Angiotensin converting enzyme-2 is protective but downregulated in human and experimental lung fibrosis

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Li X, Molina-Molina M, Abdul-Hafez A, Uhal V, Xaubet A, Uhal BD. Angiotensin converting enzyme-2 is protective but downregulated in human and experimental lung fibrosis. Am J Physiol Lung Cell Mol Physiol 295: L178–L185, 2008. First published April 25, 2008; doi:10.1152/ajplung.00009.2008.—Earlier work from this laboratory showed that local generation of angiotensin (ANG) II is required for the pathogenesis of experimental pulmonary fibrosis and that ANG peptides are expressed robustly in the lungs of patients with idiopathic pulmonary fibrosis (IPF). Angiotensin converting enzyme-2 (ACE-2) degrades the octapeptide ANG II to form the heptapeptide ANG1-7 and thereby limits ANG II accumulation. On this basis, we hypothesized that ACE-2 would be protective against experimental lung fibrogenesis and might be downregulated in human and experimental lung fibrosis. In lung biopsy specimens from patients with IPF, ACE-2 mRNA and enzyme activity were decreased by 92% (P < 0.01) and 74% (P < 0.05), respectively. ACE-2 mRNA and activity were also decreased similarly in the lungs of bleomycin-treated rats and C57-BL6 mice. In mice exposed to low doses of bleomycin, lung collagen accumulation was enhanced by intratracheal administration of either ACE-2-specific small interfering RNAs (siRNAs) or the peptide DX600, a competitive inhibitor of ACE-2 (P < 0.05). Administration of either ACE-2 siRNA or DX600 significantly increased the ANG II content of mouse lung tissue above the level induced by bleomycin alone. Coadministration of the ANG II receptor antagonist saralasin blocked the DX600-induced increase in lung collage. Moreover, purified recombinant human ACE-2, delivered to mice systemically by osmotic minipump, attenuated bleomycin-induced lung collagen accumulation. Together, these data show that ACE-2 mRNA and activity are severely downregulated in both human and experimental lung fibrosis and suggest that ACE-2 protects against lung fibrogenesis by limiting the local accumulation of the profibrotic peptide ANG II.

LOCAL TISSUE ANGIOTENSIN (ANG) generating systems have been shown to be critical regulators of fibrosis in the heart (23), kidney (10), and liver (27). Previous work from this laboratory has shown that experimental lung fibrosis in response to the antineoplastic agent bleomycin requires both angiotensinogen (AGT), the precursor of the octapeptide ANG II (15). Shortly after administration of bleomycin to rats by intratracheal instillation, ANG II levels in the lungs rise sharply, and the coadministration of ANG II receptor blockers prevents subsequent collagen accumulation in the lungs (13, 15). Consistent with those observations, studies of lung biopsy specimens from patients with idiopathic pulmonary fibrosis (IPF) have shown robust expression of AGT mRNA and protein in IPF lung tissue (11); these findings suggest that local ANG II generation is a key determinant of lung fibrosis in both human lung and animal models.

The biological half-life of the peptide ANG II is very short, on the order of 30 s to 15 min in the serum and local tissue compartments, respectively (4, 18). One reason for its short half-life is the abundant protease activities capable of degrading the peptide. One of these is the carboxypeptidase angiotensin converting enzyme-2 (ACE-2), which cleaves a single amino acid from the COOH-terminal end of ANG II to yield the heptapeptide ANG1-7 (9). ACE-2 is now known to be the receptor for binding and entry of the severe acute respiratory syndrome (SARS) coronavirus (11), which causes severe acute lung injury and death of many infected patients. Earlier studies of knockout mice have shown a clear protective effect of ACE-2 on experimental acute lung injury in response to acid aspiration or sepsis (9); the protective effect of ACE-2 was associated with reduced levels of ANG II after experimental lung injury.

On this basis, we hypothesized that ACE-2 might be protective against experimental lung fibrosis through its ability to degrade ANG II produced locally in response to bleomycin. We further theorized that the pathogenesis of lung fibrosis might involve the downregulation of ACE-2 as an integral component of the sequence of the events leading to lung collagen deposition. We report here the findings that ACE-2 mRNA, protein, and enzymatic activity are severely decreased in both human and experimental lung fibrosis. Moreover, we show that in vivo gene silencing of ACE-2 enhances bleomycin-induced lung collagen deposition in mice, whereas systemic administration of purified ACE-2 inhibits the fibrotic response.

METHODS

Materials. The fluorogenic peptide substrate MCA-YVADAPK(Dnp)-OH and purified human recombinant ACE-2 were obtained from R&D Systems (Minneapolis, MN). The peptide DX600 was synthesized by CelsTek Peptides (Nashville, TN). Antibodies against ACE-2 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Bleomycin and saralasin were purchased from Sigma-Aldrich (St. Louis, MO). Random sequence RNA and small interfering RNA...
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(sirNA) against ACE-2 were purchased from Santa Cruz Biotechnology. All other materials were of reagent grade.

**Human tissue samples and handling.** Human lung tissue was obtained by open lung biopsy or video-assisted thoracoscopic surgery from patients undergoing diagnostic evaluation at Instituto del Tórax, Hospital Clínica de Barcelona, Spain. Fibrotic lung tissue was obtained from eight patients with IPF, and lung biopsies were performed in more than one lobe in each patient. All patients had clinical, functional, and radiological features that fulfill the diagnostic criteria for IPF (1). The IPF patients had neither antecedents of any occupational or environmental exposure nor any other known cause of interstitial lung disease. None of the patients was treated with steroids or other immunosuppressant therapy at the time of sample collection. Normal human lung tissue was obtained from individuals undergoing surgical treatment for spontaneous pneumothorax with no history of pulmonary disease. No histopathological evidence of any abnormality was found in these tissue samples. Written informed consent was previously obtained from all individuals included according to institutional guidelines, and the study was approved by the Ethics Committee of Hospital Clinic de Barcelona.

Tissue samples were shock frozen in liquid nitrogen immediately after resection and were stored at −80°C until use. For ACE-2 enzyme assay, thawed tissue samples were used immediately to retain full activity. Protein and mRNA were extracted from frozen biopsy samples by direct homogenization in Nonidet P-40 lysis buffer or TRIzol.

**Animals, induction of pulmonary fibrosis, and surgical procedures.** Wild-type C57BL/6J mice were obtained from The Jackson Laboratories (Bar Harbor, ME); male Wistar rats were purchased from Charles River Laboratories (Wilmington, MA). Animals were housed in a satellite facility of University Laboratory Animal Resources, Michigan State University, and were used at 7–8 wk of age.

**Induction of lung fibrosis and administration of blockers or enhancers.** Animals under pentobarbital anesthesia received a single intratracheal instillation of bleomycin sulfate (BLEO) in 50 μl of sterile saline. In a preliminary dose-response study, a dose of 1 unit per kilogram body weight was chosen as a subfibrotic load of this particular lot of bleomycin. The 50-μl solution was instilled at end expiration, and the Bleomycin was followed immediately by 300 μl of air to insure delivery to the distal airways. Control animals were instilled with an equal volume of sterile saline. In some studies, the ACE-2 competitive inhibitor DX600 (100 μM) or the ANG receptor blocker saralasin (50 μM) was added to the intratracheal instillate with the bleomycin and also reinstilled every other day thereafter for 14 days.

For in vivo gene silencing, 10 μg of the ACE-2 sirRNAs or scrambled-sequence control RNAs were dissolved in 50 μl of sterile saline. In a preliminary dose-response study, a dose of 1 unit per kilogram body weight was chosen as a subfibrotic load of this particular lot of bleomycin. The 50-μl solution was instilled at end expiration, and the Bleomycin was followed immediately by 300 μl of air to insure delivery to the distal airways. Control animals were instilled with an equal volume of sterile saline. In some studies, the ACE-2 competitive inhibitor DX600 (100 μM) or the ANG receptor blocker saralasin (50 μM) was added to the intratracheal instillate with the bleomycin and also reinstilled every other day thereafter for 14 days.

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**RNA isolation RT-PCR.** Total RNA was extracted from human lung biopsies or frozen mouse lung with TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. First-strand cDNA was synthesized from 2 μg of total RNA with Superscript II reverse transcriptase (Invitrogen) and oligo(dT)12-18. Real-time RT-PCR was carried out with cDNA synthesized from 50 ng of total RNA, SYBR Green PCR core reagents (Applied Biosystems, Foster City, CA) according to the manufacturer’s protocol, and 0.2 μM specific primers for human ACE-2 (sense 5′-CATTTGAGCAAGTTGTGGATCTTT-3′; and antisense 5′-GAGCTA-ATGCGTCCA-TTCTCA-3′) and β-actin (sense 5′-AGGCCAACCGCG-AGAAGATGACC-3′ and antisense 5′-GAAGTCCAGGGC-GAGACGTC-3′). For mouse ACE-2, the primers were: sense 5′-GGATAACC AGCTCTCCTTACATAGC-3′ and antisense 5′-CTACACCCAGTAGCATACCAAGA GCA-3′, and for mouse β-actin, sense 5′-TCTGGTGCATCATGAAACT-3′ and antisense 5′-CTTCGTAAGCAAGCCACGTGCTA-3′. The PCR thermal profile started with 10-min activation of Taq polymerase at 95°C followed by 40 cycles of denaturation at 94°C for 60 s, annealing at 55°C for 60 s, and extension at 72°C for 60 s, ending with dissociation curve analysis to validate the specificity of the PCR products. Reactions were performed in a Mx3000P machine (Stratagene, La Jolla, CA), and threshold cycle (Ct) data were collected with MxPro-Mx3000P software version 3.0. The relative ACE-2 expression was normalized to β-actin and calculated with the comparative CT method of 2^−ΔΔCT. In all figures, the mean value for the ACE-2/β-actin ratio in the control group was set to 100% and was expressed relative to the ACE-2/β-actin ratio for the treatment group.

**Western blotting.** Human or mouse lung tissue was homogenized in ice-cold Tris-HCl buffer, supplemented with protease inhibitor (Protease Inhibitor Cocktail P840, Sigma). Soluble protein extracts (20 μg) were loaded and run on 10% Tris-HCl polyacrylamide gels, separated by SDS-PAGE, in 10× Tris/glycine/SDS buffer (BioRad). Gels were transferred to Immuno-blot PVDF blotting membrane (Bio-Rad) in Towbin buffer. Blotting membrane was blocked by 5% nonfat dry milk in 0.1% Tween 20 in Tris-buffered saline. Western blot analysis of ACE-2 was performed with anti-ACE-2 polyclonal antibody (1:1000 dilution; Santa Cruz Biotechnology). Bands were visualized by HRP-conjugated donkey anti-goat secondary antibody (1:2000 dilution; Santa Cruz Biotechnology) using the chemiluminescent substrate SuperSignal West Femto Maximum Sensitivity (Pierce, Rockford, IL). To ensure equal loading of proteins, membranes were stripped and then reprobed with an antibody against β-actin (Cell Signaling Technology).

**ACE-2 enzyme assay.** Protein was extracted from human biopsy samples or from mouse lung by homogenization in ice-cold Tris-HCl EDTA-free buffer, pH 6.5 (19, 26). The enzymatic activity of ACE-2
proteins was measured immediately after homogenization by the cleavage of fluorogenic substrate MCA-YVADAPK at 10 μM, in 45 μl of lung tissue homogenate with Tris-HCl buffer, pH 6.5, containing lisinopril (50 μg/l) to block ACE activity (26). Reactions were performed in black microtiter plates at room temperature within a fluorescence microplate reader (FL600 Biotec Fluorescence Reader; BMG, Durham, NC) during 30 min using excitation and emission wavelengths of 310/20 and 420/50 nm.

Quantitation of lung collagen. Tissues frozen in liquid N2 were dried to constant weight in preweighed tubes at 80°C. The weighed dry tissue was hydrolyzed in 6 N HCl and was subjected to determination of hydroxyproline as described earlier by Woessner (25). The efficiency of the hydrolysis was verified with rat tail collagen by comparison to standard hydroxyproline (Sigma).

RESULTS

ACE-2 assay. The fluorogenic substrate commonly used to detect ACE-2 activity also detects interleukin-1β converting enzyme (ICE; 3, 19), an enzyme that was previously shown to be activated in alveolar epithelial cells (AECs) by Fas ligand or ANG II (21). For this reason, it was essential to assay ACE-2 under conditions that do not detect ICE activity. Figure 1A shows enzyme assay of ACE-2 with the fluorogenic peptide substrate MCA-YVADAPK (26) under assay conditions that minimized the codetection of ICE. In vivo experiments discussed below make use of a peptide inhibitor of ACE-2, DX600; in Fig. 1B, the synthetic peptide DX600, a competitive inhibitor of ACE-2 (7), completely inhibited ACE-2 activity in homogenates of mouse lung with IC50 of 100 nM. These data establish that the MCA substrate is indeed detecting DX600-inhibitable ACE-2 in mouse lung.

ACE-2 in human lung fibrosis. It was hypothesized that ACE-2 activity would be reduced in human lung fibrosis and in animal models of lung fibrosis. Lung biopsy specimens were obtained at Hospital Clinic of Barcelona, Spain, from patients with IPF and from nonfibrotic controls (see METHODS for details). Table 1 reports clinical data from the patients from whom lung biopsy specimens were obtained. The Control and IPF patient groups were not significantly different with respect to age, smoking history, and sex, but IFP patients exhibited significantly decreased DLCO (diffusing capacity for carbon monoxide), KCO (DLCO normalized to lung volume), and increased A-a Po2 gradient (alveolar-arterial oxygen gradient), all indicative of advanced diffusion impairment. Figures 2, A–C, show that ACE-2 mRNA, immunoreactive protein, and enzymatic activity, respectively, are severely decreased by 92%, 71%, and 74%, respectively, in lung tissue from patients with IPF relative to nonfibrotic controls.

ACE-2 in experimental lung fibrosis. ACE-2 was also severely decreased in experimental lung fibrosis. Figure 3, A and B, shows decreases in ACE-2 enzyme activity in the lungs of Wistar rats (Fig. 3A) or C57/BL6 mice (Fig. 3B) after intratracheal bleomycin administration. Together with Fig. 2, these data show that ACE-2 is decreased in lung fibrogenesis in three different species and also suggest that the decrease in ACE-2 is progressive over time (Fig. 3A). In Fig. 4, the decreased ACE-2 activity in mouse lung was associated with a quantitatively similar decrease in ACE-2 mRNA (82%, Fig. 4A) and reduced ACE-2 immunoreactive protein (Fig. 4B).

Effect of ACE-2 inhibition or knockdown on experimental lung fibrosis. To test the role of ACE-2 in experimental lung fibrosis, ACE-2-specific siRNAs were tested for the ability to silence ACE-2 expression and enzymatic activity in mouse lung. Figure 5 shows significant decreases in ACE-2 immunoreactive protein (Fig. 5A) and total lung ACE-2 enzyme activity (Fig. 5B) in lung homogenates obtained 25 h or 7 days after intratracheal administration of the siRNAs.

In Fig. 6, the effect of ACE-2 inhibition by peptide DX600 on lung fibrogenesis in vivo was evaluated in the presence of a subfibrotic dose of bleomycin; a low bleomycin dose was chosen to maximize the possibility of detecting an effect of ACE-2 blockade that might be additive or synergistic to that of bleomycin alone. Figure 6A shows that the ACE-2 competitive inhibitor peptide DX600, administered intratracheally together with a low dose of bleomycin, enhanced bleomycin-induced lung collagen deposition in mice (P < 0.05). Coadministration of the ANG II receptor antagonist saralasin (SAR) blocked the enhancing effect of the DX600. Figure 6, B–E, shows histological changes in the lungs of mice treated identically to those used to generate Fig. 6A. The low dose of bleomycin alone (Fig. 6C) caused small and focal lesions that did not increase lung collagen significantly (see hydroxyproline data in Fig. 6A), and the DX compound alone caused no apparent lesions.

Fig. 1. Assay of angiotensin converting enzyme-2 (ACE-2) enzyme activity and inhibition by peptide DX600. A: purified recombinant human ACE-2 was assayed in a Tris-HCl buffer system (19) with the fluorogenic substrate MCA-YVADAPK at 10 μM (see METHODS). Similar amounts of purified ACE-2 and interleukin-1β converting enzyme (ICE) were assessed under the same assay conditions; note failure of assay to detect ICE in the buffer system used. Bars are the means ± SE. B: blockade of mouse lung ACE-2 with a synthetic peptide inhibitor. ACE-2 activity in mouse lung homogenates was assayed in the presence or absence of the synthetic peptide DX600 (7), a competitive inhibitor of ACE-2 (see METHODS). Note essentially complete inhibition at 10−6 M DX600. Bars are the means of 2 replicates.
ACE-2 is protective but downregulated in lung fibrosis

**DISCUSSION**

Lentiviral overexpression of the enzyme ACE-2 has been shown to protect against experimental cardiac fibrosis in response to ANG II infusion (8) or as a consequence of hypertension in spontaneously hypertensive rats (2). ACE-2 and its product ANG1-7 also have been speculated to play protective roles in liver fibrosis (21). To our knowledge, the present study is the first to demonstrate a protective role for ACE-2 in pulmonary fibrosis and is the first to document severe downregulation of ACE-2 in human IPF and in two animal models of this disease.

In experimental fibrosis models involving organs other than the lungs, ACE-2 has been reported to be either upregulated or (not shown). In contrast, the addition of DX600 to the low-dose bleomycin (Fig. 6D) increased the extent and severity of lung lesions, which contained mononuclear infiltrates and thickened alveolar septa throughout the lung parenchyma. Addition of saralasin to the bleomycin + DX600 treatment (Fig. 6E) reduced the extent and severity of lesions to a level similar to that caused by low-dose bleomycin alone (Fig. 6C).

In Fig. 7, the siRNAs used in Fig. 5 to silence ACE-2 were administered in vivo in a manner similar to that for DX600 in Fig. 6, except that intratracheal administrations were facilitated by using mouse lung surfactant as vehicle (see METHODS). In Fig. 7A, the low dose of bleomycin, which did not increase lung collagen in Fig. 6, significantly increased lung hydroxyproline content when administered here in the mouse lung surfactant vehicle (P < 0.05). More importantly, the ACE-2 siRNAs enhanced bleomycin-induced mouse lung collagen accumulation, whereas random-sequence control RNAs did not (siCTL, Fig. 7A). Figure 7, B–E, shows histological changes in the lungs of mice treated identically to those shown in Fig. 7A. The instillation of siRNAs against ACE-2 together with bleomycin (Fig. 7D) caused more extensive lesions than those induced by the bleomycin + RNA vehicle alone (Fig. 7C), but control siRNAs did not increase the extent or severity of lung lesions (Fig. 7E).

**Effect of ACE-2 inhibition or knockdown on lung ANG II levels.** Earlier work from this laboratory showed that bleomycin increases local ANG II synthesis in the lungs (14, 15). In Fig. 8, steady-state ANG II levels were increased further by the addition of either the ACE-2 inhibitor DX600 (Fig. 8A) or the ACE-2-specific siRNAs (Fig. 8B) to low doses of bleomycin that were applied, respectively, to cultured lung epithelial cells or to whole mouse lung by intratracheal administration.

**Effect of systemic administration of ACE-2 on experimental lung fibrosis.** Others have shown that systemic administration of purified ACE-2 is protective against acute lung injury (9). To determine if administration of ACE-2 might protect against bleomycin-induced lung fibrosis, purified ACE-2 was administered to mice by osmotic minipump. Figure 9 shows that recombinant human ACE-2, when administered systemically by subcutaneous minipump at a dose of 20 μg·kg⁻¹·day⁻¹, reduced bleomycin-induced lung collagen accumulation to a value not significantly different from the control.

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**Table 1. Clinical data from patients donating lung biopsies**

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<th>Parameter</th>
<th>Control Patients</th>
<th>IPF Patients</th>
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<tr>
<td>Age, yearsa</td>
<td>45.0 ± 2.0</td>
<td>59.9 ± 9.4</td>
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<td>Sex, % male</td>
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<td>62.7</td>
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<tr>
<td>FVC, %predicted</td>
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<td>97.7 ± 3.5</td>
<td>71.7 ± 24.3</td>
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<td>DLCO, %</td>
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<td>45.6 ± 13.7b</td>
</tr>
<tr>
<td>KCO, %</td>
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<td>62.4 ± 17.5c</td>
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<td>29.7 ± 10.4d</td>
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<td>Smoking, %</td>
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<td>37.5</td>
</tr>
<tr>
<td>n</td>
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<td>7</td>
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*All data are the means ± SD. *P < 0.0005 vs. controls by unpaired t-test. *P < 0.03 vs. controls by unpaired t-test. *P < 0.02 vs. controls by unpaired t-test. *S*Smoking, % is the % of patients with a history of smoking. IPF, idiopathic pulmonary fibrosis. *Clinical data were available on only 3 control patients, but more than three donated biopsies.
downregulated during disease pathogenesis. In a rat model of biliary fibrosis induced by bile duct ligation (6), experimental collagen deposition in the liver was associated with upregulation of hepatic ACE-2 mRNA and enzyme activity. In contrast, in an adult pig model, the cardiac fibrosis associated with experimental pacing-induced atrial fibrillation was associated with downregulation of ACE-2 mRNA and protein (16). Although the degree of decline in ACE-2 activity in response to atrial fibrillation was not as great as that shown here, that study is similar to the present report in its demonstration of decreases in both ACE-2 mRNA and protein during the development of the fibrotic response. However, the mechanisms that might be responsible for decreased ACE-2 mRNA and activity are poorly understood.

To date, only one study has reported examinations of the molecular control of ACE-2 gene expression. In a study of cultured rat astrocytes, Gallagher et al. (5) showed that exposure to ANG II could decrease ACE-2 mRNA by 60% in astrocytes isolated from cerebellar or medullary but not other brain regions. The decrease in ACE-2 mRNA was blocked by ANG1-7 through its receptor mas; this finding implied the existence of an intriguing feedback system involving ANG II and its degradation product ANG1-7 in the brain. Although the decrease in ACE-2 mRNA by ANG II was suggested to be the result of repression of ACE-2 gene transcription, no measurements of ACE-2 mRNA stability or transcription rate were offered to support that theory.

The molecular mechanism that downregulates ACE-2 in lung cells may also be mediated by ANG II; earlier work showed that intratracheal bleomycin administration rapidly increases local pulmonary ANG II concentrations (15). Moreover, earlier work by Li et al. (14) showed that exposure of
lung AECs to bleomycin in vitro activates angiotensinogen gene transcription and secretion of the mature peptide ANG II into the extracellular space. The present finding that ACE-2 inhibition further increases extracellular ANG II in primary cultures of AECs (Fig. 8A) supports the theory that ACE-2 limits the accumulation of ANG II produced by AECs in response to bleomycin. Recent studies by Weiner et al. (24) showed that AECs are the main source of lung tissue ACE-2 in the mouse. Whether or not the decrease in ACE-2 mRNA induced by bleomycin is mediated by autocrine production of ANG II (14) will be an interesting and compelling question for further study.

Fig. 6. In vivo inhibition of pulmonary ACE-2 with peptide DX₆₀₀ enhances experimental lung collagen accumulation and fibrogenesis by a mechanism dependent on ANG II receptors. A: peptide DX₆₀₀ (DX) or the ANG II receptor blocker saralasin (SAR) were instilled intratracheally into mice together with a low dose of BLEO or sterile saline vehicle (CTL). DX and SAR were also reinstitled every other day thereafter. Fourteen days later, lung hydroxyproline (HP) content was measured in whole lung homogenates. Note the failure of low-dose BLEO alone to increase HP, in contrast to the significant increase in the presence of DX that was blocked by SAR. Bars are the means ± SE of at least 3 animals; *P < 0.05 vs. CTL by ANOVA and Student-Newman-Keuls test. B–E: mice were treated as described in A with bleomycin, saline vehicle, ACE-2 inhibitor, or saralasin, all administered intratracheally. Fourteen days later, histology was performed as described in METHODS. B: normal mouse lung control. C: low-dose bleomycin alone, administered intratracheally. D: bleomycin + DX₆₀₀ inhibitor. E: bleomycin + DX₆₀₀ + saralasin.

Fig. 7. In vivo silencing of pulmonary ACE-2 with siRNA enhances experimental lung collagen accumulation and fibrogenesis. A: specific siRNAs against ACE-2 (siACE2) or scrambled-sequence control RNAs (siCTL) were dissolved in purified mouse lung surfactant and instilled intratracheally into mouse lungs together with BLEO or saline. The siACE2 and siCTL were reinstitled 7 days later. Fourteen days after BLEO administration, mice were killed for measurement of lung HP as in Fig. 6. Note the enhancement of BLEO-induced lung HP accumulation by siACE2 but not by siCTL. Bars are the means ± SE of at least 5 animals; *P < 0.05 and **P < 0.01 vs. CTL by ANOVA and Student-Newman-Keuls test. B–E: mice were treated as described in A with bleomycin, vehicle, or ACE-2 inhibitors, all administered in surfactant intratracheally (see METHODS). Fourteen days later, histology was performed as described in METHODS. B: normal mouse lung control. C: low-dose bleomycin, delivered in surfactant intratracheally. D: bleomycin + siACE2 inhibitor. E: bleomycin + control siRNA.
The enhancement of bleomycin-induced lung collagen deposition by inhibition or silencing of ACE-2 in mouse lung (Figs. 6 and 7) strongly suggests a protective role for local pulmonary ACE-2 in lung fibrogenesis. Moreover, the ability of the ANG II receptor nonselective antagonist saralasin to block the enhancement of collagen deposition is mediated by a mechanism dependent on ANG II and its receptors. This theory is further supported by the findings that either ACE-2 inhibition or gene silencing could increase the steady-state ANG II concentration to a level above that attained in response to bleomycin alone (Fig. 8). Although inhibition of ACE-2 activity in vivo was not fibrogenic by itself, it did induce fibrosis in the presence of a low dose of bleomycin (Fig. 6A); this finding might be explained by the ability of bleomycin to activate gene transcription of angiotensinogen (14) and thereby supply the substrate required for increased ANG II generation in the immediate microenvironment of the AEC.

The notion that ACE-2 protects against the profibrotic effect of bleomycin is further supported by the trend of systemically administered recombinant human ACE-2 to affect bleomycin-induced collagen deposition in mice (Fig. 9), which was reduced by rhACE-2 to a value not significantly different from the control. On the other hand, the lung collagen level observed with rhACE-2 administration was not significantly different from that observed for bleomycin alone, and therefore the success of the ACE-2 administration protocol tested here is of questionable significance. The modest attenuating effect of purified ACE-2 might be due to an ACE-2 dose that was too low; the dose used was estimated on the basis of earlier work in which systemically delivered purified ACE-2 inhibited acid aspiration-induced acute lung injury (9). In that study, a single dose of ACE-2 (100 µg/kg) was administered intraperitoneally; in the present study, the minipumps were loaded to deliver \( -20 \mu g \cdot kg^{-1} \cdot day^{-1} \) over 16 days. In light of these differences in dosing and other factors affecting bioavailability, future experiments testing local delivery (intratracheal) or overexpression of ACE-2 might show greater efficacy at blocking experimental lung fibrosis, particularly if targeted to AECs. For relevance to IPF patients, delayed administration of ACE-2 (i.e., after the onset of lung fibrogenesis) might also need to be considered.

On the basis of the findings reported here and previously, several potential mechanisms might be envisioned to mediate the progression from alveolar epithelial injury to fibrosis in the lungs. Previous work has shown that apoptosis inducers, both xenobiotic (14) and endogenous (20), upregulate angiotensinogen expression in AECs and increase secretion of ANG II, which itself is proapoptotic for AECs (21). The induction of apoptosis in AECs could, by itself, reduce total lung ACE-2 activity because AECs are believed to be the major site of ACE-2 expression (24), at least in adult mouse lung. In addition, ANG II has been shown to reduce ACE-2 mRNA expression in astrocytes (5), which raises the possibility that autocrine production of ANG II might further downregulate ACE-2 expression by AECs independently of its effect on apoptosis. Whether or not ANG II directly influences ACE-2 expression by AECs independently of its effect on apoptosis inducers, both xenobiotic and endogenous, upregulate angiotensinogen expression in AECs and increase secretion of ANG II, which itself is proapoptotic for AECs (21). The induction of apoptosis in AECs could, by itself, reduce total lung ACE-2 activity because AECs are believed to be the major site of ACE-2 expression (24), at least in adult mouse lung. In addition, ANG II has been shown to reduce ACE-2 mRNA expression in astrocytes (5), which raises the possibility that autocrine production of ANG II might further downregulate ACE-2 expression by AECs independently of its effect on apoptosis. Whether or not ANG II directly influences ACE-2 expression by AECs independently of its effect on apoptosis inducers, both xenobiotic and endogenous, upregulate angiotensinogen expression in AECs and increase secretion of ANG II, which itself is proapoptotic for AECs (21). The induction of apoptosis in AECs could, by itself, reduce total lung ACE-2 activity because AECs are believed to be the major site of ACE-2 expression (24), at least in adult mouse lung. In addition, ANG II has been shown to reduce ACE-2 mRNA expression in astrocytes (5), which raises the possibility that autocrine production of ANG II might further downregulate ACE-2 expression by AECs independently of its effect on apoptosis.

**Fig. 8.** Inhibition or silencing of ACE-2 increases steady-state ANG II in vitro by BLEO alone. Bars are the means ± SE of at least 3 cultures; \(* P < 0.05\) vs. BLEO by ANOVA and Student-Newman-Keuls test. B: C57/BL6 mice received intratracheal instillations of BLEO, siACE2, or siCTL as in Fig. 7. Fourteen days later, ANG II was measured in whole lung homogenates by specific ELISA (see METHODS). Bars are the means ± SE of at least 5 animals; \(* P < 0.05\) vs. BLEO by ANOVA and Student-Newman-Keuls test.

**Fig. 9.** Systemic administration of purified ACE-2 inhibits experimental lung fibrosis. C57/BL6 mice received a single intratracheal instillation of BLEO (2 U/kg) or saline vehicle (CTL). Two days earlier, all mice were implanted with a subcutaneous minipump that contained either sterile saline or purified recombinant human ACE-2 (see METHODS). Fourteen days after BLEO administration, mice were killed for measurement of lung collagen by HP assay. Bars are the means ± SE of at least 4 animals; \(* P < 0.01\) vs. CTL; N.S., not statistically significant vs. CTL or BLEO by ANOVA and Tukey-Kramer multiple comparisons test.
mRNA and protein in AECs independently of apoptosis is currently under investigation.

The proapoptotic and profibrotic effects of ANG II in the lungs and other organs have been discussed earlier (17, 23, 27) and include AT1 receptor-mediated transactivation of caspases in epithelial cells and TGF-β1 and collagen gene expression in fibroblasts and other mesenchymal cells. These profibrotic effects of ANG II have been suggested by other authors to be a common final pathway in fibrogenesis of many organs (22, 23, 27). This pathway and the findings reported herein suggest the existence of a complex feedback system in which high ACE-2 expression normally limits local pulmonary ANG II generation, but also suggest that ACE-2 and its protective effects can be downregulated by proapoptotic and profibrotic stimuli including ANG II itself. Together, these data suggest a critical role for ACE-2 in lung fibrogenesis.

In summary, ACE-2 mRNA, protein, and enzymatic activity were severely decreased in human IPF and in both rat and mouse models of experimental lung fibrosis induced by bleomycin. In vivo inhibition or gene silencing of ACE-2 in mice increased pulmonary ANG II levels and enhanced bleomycin-induced lung fibrosis through a mechanism dependent on ANG II receptors. In contrast, systemic administration of purified recombinant ACE-2 reduced bleomycin-induced lung collagen deposition to a value not different from the control. These data indicate that ACE-2 protects against lung fibrogenesis by limiting local accumulation of ANG II. They also suggest that downregulation of ACE-2 may be a critical profibrotic event in IPF. The molecular mechanisms responsible for the loss of ACE-2 in experimental lung fibrosis are currently under investigation.

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