Enalapril protects mice from pulmonary hypertension by inhibiting TNF-mediated activation of NF-κB and AP-1

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Objectives—Enalapril protects mice from pulmonary hypertension by inhibiting TNF-mediated activation of NF-κB and AP-1.

Methods—Endotracheal instillation of bleomycin in mice results in pulmonary fibrosis. Pulmonary arterial pressure (PAP) was 26.4 ± 2.5 mmHg (P < 0.05) vs. 15.2 ± 3 mmHg, respectively. Bleomycin induced significant pulmonary hypertension in C57BL/6, but not in BALB/c, mice; average pulmonary arterial pressure (PAP) was 26.4 ± 2.5 mmHg (P < 0.05) vs. 15.2 ± 3 mmHg, respectively. Bleomycin treatment induced activation of nuclear factor (NF)-κB and activator protein (AP)-1 and enhanced collagen and TNF mRNA expression in the lung of C57BL/6 but not in BALB/c mice. Double TNF receptor-deficient mice (in a C57BL/6 background) that do not activate NF-κB or AP-1 in response to bleomycin did not develop bleomycin-induced pulmonary hypertension (PAP 14 ± 3 mmHg). Treatment of C57BL/6 mice with enalapril significantly (P < 0.05) inhibited the development of pulmonary hypertension after bleomycin exposure. Enalapril treatment inhibited NF-κB and AP-1 activation, the enhanced TNF and collagen mRNA expression, and the deposition of collagen in bleomycin-exposed C57BL/6 mice. These results suggest that ACE inhibitor treatment decreases lung injury and the development of pulmonary hypertension in bleomycin-treated mice.

Results—Enalapril significantly alters survival (47). In these disorders, pulmonary vascular pathology characterized by concentric intimal fibrosis, medial hypertrophy, and adventitial fibrosis can be identified (47). Traditionally, it has been assumed that hypoxic vasoconstriction is responsible for the development of pulmonary hypertension associated with ILD (47). Growing evidence suggests that the upregulation of cytokines and growth factors in the lung contribute to the development of the pulmonary hypertension associated with these diseases (47).

Bleomycin induction of lung injury in mice is a well-established model of ILD, resulting in pulmonary fibrosis (14, 43). Endotracheal instillation of bleomycin in mice is followed by upregulated expression of lung cytokines, development of lung inflammation, and accumulation of collagen in the lung (14, 43). Among the cytokines upregulated in the lung of bleomycin-treated mice, tumor necrosis factor (TNF)-α plays a fundamental role in the pathogenesis of bleomycin-induced pulmonary fibrosis (32, 36, 37). The upregulation of TNF expression correlates with the murine strain sensitivity to bleomycin (32). Inbred mice of the C57BL/6 strain, which are sensitive to bleomycin, upregulate lung TNF expression, develop lung inflammation, and accumulate collagen in their lung in response to bleomycin exposure (32–34). In contrast, BALB/c mice, a strain resistant to bleomycin, do not upregulate lung TNF expression or develop pulmonary fibrosis in response to bleomycin (32–34).

ANG II is the product of the proteolytic cleavage of ANG I by angiotensin-converting enzyme (ACE). Increased concentrations of ANG II are found in bleomycin-treated or irradiated animals preceding the development of fibrosis (20, 44). Elevated levels and activity of ACE have been found in serum and bronchoalveolar lavage fluid of patients with fibrotic lung diseases such as sarcoidosis, asbestosis, silicosis, and IPF (8, 19, 45) and in animal models of pulmonary hypertension (3, 24). The biological effects of enhanced ACE expression

PULMONARY HYPERTENSION is a prominent feature of interstitial lung diseases (ILD), including systemic sclerosis, granulomatous diseases, and idiopathic pulmonary fibrosis (IPF) (47). In ILD, the presence of pulmonary hypertension significantly alters survival (47). In these disorders, pulmonary vascular pathology characterized by concentric intimal fibrosis, medial hypertrophy, and adventitial fibrosis can be identified (47). Traditionally, it has been assumed that hypoxic vasoconstriction is responsible for the development of pulmonary hypertension associated with ILD (47). Growing evidence suggests that the upregulation of cytokines and growth factors in the lung contribute to the development of the pulmonary hypertension associated with these diseases (47).

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in the injured lung appear to be the result of a local rather than a global action (38, 41). This is suggested by data demonstrating isolated enhanced expression of the ACE gene and protein at the site of cell proliferation in the intima of pulmonary arteries of patients with pulmonary hypertension (38, 41).

In animal models of pulmonary hypertension, the inhibition of ACE attenuates the fibroproliferative effects of chronic hypoxia and partially reverses the vascular neointimal formation and smooth muscle cell proliferation (27, 29). The mechanisms responsible for the ability of ACE inhibitors to decrease the fibroproliferative reaction observed in pulmonary hypertension are not completely understood. Recently, we have shown that bleomycin-induced lung injury is characterized by nuclear factor (NF-κB) activation in mouse lung (30). This enhanced NF-κB activation appears to promote expression of inflammatory mediators and collagen deposition, and interventions that inhibit NF-κB activation have been shown to ameliorate bleomycin-induced lung injury (7, 9). Recent studies have shown that ACE inhibitors are capable of regulating the activation of the NF-κB transcription factor in animal models of atherosclerosis (15).

Therefore, we hypothesized that ACE inhibition would decrease activation of transcription factors and inhibit TNF and collagen expression in the mouse lung, thus ameliorating bleomycin-induced lung injury and pulmonary hypertension. In the present investigation, we studied the effect of enalapril on the activation of the transcription factors NF-κB and activator protein (AP)-1, the expression of TNF and α1(I)-procollagen mRNA, the induction of inflammation, and the accumulation of collagen in the lung of bleomycin-treated mice. We have also measured pulmonary hemodynamics, by right heart catheterization, in intact spontaneously breathing mice.

**METHODS**

**Chemicals.** Stock solutions (5 U/ml) of bleomycin (Blenoxan; kindly donated by Bristol-Meyer-Squibb Pharmaceuticals, Princeton, NJ) were prepared immediately before use with endotoxin-free water. All other chemicals were of the highest grade commercially available (specific vendors and their locations denoted).

**Animals.** Animal protocols were approved by the Tulane University Committee on the Use and Care of Animals. Specific pathogen-free female C57BL/6 and BALB/c (Charles River Laboratories, Kingston, NY) mice weighing 20–25 g (6–10 wk old) were housed in pathogen-free cabinets and provided with water ad libitum. Mice genetically deficient in both p55 and p75 receptors [p55(−/−)-p75(−/−)] were generated, on a C57BL/6 genetic background, by gene targeting in embryonic stem cells at Immunex (Seattle, WA) and have been described previously (33, 35).

**Bleomycin exposure.** Animals were anesthetized with intraperitoneal tribromoethanol (250 mg/kg; Aldrich, Milwau-kee, WI) and exposed to bleomycin as previously described (33). Briefly, the trachea was exposed, and 4 U/kg bleomycin in 0.05 ml of 0.9% NaCl was slowly instilled in the tracheal lumen. Control mice received the same volume of sterile saline. After exposure, the skin incision was closed, and the animals were allowed to recover on a warming plate. Hemodynamic evaluations were performed 14 days after bleomycin exposure (33). After hemodynamic evaluation, the thorax was opened, and the descending aorta was severed. The left lungs were removed and stored at −80°C for subsequent analysis. The right lungs were perfused with formalin in situ as described below.

**Enalapril treatment.** In preliminary studies, a dose of 5 mg/kg enalapril (Merck, West Point, PA) was found to attenuate 95% of the systemic and pulmonary vascular pressor response to ANG I (dose range 0.1–1 μg/kg iv). Enalapril was given daily, at a dose of 5 mg/kg as an intraperitoneal injection, for 14 days after bleomycin exposure. The first enalapril dose was given 1 h after the endotracheal administration of bleomycin.

**Measurements of pulmonary vascular responses and cardiac output.** The techniques for measurement of pulmonary hemodynamics have been described previously (4). After administration of bleomycin or vehicle (14 days), mice were anesthetized with thiopentobarbital (85–95 μg/g ip) and ketamine (3 μg/g ip) and placed on a thermoderegulated surgical table. Body temperature was monitored and maintained at 37°C with a water-jacketed heating blanket. The trachea was cannulated (PE-90 tubing) to maintain a patent airway, and the animals breathed air enriched with 95% O2-5% CO2. A femoral artery was cannulated for the measurement of systemic arterial pressure. Systemic arterial pressure was measured with a Viggo-Spectramed transducer (Viggo Spectramed, Oxnard, CA) attached to a polygraph (model 7; Grass Instruments, Quincy, MA). Heart rate was monitored electronically from the systolic pressure pulses with a tachometer (model 7P44A; Grass).

Pulmonary arterial pressure (PAP) was measured in anesthetized mice with the use of a single-lumen catheter (NuMed, Hopkinton, NY). The catheter (145 mm in length, 0.25 mm OD) has a specially curved tip to facilitate passage through the right heart, main pulmonary artery, and the left or right pulmonary artery. Immediately after placement of the pulmonary catheter (30 min in average), pressure in the main pulmonary artery was measured with a pressure transducer (Schneider/Namic, Glenns Falls, NY), and mean PAP was derived electronically and recorded continuously. For the determination of pulmonary arterial wedge pressure, the catheter was advanced to the left or right pulmonary artery and wedged with continuous measurement of the pressure waveform.

Cardiac output was measured by the thermodilution technique. A known volume (20 μl plus catheter dead space) of 0.9% NaCl solution at 23°C was injected in the right atrium, and changes in blood temperature were measured in the root of the aorta. A cardiac output computer (Cardiotherm 500; Columbus Instruments, Columbus, OH) equipped with a small animal interface was used. Thermodilution curves were recorded on a chart recorder (Western Graphtec, Irvine, CA), and pulmonary and systemic arterial pressures were monitored continuously. Catheter placement was verified at postmortem examination. Immediately after bleomycin exposure, the descending aorta was severed. Thermodilution curves were recorded on a chart recorder (Western Graphtec, Irvine, CA), and pulmonary and systemic arterial pressures were monitored continuously. Catheter placement was verified at postmortem examination. Immediately after bleomycin exposure, the descending aorta was severed. Thermodilution curves were recorded on a chart recorder (Western Graphtec, Irvine, CA), and pulmonary and systemic arterial pressures were monitored continuously. Catheter placement was verified at postmortem examination. Immediately after bleomycin exposure, the descending aorta was severed. Thermodilution curves were recorded on a chart recorder (Western Graphtec, Irvine, CA), and pulmonary and systemic arterial pressures were monitored continuously. Catheter placement was verified at postmortem examination. Immediately after bleomycin exposure, the descending aorta was severed. Thermodilution curves were recorded on a chart recorder (Western Graphtec, Irvine, CA), and pulmonary and systemic arterial pressures were monitored continuously. Catheter placement was verified at postmortem examination. Immediately after bleomycin exposure, the descending aorta was severed. Thermodilution curves were recorded on a chart recorder (Western Graphtec, Irvine, CA), and pulmonary and systemic arterial pressures were monitored continuously. Catheter placement was verified at postmortem examination. Immediately after bleomycin exposure, the descending aorta was severed. Thermodilution curves were recorded on a chart recorder (Western Graphtec, Irvine, CA), and pulmonary and systemic arterial pressures were monitored continuously. Catheter placement was verified at postmortem examination. Immediately after bleomycin exposure, the descending aorta was severed. Thermodilution curves were recorded on a chart recorder (Western Graphtec, Irvine, CA), and pulmonary and systemic arterial pressures were monitored continuously. Catheter placement was verified at postmortem examination. Immediately after bleomycin exposure, the descending aorta was severed. Thera...
scribed elsewhere (32, 33). The murine αI(1)-procollagen cDNA was a kind gift from Dr. Eero Vuorio (Turku University, Turku, Finland) and has been described previously (23). The murine 18S cDNA was used for loading control, as previously described (32, 33). Northern analysis. After hemodynamic studies, the mice were killed by exanguination. Lung tissue was isolated and immediately snap-frozen in liquid nitrogen. Total RNA was extracted from the lung using a cesium chloride method for Northern analysis (32, 33). RNA was separated by electrophoresis (20 μg/lane) on a 1.2% formaldehyde-agarose gel, transferred to an Immobilon-N transfer membrane (Millipore, Bedford, MA), and hybridized overnight at 62°C with [32P]dCTP-labeled (ICN, Irvine, CA) random-primed cDNA probes as previously described (32–34). Membranes were probed first for TNF, then stripped and reprobed for αI(1)-procollagen and, as a loading control, for 18S. Blots were developed for 72 h using Biomax film and intensifying screens (Kodak). To quantitate mRNA, membranes were exposed to a Fuji PhosphorImager (Fujix BAS 1000; Fuji, Stamford, CT) plate overnight and scanned. Quantitative analysis was determined with the use of McBAS 2.5 software (Fuji USA). For each mRNA band, the results were normalized to the internal control (18S) and expressed as a degree of increase between the bands for the control and the bands for the bleomycin- and bleomycin- and enalapril-treated animals.

Lung hydroxyproline content. Lung hydroxyproline concentration was determined spectrophotometrically according to the method of Kivirikko et al. (18). Briefly, left lungs from control-, enalapril-, bleomycin-, and bleomycin- and enalapril-treated mice were homogenized in 5% trichloroacetic acid (1.9 wt/vol) and centrifuged for 10 min at 4,000 g. The pellet was then washed two times with distilled water and hydrolyzed for 16 h at 100°C in 6 N HCl. Hydroxyproline in the hydrolyzate was assessed colorimetrically at 561 nm with p-dimethylaminobenzaldehyde. Hydroxyproline content was computed as micrograms hydroxyproline per whole left lung.

Lung morphology and evaluation of right ventricular hypertrophy. The heart was perfused with 0.9% NaCl to remove residual blood, and the right lung was fixed in situ for 2 h by intratracheal instillation of 10% neutral formalin (Sigma, St. Louis, MO) at a constant pressure of 30 cmH2O and was preserved in fixative for 24 h. Lung tissues were then sectioned sagittally and embedded in paraffin. Sections (4 μm thick) were generated and mounted on positively charged slides (Fisher Scientific, Pittsburgh, PA). Slides were stained with hematoxylin-eosin for light microscopic examination. Immediately after death, hearts were resected to evaluate right ventricular hypertrophy. The atria were removed up to the plane of the atrial-ventricular valves. The right ventricle (RV) free wall was then dissected free of the left ventricle (LV) and septum. The RV and LV plus septum were weighed and the RV-to-LV + septum ratio was calculated.

Electrophoretic mobility shift assays. To generate nuclear extracts, lungs were minced on ice and homogenized with a Dounce homogenizer in 2 ml buffer [0.32 M sucrose, 3 mM magnesium chloride, 0.5 mM EGTA, 1 mM HEPES, 0.1% Triton X, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml aprotinin, and 10 μg/ml leupeptin]. Homogenates were filtered and centrifuged at 4°C at 700 g for 10 min. Pellets were resuspended in 200 μl lysis buffer [20 mM HEPES, 125 mM sodium chloride, 5 mM magnesium chloride, 12% vol/vol glycerol, 5 mM dithiothreitol (DTT), 0.5 mM PMSF, 10 μg/ml aprotinin, and 10 μg/ml leupeptin], sonicated on ice for 15 s, centrifuged at 14,000 rpm at 4°C, and frozen at −70°C until used.

For electrophoretic mobility shift assays, NF-κB consensus oligonucleotide 5′-GGGAGCTTCCCC-3′ and AP-1 consensus oligonucleotide 5′-CGGTGATGACTCAAGCCGAA-3′ (Santa Cruz Biotechnology, Santa Cruz, CA) were end labeled with [γ-32P]ATP and T4 polynucleotide kinase (GIBCO-BRL Life Technologies, Gaithersburg, MD) and purified by G-25 Sephadex columns (Pharmacia Biotech, Piscataway, NJ). Crude nuclear extract protein (5 μg) was mixed with the labeled probe and buffer [10 mM HEPES, 7.2% vol/vol glycerol, 3 mM magnesium chloride, 3 mM DTT, 3 μl Nonidet, 60 μg BSA, 360 μg spermidine, and 1.5 μg poly(dI-dC); Pharmacia Biotech] in a 20-μl total volume and incubated to allow NF-κB and AP-1 binding to the probe. DNA-protein complexes were separated on 6% polyacrylamide gel (Novex, San Diego, CA). The gels were vacuum-dried, and labeled complexes were detected by autoradiography.

Competition assays were performed using 400-fold excess of unlabeled probe or NF-κB mutant oligonucleotide (Santa Cruz). Supershift assays were performed by adding to the binding mixture antibodies to p50, p65, or c-Jun (Santa Cruz).

Statistics. All values are expressed as means ± SE. Differences between murine strains were analyzed using ANOVA with Fisher’s protected least-significant difference test for pair-wise comparison (Statview 4; Abacus Concept, Berkeley, CA). A P value <0.05 was considered significant.

RESULTS

Enalapril treatment blocks the systemic and pulmonary vascular hemodynamic effects of ANG I. Enalapril (5 mg/kg ip) significantly decreased the mean arterial pressure in C57BL/6 mice. The mean arterial blood pressure of control- and enalapril-treated mice was 94 ± 9 and 83 ± 7 mmHg, respectively. Enalapril attenuated 95% of the systemic and pulmonary vascular pressor response to ANG I in C57BL/6 mice. Control-treated mice responded to ANG I (doses of 0.3 and 1 μg/kg iv) with significant increases in arterial blood pressure (+38.3 ± 8 and +48 ± 8 mmHg, respectively). This pressor response to ANG I was significantly decreased by enalapril treatment (+6 ± 4 and +7 ± 5 mmHg, respectively).

Enalapril treatment also inhibited the pressor effect of ANG I on the pulmonary vasculature in C57BL/6 mice. The mean pulmonary arterial blood pressure of control-treated mice was 12.7 ± 1 mmHg. Control-treated mice responded to ANG I (doses of 0.3 and 1 μg/kg iv) with significant increases in mean pulmonary pressure (+3.4 ± 0.6 and +4.3 ± 0.4 mmHg, respectively). These pressor responses were significantly (P < 0.001) inhibited by enalapril treatment (+0.8 ± 0.4 and +0.7 ± 0.6, respectively).

Bleomycin induces pulmonary hypertension in C57BL/6, but not in BALB/c, mice. Bleomycin treatment resulted in the development of pulmonary hypertension in a strain-specific manner (Figs. 1 and 2). Compared with vehicle-treated C57BL/6 mice, bleomycin treatment significantly increased right atrial pressure (RA) (4.2 ± 0.4 vs. 7.9 ± 0.8 mmHg), PAP (13.2 ± 1.9 vs. 26.4 ± 2.5 mmHg), pulmonary vascular resistance (PVR; 0.9 ± 0.08 vs. 2.0 ± 0.15 mmHg·ml−1·min−1), and RV free wall weight (0.23 ± 0.02 vs. 0.39 ± 0.04) in C57BL/6 mice (Figs. 1 and 2). In contrast, bleomycin...
exposure did not significantly alter RA (4.2 ± 0.8 vs. 4.3 ± 0.9 mmHg), PAP (13.2 ± 2.1 vs. 15.2 ± 3 mmHg), PVR (0.90 ± 0.1 vs. 0.92 ± 0.11 mmHg·ml⁻¹·min⁻¹), or RV free wall weight (0.23 ± 0.02 vs. 0.21 ± 0.05) in BALB/c mice compared with vehicle-treated BALB/c mice (Fig. 2). Bleomycin did not significantly alter cardiac output in either murine strain (Fig. 2).

**Bleomycin-induced pulmonary hypertension is a TNF receptor-mediated event.** Because bleomycin-induced pulmonary hypertension develops only in the C57BL/6 mouse strain, compared with the BALB/c strain, which is similar to what has been previously reported for bleomycin-induced TNF expression in the lung, we studied whether TNF interaction with its receptors was an important determinant of the changes in pulmonary hypertension. To test this, we exposed double [p55(−/−)-p75(−/−)] TNF receptor-deficient mice (developed on a C57BL/6 genetic background) to bleomycin and measured the pulmonary pressure and vascular resistance in these mice. As seen in Figs. 1 and 2 and in contrast to bleomycin-exposed C57BL/6 mice, bleomycin exposure did not significantly alter RA (4.1 ± 0.8 vs. 3.9 ± 0.9 mmHg), PAP (13.4 ± 2.1 vs. 14 ± 3 mmHg), PVR (0.91 ± 0.1 vs. 0.89 ± 0.11 mmHg·ml⁻¹·min⁻¹), or RV free wall weight (0.23 ± 0.04 vs. 0.22 ± 0.03) in p55(−/−)-p75(−/−) TNF receptor-deficient mice compared with p55(−/−)-p75(−/−) TNF receptor-deficient mice exposed to vehicle alone (Figs. 1 and 2).

**TNF receptors mediate NF-κB and AP-1 activation in response to bleomycin.** An important effect of the interaction of TNF with its receptors is the activation of transcription factors (such as NF-κB and AP-1) that regulate the expression of TNF-sensitive genes such as collagen, matrix metalloproteinases, and other cytokines involved in fibrosis such as transforming growth factor (TGF)-β (31, 32, 50). Gel shift electrophoresis of nuclear extracts obtained from the lungs of bleomycin-treated mice 14 days after exposure demonstrated evidence of NF-κB activation in the lungs from C57BL/6 mice.
mice. In contrast, no evidence of NF-κB activation was found in nuclear extracts isolated from the lungs of BALB/c or double [p55(−/−)-p75(−/−)] TNF receptor-deficient mice exposed to bleomycin (Fig. 3). The enhanced NF-κB activation observed in bleomycin-exposed C57BL/6 mice was found as early as 30 min after bleomycin exposure (data not shown). The specificity of the observed NF-κB binding was demonstrated by the fact that the binding was inhibited by addition of a nonlabeled NF-κB oligonucleotide. Also the use of an antibody specific to the p50, but not the p65, subunit of NF-κB caused a shift of the NF-κB complexes (Fig. 3).

Bleomycin treatment also resulted in AP-1 activation in the lungs of C57BL/6 mice at 14 days postexposure (Fig. 4). This response was greatly decreased in the lungs of BALB/c and double [p55(−/−)-p75(−/−)] TNF receptor-deficient mice (Fig. 4). Use of antibodies against c-jun, but not antibodies against c-fos, depleted the corresponding AP-1 band, confirming AP-1 binding specificity (Fig. 4).

Enalapril ameliorates bleomycin-induced lung injury in C57BL/6 mice. To evaluate the effects of persistent ACE inhibition on bleomycin-induced lung injury and bleomycin-induced pulmonary hypertension in bleomycin-sensitive (C57BL/6) mice, we studied the effects of enalapril treatment on the bleomycin-induced loss of weight in C57BL/6 mice, as shown in Table 1. Bleomycin treatment induced a significant (P < 0.05) loss in body weight (measured 14 days after intratracheal exposure) in C57BL/6 mice (4.1 ± 0.3 g). No significant weight loss was found in control- or enalapril-treated mice exposed to bleomycin (21.7 ± 0.3 and 20.4 ± 0.2 g, respectively).

The effects of enalapril on lung histology and lung collagen accumulation were also evaluated in C57BL/6 mice 14 days after bleomycin exposure. Compared with

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**Fig. 2.** Effect of bleomycin exposure on cardiac output and right ventricular hypertrophy in bleomycin-sensitive, bleomycin-resistant (BALB/c), and double [p55(−/−)-p75(−/−)] TNF receptor-deficient mice. Influence of bleomycin administration on cardiac output, PVR, and right ventricular hypertrophy (RVH; as measured by the right ventricle (RV)-to-left ventricle (LV)+ septum (S) ratio) in C57BL/6, BALB/c, and double [p55(−/−)-p75(−/−)] TNF receptor-deficient mice. Comparisons are made in mice 2 wk after administration of vehicle (saline) or bleomycin (4 U/kg it). *P < 0.05.
vehicle-treated mice (data not shown), the intrathoracic exposure of C57BL/6 mice to bleomycin resulted in the development of subpleural areas of inflammation (accumulations of macrophages, lymphocytes, and fibroblasts) that extended into the lung parenchyma and involved the bronchi and vasculature (Fig. 5A).

Fig. 3. Bleomycin (BLM) induces nuclear factor (NF)-κB activation in mouse lung. DNA-binding activity of NF-κB in crude nuclear extracts from whole lung isolated from C57BL/6, BALB/c, and double [p55(−/−)-p75(−/−)] TNF receptor-deficient mice 14 days after bleomycin exposure. Cold κB represents NF-κB binding in lung nuclear extract of a C57BL/6 bleomycin-treated mouse, assayed in the presence of excess unlabeled oligonucleotide as a competitor. Antibody supershifts were performed with the nuclear extract of a C57BL/6 bleomycin-treated mouse as described in METHODS. Gel is representative of 4 experiments. ns, Not significant.

Fig. 4. Activator protein (AP)-1 activation in murine lung after bleomycin exposure. DNA-binding activity of AP-1 in crude nuclear extracts from whole lung isolated from C57BL/6, BALB/c, and double [p55(−/−)-p75(−/−)] TNF receptor-deficient mice 14 days after bleomycin exposure. Cold AP-1 represents AP-1 binding in lung nuclear extract of a C57BL/6 bleomycin-treated mouse, assayed in the presence of excess unlabeled oligonucleotide as a competitor. Antibody supershifts were performed with the nuclear extract of a C57BL/6 bleomycin-treated mouse as described in METHODS. Gel is representative of 4 experiments.

Bleomycin treatment induced thickening of the medium-sized and intra-alveolar pulmonary arteries (Fig. 5A). This thickening appears to be the result of an increase in smooth muscle cell mass in the media, as only one layer of endothelial cells could be appreciated, and was more evident in areas of bleomycin-induced pneumonia (Fig. 5A). However, medial thickening of intra-alveolar arteries could also be observed in subpleural areas of the lung and in areas free of bleomycin-induced inflammation (Fig. 5B). In contrast, the lung histology in mice treated with enalapril was comparable to that of vehicle-treated mice, and no change in the histology of intra-alveolar arteries was observed (Fig. 5C).

We evaluated the effect of enalapril treatment on collagen deposition by measuring lung hydroxyproline content 14 days after bleomycin exposure. The results are shown in Fig. 6. The administration of enalapril alone did not result in a difference in lung collagen content (48 ± 3 μg/lung) compared with vehicle-treated mice (40 ± 1 μg/lung). Compared with vehicle-treated mice, there was a statistically significant increase (P < 0.05) in hydroxyproline content in the lungs of bleomycin-treated mice (82 ± 11 μg/lung). Enalapril treatment significantly (P < 0.05) decreased the lung hydroxyproline content in the bleomycin-treated mice (59 ± 5 μg/lung).

We then studied whether this enalapril effect on hydroxyproline content could be explained by a decreased expression of the collagen gene in the lungs of bleomycin-treated C57BL/6 mice. Bleomycin treatment enhanced α1(I)-procollagen mRNA expression in the lungs of C57BL/6 mice 14 days after bleomycin exposure (Fig. 7A). Compared with control-treated animals [2.1 ± 0.2 arbitrary units (AU)], bleomycin treatment resulted in a significant (P < 0.05) increase (5.4 ± 1 AU) in α1(I)-procollagen mRNA expression in the lung (Fig. 7B). This bleomycin-induced upregulation in α1(I)-procollagen mRNA expression was significantly (P < 0.05) inhibited in enalapril-treated animals (1 ± 0.4 AU; Fig. 7B).

Enalapril inhibits bleomycin-induced pulmonary hypertension in C57BL/6 mice. As described above, enalapril treatment inhibited the bleomycin-induced remodeling of the pulmonary circulation in C57BL/6 mice (Fig. 5). The effects of enalapril treatment on the bleomycin induction of pulmonary hypertension in C57BL/6 mice are shown in Table 2. Enalapril treat-

Table 1. Effect of enalapril on body weight during bleomycin-induced lung injury in C57BL/6 mice

<table>
<thead>
<tr>
<th>Treatment Regimen</th>
<th>n</th>
<th>Baseline Body Weight, g</th>
<th>Body Weight 14 Days After Bleomycin Exposure, g</th>
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<tr>
<td>Control</td>
<td>11</td>
<td>20.1 ± 0.3</td>
<td>21.7 ± 0.3</td>
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<tr>
<td>Enalapril alone</td>
<td>10</td>
<td>20.1 ± 0.1</td>
<td>21.1 ± 0.2</td>
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<tr>
<td>Bleomycin</td>
<td>9</td>
<td>20.3 ± 0.2</td>
<td>17.6 ± 0.8*</td>
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<tr>
<td>Bleomycin + enalapril</td>
<td>9</td>
<td>20.1 ± 0.2</td>
<td>20.4 ± 0.2*</td>
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</tbody>
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Values are means ± SE; n, no. of mice. *Significantly different from baseline weight. †Significantly different from bleomycin-treated alone.

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ment significantly ($P < 0.05$) attenuated the increases in RA, PAP, and PVR observed in C57BL/6 mice in response to bleomycin. Enalapril treatment did not alter the mean pulmonary artery wedge pressure or the cardiac output in bleomycin-treated mice.

Enalapril treatment inhibits TNF mRNA expression in the lungs of bleomycin-treated C57BL/6 mice. Because bleomycin-induced lung injury correlates with enhanced TNF expression in the mouse lung, we evaluated whether enalapril treatment decreased TNF expression in the lungs of bleomycin-treated C57BL/6 mice. Bleomycin exposure resulted in an enhanced TNF mRNA expression in the lungs of C57BL/6 mice 14 days after bleomycin exposure, as assessed by Northern analysis (Fig. 7A). Compared with control-treated animals ($1.8 \pm 0.2$ AU), bleomycin-treated mice demonstrated a significant ($P < 0.05$) increase ($2.9 \pm 0.4$ AU) in TNF mRNA expression in their lungs (Fig. 7C). This enhanced TNF expression was significantly inhibited ($1.1 \pm 0.3$ AU) by enalapril treatment (Fig. 7C).

Enalapril inhibits NF-κB and AP-1 activation in the lungs of bleomycin-treated C57BL/6 mice. We studied whether the inhibitory effect of enalapril on TNF and collagen mRNA expression is associated with inhibition of the activity of NF-κB and AP-1 transcription factors. As shown in Figs. 8 and 9, bleomycin exposure resulted in enhanced NF-κB (Fig. 8A) and AP-1 (Fig. 9A) activation in the lungs of C57BL/6 mice 14 days after exposure. In contrast, enalapril treatment significantly inhibited NF-κB (Fig. 8B) and AP-1 (Fig. 9B) activation in response to bleomycin.
DISCUSSION

In this study, pulmonary hypertension is a prominent feature of ILD in humans (47). Here we demonstrate the development of pulmonary hypertension in a murine model of bleomycin-induced lung injury. Mice treated with bleomycin develop fibroproliferative responses characterized by enhanced expression of cytokines, infiltration of lung parenchyma by inflammatory cells, apoptosis of alveolar epithelial cells, fibroblast proliferation, upregulated collagen gene expression, and excess deposition of collagen in the lung (1, 10, 32, 36, 43). The sequences of events leading to bleomycin-induced pulmonary hypertension are less well understood. Traditionally, it has been thought that hypoxic vasoconstriction resulting from severe lung damage induces pulmonary vascular remodeling (47). However, a more plausible explanation is that endothelial and smooth muscle cell proliferation observed in the pulmonary circulation is induced as a result of the same inflammatory environment that is present in the lung.

Fig. 7. Effect of enalapril (Enalp and Enalapr) on TNF and collagen mRNA expression in the lung of bleomycin-treated C57BL/6 mice. A: Northern blot analysis of TNF, α1(I)-collagen, and 18S (loading control) mRNA expression in mouse lung 14 days after intratracheal injection of saline as control, bleomycin alone, or bleomycin followed by enalapril (5 mg·kg⁻¹·day⁻¹ ip for 14 days), as described in METHODS. B and C: densitometry analysis of Northern blots as described in METHODS. Bleomycin significantly (P < 0.05) induced expression of α1(I)-collagen (B) and TNF (C) mRNA in C57BL/6 mice. Enalapril treatment significantly inhibited (P < 0.05) bleomycin-induced TNF and α1(I)-collagen mRNA expression in the lungs of C57BL/6 mice. Blots show results obtained from one set of mice exposed to vehicle (saline), bleomycin, or bleomycin + enalapril. The same results were obtained using three additional sets of animals. AU, arbitrary units. P < 0.05, strains significantly different compared with vehicle-treated mice (*) and bleomycin-treated C57BL/6 mice (†).
Enalapril inhibits bleomycin-induced pulmonary hypertension in C57BL/6 mice

Table 2. Enalapril inhibits bleomycin-induced pulmonary hypertension in C57BL/6 mice

<table>
<thead>
<tr>
<th>Hemodynamic Parameter</th>
<th>Control</th>
<th>Bleomycin + Vehicle</th>
<th>Bleomycin + Enalapril</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean pulmonary artery pressure, mmHg</td>
<td>12.7 ± 1</td>
<td>29.4 ± 2.9*</td>
<td>19.1 ± 2.7†</td>
</tr>
<tr>
<td>Pulmonary wedge pressure, mmHg</td>
<td>4.6 ± 0.8</td>
<td>4.1 ± 0.9</td>
<td>4.2 ± 0.8</td>
</tr>
<tr>
<td>Pulmonary vascular resistance, mmHg·ml⁻¹·min⁻¹</td>
<td>0.78 ± 0.04</td>
<td>2.75 ± 0.14*</td>
<td>1.4 ± 0.03†</td>
</tr>
<tr>
<td>Cardiac output, ml/min</td>
<td>10.2 ± 0.7</td>
<td>8.9 ± 1</td>
<td>9.7 ± 1</td>
</tr>
<tr>
<td>Mean right atrial pressure, mmHg</td>
<td>4.2 ± 0.4</td>
<td>7.8 ± 0.9*</td>
<td>5.2 ± 0.4†</td>
</tr>
</tbody>
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Values are means ± SE; n = 10 mice. *P < 0.05 vs. control. †P < 0.05 vs. bleomycin + vehicle.

Parenchyma (47). Consistent with this hypothesis is our observation that the development of bleomycin-induced pulmonary hypertension correlates well with the murine strain sensitivity to bleomycin (32–34). C57BL/6 mice are sensitive, whereas BALB/c mice are resistant, to bleomycin-induced lung injury (32–34). In this study, we found that pulmonary hypertension develops in C57BL/6 mice exposed to bleomycin and that it is absent in BALB/c mice.

TNF plays a fundamental role in the pathogenesis of bleomycin-induced lung injury, and antagonism of TNF with the use of anti-TNF antibodies or soluble TNF receptors ameliorates bleomycin-induced lung injury (32, 36, 37). We have previously reported that TNF expression in the mouse lung is a major determinant of the difference in murine strain sensitivity to bleomycin (32, 34). We have reported that animals deficient in both TNF receptors [p55(−/−)-p75(−/−)] are protected from bleomycin-induced lung injury (33). These double TNF receptor-deficient mice were developed on a bleomycin-sensitive (C57BL/6) genetic background and exhibited enhanced TNF expression in their lungs in response to bleomycin (33). In the present work, we report that TNF receptors appear to also be important contributors to the development of bleomycin-induced pulmonary hypertension, since double TNF receptor-deficient mice are protected from bleomycin-induced pulmonary hypertension (Fig. 3). These observations complement recent published reports suggesting that TNF overexpression in the lung of rodents is associated with lung inflammation and fibrosis and the spontaneous development of pulmonary hypertension (6, 42).

An important finding of the present study is that treatment with the ACE inhibitor enalapril significantly reduced bleomycin-induced lung injury (Table 1 and Fig. 5) and prevented the development of bleomycin-induced pulmonary hypertension (Table 2) in C57BL/6 mice. The protection against bleomycin-induced lung injury and pulmonary hypertension by enalapril was associated with reduced transcription factor (NF-κB and AP-1; Figs. 8 and 9) activation and expression of inflammatory (TNF) and fibrogenic [αI(I)-collagen] genes (40). These effects of enalapril translated into decreased lung inflammation, reduced collagen deposition, and decreased muscle thickness in the media of small vessels from bleomycin-treated mice (Fig. 5B). The selection of enalapril in the present work was based on its long half-life, which allows for daily administration. Whether similar results can be achieved by using other ACE inhibitors or ANG II receptor antagonists may be determined in future studies.

The mechanisms responsible for modulation of TNF and collagen gene expression by enalapril in response to bleomycin appear to involve attenuation of transcription factor activation in the lung. Prominent among the transcription factors activated by bleomycin in the mouse lung are NF-κB and AP-1, which regulate the transcription of multiple genes involved in the inflammatory and fibroproliferative responses (2, 31, 50). Bleomycin has been proposed to activate NF-κB by promoting expression of inflammatory cytokines such as TNF or via production of reactive oxygen species (7). Consistent with this concept, our data demonstrate decreased NF-κB and AP-1 activation in the lungs of bleomycin-resistant (BALB/c) or double [p55(−/−)-p75(−/−)] TNF receptor-deficient mice. Furthermore, our data also demonstrate that enalapril treatment reduced TNF mRNA expression and NF-κB and AP-1 activation in the lungs of bleomycin-treated C57BL/6 mice. Therefore, these data suggest that enalapril inhibition of TNF expression greatly reduced activation of transcription factors and subsequently decreased the downstream effects of TNF on fibrogenic genes (30–37). In addition to enhancing TNF expression, bleomycin has been proposed to activate NF-κB by increasing the production of reactive oxygen species (7). The use of antioxidant agents inhibits bleomycin-induced inhibitor factor κB (IkB) degradation, prevents NF-κB activation, and decreases the expression of inflammatory cytokines and the accumulation of collagen in response to bleomycin (9, 12). The mechanisms leading to bleomycin-induced activation of AP-1 are not well understood. Bleomycin has been shown to activate the c-Jun NH2-terminal kinase, a member of the mitogen-activated protein kinase (MAPK) family involved in AP-1 activation (40). However, the present work suggests that most of the activation of AP-1 in the lungs of C57BL/6 mice can be explained by the enhanced TNF expression observed in the lungs in response to bleomycin. This is suggested by the fact that neither BALB/c, which do not upregulate TNF (32, 33), nor double [p55(−/−)-p75(−/−)] TNF receptor mice demonstrated enhanced AP-1 activation in response to bleomycin (Figs. 3 and 4).

The specific mechanisms by which enalapril attenuates NF-κB and AP-1 activation are not completely understood (13). The most accepted explanation to address the inhibitory effects of enalapril treatment on
the activation of NF-κB and AP-1 is that there may be common elements between the signal transduction for ANG II and signaling events leading to NF-κB and AP-1 activation. The ACE inhibitor might in turn downregulate these elements. A potential target is MAPK kinase (MAPKK), which has been shown to induce activation of the IκB kinase (IKK) kinases, leading to IκB phosphorylation and NF-κB activation (16, 17, 26, 56). Consistent with this hypothesis, we have demonstrated in preliminary studies that endotracheal administration of bleomycin to mice is followed by an enhanced activity of the IKK-α, but not IKK-β, subunit of the IKK (30). Activation of the AT1 receptor is followed by receptor endocytosis and rapid tyrosine phosphorylation of platelet-derived growth factor and epidermal growth factor receptors that provide docking sites for the upstream activation of the Src family of tyrosine kinases and the formation of downstream complexes leading to Ras activation (13). This scaffolding step facilitates activation of MAPK and can be inhibited by AT1 receptor antagonists as described above (5, 25).

In addition to inhibiting the expression of inflammatory mediators, enalapril may prevent the well-known effects of ANG II on cell proliferation and apoptosis (21, 25, 46). In general, activation of AT1 receptors mediates cell proliferation (21, 25), whereas inhibition of cell growth and induction of apoptosis follow activation of AT2 receptors (28, 46, 54). Therefore, it has been proposed that the balance between the expression of these two receptors is an important determinant of the response to ANG II (21). In human lung fibroblasts, ANG II exerts a mitogenic response via AT1 receptors, which are the only ANG II receptors present on these cells (21). The mitogenic response of ANG II on human lung fibroblasts appears to be mediated via an autocrine production of TGF-β and can be inhibited by treatment of these cells with the AT1 receptor antagonist losartan (21). ANG II also induces DNA synthesis and cell growth in human pulmonary artery smooth muscle cells (25). ANG II mediates this mitogenic effect on smooth muscle cells by triggering activation of MAPK, and pretreatment of these cells with the specific MAPK inhibitor PD-98059 inhibits ANG II-induced DNA synthesis (25). MAPK activation in response to ANG II is mediated via AT1 receptors and can be readily inhibited by losartan treatment (25). Our results demonstrating attenuated AP-1 activation in enalapril-treated C57BL/6 mice correlate well with these observations, since activation of MAPKs constitutes the primary signal leading to AP-1 activation (25).

Fig. 8. Enalapril inhibits NF-κB activation in the lungs of bleomycin-treated C57BL/6 mice. Effect of enalapril on NF-κB activation in murine lung after bleomycin exposure. A: DNA-binding activity of NF-κB in crude nuclear extracts from whole lung isolated from C57BL/6 mice 14 days after bleomycin exposure. Cold κB represents NF-κB binding in lung nuclear extract of a C57BL/6 bleomycin-treated mouse, assayed in the presence of excess unlabeled oligonucleotide as a competitor. B: densitometry analysis of gel shifts as described in METHODS. Values represent means ± SE. Gel is representative of 4 experiments. P < 0.05, significantly different compared with vehicle-treated animals (*) and with bleomycin-treated C57BL/6 mice (†).
In addition to the induction of cell proliferation, ANG II is also capable of inducing apoptosis (54). Excessive apoptosis has been closely linked to the pathogenesis of lung fibrosis and bleomycin-induced lung injury (10, 11, 34). Uhal and associates (49) reported that myofibroblasts isolated from fibrotic human lung secreted soluble mediators capable of inducing apoptosis in alveolar epithelial cells. These mediators were identified as angiotensinogen and its active peptide ANG II (52). In subsequent reports, these authors demonstrated that the ANG II-induced apoptosis in human and rat epithelial cells could be modulated by treatment with the ACE inhibitor captopril (48). Furthermore, these investigators reported that the capacity of TNF to induce apoptosis in epithelial cells depends on the ability of the target cells to generate ANG II de novo (51, 53). These observations are important, since we have indicated that TNF is required for the development of bleomycin-induced lung fibrosis in mice (1, 32, 33, 36). TNF induction of apoptosis depends on its interaction with TNF receptors, and double TNF receptor-deficient mice demonstrated significantly less apoptosis in alveolar macrophages than did C57BL/6 or BALB/c mice in response to bleomycin (34). In the present work, we found that double TNF receptor-deficient mice do not develop pulmonary hypertension in response to bleomycin. Therefore, it is possible that, by inhibiting TNF expression, enalapril modulates the induction of TNF-mediated apoptosis in the endothelium of pulmonary vessels. This effect may be important, since it has been reported that the ability of the endothelial cell to survive apoptosis confers a survival advantage that allows for the uncontrolled endothelial cell proliferation observed in pulmonary hypertension (47).

In summary, enalapril treatment protects mice from the development of bleomycin-induced fibrosis and pulmonary hypertension. Enalapril treatment promotes lung homeostasis by inhibiting cell proliferation, decreasing collagen expression, and reducing TNF-mediated inflammation. Enalapril inhibition of TNF and collagen expression appears to be mediated via attenuation of NF-kB and AP-1 activation in the mouse lung. The protective effects of enalapril may involve inhibition of kinases located upstream of the IKK and MAPKK. Further studies are necessary to identify these kinases and reactions. The present data also suggest a need for further studies looking at the effects of ACE inhibitors in the treatment of pulmonary hypertension and fibrotic lung diseases in humans.

Fig. 9. Enalapril inhibits AP-1 activation in lungs of bleomycin-treated C57BL/6 mice. Effect of enalapril on AP-1 activation in murine lung after bleomycin exposure. A: DNA-binding activity of AP-1 in crude nuclear extracts from whole lung isolated from C57BL/6 mice 14 days after bleomycin exposure. Cold AP-1 represents AP-1 binding in lung nuclear extract of a C57BL/6 bleomycin-treated mouse, assayed in the presence of excess unlabeled oligonucleotide as a competitor. B: densitometry analysis of gel shifts as described in METHODS. Values represent means ± SE. Gel is representative of 4 experiments. P < 0.05, significantly different compared with vehicle-treated animals (*) and bleomycin-treated C57BL/6 mice (†).
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