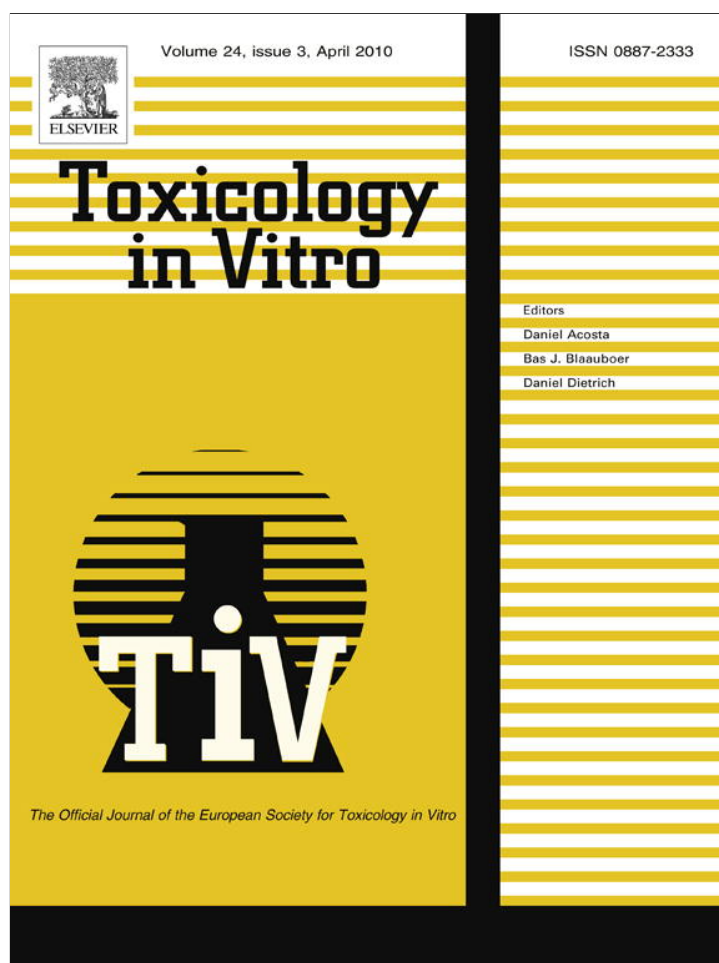




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Paraquat increases connective tissue growth factor and collagen expression via angiotensin signaling pathway in human lung fibroblasts

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ABSTRACT

Survivors of paraquat poisoning are left with pulmonary fibrosis which results in a restrictive type of long-term pulmonary dysfunction. Connective tissue growth factor (CTGF) is a key growth factor that initiates tissue repair and underlies the development of lung fibrosis. Angiotensin (ANG) II may induce CTGF expression in the heart and kidney and plays an important role in the pathogenesis of lung fibrosis. The biological effects of ANG II are mediated by ANG II type 1 receptor (AT1R) and AT2R. The aims of this study were to investigate the effects of paraquat on ANG II, ANG II receptors, CTGF, and collagen expressions and to assess the role of ANG II receptors in paraquat-induced collagen synthesis in human lung fibroblasts (MRC-5). MRC-5 cells were incubated with various concentrations of paraquat with or without the ANG II receptor antagonist, saralasin. Paraquat increased ANG II production and AT1R mRNA and protein expression and decreased AT2R mRNA expression. Furthermore, paraquat treatment increased CTGF and collagen mRNA and protein expression in a dose-dependent manner and saralasin inhibited these effects. These results indicate that paraquat increases CTGF and collagen expression by activating angiotensin signaling pathway in human lung fibroblasts.

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1. Introduction

Paraquat dichloride (1,1'-dimethyl-4,4'-bipyridilium dichloride; methyl viologen) is an effective and widely used herbicide. The intentional and accidental ingestion of commercial liquid formulations of paraquat has caused a large number of human fatalities in Taiwan during 1985 and 1997 (Satoh and Hosokawa, 2000). The lungs are one of the primary target organs in paraquat-induced toxicity in rats and humans (Chen and Lua, 2000; Dinis-Oliveira et al., 2008). The acute toxic effects of paraquat are pulmonary edema, hypoxia, and respiratory failure. Survivors of paraquat poisoning may be left with pulmonary fibrosis which results in a restrictive type of long-term pulmonary dysfunction (Yamashita et al., 2000).

Connective tissue growth factor (CTGF) is an important growth factor that initiates lung tissue repair and fibrosis (Bogatkevich et al., 2008). CTGF is a member of the CCN (CTGF, Cyr61/Cef10, Nov) family and was originally identified in conditioned media from human umbilical vein endothelial cells and mice fibroblasts; it has been implicated in fibroblast proliferation, cellular adhesion, angiogenesis, and extracellular matrix synthesis (Moussad and Brigstock, 2000). Angiotensin (ANG) II induces CTGF expression in the heart and kidney (Finckenberg et al., 2003; Rupérez et al., 2003) and plays an important role in the pathogenesis of lung fibrosis (Marshall et al., 2004). The biological effects of ANG II are mediated by its interaction with two distinct high-affinity G protein-coupled receptors now designated ANG II type 1 receptor (AT1R) and AT2R (DeGasparo et al., 2000). Most physiological and pathophysiological effects of ANG II are mediated via the AT1R (Iwanciw et al., 2003). Although CTGF has been reported to play a role in pulmonary fibrosis induced with bleomycin and hyperoxia (Lasky et al., 1998; Bonniaud et al., 2003; Chen et al., 2007), its relationship with ANG II has not yet been confirmed in paraquat-induced collagen production. The aims of this study were to investigate the effects of paraquat on ANG II, ANG II receptors, CTGF, and collagen expressions and to assess the role of ANG II receptors in paraquat-induced collagen synthesis in human lung fibroblasts.

Abbreviations: ANG, angiotensin; ATR, ANG II receptor; CTGF, connective tissue growth factor; MTT, 3-(4,5-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

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2. Materials and methods

2.1. Cell culture

MRC-5 cells (human lung fibroblasts; ATCC, Manassas, VA, USA) were maintained in Dulbecco's minimal essential medium (DMEM, GIBCO Invitrogen Life Technologies, Grand Island, NY, USA) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, and 10% heat-inactivated fetal calf serum (FCS; GIBCO Invitrogen Life Technologies), and incubated at 37 °C in 5% CO₂. Fibroblasts between passages 25 and 35 were used for all experiments. For collagen expression induced by paraquat, 50 µg/ml ascorbic acid and 50 µg/ml β-aminopropionitrile fumarate (Sigma–Aldrich, Saint Louis, MO, USA) were added to the culture medium. Cells were grown to confluence and then incubated in fresh medium with 0.2% fetal calf serum for 24 h, followed by administration of paraquat at indicated concentrations for 48 h. The cytotoxic effects of paraquat on incubated MRC-5 cells were measured using the 3-(4,5-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Mosmann, 1983) over a range of doses (100–900 µM) for 48 h. The cell viability was maintained at ~90% below 500 µM paraquat, but decreased significantly above 700 µM paraquat. Therefore, paraquat concentrations between 0 and 500 µM were used in this study. In order to verify the role of ANG II receptor signaling in paraquat-induced collagen synthesis, saralasin (10 µM) was added 1 h before paraquat treatment. The dose has been shown to inhibit ANG II-induced extracellular matrix production in mesangial cells (Davis et al., 2008). The conditioned media were used to determine ANG II and collagen concentration, while the cell pellets were used to examine gene expression by quantitative RT-PCR and protein expression by Western blot.

2.2. ANG II levels

The ANG II in culture supernatant was measured by an ELISA kit (Phoenix Pharmaceuticals, Burlingame, CA, USA). The standards or samples were incubated with anti-ANG II antibody and biotinylated ANG II, the bound biotinylated ANG II was reacted with streptavidin-horseradish peroxidase using tetramethyl benzidine and hydrogen peroxide as a substrate. The reaction was terminated by the addition of hydrogen chloride and absorbance was measured at 450 nm.

2.3. Quantitative RT-PCR

The abundance of mRNA was determined by reverse transcription, followed by real-time PCR using appropriate primers (Table 1). Total cellular RNA was isolated with TRIzol (Invitrogen Life technologies). DNase-treated RNA samples were reverse transcribed with poly (dT) primers using a first-strand cDNA synthesis

kit (GE Healthcare, Piscataway, NJ, USA). Gene expression was quantified by SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and carried out using the ABI Prism 7300 Sequence Detection System. The relative quantification of gene expression was normalized to 18S rRNA as internal standard. Triplicate experiments were done for each sample.

2.4. Immunofluorescence assay

MRC-5 cells were fixed in PBS with 4% formaldehyde and permeabilized with 0.1% Triton X-100. The fixed cells were incubated with an anti-AT1R polyclonal antibody (1:200, Abcam Inc., Cambridge, MA, USA), then indirect immunolabeling was performed by incubation with a Cy3-conjugated anti-mouse IgG antibody (1:200, Zymed). 4',6-Diamidino-2-phenylindole (DAPI, Sigma–Aldrich) was used for nuclear staining. Images of marked cells were visualized using a fluorescence microscope (Leica Microsystems, Exton, PA, USA).

2.5. Western blot analysis

In total, 50 µg of protein was separated on a 12% SDS–PAGE and transferred to a polyvinylidene fluoride membrane. The primary antibodies used in this study were rabbit anti-AT1R (Abcam, 1:1000), rabbit anti-CTGF (Abcam, 1:1000) and mouse anti-β-actin (1:100,000; Sigma–Aldrich). After incubation with the primary antibody, the membranes were probed with the appropriate horseradish peroxidase-conjugated secondary antibody (anti-mouse or anti-rabbit, 1:20,000; Pierce, Rockford, IL, USA). Immune complexes were visualized using ECL plus detection reagents (Pierce). Quantitative comparison of the fluorescent images was achieved with a densitometer. Densitometric analysis was performed to measure the intensity of Western blot bands using AIDA software (Advanced Image Data Analyzer; Raytest Izotopenmessgeraete, Straubenhardt, Germany).

2.6. Collagen measurement

Total soluble collagen was measured in cultured supernatant using the Sircol Collagen Assay Kit (Biocolor, Belfast, UK). Briefly, 0.3 ml of Sirius red reagent was added to an equal volume of test sample and mixed. The collagen–dye complex was dissolved in 0.5 M sodium hydroxide; the absorbance was measured at 540 nm. The collagen level in each specimen was obtained as an average of three readings.

2.7. Statistical analysis

Data are expressed as the mean ± SD. Differences among groups were evaluated by one-way ANOVA with *post hoc* Sheffe's test. A *p* value of <0.05 was considered statistically significant.

3. Results

3.1. Paraquat induces ANG II production and ANG II type I receptor (AT1R) mRNA and protein expression and reduces AT2R mRNA expression

Forty-eight hours after paraquat treatment, the ANG II levels and AT1R mRNA and protein expression increased, while AT2R mRNA expression decreased in a dose-dependent manner, and the values were significantly higher or lower in 100, 300, and 500 µM paraquat-exposed cells when compared with the 0 µM paraquat-exposed control, respectively (Fig. 1). Fig. 1A and B shows that fibroblasts incubated 48 h with 100, 300, 500 µM paraquat re-

Table 1
Primers used for RT-PCR.

Primer	Sequence 5' → 3'	Accession No.
AT1R	1422 ATCCACCAAGAAGCCTGCAC ¹⁴⁴¹ 1533 TGAAGTGCTGCAGGAATC ¹⁵¹⁴	NM_000685
AT2R	417 CCTCGCTGTGGCTGATTACTCCTT ⁴⁴¹ 517 CTTTGACATCACAGGTCCAA ⁴⁹⁷	NM_000686
Collagen I	1310 GTGCTAAAGGTGCCAATGGT ¹³²⁹ 1437 ACCAGGTTACCGCTGTTAC ¹⁴¹⁸	NM_000088
Collagen III	4413 AACACGCAAGGCTGTGAGACT ⁴⁴³³ 4500 GCCAACGTCCACACAAATT ⁴⁴⁸¹	NM_000090
CTGF	729 TTAGAGCCAACCTGCTGGTC ⁷⁴⁸ 828 CAGGAGCGTTGTCTATTGGTA ⁸⁰⁸	NM_001901
18S rRNA	70 GGACACGGACAGGATTGACA ⁸⁹ 119 ACCCACGGAATCGAGAAAGA ¹⁰⁰	AJ_844646

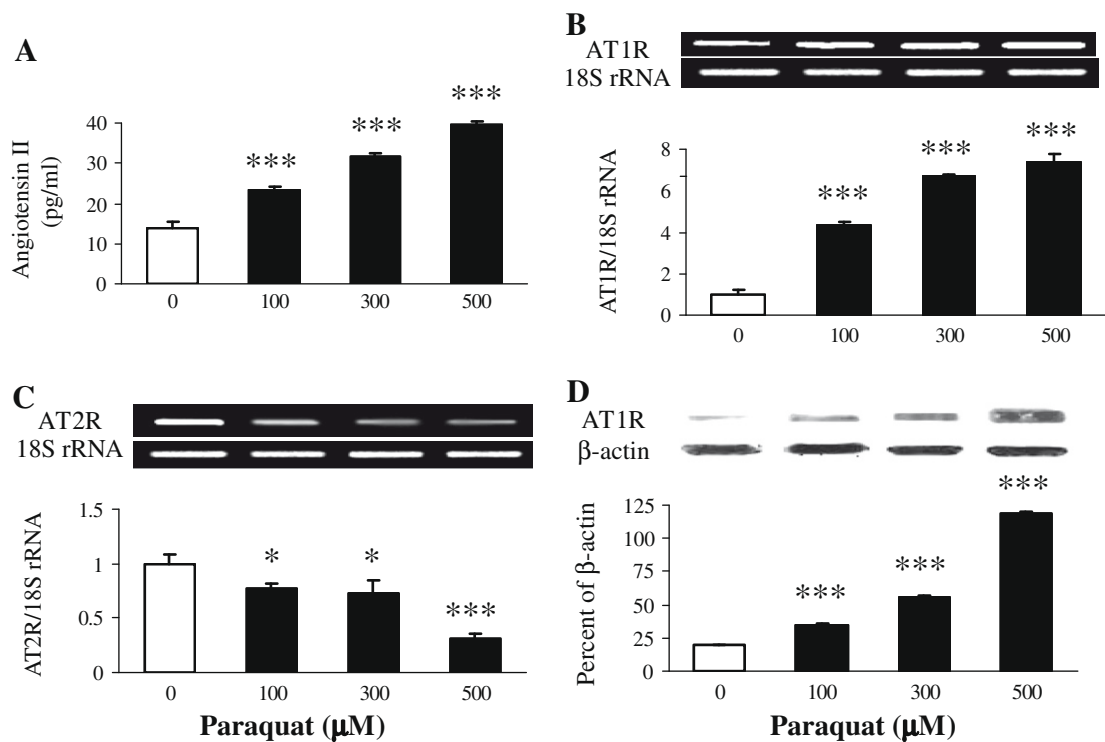


Fig. 1. Effects of paraquat on (A) angiotensin (ANG) II level, (B) ANG II type 1 receptor (AT1R) mRNA, (C) AT2R mRNA, and (D) AT1R protein in MRC-5 cells. MRC-5 cells were treated with the indicated concentrations of paraquat for 48 h. The ANG II level and AT1R mRNA and protein expressions increased after paraquat treatment in a dose-dependent manner. AT2R mRNA expression decreased after paraquat treatment. Data are presented as the mean $\pm$ SD ($n = 3$). * $p < 0.05$ and *** $p < 0.001$ vs. 0 μ M paraquat.

sulted in ~ 0.5 -, 1-, and 2-fold increase in ANG II level and 4-, 6-, and 7-fold increase in AT1R mRNA expression, respectively (all $p < 0.001$).

3.2. Immunofluorescent analysis of AT1R in human lung fibroblasts

The immunostaining assay for AT1R was performed to assess its expression and cellular localization in the fibroblast cultures. Expression of AT1R and assumption of a fibroblast-like morphology was induced in MRC-5 monolayer exposed to paraquat (0–500 μ M) for 48 h (Fig. 2). Immunofluorescence microscopy shows that AT1R was diffusely and uniformly distributed on the cell surface and in the cytoplasm. The immunoreactivity of AT1R increased as the paraquat dosage increased.

3.3. Paraquat induces CTGF mRNA and protein expression and saralasin inhibits these effects

CTGF mRNA expression significantly increased at 300 and 500 μ M paraquat-treated cells for 48 h when compared with 0 μ M paraquat-exposed controls ($p < 0.01$ and $p < 0.001$, respectively) and the addition of 10 μ M saralasin decreased CTGF mRNA expression (Fig. 3A). Paraquat treatment also significantly increased CTGF protein expression at doses of ≥ 100 μ M ($p < 0.001$) and saralasin completely inhibited its expression (Fig. 3B).

3.4. Paraquat increases collagen type I and III mRNA expressions and total collagen content and saralasin inhibits these effects

The experiments were performed to determine whether the fibroblast cultures respond to stimulation with paraquat by increasing collagen synthesis. Paraquat increased collagen type I and III mRNA expression and total collagen content after 48 h exposure (Fig. 4). The effects of paraquat on collagen expression

were observed at doses of ≥ 100 μ M tested fibroblast cultures ($p < 0.001$). The concentration-dependent increase in paraquat-stimulated collagen mRNA expression and total collagen content was reduced in cells treated with saralasin, compared with cells that were treated with paraquat alone.

4. Discussion

Paraquat may cause acute respiratory distress syndrome and the final clinical course is characterized by collagen deposition and pulmonary fibrosis that lead to reduced expansibility and vital capacity, and eventually impaired gas exchange. Death usually occurs due to respiratory failure (Dinis-Oliveira et al., 2008). Survivors of paraquat poisoning may be left with a restrictive type of long-term pulmonary dysfunction (Yamashita et al., 2000). The signaling pathway that leads to pulmonary fibrosis is not clear. This study showed the existence of an ANG II and CTGF pathway in paraquat-induced collagen production, which is sensitive to saralasin. This evidence supports a functional role for ANG II receptor antagonist in the treatment of paraquat-induced lung fibrosis.

ANG II was formerly described as a potent vasoconstrictor; it is now recognized as a profibrotic and plays a role in the pathogenesis of pulmonary fibrosis (Marshall et al., 2004; Otsuka et al., 2004). ANG II can be generated locally in lung tissues and may have autocrine and paracrine actions at the cellular level (Filippatos et al., 2001). The biological effects of ANG II are mediated by its interaction with two distinct high-affinity G protein-coupled receptors now designated AT1R and AT2R (DeGasparo et al., 2000). Most physiological and pathophysiological effects of ANG II are mediated via the AT1R (Iwanciw et al., 2003). Immunohistochemical studies have demonstrated that AT1R is expressed on alveolar type II cells, bronchiolar epithelial cells, vascular smooth muscle cells, endothelial cells, and fibroblasts (Otsuka et al., 2004). Lung AT1R expression was markedly increased in a rat model of bleomycin-in-

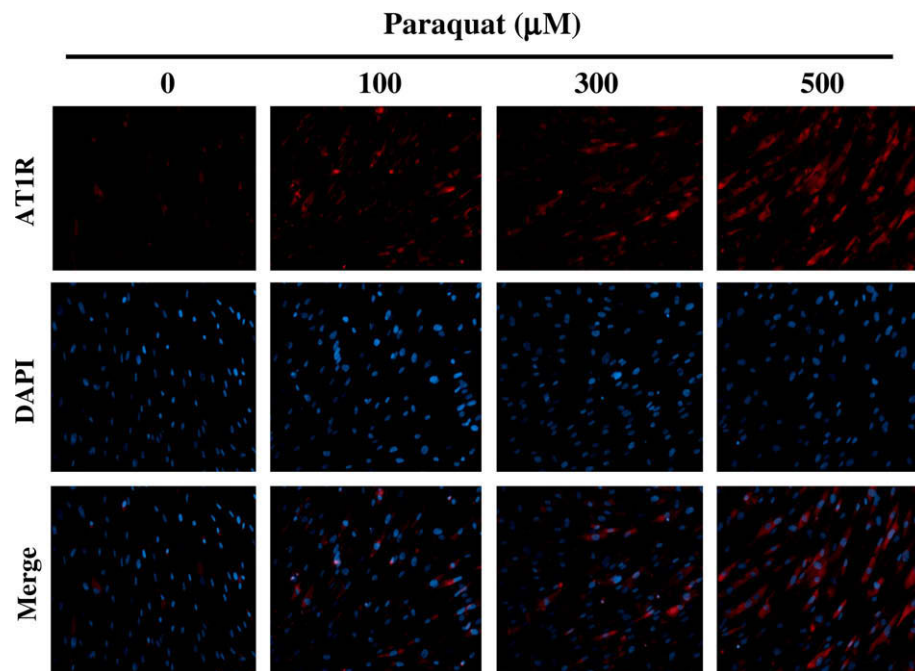


Fig. 2. Effects of paraquat on angiotensin II type I receptor (AT1R) immunoreactivity in MRC-5 cells ($\times 400$). MRC-5 cells were treated with paraquat at indicated concentrations for 48 h and then processed for immunofluorescence detection. Upper panel: Monolayer in media reacted with the anti-AT1R monoclonal antibody. Red staining represents AT1R immunoreactivity and the staining increased as the paraquat dosage increased. Middle panel: DAPI nuclear staining. The lower panel shows a merged image. Photographs are representative of >12 cultures from more than three separate experiments. Staining was performed in fibroblasts on at least four occasions in each case with consistent results. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

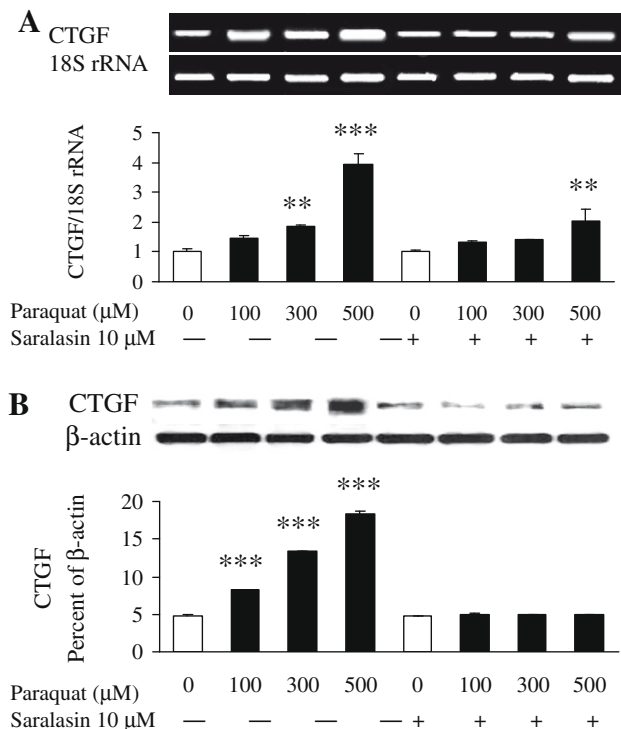


Fig. 3. Paraquat induced (A) connective tissue growth factor (CTGF) mRNA, and (B) CTGF protein expressions, and the angiotensin receptor antagonist, saralasin, inhibited these expressions. Confluent monolayers of MRC-5 cells were exposed to various concentrations of paraquat for 48 h with or without saralasin (10 μ M). The cell pellets were used to examine CTGF mRNA expression by quantitative RT-PCR and protein expression by Western blot. The same membrane was probed with an anti- β -actin antibody to assess equal loading of the gel. Data are presented as the mean $\pm$ SD ($n = 3$). ** $p < 0.01$ and *** $p < 0.001$ compared with 0 μ M paraquat with or without saralasin.

duced pulmonary fibrosis (Otsuka et al., 2004). In this study, we found that paraquat significantly increased ANG II and AT1R and collagen mRNA and protein expressions and ANG II receptor antagonist inhibited collagen synthesis in human lung fibroblast. These studies suggest that increased signaling through the ANG II receptor may be involved in mediating paraquat-induced pulmonary fibrosis.

CTGF is a multifunctional cytokine that plays an important role in lung fibrosis. CTGF has been reported to be increased in patients with scleroderma with severe pulmonary fibrosis (Sato et al., 2000) and associated with an animal model of pulmonary fibrosis (Chen et al., 2007). A previous in vitro study demonstrated that ANG II stimulation increased CTGF expression in human lung fibroblasts (Huang et al., 2006). However, whether ANG II signaling regulates CTGF expression in paraquat-induced collagen synthesis is unclear. In this study, we found that paraquat increased ANG II and CTGF mRNA and protein expression in a dose-dependent manner and inhibition of angiotensin signaling pathway reduced CTGF and collagen expression. These results suggest the existence of an ANG II and CTGF pathway in paraquat-induced collagen production. We previously demonstrated that paraquat-induced lung fibrosis is independent of ANG II in rats (Chen et al., 2005). Tissue fibrosis is a complex response initiated to protect the host from an injurious event and involves massive deposition of matrix. The difference in response of ANG II to paraquat in MRC-5 fibroblasts and in rats remains unclear, but it may be due to other lung mesenchymal cells that regulate collagen production in vivo.

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 tree, the interlobular septa, the bronchial lamina propria, and the alveolar interstitium. Although acute lung injury presents as three consecutive: exudative, proliferative, and fibrotic phases, recent evidence

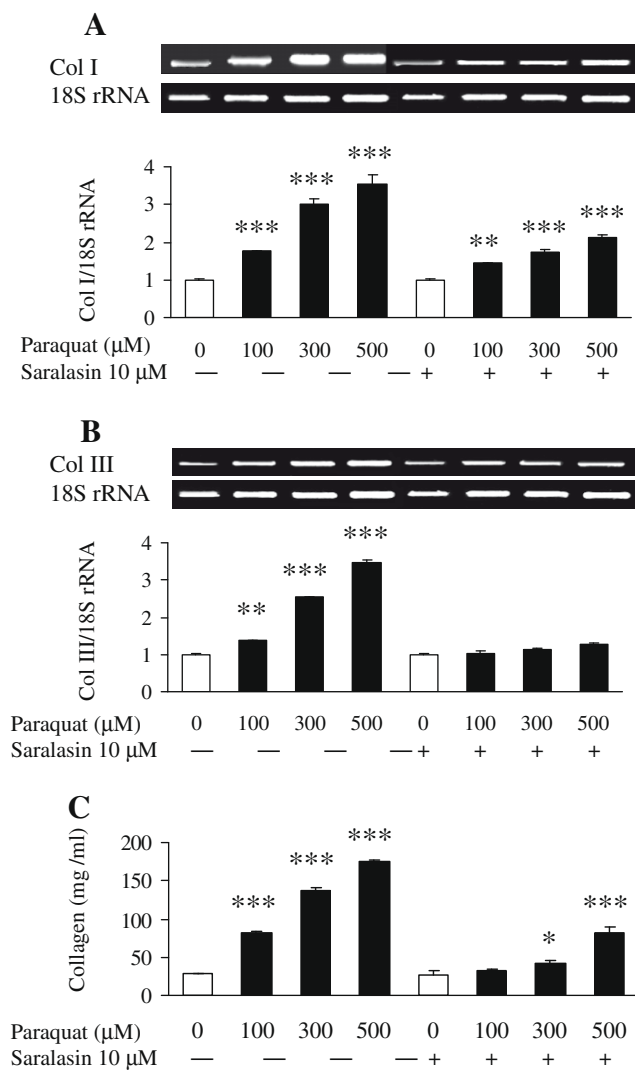


Fig. 4. Effects of paraquat alone or combined with saralasin on (A) type I collagen (Col I) mRNA expression, (B) type III collagen (Col III) mRNA expression, and (C) total collagen content in MRC-5 cells. MRC-5 cells were treated with paraquat at the indicated concentrations for 48 h. Saralasin (10 μM) was added 1 h before paraquat treatment. The cells were harvested and real-time RT-PCR was applied to examine the mRNA levels of Col I and Col III. Conditioned medium was collected for measurement of collagen content by a Sircol Collagen Assay Kit. Type I and III collagen mRNA expression and total collagen content increased after paraquat treatment in a dose-dependent fashion. The concentration-dependent increase in paraquat-stimulated collagen mRNA expression was reduced by the addition of saralasin. Data are presented as the mean $\pm$ SD ($n = 3$). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ vs. 0 μM paraquat alone or plus saralasin.

suggests that there is an overlap of the inflammatory and fibroproliferative phases (Marshall et al., 2000). The amounts of collagen type I (Liebler et al., 1998) and III (Chesnutt et al., 1997; Meduri et al., 1998) and the number of collagen fibers (Rocco et al., 2001) increase early in the course of acute lung injury and influence the respiratory mechanics. In this study, we found that collagen increased 48 h after paraquat treatment in human lung fibroblasts. Those findings and our in vitro results suggest that the proliferative phase begins early in the evolution of the lesions.

In conclusion, our data showed paraquat increased ANG II production, AT1R, CTGF and collagen mRNA and protein expressions in a dose-dependent manner and saralasin inhibited CTGF and collagen production in human lung fibroblasts. These results indicate these effects involve the activation of CTGF and angiotensin signaling pathways via the AT1R. With an understanding of these signal

transduction pathways, we can design therapeutic strategies to reduce fibrosis caused by paraquat intoxication.

Conflict of interest statement

None declared.

Acknowledgment

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