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Transforming growth factor-β1 upregulation is independent of angiotensin in paraquat-induced lung fibrosis

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Abstract

Transforming growth factor-β1 (TGF-β1) contributes to the fibrosis of injured organs. Angiotensin II (Ang II) is an inducer of TGF-β1 in cells of the heart and kidneys, and the regulation of TGF-β1 by Ang II has not yet been confirmed in lung tissue. We evaluated the role of TGF-β1 and its relationship with Ang II in paraquat-induced lung fibrosis. Adult male Sprague–Dawley rats were treated intraperitoneally with paraquat (20 mg/kg) or saline in the control group. On days 1, 3, 7, and 21 after paraquat treatment, TGF-β1 and collagen gene expressions, TGF-β1 protein, angiotensin-converting enzyme (ACE) activity, Ang II, and hydroxyproline contents were measured in lung tissue. Lung TGF-β1 mRNA expression progressively increased and reached a peak on day 7 after paraquat treatment. Increases in TGF-β1 mRNA expression and TGF-β1 levels preceded the onset of increased collagen I mRNA expression and hydroxyproline contents. c-myc mRNA expressions were inversely correlated with TGF-β1 protein levels in paraquat-treated lungs. Lung ACE activity decreased after paraquat administration and the decrement was maximal on day 7. Lung Ang II concentrations immediately decreased after paraquat administration and the values were not related to TGF-β1 levels. We conclude that TGF-β1 is upregulated and contribute to the paraquat-induced lung fibrosis and this effect is independent of the renin–angiotensin system.

Keywords: Paraquat; Angiotensin-converting enzyme; Angiotensin; Transforming growth factor; Hydroxyproline

1. Introduction

Paraquat dichloride (1,1′-dimethyl-4,4′-bipyridilium dichloride; methyl viologen) is an effective and widely used herbicide in most countries. The intentional and accidental ingestion of commercial liquid formulations of paraquat has caused a large number of human fatalities. According to epidemiological data in the National Poison Center in Taiwan during 1985 and 1997, paraquat poisoning was the leading cause of poisoning-induced death in Taiwan (Satoh and Hosokawa, 2000). Paraquat produces toxicity in humans and the lungs are one of the primary target organs (Forman et al., 1982). The toxic effects of paraquat on the lungs result in pulmonary edema, hypoxia, respiratory failure, and pulmonary fibrosis. Survivors of paraquat poisoning may be left with a restrictive type of long-term pulmonary dysfunction (Yamashita et al., 2000).

Transforming growth factor-β1 (TGF-β1) is a key growth factor that initiates tissue repair and its sustained

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production underlies the development of tissue fibrosis (Border and Ruoslahti, 1992). In experimental models of lung fibrosis, TGF-β1 is an important upstream effector of collagen gene expression by a variety of approaches, including the administration of the TGF-β1 gene and TGF-β1 to the lung (Gauldie et al., 1999; Kenyon et al., 2003). Angiotensin II (Ang II) produced from proteolytic processing of angiotensinogen is documented to be an inducer of TGF-β1 expression in cells of the heart and kidneys (Campbell and Katwa, 1997; Klahr and Morrissey, 1998; Kupfahl et al., 2000). Although TGF-β1 has been reported to play a role in pulmonary fibrosis induced with paraquat and hyperoxia (Ruiz et al., 2003), its relationship with Ang II has not yet been confirmed in lung tissue. The aims of this study were to evaluate the role of TGF-β1 and to determine its relationship with angiotensin in paraquat-induced lung fibrosis.

2. Materials and methods

2.1. Animals

This study was approved by the Institutional Animal Use Committee at Taipei Medical University and was performed using adult male Sprague–Dawley rats (with approximate body weights of 230–250 g) maintained on a standard laboratory diet and water ad libitum. Rats were treated intraperitoneally with paraquat (20 mg/kg, Sigma Chemical, St. Louis, MO, USA) or saline in the control group. On days 1, 3, 7, and 21 after paraquat treatment, rats were anesthetized by intraperitoneal pentobarbital (50 mg/kg), and the lungs were removed from the chest and immediately frozen in liquid nitrogen for determination of TGF-β1 and collagen gene expressions, angiotensin converting-enzyme (ACE) activity, the Ang II concentration, and TGF-β1 contents. Another set of rats was used for measurement of lung hydroxyproline as an estimate of collagen content.

2.2. TGF-β1, collagen, and c-myc gene expressions by reverse transcription-polymerase chain reaction (RT-PCR)

Lung tissue was ground into a powder in liquid nitrogen, and the gene expressions of TGF-β1, collagen I, collagen III, c-myc were measured using RT-PCR. Total RNA was extracted using the TRIzol Reagent (Invitrogen Life Technologies, Paisley, UK) according to the manufacturer's instructions. Yield and purity of the isolated RNA solution were determined by A260 and A280 readings on a spectrophotometer. Reverse transcription was performed on 3 μg of RNA with oligo-dT primers and avian myeloblastosis virus reverse transcriptase (Roche, Indianapolis, IN, USA). The PCR were carried out with the primers shown in Table 1. The PCR products were analyzed by electrophoresis on an agarose gel, stained with ethidium bromide, and photographed. To determine the linear range of the PCR, the intensity of the amplified products was plotted against the cycle number. At least three samples (range 3–6) on each day were analyzed in each group.

2.3. Measurements of ACE activity, Ang II, and TGF-β1 levels in lung tissue

Lung tissue was homogenized in lysis buffer and centrifuged at speeds according to the manufacturer's instructions. The supernatant solution was used for measurements of ACE activity, Ang II, and TGF-β1 levels with enzyme-linked immunosorbent assay kits from Buhlmann Labs AG, Switzerland; SPI-BIO, Massy Cedes, France; and Biosource, Camarillo, CA, USA, respectively. One unit of ACE activ-

Table 1
Oligonucleotide sequences of the primers used

Primer	Sequence	Product size (bp)
TGF-β1		
Sense	5′-GCT CGC TTT GTA CAA CAG CA-3′	280
Antisense	5′-GAG TTC TAC GTG TTG CTC CA-3′	
Collagen I		
Sense	5′-GCT GCC TTT TCT GTT CCT TT-3′	185
Antisense	5′-GGA TTT GAA GGT GCT GGG TA-3′	
Collagen III		
Sense	5′-GCC ACC CTG AAC TCA AGA GT-3′	446
Antisense	5′-GCC ATC CTC TAG AAC TGT GT-3′	
c-myc		
Sence	5′-AGG AAC TAT GAC CTC GAC TAC G-3′	293
Antisense	5′-AGT AGC TCG GTC ATC ATC TCC AG-3′	
β-Actin		
Sense	5′-TTG TAA CCA ACT GGG ACG ATA TGG-3′	764
Antisense	5′-GAT CTT GAT CTT CAT GGT GCT AGG-3′	

ity was defined as the amount of enzyme required to release 1 μmol/min of hippuric acid. TGF-β1 and Ang II were expressed as μg/g of protein and ng/g of protein, respectively.

2.4. Hydroxyproline assay of lung tissue

The hydroxyproline contents of lung tissues were determined and the data were expressed as μg/g wet lung tissue (Reddy and Enwemeka, 1996). Total lung tissues from control and paraquat-treated rats were frozen in liquid nitrogen and lyophilized using a freeze-dry system (Labconco, Kansas City, MO, USA). The lyophilized lung tissue was thoroughly homogenized in distilled water using a polytron homogenizer. Aliquots of standard hydroxyproline and lung tissue samples were hydrolyzed and mixed with a buffered chloramine-T reagent, and oxidation was allowed to proceed at room temperature. The chromophore was developed with the addition of Ehrlich's aldehyde and was incubated. Absorbance of each sample was read at 550 nm using a spectrophotometer and was plotted against the concentration of standard hydroxyproline.

2.5. Histological evaluation

After sacrifice, right lung was isolated and inflation-fixed in formalin at a pressure of 20 H_2O. Subsequently, the lobes of the lung were separated and sectioned sagittally. The sagittal sections were embedded in paraffin, and 5-μm-thick sections were made and stained with hematoxylin and eosin. The sections were examined by light microscopy and assessed for the presence of hemorrhage, intra-alveolar edema, and fibrosis.

2.6. Statistical analysis

Results are presented as the mean ± S.E.M. Comparisons between control and paraquat-treated groups at each time point were made using unpaired Student's *t*-test. Differences were considered significant at $P < 0.05$.

3. Results

Sixty rats received paraquat treatment in this study. Three deaths occurred between 1 and 3 days after paraquat treatment. Between days 3 and 21 no deaths occurred. Twenty-five rats were used for hydroxyproline measurements. The rest were used for measurements of TGF-β1 and collagen gene expressions, ACE activity, Ang II concentration, and TGF-β1 content.

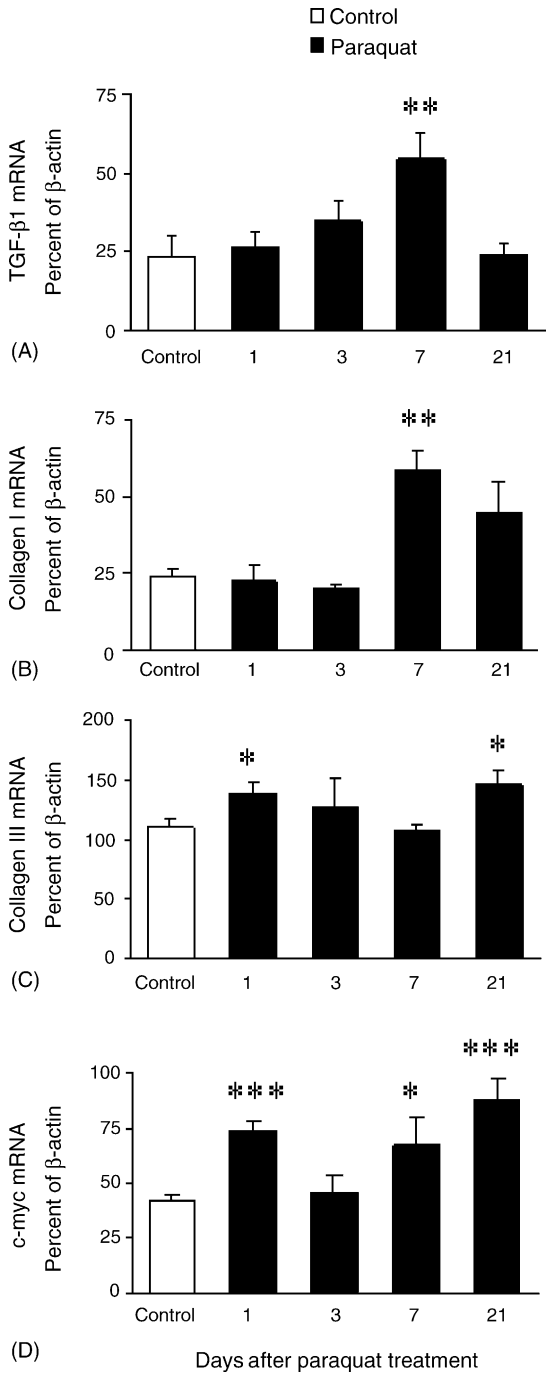

Fig. 1. TGF-β1, collagen I, collagen III, and c-myc gene expressions in control and paraquat-treated rat lungs. (A) Lung TGF-β1 mRNA expression progressively increased after paraquat treatment and the value had reached a peak and statistical significance on day 7. (B) Collagen type I mRNA expressions were comparable between the control and paraquat-treated lungs on days 1 and 3, and values had significantly increased on day 7 after paraquat treatment. (C) Collagen type III mRNA expressions had significantly increased on days 1 and 21 after paraquat treatment when compared with the control group. (D) c-myc mRNA expressions were increased in paraquat-treated lungs and the values were significantly higher on days 1, 7, and 21 when compared with the control group ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

3.1. TGF-β1, collagen, and c-myc gene expressions in control and paraquat-treated rat lungs

Lung TGF-β1 mRNA expression progressively increased after paraquat treatment and the value reached a peak and statistical significance on day 7 when compared with the control group (Fig. 1A). Collagen type I mRNA expressions were comparable between the control and paraquat-treated lungs on days 1 and 3, and the values had significantly increased on day 7 (Fig. 1B). Collagen type III mRNA expressions had significantly increased on days 1 and 21 after paraquat treatment when compared with the control group (Fig. 1C). c-myc mRNA expressions were increased in paraquat-treated lungs and the values were significantly higher on days 1, 7, and 21 when compared with the control group (Fig. 1D).

3.2. Effects of paraquat treatment on lung ACE activity and Ang II concentration

Lung ACE activity gradually decreased after paraquat administration and the values were significantly lower in paraquat-treated group on days 3 and 7 when compared with the control group (Fig. 2A). Lung Ang II concentrations immediately decreased after paraquat administration and the values reached statistical significance on days 1, 7, and 21 (Fig. 2B).

3.3. Effects of paraquat treatment on lung TGF-β1 and hydroxyproline contents

Paraquat-treated rats exhibited a progressive increase in lung TGF-β1 levels and the values reached statistical significance on days 3 and 7 (Fig. 3A). The values then decreased after day 7 and returned to the control level by day 21 after paraquat treatment. Hydroxyproline contents of the lung tissue were comparable among control and paraquat-treated rats on days 1, 3, and 7 and the value reached a maximum on day 21 (Fig. 3B).

3.4. Histology

The histological appearance of the lungs is illustrated in Fig. 4. Examination of random fields under a light microscopy revealed lung structure progressively disorganized and inflammatory cellular infiltrate increased in the interstitium and airspaces as paraquat-treated rats aged. Alveolar hemorrhage and capillary stasis were found mostly in rats on day 3 after paraquat treatment (Fig. 4C).

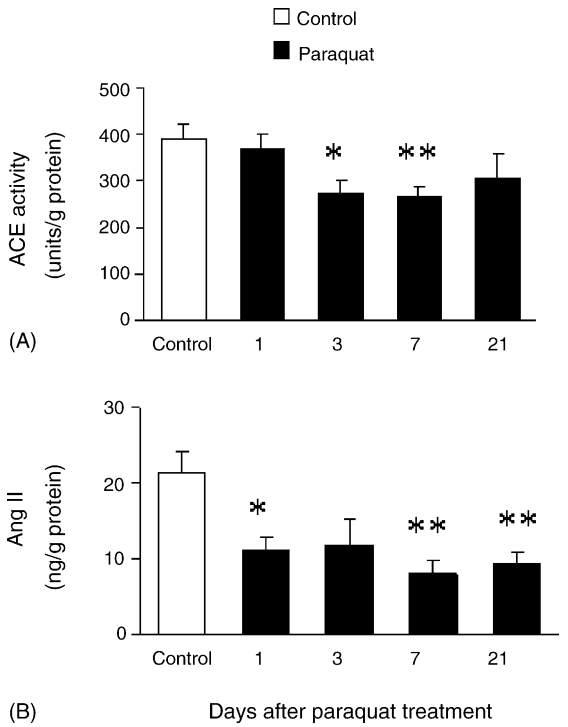

Fig. 2. ACE activities and Ang II levels in lung tissues of control and paraquat-treated rats. (A) Lung ACE activity progressively decreased after paraquat treatment, and the activities were significantly lower in paraquat-treated groups on days 3 and 7 when compared with the control group. (B) Lung Ang II concentrations immediately decreased after paraquat administration and the values had reached statistical significance on days 1, 7, and 21 when compared with the control group ($^{*}P < 0.05$, $^{**}P < 0.01$).

4. Discussion

Acute respiratory distress syndrome (ARDS) is a rather heterogenous disorder, and the clinical course is divided into three phases: (1) an early exudative phase of lung inflammation and edema; (2) a proliferative phase with pneumocyte and fibroblast proliferation; and (3) a final fibrotic phase with collagen deposition and pulmonary fibrosis (Meduri, 1996). We previously reported that an intraperitoneal paraquat injection (35 mg/kg) in rats produced a picture resembling the initial inflammatory phase of ARDS; rats exhibit increased wet lung weight, inflammatory responses, and total protein content in bronchoalveolar lavage fluid (Chen and Lua, 2000). However, pulmonary fibrosis is a major determinant of the prognosis associated with ARDS.

Many inflammatory cytokines, particularly TGF-β1, are involved in the pathogenesis of ARDS (Dhainaut et al., 2003). TGF-β1 is mitogenic and chemotactic for fibroblasts, monocytes, and macrophages, and promotes accumulation of the extracellular matrix (Blobe et al.,

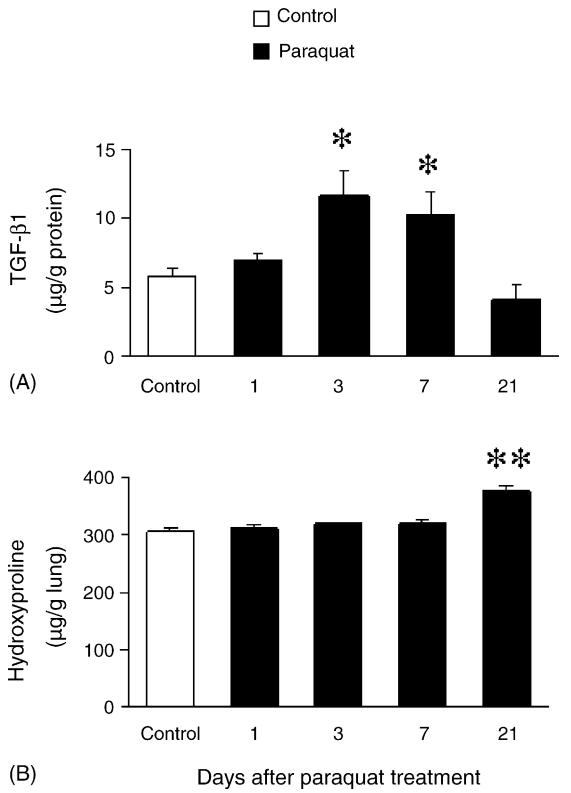

Fig. 3. TGF-β1 and hydroxyproline contents in control and paraquat-treated rat lungs. (A) Lung TGF-β1 levels progressively increased after paraquat treatment and values reached statistical significance on days 3 and 7. Values then decreased after day 7 and had returned to the control level by day 21 after paraquat treatment. (B) Hydroxyproline contents of lung tissues were significantly higher in paraquat-treated rats than control rats on day 21 ($^{*}P < 0.05$, $^{**}P < 0.01$).

2000). TGF-β1 not only participates in the active early phase of acute lung injury and contributes to the development of pulmonary edema, but is also associated with the late phase of acute lung injury and leads to pulmonary fibrosis (Dhainaut et al., 2003). Collagen is the major extracellular matrix component of the lungs and is vital for maintaining the normal lung architecture. Types I and III collagen are the most abundant collagen subtypes in the lungs (Kirk et al., 1984). They are present in the adventitia of pulmonary arteries, the interstitium of the bronchial tee, the interlobular septa, the bronchial lamina propria, and the alveolar interstitium. In this study, we found that increased TGF-β1 mRNA expression preceded the increase in collagen I mRNA expression and

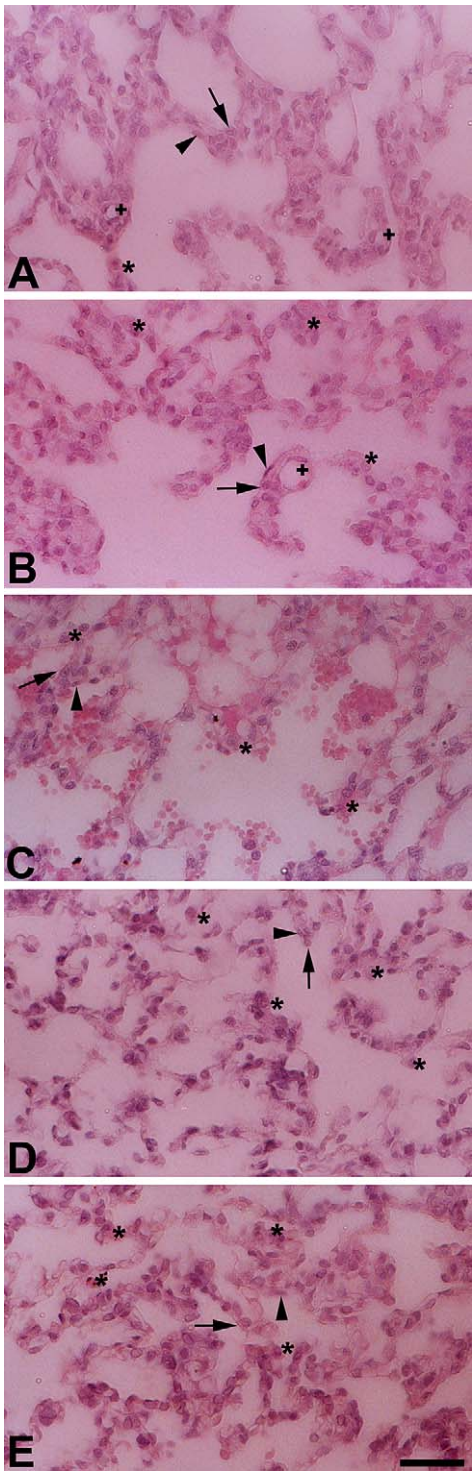

Fig. 4. Light micrographs of lung sections stained with hematoxylin and eosin from control (A) and paraquat-treated rats on days 1 (B), 3 (C), 7 (D), and 21 (E) after paraquat treatment (×400). The central component of the alveolar wall is the capillary (+) and its associated connective tissue. On each side faces the alveolus, flat, squamous pneumocyte type I cell (arrowhead) is interposed between the capillary and air spaces. Pneumocyte type II cell (arrow) lines the alveolus which shows a round shaped nucleus and is surrounded by a noticeable amount of cytoplasm. Large alveolar macrophage (⋆) were found in the alveolar wall or free in the alveolar space. Bar = 50 μm.

increased hydroxyproline content following the increase in TGF-β1 levels of lung tissues. Collagen III mRNA expression was significantly increased on day 1 after paraquat treatment, but the result was not related to TGF-β1 mRNA expression. This finding implies the presence of other pathways for collagen production and is consistent with the observations of Batra et al. (2003), who found that TGF-β1 does not increase collagen III synthesis in human lung fibroblasts. Previous studies also found that N-terminal procollagen peptide-III is elevated in tracheal aspirate, serum, and bronchoalveolar lavage fluid from ARDS patients within 24 h of diagnosis (Chesnutt et al., 1997; Marshall et al., 2000).

Transforming growth factors are multifunctional growth factors that are also involved in lung organogenesis. c-myc, a member of the basic helix-loop-helix leucine zipper family of transcription factors, is important for lung differentiation (Kim et al., 2003). In this study, we found that higher c-myc expressions in paraquat-treated lungs and the values were inversely correlated with TGF-β1 protein levels. These data are consistent with the findings of Kim et al. (2003) and indicate that lung differentiation was diminished during the fibrotic stage of acute lung injury. Oxidative agents generated from xanthine and xanthine oxidase induces c-myc expression (Shibanuma et al., 1988) and 2-h paraquat treatment increases in vivo lung xanthine oxidase activity (Waintrub et al., 1990). We speculate that higher c-myc expressions in paraquat-treated lungs than those in control lungs might be due to paraquat-induced xanthine oxidase.

ACE is distributed along the luminal pulmonary endothelial surface and hydrolyzes Ang I to Ang II. ACE activity decreases at an early stage of acute lung injury and can be used as a marker of underlying pulmonary capillary endothelial dysfunction (Orfanos et al., 2000). In this study, we used 20 mg/kg of paraquat and found that ACE activity had decreased on days 3 and 7 after paraquat treatment. This finding is consistent with the observations of Roth et al. (1979) and Venkatesan (2000), who found that lung ACE activity had significantly decreased on days 1 and 4 after 50 and 25 mg/kg paraquat treatment, respectively. These studies indicate that the paraquat-mediated decrease in lung ACE activity is dose dependent. The reduction in ACE activity was secondary to necrosis of pulmonary capillary endothelial cells (Hollinger et al., 1980). In this study, the alteration in ACE activity was concordant with the histological appearance that showed prominent pulmonary hemorrhage on day 3 after paraquat treatment.

Ang II is documented to be an inducer of TGF-β1 expression in the heart and kidneys (Campbell and Katwa, 1997; Klahr and Morrissey, 1998; Kupfahl et al., 2000). However, the role of Ang II in pulmonary fibrosis has not yet been clarified. In this study, we found that paraquat treatment decreased lung Ang II levels before the rise in TGF-β1 and hydroxyproline levels and the fall in ACE activity. These data indicate that Ang II is not an upstream activator of TGF-β1 in paraquat-induced lung injury, and lower lung Ang II levels may result from decreased angiotensinogen synthesis. These findings are inconsistent with the observations of Marshall et al. (2004), who found increased lung Ang II concentrations in bleomycin-induced lung injury. Several mechanisms may activate TGF-β1, including pathways involving interleukin-13 (Lee et al., 2001), CD36 and thrombospondin-1 (Yehualaeshet et al., 1999), as well as reactive oxygen intermediates including the superoxide anion and hydrogen peroxide (Bellocq et al., 1999). Paraquat treatment increases lung xanthine oxidase activity and generates superoxide anion and hydrogen peroxide (Waintrub et al., 1990; Suntres, 2002). In addition to the lungs, the kidneys are another target organ for paraquat toxicity in rats. The nephrotoxicity caused by paraquat is prominent and appears to involve convoluted renal tubules and proximal tubular cells (Murray and Gibson, 1972; Mølck and Friis, 1997). We speculate that the discrepancy in lung Ang II levels between paraquat and bleomycin studies might be due to other mechanisms activating TGF-β1 and the nephrotoxicity associated with paraquat treatment that might alter the activity of the renin–angiotensin system.

In conclusion, we found that increase in TGF-β1 mRNA expression and TGF-β1 levels preceded the onset of increased collagen I mRNA expression and hydroxyproline content and decreased Ang II levels in lung tissues with paraquat-induced lung injury. These results confirm previous study that TGF-β1 plays an important role in the fibroproliferative phase of paraquat-induced lung injury and this effect is independent of the renin–angiotensin system.

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