Extracellular cysteine/cystine redox potential controls lung fibroblast proliferation and matrix expression through upregulation of transforming growth factor-β

Allan Ramirez,1 Bassel Ramadan,1 Jeffrey D. Ritzenthaler,1 Hilda N. Rivera,2 Dean P. Jones,1 and Jesse Roman1,2

1Division of Pulmonary, Allergy, and Critical Care Medicine, Department of Medicine, Emory University School of Medicine and 2Atlanta Veterans Affairs Medical Center, Atlanta, Georgia

Submitted 5 January 2007; accepted in final form 19 July 2007

Ramirez A, Ramadan B, Ritzenthaler JD, Rivera HN, Jones DP, Roman J. Extracellular cysteine/cystine redox potential controls lung fibroblast proliferation and matrix expression through upregulation of transforming growth factor-β. Am J Physiol Lung Cell Mol Physiol 293: L972–L981, 2007. First published July 20, 2007; doi:10.1152/ajplung.00010.2007.— Oxidant stress has been implicated in the pathogenesis of chronic lung disorders like idiopathic pulmonary fibrosis. However, mechanisms that link oxidant stress to fibrogenesis remain partially elucidated. Emerging data suggest an important role for the extracellular thiol/disulfide redox environment. The cysteine (Cys)/cystine (CySS) redox couple represents the predominant low-molecular-weight thiol/disulfide pool found in plasma and is sensitive to aging, smoking, and other host factors. We hypothesized that an oxidized extracellular Cys/CySS redox potential (E<sub>a</sub> Cys/CySS) affects lung fibroblasts by inducing intracellular signals that stimulate proliferation and matrix expression. We tested this hypothesis in primary murine lung fibroblasts and found that an oxidized E<sub>a</sub> Cys/CySS (−46 mV) stimulates lung fibroblast proliferation. Furthermore, it stimulated their expression of fibronectin, a matrix glycoprotein highly expressed in fibrotic lung diseases and implicated in lung injury. This stimulatory effect was dependent on protein kinase C activation. Oxidant stress also increased the phosphorylation of cAMP response element binding protein, a transcription factor known for its ability to stimulate fibronectin expression, and increased the expression of mRNAs and proteins coding for the transcription factors nuclear factor (NF)-κB and mothers against decapentaplegic homolog 3. Fibroblasts cultured in normal (−80 mV) or reduced (−131 mV) E<sub>a</sub> Cys/CySS showed less induction. Furthermore, fibronectin expression in response to an oxidized E<sub>a</sub> Cys/CySS was associated with expression of transforming growth factor-β1 (TGF-β1) and was inhibited by an anti-TGF-β1 antibody and SB-431542, a TGF-β1 receptor inhibitor. These studies suggest that extracellular oxidant stress activates redox-sensitive pathways that stimulate lung fibroblast proliferation and matrix expression through upregulation of TGF-β1.

redox potential; oxidant stress; fibroblasts; fibrosis; fibronectin; proliferation; redox signaling

IT IS A COMMON BELIEF THAT oxidant stress represents an important mechanism of chronic lung disorders (5, 13, 35, 45, 46). The relevance of this idea in fibrotic lung disease is supported by a number of studies showing decreased levels of the antioxidant glutathione (GSH), increased levels of glutathione disulfide (GSSG), and/or increased generation of superoxide anions in clinical samples of subjects with idiopathic pulmonary fibrosis (3, 15, 35, 36, 38, 45, 53, 55). These and other studies suggest that redox reactions of thiol/disulfide couples play important roles in the progression of pulmonary fibrosis, but the mechanisms that link these reactions to fibroblast proliferation and tissue fibrogenesis remain unclear.

Several studies in vitro demonstrate that reversible redox reactions of thiol/disulfide couples play roles in the regulation of important cellular processes such as proliferation, differentiation, and apoptosis and have been implicated in human disease (18, 26, 29, 52). Also, various growth factor receptors are sensitive to thiol content and redox (17, 40). Of note, most of the available research on thiol/disulfide couples and oxidative stress has focused on intracellular GSH/GSSG, the most abundant intracellular low-molecular-weight thiol/disulfide couple. However, the extracellular thiol/disulfide redox environment consisting of cysteine (Cys)/cystine (CySS) also appears to be important. This thiol/disulfide redox couple is the predominant low-molecular-weight thiol/disulfide pool found in plasma. In humans, the physiological Cys/CySS redox potential (E<sub>a</sub> Cys/CySS) in healthy subjects is around −80 mV, whereas in subjects with disease, this redox state becomes oxidized to between −62 to −20 mV (28). Oxidized E<sub>a</sub> Cys/CySS has been documented in subjects with diets low in Cys, alcohol abuse, diabetes, and cigarette smoking (1, 25, 26, 28). These observations are intriguing particularly when considered in conjunction with data showing that alterations in extracellular E<sub>a</sub> Cys/CySS redox can drive signal transduction. This is highlighted in the work of Nkabyo and colleagues (40), among that of others, showing activation of epidermal growth factor receptor signaling and induction of the p44/p42 mitogen-activated protein kinase pathway followed by increased proliferation in Caco-2 cells exposed to reduced extracellular E<sub>a</sub> Cys/CySS redox. Relevant to our work is the finding that GSH (at physiological concentrations of 0–500 μM) has been found to suppress the proliferation of cultured lung fibroblasts (9). Of note, other sulphhydryl-containing compounds like Cys, N-acetylcysteine, 2-mercaptoethanol, and dithiothreitol also reduce fibroblast proliferation. However, to our knowledge, the effect over the pathophysiological range occurring in vivo of oxidized E<sub>a</sub> Cys/CySS on lung fibroblast functions remains unknown.

Together, these studies suggest that extracellular oxidant stress, through oxidation of the Cys/CySS thiol disulfide couple, might directly activate redox-sensitive pathways that stimu-
ulate the differential expression of genes that control fibroblast proliferation and matrix deposition. To explore this hypothesis, we cultured lung fibroblasts in the setting of oxidized \(E_h\) Cys/CySS and tested for cell proliferation and the expression of fibronectin, a matrix glycoprotein implicated in injury and repair and highly expressed by lung fibroblasts in the setting of fibrotic lung disorders.

**MATERIALS AND METHODS**

Preparation of media and measurement of \(E_h\) Cys/CySS. Different extracellular thiol/disulfide redox potentials were established by varying the concentrations of Cys and CySS added to Cys-free DMEM containing 4 mM l-glutamine, 10 U/ml penicillin, and 10 \(\mu\)g/ml streptomycin (catalog no. D0422 from Sigma-Aldrich, St. Louis, MO), as previously reported (39). Stock solutions for Cys and CySS (10 mM; \(pH\) 7.4) were made fresh before each experiment and filtered through a 0.2-\(\mu\)m syringe filter. The amount of Cys/CySS added to the redox media for the various redox potential states is listed in Table 1. Direct redox potential measurements were made using a platinum redox electrode (Thermo Electron, Beverly, MA). The electrode was calibrated before each use with a standardization solution at 25°C (Thermo Electron). Cys and CySS concentrations were measured by HPLC with fluorescence detection (26).

**Cell culture.** Murine NIH/3T3 and primary lung fibroblasts were maintained in DMEM with 4.5 g/l glucose supplemented with 10% heat-inactivated FBS and 1% antibiotic-antimycotic solution (100 U/ml penicillin G sodium, 100 U/ml streptomycin, and 0.25 \(\mu\)g/ml amphotericin B) and incubated in a humidified 5% CO2 incubator at 37°C. Primary murine fibroblasts were obtained from the lungs of wild-type or transgenic mice expressing the full-length human fibronectin promoter connected to the luciferase reporter vector as previously described (56). Mice transgenic for the human fibronectin promoter connected to a luciferase reporter gene were developed by the Emory University Winship Cancer Center Transgenic Mouse Core Facility. Briefly, the animals were created by direct microinjection of a 1.2-kb human fibronectin promoter fragment connected to a 1.9-kb luciferase gene fragment (pFN[1.2kb]LUC) into 200 fertilized one-cell (C57BL/6 × SJL) F2 hybrid mouse eggs. All surviving eggs were reinplemented in pseudopregnant recipient female mice to generate the pFN[1.2kb]LUC founder transgenic mice. These mice do not show phenotypic abnormalities, and their expression of endogenous fibronectin and the pFN[1.2kb]LUC transgene resembles that observed in unchallenged wild-type mice and mice stimulated with nicotine (51 and unpublished observations). As expected, primary lung fibroblasts isolated from the transgenic mice showed increased expression of luciferase driven by the human fibronectin promoter when exposed to nicotine (56 and data not shown).

**Cells were cultured in complete serum-free media with l-glutamine and 1 g/l BSA (Cellgro Complete 1× Serum Free/Low Protein Media; Catalog no. 40–101-cv; Mediatech, Herndon, VA) for 24 h before the addition of the redox media. The redox medium was prepared fresh every day and changed every 24 h. mRNA analysis.** Primary wild-type mouse lung fibroblasts (10\(^6\) cells/ml) were plated on six-well plates and incubated in complete serum-free media for 24 h before the addition of the redox media for 6 h. Total RNA was isolated as previously described (11). The reverse transcription reactions of the extracted RNA were performed by combining the following reagents in a PCR reaction tube: 0.625 \(\mu\)m dNTPs, 16 nmol random hexamer oligonucleotides (Roche Diagnostics, Indianapolis, IN), 5 \(\mu\)l first-strand buffer (50 mM Tris-HCl, \(pH\) 8.3, 75 mM KCl, and 3 mM MgCl2; Invitrogen, Carlsbad, CA), 20 mM DTT, 200 units reverse transcriptase enzyme, 0.5 \(\mu\)l RNasin (ribonuclease inhibitor; Promega, Madison, WI), and 1 \(\mu\)g extracted RNA in a total volume of 25 \(\mu\)l. Primers for PCR reactions were based on GenBank published sequences and are as follows: mouse fibronectin forward primer (CTGGGAACACCTGGCATAG), reverse primer (CAGCGGCTCCAGCAGT), probe (ACCAAGGTTCACTTCA-CAC); mouse \(\beta\)-actin forward primer (ATGGATGAGATCATGATCG), reverse primer (ATGATGGATCTCGTCAGGG), probe (GGATGGTACGTACATGGCT); mouse nuclear factor (NF)-\(\kappa\)B p65 forward primer (CTGATGGTGTCATCTAGG), reverse primer (TGCTGGGAAGGTAGTACAG), mouse mothers against decapentaplegic homolog 3 (Smad3) forward primer (GCAT GACGCTGGTTCGAGT), reverse primer (TTGATCCCTGATGATGATG); RT-PCR reactions were performed using the following PCR protocol: 95°C for 30 s, 55°C for 30 s, 72°C for 1 min for 35 cycles. PCR products for NF-\(\kappa\)B (p65) and Smad3 were resolved on 1% agarose gels and stained with ethidium bromide, and band sizes were verified. \(\beta\)-Actin mRNA was used as an internal standard.

Real-time RT-PCR reactions were set up by adding the following reagents to Smart Cycler Reaction Tubes: 5 mM MgCl2, 0.2 \(\mu\)M forward and reverse primer (for sequences, see above), 10\(^\times\) Master Mix (Roche LightCycler FastStart Master SYBR Green I), and 500 ng of template cDNA. Samples were briefly centrifuged and processed using the following cycle program using the Cepheid Smart Cycler (Sunnyvale, CA): hold at 95°C for 120 s followed by 35 cycles at temperatures of 95°C for 15 s, 68°C for 30 s, and 72°C for 30 s. Results of the log-linear phase of the growth curve were analyzed by use of the mathematical equation of the second derivative, and relative quantification was performed using the 2\(^{-}\Delta\DeltaCT\) method (34).

**Western blot analysis.** Primary wild-type mouse lung fibroblasts (1 \(\times\) 10\(^6\) cells/ml) were incubated in complete serum-free media for 24 h before exposure to various media redox states ranging from −46 to −131 mV for another 48 h. Cells were then washed and lysed, and the resulting homogenate was submitted to Western blotting. Protein concentration was determined by the Bradford method (6). Blots were incubated with a polyclonal antibody raised against human fibronectin (antibody F3648; 1:1,000 dilution; Sigma), an antibody specific for phosphor (p)-cAMP response element binding protein (CREB) or total CREB (1:1,000 dilution; Cell Signaling Technology), primary antibodies against Erk-1/2 and p-extracellular signal-regulated kinase (Erk; Tyr423/422), p-protein kinase C (PKC)-\(\alpha\) (Ser67/68; 1:1,000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), a primary antibody against Smad3 (Upstate, Lake Placid, NY), or a primary antibody against the COOH-terminal region of PKC\(\delta\) (antibody P-4334; 1:3,000 dilution; Sigma). Identified loaded gels were run and incubated with a primary antibody against actin (abcam 1801; 1:1,000 dilution) to control for loading. Blots were incubated with a secondary rabbit antibody raised against goat IgG conjugated to horseradish peroxidase (1:20,000 dilution). The blots were transferred to freshly made ECL solution (Amersham, Arlington, IL) for 1 min and exposed to X-ray film. Protein bands were quantified by densitometric scanning using a GS-800 Calibrated laser densitometer (Bio-Rad, Hercules, CA).

<table>
<thead>
<tr>
<th>Table 1. Concentrations of Cys and CySS for testing redox</th>
<th>CySS</th>
<th>Cys</th>
</tr>
</thead>
<tbody>
<tr>
<td>(E_h), mV</td>
<td>(\mu M)</td>
<td>(10 , M) Stock</td>
</tr>
<tr>
<td>0</td>
<td>99.75</td>
<td>10</td>
</tr>
<tr>
<td>−46</td>
<td>98</td>
<td>9.8</td>
</tr>
<tr>
<td>−80</td>
<td>93</td>
<td>9.3</td>
</tr>
<tr>
<td>−109</td>
<td>80</td>
<td>8</td>
</tr>
<tr>
<td>−131</td>
<td>60</td>
<td>6</td>
</tr>
<tr>
<td>−150</td>
<td>10</td>
<td>0</td>
</tr>
</tbody>
</table>

CySS, cystine; Cys, cysteine. Units for 10 M stock are \(\mu\)l/1 ml. \(E_h\) = \(E_o \pm RT/2F\ln ([Cys]/[CySS])\), where \(E_o\) (given in V or mV) represents the electromotive relative to a standard hydrogen electrode, \(R\) is the gas constant, \(T\) is the absolute temperature, \(F\) is Faraday’s constant, and \(E_h\) is the standard electrode potential (−0.250 V at \(pH\) 7.4) for the Cys/Cyss redox couple.
Cys/CySS redox potential controls fibroproliferation and matrix expression

Examination of fibronectin gene transcription. To evaluate fibronectin gene transcription, primary lung fibroblasts and NIH/3T3 cells expressing pFN(1.2kb)LUC were studied (37, 56). The DNA construct pFN(1.2kb)LUC contains ∼1,200 bp of the 5′-flanking region of the human fibronectin gene isolated from the human fibrosarcoma cell line HT1080. This construct includes 69 bp of exon 1, a CAAT site located at −150 bp, and the sequence ATATAA at −25 bp from the transcription start site. It also contains several previously identified regulatory elements such as three cAMP response elements located at −415, −270, and −170 bp and an SP-1 site at −102 bp from the transcription start site. The promoter was subcloned in the Sma I site of pGL3 Basic Luciferase Reporter Vector (Promega). The pFN(1.2kb)LUC promoter construct was introduced into murine NIH/3T3 fibroblasts via electroporation to create stable transfectants (37).

Both primary fibroblasts and stably transfected NIH/3T3 fibroblasts were maintained in DMEM with 4.5 g/l glucose supplemented with 10% heat-inactivated FBS and 1% antibiotic-antimycotic solution (100 U/ml penicillin G sodium, 100 U/ml streptomycin, and 0.25 μg/ml amphotericin B) and incubated in a humidified 5% CO2 incubator at 37°C. The cells were harvested by trypsinization with 2.5× trypsin and 5.3 mM EDTA (Sigma Chemical, St. Louis, MO), washed with PBS, counted, and plated at 1.5 × 105 cells/ml in 12-well tissue culture dishes. The cells were then incubated in complete serum-free media for 24 h before treatment with various redox state media. Concurrently, for some experiments, cells were treated with nicotine (50 μg/ml) as a positive control (51) for various periods of time in different redox media. In experiments involving 4-acetamidamide-4′-amleimidylstilbene-2, 2′-disulfonic acid, disodium salt (AMS; Invitrogen), cells were pretreated with AMS (0.5 mM) for 2 h before incubation with the redox state media. Afterward, the cells were tested for luciferase activity. For this, the cells were harvested by scraping, washed with PBS, resuspended in 100 μl of cell lysis buffer (Promega), and sonicated, and a 10-μl aliquot was tested by adding 50 μl Luciferase assay reagent (Promega). Light intensity was measured using a Labsystems Luminoskan Ascent Plate Luminometer. Results were recorded as luciferase units adjusted for total protein content that was measured using the Bradford method (6).

![Graphs and images](http://ajplung.physiology.org/).
**Statistical evaluation.** The data shown represent results from a single representative experiment with four to eight replicates within a single experiment. Each experiment was performed at least three times. Means ± SE were calculated for all experimental values. Significance was assessed by ANOVA followed by Student’s t-test.

**RESULTS**

Oxidized $E_h$ Cys/CySS stimulates fibronectin expression in lung fibroblasts. To explore the cellular effects of variable extracellular $E_h$ Cys/CySS states, we established a reliable cell culture-based system to study lung fibroblast functions. Six states of redox, from oxidized (0 mV) to reduced ($-150$ mV), were established by adding different concentrations of Cys and CySS to the culture media, followed by measuring the redox potential of the media at time 0 using a platinum redox electrode. Cys and CySS concentrations using this method were verified by HPLC. As depicted in Fig. 1A, we found a high correlation between the calculated $E_h$ Cys/CySS and that measured by potentiometry.

We then tested the effects of $E_h$ Cys/CySS on fibronectin expression (Fig. 1, B–E). For this, both primary lung fibroblasts [isolated from wild-type or transgenic mice expressing the pFN(1.2 kb)LUC human fibronectin promoter-luciferase reporter gene construct] and NIH/3T3 fibroblasts [permanently transfected with pFN(1.2 kb)LUC] were cultured in serum-free media for 24 h. Afterward, the fibroblasts were incubated in $E_h$ Cys/CySS redox media for an additional 24 h, harvested, and processed to determine luciferase activity. As shown in Fig. 1, B and C, fibronectin gene transcription increased with progressive oxidation of the extracellular $E_h$ Cys/CySS. The changes in gene transcription were accompanied with concordant alterations in fibronectin mRNA accumulation (Fig. 1D) and pro-

**Fig. 2.** Induction of fibronectin expression by oxidized $E_h$ Cys/CySS inhibited by blockade of protein kinase C (PKC) activation, but not by an inhibitor of the mitogen/eritocyte signal-regulated kinase (MEK)-1/extracellular signal-regulated kinase (Erk) pathway. A: primary wild-type murine lung fibroblasts ($1.5 \times 10^5$ cells/ml) were incubated in complete serum-free media for 24 h before culture under varying $E_h$ Cys/CySS redox conditions in the presence or absence of a blocker of PKC activity (calphostin C; $1 \times 10^{-7}$ M) for 24 h followed by Western blot analysis for PKCα and the active phosphorylated form of PKCα, a PKC isoform that mediates serum-induced fibronectin expression (38). Duplicate blots were analyzed for actin expression and used as loading controls (top). Primary murine lung fibroblasts isolated from mice transgenic for the human fibronectin promoter fused to a luciferase gene ($1.5 \times 10^6$ cells/ml) were incubated in complete serum-free media for 24 h before culture under varying $E_h$ Cys/CySS redox conditions in the presence or absence of a blocker of PKC activity (calphostin C; $1 \times 10^{-7}$ M) for 24 h followed by measurement of fibronectin gene expression by luciferase assay. Note that the PKC inhibitor blocked the induction of fibronectin (bottom, $n = 4$). B: a second blocker of PKC activity, chelerythrine chloride ($1 \times 10^{-6}$ M), was also tested in experiments, similar to those for calphostin C, for 24 h followed by the measurement of fibronectin gene expression by luciferase assay. C: primary wild-type murine lung fibroblasts ($1.5 \times 10^6$ cells/ml) were incubated in complete serum-free media for 24 h before culture under varying $E_h$ Cys/CySS redox conditions in the presence or absence of a blocker of MEK-1/Erk inhibitor (PD-98059; $10 \mu$M) for 4 h followed by Western blot analysis for total Erk 1/2 and its active phosphorylated (p) forms. Duplicate blots were analyzed for actin expression and used as loading controls (top). Primary murine lung fibroblasts isolated from mice transgenic for the human fibronectin promoter fused to a luciferase gene ($1.5 \times 10^6$ cells/ml) were incubated in complete serum-free media for 24 h before culture under varying $E_h$ Cys/CySS redox conditions in the presence or absence of a MEK-1/Erk inhibitor (PD-98059) for 24 h followed by measurement of fibronectin gene expression by luciferase assay. Note that the MEK-1/Erk inhibitor did not block the induction of fibronectin (bottom, $n = 4$).
tein production (Fig. 1E) as determined by real-time RT-PCR and Western blot, respectively. Nicotine was used as positive control since it also stimulates fibronectin expression (51).

**Induction of redox signaling in lung fibroblasts.** To study the signaling events elicited by Eh Cys/CySS, we studied lung fibroblasts and measured fibronectin gene transcription. Previously, we found that fibronectin expression is controlled by activation of PKC and mitogen-activated protein kinase Erk 1/2, and by induction of the transcription factor CREB (37); therefore, we focused on these signals for further study. As demonstrated in Fig. 2A, we found that an oxidized Eh Cys/CySS redox potential enhanced the expression of total and phosphorylated forms of PKC and that an inhibitor of PKC activation (calphostin C) blocked redox stimulation of fibronectin. A second inhibitor of PKC activation, chelerythrine chloride, also blocked this effect (Fig. 2B). In contrast, an inhibitor of mitogen/extracellular signal-regulated kinase (MEK)-1 (PD-98059) did not inhibit the stimulation of fibronectin by an oxidized Eh Cys/CySS redox potential (Fig. 2C). Instead, in cells treated with the MEK-1/Erk inhibitor, fibronectin gene transcription remained elevated in normal and reduced states, suggesting that this pathway might control baseline expression of the fibronectin gene.

Next, we examined the ability of the Eh Cys/CySS redox couple to activate transcription factors known to affect fibronectin gene transcription. As shown in Fig. 3A, oxidized Eh Cys/CySS redox potential (−46 mV) stimulated the phosphorylation of CREB, a transcription factor that is important for induction of fibronectin by TGF-β1, serum, phorbol 12-myristate, 13-acetate, and several other stimulants (16, 37).

To further determine the role of CREB, fibroblasts were transfected with competing CREB oligonucleotides and tested under oxidizing conditions. Again, fibroblasts cultured at −46 mV showed increased fibronectin gene transcription but not at −80 or −131 mV (Fig. 3B). The induction of fibronectin was greatly diminished by the competing CREB oligonucleotide; no effects were noted with the control oligonucleotide (data not shown). These results suggest that a role for CREB in the fibronectin response triggered by an oxidized Eh Cys/CySS as has been demonstrated for fibronectin induction by serum and other stimulants (16, 25, 28, 37).

We then determined that the effects of oxidized Eh Cys/CySS conditions were indeed related to alterations in redox signaling caused by changes in the extracellular relative concentrations of Cys and CySS. This was accomplished by treating fibroblasts with varying redox conditions in the presence or absence of a non-cell-permeable thiol-reacting reagent, AMS. AMS is a reagent that conjugates to thiols and blocks thiol/disulfide interactions (19). As depicted in Fig. 4, an oxidized Eh Cys/CySS redox media stimulated fibronectin gene transcription, whereas the control and reduced media had no effect. Pretreatment with AMS inhibited the induction of fibronectin in the setting of oxidized Eh Cys/CySS.
Extracellular oxidized Eh Cys/CySS stimulates fibronectin expression via induction of TGF-β1. TGF-β1 is a fibroblast-derived factor that can stimulate fibronectin expression (16). To test the role of TGF-β1 in redox induction of fibronectin, we exposed lung fibroblasts to various redox states for 24 h and then tested the media for TGF-β1 protein. As shown in Fig. 5A, oxidized Eh Cys/CySS redox potential stimulated TGF-β1 protein accumulation in the media. Specifically, cells showed increased expression of latent (40 kDa) and active (23 kDa) TGF-β1 protein. Most importantly, an antibody against TGF-β1 inhibited redox induction of fibronectin, whereas a control antibody had no effect (Fig. 5B). Similar results were obtained with the use of SB-431542, a potent and selective TGF-β1 receptor inhibitor (Fig. 5C).

Extracellular oxidized Eh Cys/CySS stimulates the proliferation of lung fibroblasts and the expression of NF-κB and Smad3. In fibrotic lung disease, fibrosis can be increased by the rate of matrix deposition along with the remodeling of the existent extracellular matrix. Fibrosis can also be affected by an increase in the number of fibroblasts. Therefore, we examined whether Eh Cys/CySS affected cell proliferation in thymidine incorporation assays. We found that, in addition to stimulation of fibronectin expression, oxidized Eh Cys/CySS stimulates fibroblast proliferation. As depicted in Fig. 6, fibroblasts cultured for up to 48 h in oxidized Eh Cys/CySS media showed stimulation of growth, whereas proliferation in normal or reduced media did not differ from control. Interestingly, an oxidized Eh Cys/CySS can also stimulate the expression of important transcription factor mRNAs and proteins capable of affecting the expression of genes coding for

Fig. 5. Oxidized Eh Cys/CySS stimulates transforming growth factor (TGF)-β1 protein expression in primary lung fibroblasts. A: primary wild-type mouse lung fibroblasts (1 × 10⁴ cells/ml) were incubated in complete serum-free media for 24 h before incubation in oxidized media (~46 mV), normal media (~80 mV), and reduced (~131 mV) Eh Cys/CySS redox for 24 h. The media was concentrated using centrifugal filter devices (Millipore, Bedford, MA) followed by Western blotting for TGF-β1. Oxidized Eh Cys/CySS redox stimulated TGF-β1 protein expression, and this effect was associated with an increase in the active form of TGF-β1 (23 kDa). A duplicate gel stained with Coomassie was analyzed for total protein expression and used for gel loading control (top). Note that there was no activation of TGF-β1 in control or in the other redox potential (~80 and ~131 mV) samples. B: primary murine lung fibroblasts isolated from mice transgenic for the human fibroblastin promoter fused to a luciferase gene (1.5 × 10⁵ cells/ml) were incubated in complete serum-free media for 24 h before culture under varying Eh Cys/CySS redox conditions with and without IgG or TGF-β1 antibody (10 μg/ml) for 24 h followed by measurement of fibroblastin gene expression by luciferase assay. Note that TGF-β1 antibody (Ab) inhibited the effects of oxidized Eh Cys/CySS redox on fibroblastin gene transcription, but IgG did not.

*Significant difference of P < 0.05; n = 4. C: primary murine lung fibroblasts isolated from mice transgenic for the human fibroblastin promoter fused to a luciferase gene (1.5 × 10⁵ cells/ml) were incubated in complete serum-free media for 24 h before culture under varying Eh Cys/CySS redox conditions with and without SB-431542 (SB; 10 μM), a TGF-β1 receptor inhibitor, for 24 h followed by measurement of fibroblastin promoter expression by luciferase assay. Note that SB-431542 inhibited the effects of oxidized Cys/CySS redox on fibroblastin promoter expression; n = 4.

Fig. 6. Oxidized Eh Cys/CySS stimulates fibroblast proliferation. Primary wild-type mouse lung fibroblasts (1 × 10⁴ cells/ml) were incubated in complete serum-free media for 24 h before culture under varying Cys/CySS redox conditions that included 1 μCi/ml [3H]thymidine for 24–48 h. Cells were washed and harvested, and proliferation was quantified by counting cell extracts in a Beckman LS 6500 liquid scintillation counter. Note that SB-431542 inhibited the effects of oxidized Cys/CySS redox showed more proliferation at 48 h compared with cells grown under more reduced conditions. cpm, Counts/min. *Significant difference of P < 0.05 compared with control; n = 5.
performed, and mRNA was quantified using real-time RT-PCR and the 2^-ΔΔCT method to determine NF-κB and Smad3 protein expression.

Primary wild-type mouse lung fibroblasts (1 × 10^5 cells/ml) were incubated in complete serum-free media for 24 h before culture under varying Cys/CySS redox conditions for 6 h. Cells were harvested, washed, and lysed, and the resulting homogenate was submitted to Western blotting using a polyclonal antibody raised against NF-κB (p65 subunit; 1:1,000 dilution) or Smad3 (1:1,000 dilution). Note that oxidized Eh Cys/CySS (−46 mV) induced both NF-κB and Smad3 protein expression.

**DISCUSSION**

In fibrosing lung disorders, fibroblasts proliferate and accumulate within the pulmonary interstitium and airspaces where they deposit extracellular matrices, leading to the effacement of the alveolar capillary units with fibrosis. In idiopathic pulmonary fibrosis, for example, accumulations of fibroblasts in lesions termed “fibroblastic foci” are believed to represent the main source of disease activity (14). In these disorders, fibroblasts seem to have a profibrotic phenotype characterized by increased proliferation, increased matrix expression, and myofibroblastic differentiation (44, 58). The mechanisms involved and the factors responsible for this switch in fibroblast phenotype remain incompletely elucidated, but soluble factors (e.g., growth factors, chemokines, cytokines) produced during tissue injury are considered important mediators of these events.

The studies presented herein point to oxidant stress produced by an oxidized extracellular Eh Cys/CySS redox potential as an important modulator of fibroblast proliferation and matrix expression. The Eh Cys/CySS redox pool is sensitive to many host factors (e.g., aging and smoking) and can activate genes and transcription factors that control cell proliferation, differentiation, and apoptosis (27, 39). Because the Eh Cys/CySS redox couple represents the predominant low-molecular-weight thiol/disulfide pool found in plasma (28), it makes sense to study how alterations in this redox couple may promote fibroproliferation and matrix expression.

We found that an oxidized extracellular Eh Cys/CySS stimulates the expression of fibronectin through specific signaling pathways that include phosphorylation of CREB and induction of fibronectin gene transcription. The ability of oxidized extracellular Eh Cys/CySS to stimulate fibronectin expression is significant for the following reasons. Fibronectin is a cell-adhesive extracellular matrix glycoprotein that is rapidly expressed after tissue injury. It has been found to be elevated in most acute and chronic forms of clinical and experimental lung injury, especially in fibrotic lung disorders, and it is for this reason that fibronectin is considered a sensitive marker of tissue injury (32, 48, 49). Unfortunately, the study of fibronectin has been hampered by the fact that knockout mutations of fibronectin lead to early embryonic lethality (18). Nevertheless, its functions have been surmised through many studies in vitro revealing the effects of fibronectin in diverse cell types. Fibronectin promotes the proliferation of fibroblasts, aids the deposition of collagen, stimulates the chemotaxis of monocytes and other immune cells, and induces cytokine production in macrophages (4, 10, 12, 20, 41, 42). With regard to the latter, we have reported that fibronectin induces the transcription factors activator protein-1 and NF-κB in human monocytic cells (50). These factors stimulate the transcription of genes coding for cytokines with proinflammatory capabilities, including TNF-α and interleukin-1β (10, 50). These and other observations have implicated fibronectin in complex processes involved in angiogenesis, oncogenesis, inflammation, and
wound healing (32, 49). Because fibronectin stimulates the proliferation and decreases the apoptosis of nonsmall cell lung carcinoma cells (21, 22), the present findings also suggest that increased expression of fibronectin in lung (as seen in tobacco-related and fibrotic chronic lung disorders) might provide a fertile substrate for the proliferation of tumors. This, among other factors, might help to explain the higher incidence of lung cancer found in patients with fibrotic lung diseases (57).

We (37) and others (16, 31, 54) have demonstrated that fibronectin induction in fibroblasts is dependent on distinct signals that include the activation of protein kinases and the induction of the transcription factor CREB. The growth factor transforming growth factor (TGF)-β1, phorbol esters, and serum, among other stimulants, also increase CREB (16, 37). In the current study, an oxidized extracellular Eh Cys/CySS was found to stimulate fibronectin expression through activation of PKC and induction of CREB. Other signals stimulated include induction of NF-κB and Smad3, transcription factors also known for their ability to promote matrix expression. These findings suggest that the extracellular Eh Cys/CySS state can stimulate intracellular signaling termed “redox signaling.” Redox signaling is known to control cellular growth and other processes through at least three different redox-dependent processes. One of these processes involves cellular GSH (23), whereas the second involves reactive oxygen species, which are generated during growth signaling in experiments activating receptor tyrosine kinases (30). The third process involves Cys and is the one tested here. Unlike the other two processes, it is extracellular, and it is mediated by the redox state of the Cys/CySS pool (24, 39). Cys is a precursor of GSH, and Cys or CySS can independently affect cell proliferation by altering the available Cys and CySS pool.

We also explored whether the stimulatory effects of Eh Cys/CySS were the result of indirect induction of soluble factors capable of stimulating fibronectin expression. We focused on TGF-β1 because it has been shown to be highly expressed in injured lungs and because it affects both cellular proliferation and matrix expression (7, 33). Our studies show that an oxidized Eh Cys/CySS stimulates TGF-β1 expression and that an antibody against TGF-β1 and a TGF-β1 receptor inhibitor blocked redox induction of fibronectin. This suggests that TGF-β1 mediates the effects of extracellular oxidized Eh Cys/CySS on fibronectin expression. This finding further strengthens our idea that the extracellular Eh Cys/CySS may modulate fibroblast phenotype since TGF-β1, considered a master switch for tissue remodeling, has been shown to promote myofibroblastic transdifferentiation (44, 58). In preliminary work, we have shown that an oxidized Eh Cys/CySS can promote α-smooth muscle actin expression in lung fibroblasts (unpublished observations).

It should be noted that, although the induction of fibronectin (and other variables) was consistent in fibroblasts cultured in the setting of oxidized Eh Cys/CySS (i.e., ~46 mV), we observed variations in the intensity of this response. We also observed some induction of fibronectin, albeit less, in the other redox states tested (~80 and ~131 mV), but these changes were not consistent. We believe that these findings can be explained by at least two sources of variation in our system. First, this work was conducted in primary lung fibroblasts. Such systems often show some variability when tested repeatedly. It is for this reason that we always conducted the studies multiple times and with the inclusion of appropriate controls. We also used cells between passages 2 and 8, since the variation seemed to intensify when using higher passage number cells. The second source of variability relates to the control of the redox state since it is difficult to consistently control the redox state over prolonged periods of time because of the many variables that affect it. Despite the above, our results are consistent in showing that the more oxidized the Eh Cys/CySS is, a more intense upregulation of fibroblast gene transcription is observed. Finally, although the studies performed with AMS suggest that Cys/CySS signaling results as a consequence of thiol/thiol interactions, it is possible that Cys/CySS themselves (not the redox potential) could also promote signaling events. This possibility cannot be entirely discarded at this point in time.

Having examined the effects of Eh Cys/CySS on fibronectin expression, we tested its effects on lung fibroblast proliferation. This finding might explain the fibroproliferative response observed in chronic lung fibrosing disorders. This stimulatory effect might represent a direct effect of redox signaling on genes involved in the control of the cell cycle or might have been induced indirectly through the induction and/or activation of growth factors. Because fibronectin was stimulated, fibronectin itself might have contributed to the observed fibroblast proliferation, since it has been shown to stimulate the proliferation of these and other lung cells (4).

The implications of these in vitro findings to the clinical arena are significant. In humans, physiological extracellular Eh Cys/CySS in healthy subjects is around ~80 mV, whereas, in subjects with disease, this redox may vary between ~62 to ~20 mV (2). The Jones group and others have documented oxidized Eh Cys/CySS in subjects with diets low in Cys, alcohol abuse, diabetes, and cigarette smoking (1, 8, 9, 25, 28). Because we have examined physiologically relevant redox potentials, one can postulate that oxidation of the Eh Cys/CySS (through deficient diet, drugs, disease) contributes to phenotypic alterations in lung fibroblasts and the generation of a microenvironment that promotes fibroproliferation and lung scarring. Further studies are needed to test the exact contribution of this process in the clinical arena and to determine whether interventions to normalize oxidized Eh Cys/CySS can have therapeutic effects.

In conclusion, we found that extracellular oxidant stress, through oxidation of the thiol/disulfide couple Cys/CySS, activates redox-sensitive pathways that stimulate the differential expression of genes that control the phenotype of fibroblasts in ways that enhance fibroblast proliferation and matrix deposition. This “altered” fibroblast deposits an aberrant matrix characterized by increased relative concentrations of fibronectin through upregulation of TGF-β1.

GRANTS
This work was supported by grants from the Roche Organ Transplant Research Foundation to A. Ramirez and Program Project Grant P50 AA 135757 to J. Roman.

REFERENCES


