by demonstrating that PGE₂ production is increased in COPD fibroblasts after exogenous stimulation. The magnitude of the stimulation in COPD fibroblasts also showed a greater relative increase, despite the COPD cells having a greater baseline PGE₂ production.

The current study also suggests that increased PGE₂ production in response to IL-1 β and TNF- α likely results from different mechanisms that account for increased baseline production. Specifically, after cytokine stimulation, there was an increase in the expression of COX-2 in both COPD and control fibroblasts, but the increase was much greater in the COPD cells. This contrasts with the lack of change in COX-1 expression after stimulation with IL-1 β and TNF- α . Measurement of COX enzymatic activity demonstrated a trend toward an increase in basal conditions that was entirely caused by COX-1. After cytokine stimulation, the significant increase in COX activity, which was greater in the COPD fibroblasts, was caused by COX-2. This suggests that COX-1 may account for the increased basal production of PGE₂ but that increased PGE₂ production in response to IL-1 β and TNF- α is caused by COX-2. The increase in COX-2 seems to be essential in mediating the increased PGE₂ production by COPD fibroblasts after IL-1 β and TNF- α stimulation because inhibition of COX-2 by either a pharmacologic inhibitor or by siRNA suppression blocks the stimulatory effect of these cytokines. A role for COX-1 in COPD cells is not excluded: the lack of effect of the siRNA could be caused by incomplete suppression; alternatively, the partial inhibition by SC-560 could have been caused by a nonspecific effect of the inhibitor, although this was not seen in control cells.

With regard to PGE₂ biosynthesis, several miRNAs have been reported to regulate COX-2 including miR-16 (32), miR-101 (28, 33), and miR-199a* (33). The current study extends these studies by demonstrating that miR-146a can also modulate COX-2. Interestingly, miR-16, which has been reported to regulate COX-2 mRNA (32), lacks a defined homology with COX-2 through the *in silico* assessment, suggesting that miRNA regulation has as yet undefined complexities. The current study, however, supports a direct effect of miR-146a on COX-2 mRNA levels, rather than an indirect effect mediated by altered transcription factor expression. In the current study, miR-101, miR-199a, and miR-16 were not evaluated, but their expression indicated by the microarray analyses was not noted to be altered after IL-1 β and TNF- α stimulation in COPD or control fibroblasts. In addition, miR-146a has homologies with two other enzymes that could contribute to PGE₂ biosynthesis: phospholipase A₂ (type IV) and microsomal PGE₂ synthase-1 (TargetScan). Whether these enzymes are altered in COPD fibroblasts or altered by miR-146a remains undefined.

In the current study, LUC reporter assays were performed to confirm interactions between miR-146a and COX-2 mRNA. The type of assay is often performed to assess the miRNA interactions with target mRNAs (28, 33). The data generated

confirm that miR-146a modulates the expression of a reporter driven by the COX-2 promoter to which it has homology. Although this is consistent with direct binding, a role for an intermediate is not excluded.

In addition, inflammation in COPD has been described as being abnormal, particularly in its persistence long after removal of the inciting stimulus, most commonly cigarette smoking. The current study suggests that underproduction of an miRNA, specifically miR-146a, may be a mechanism for abnormal inflammation in COPD. In this context, miR-146a is known to be induced by IL-1 β and TNF- α (34) and is thought to be a feedback control mechanism that limits the intensity and duration of the inflammatory response by inducing degradation of key mRNAs. Based on homologies, miR-146a has the potential to regulate several thousand potential mRNAs. The current study focused on miR-146a regulation of COX-2 and the production of PGE₂. In this context, PGE₂ is a mediator released during the inflammatory response that has pleiotropic effects on inflammation and repair. It was the focus of the current study because our previous work demonstrated that increased PGE₂ production by COPD fibroblasts contributes to their altered repair function (14). Other effects on the inflammatory response are likely. For example, miR-146a has reported homologies with IL-1 receptor-associated kinase and TNF receptor-associated factor 6 and negatively regulates the IL-1 β -induced inflammatory response in human lung alveolar epithelial cells (34, 35). The reduced production of miR-146a, therefore, could also contribute to other aspects of the abnormal inflammatory response in COPD.

In addition to advancing the understanding of COPD pathogenesis, identifying altered miR-146a expression in COPD fibroblasts raises several novel strategies for developing therapies for COPD. miRNA mimics are being developed for therapeutic use. By targeting and reducing the augmented PGE₂ production, miRNA-based therapy could restore normal tissue repair and remodeling. In addition, miR-146a may target other aspects of the inflammatory response. An miRNA-based therapy could, therefore, address several aspects of COPD pathogenesis. Finally, because miRNAs generally modulate, but do not completely inhibit their target pathways, these therapeutic strategies may be able to restore "balance" without severe untoward side effects.

The current study has several limitations. We focused on cultured fibroblasts. Whether miR-146a is altered in other cell types in the lung, or in the lung as a whole, and whether this results in altered inflammation remains undefined. In addition, the current study did not define the mechanisms for reduced expression of miR-146a in COPD fibroblasts. However, a common G/C polymorphism (rs2910164) within miR-146a precursor has been reported to be associated with reduced processing and expression of mature miR-146a and the genetic predisposition to papillary thyroid carcinoma (36).

Conclusion

The current study demonstrates that fibroblasts from patients with COPD underexpress miR-146a after stimulation with IL-1 β and TNF- α . This leads to reduced degradation of COX-2 mRNA and overproduction of PGE₂. The reduced expression of miR-146a offers a novel pathogenetic mechanism that can contribute to the abnormal inflammatory response in COPD and links the inflammatory response to altered tissue repair in this disease.

Author Disclosure: T.S. has a pending patent through the University of Nebraska Medical Center covering microRNA applications for the treatment of diseases, which is currently marketing to various commercial entities. X.L. has a pending patent through the University of Nebraska Medical Center covering microRNA applications for the treatment of diseases, which is currently marketing to various commercial entities. A.N. does not have a financial relationship with a commercial

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S.I.R. has had or currently has a number of relationships with companies who provide products or services relevant to outpatient management of chronic obstructive pulmonary disease. These relationships include serving as a consultant, advising regarding clinical trials, speaking at continuing medical education programs, and performing funded research both at basic and clinical levels. He does not own any stock in any pharmaceutical companies. S.I.R. has received compensation for consultancies for Abbott (\$1,001–\$5,000), KOL Connection (Up to \$1,000), Able Associates (\$1,001–\$5,000), Leerink Swan (Up to \$1,000), Almiral (\$10,001–\$50,000), MedaCorp (Up to \$1,000), Almiral/Forest (Up to \$1,000), Mpx (Up to \$1,000), Altana (\$1,001–\$5,000), Novartis (\$10,001–\$50,000), Anthera (Up to \$1,000), Otuska (\$1,001–\$5,000), APT Pharma/Britnall (\$1,001–\$5,000), Pfizer (\$1,001–\$5,000), Aradigm (\$1,001–\$5,000), Propagate (Up to \$1,000), AstraZeneca (\$1,001–\$5,000), Pulmatrix (Up to \$1,000), Boehringer Ingelheim (\$5,000–\$10,000), Quintiles (Up to \$1,000), Britnall and Nicolini (Up to \$1,000), Roche (\$1,001–\$5,000), Defined Health (Up to \$1,000), Scimed (Up to \$1,000), Dunn Group (Up to \$1,000), TargeGen (\$1,001–\$5,000), Eaton Associates (Up to \$1,000), Theravance (\$1,001–\$5,000), Gerson (Up to \$1,000), UBS (\$1,001–\$5,000), GlaxoSmithKline (\$10,001–\$50,000), VantagePoint (Up to \$1,000), Infomed (Up to \$1,000), VantagePoint Mgmt (\$5,000–\$10,000), and Johnson & Johnson (\$1,001–\$5,000). He has served on Advisory Boards for Abbott (\$1,001–\$5,000), Nycomed (\$10,001–\$50,000), Almiral (\$10,001–\$50,000), Nycomed/Strategicare (\$1,001–\$5,000), Boehringer Ingelheim (\$1,001–\$5,000), Pfizer (\$5,000–\$10,000), COPDForum (\$1,001–\$5,000), Pharmaxis (\$1,001–\$5,000), Dey (\$1,001–\$5,000), Schering-Plough (\$1,001–\$5,000), GlaxoSmithKline (\$10,001–\$50,000), TargeGen (\$5,001–\$10,000), and Novartis (\$10,001–\$50,000). He has received compensation for lectures from the American Thoracic Society, ACCP (Up to \$1,000), AstraZeneca (\$10,001–\$50,000), Boehringer Ingelheim (\$5,001–\$10,000), the California Allergy Society (\$1,001–\$5,000), Creative Educational Concept (\$10,001–\$50,000), France Foundation (\$5,000–\$10,000), Information TV, Network for Continuing Ed (\$1,000–\$5,000), Novartis (\$10,001–\$50,000), Pfizer, and SOMA (\$1,001–\$5,000). 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Specifically, he has received funding from the tobacco industry for studies relating to harm reduction and to the impact of tobacco smoke on stem cells. He has also consulted with RJ Reynolds without personal fee on the topic of harm reduction. He received funding from RJ Reynolds to evaluate the effect of a harm reduction product in a normal smoker (1996) and in subjects with chronic bronchitis (1999), and to assess the effect of smoking cessation on lower respiratory tract inflammation (2000). He participated in a Philip Morris multicenter study to assess biomarkers of smoke exposure (2002). He received funding for a clinical trial from the Institute for Science and Health (2005), which receives support from the tobacco industry to evaluate biomarkers in exhaled breath associated with smoking cessation and reduction. This study was supplemented with funding from Lorillard and RJ Reynolds. He has received a grant from the Philip Morris External Research Program (2005) to assess the impact of cigarette smoking on circulating stem cells in the mouse. He has consulted with RJ Reynolds on the topic of harm reduction until 2007, but did not receive personal remuneration for this. There are no active tobacco-industry funded projects. All ties with tobacco industry companies and entities supported by tobacco companies were terminated in 2007.

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