Original Research

Expression and significance of HIF-1α in pulmonary fibrosis induced by paraquat

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Abstract

It is commonly accepted that epithelial–mesenchymal transition contributes to fibrotic remodeling, but the molecular pathways involved in paraquat (PQ)-induced epithelial–mesenchymal transition remain uncharacterized. The objective of this study was to evaluate the potential involvement of HIF-1α in TGF-β1/β-Catenin and Snail pathway after PQ poisoning. In our study, 86 Spragne-Dawley rats were randomly divided into control group and PQ group, which received intragastric infusion of 20% PQ solution 50 mg/kg. Rats in the PQ group were subsequently divided into eight subgroups (10 for each subgroup) and samples were collected at different predetermined time points (2, 6, 12, 24, 48, 72, 96 h and 7 d). Fibrosis markers, including β-catenin, snail and α-SMA, were measured by western blot. The activity of HIF-1α was determined by western blot and immunofluorescence. We found that in PQ-induced pulmonary fibrosis, the level of PaO_2 was significantly reduced in the 6-h subgroup, when compared to the control group ($P < 0.01$). Interestingly, between 6 and 72 h, there was no significant difference in PaO_2. On the other hand, the level of $PaCO_2$ started to increase from 72-h subgroup ($P < 0.01$). Fibrosis markers including β-catenin, snail and α-SMA, measured by western blot, were significantly increased at 2 h, while the level of p-GSK-3β was increased at 6 h. And the level of GSK-3β showed significant reduction beginning at 24 h. The activity of HIF-1α measured by western blot assays was significantly increased starting from 2 h with sustained expression. The result of Pearson coefficient analysis showed that HIF-1α was positively correlated with Snail ($r = 0.935$, $P < 0.01$) and β-catenin ($r = 0.761$, $P < 0.05$). Meanwhile, immunofluorescent analysis of HIF-1α revealed partial staining appearing from 2 h. Our data illustrated a positive correlation between Snail, β-catenin signaling and HIF-1α, suggesting a potential synergistic role of HIF-1α in PQ-induced pulmonary fibrosis, which may be independent of GSK-3β. It might also represent a potential therapeutic window for treatment of paraquat poisoning.

Keywords: Paraquat, pulmonary fibrosis, epithelial–mesenchymal transition, hypoxia-inducible factor 1α, transforming growth factor β1

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Introduction

Paraquat (PQ), widely used as herbicide in developing countries, accumulates mainly in the lungs when ingested. Unlike most other poisons, the clinical course of paraquat intoxication is often protracted, and there is no known antidote for it. The outcome is often unsatisfactory. PQ leads to destruction of the alveolar epithelium, followed by acute lung injure (ALI) or acute respiratory distress syndrome (ARDS) and finally results in progressive, inexorable pulmonary interstitial fibrosis.[1] Studies on mechanism of fibrosis induced by PQ are vital in improving treatment and reducing mortality.

The existing literatures have suggested that the pathological hallmark of pulmonary fibrosis is excessive accumulation of extracellular matrix (ECM). Several possible origins of ECM-producing cells have been described: accumulation of resident lung fibroblasts, homing and fibroblastic differentiation of bone marrow-derived cells such as circulating fibrocytes or monocytes[2,3] and/or epithelial–mesenchymal transition (EMT).[4,5] Through EMT, epithelial cells lose intercellular adhesion and transform from the epithelial phenotype into mesenchymal phenotype. This allows them to participate in the synthesis of the fibrotic matrix. Although it remains uncertain to what degree EMT contributes directly to ECM, signaling pathways critical to EMT are important for tissue repair and fibrosis. Studies reported that EMT can be triggered by multiple signaling molecules, such as transforming growth factor β1 (TGF-β1), epidermal growth factor (EGF),

fibroblast growth factor (FGF), bone morphogenetic proteins (BMPs), WNTs and so on.[6,7]

Generally, it is the level of TGF-β1 that best correlates with the extent of fibrosis and TGF-β1 signaling pathway is involved in cell proliferation, recognition, differentiation, apoptosis, and specification of developmental fate.[8] It is widely recognized as a key profibrotic cytokine and does not proceed linearly but rather induces a complex network of cascades. These pathways mutually influence each other and cross-talk with multiple signaling pathways,[9] such as TGF-β1/β-Catenin and Snail pathways to successfully induce tissue fibrosis.[10–12] Nevertheless, the molecular mechanisms underlying this effect are largely unknown.

As widely known, hypoxemia, a common feature of ALI/ARDS, is a result of vascular damage and contributes to the development of fibrosis. In order to cope with hypoxic stress, the primary transcriptional response is mediated by the hypoxia-inducible factor 1 (HIF-1) which receives growing recognition as a common link between hypoxia, inflammation, metabolic adaptation, and tumor progression.[13] HIF-1 is a transcription factor composed of HIF-1α and HIF-1β subunits with the expression and activation of the HIF-1α subunit strictly regulated by the cellular O_2.[14] Moreover, research reported that during chronic kidney fibrosis, the degree of HIF-1α expression is correlated with the degree of tissue injury and fibrosis.[15] Studies also demonstrated that hypoxia stimulates hepatocyte fibrosis by a HIF-1α and TGF-β1-dependent mechanism.[16] As previously reported,[17] we also observed up-regulated expression of HIF-1α in PQ-induced pulmonary fibrosis. These reports prompt that HIF-1α, cooperating with TGF-β1/β-Catenin and Snail, may play a role as cross-linking agency in pulmonary fibrosis induced by PQ. But the mechanism of HIF-1α in PQ-induced lung fibrosis is rarely reported. So we hypothesized that, after PQ poisoning, HIF-1α plays a role in fibrogenesis in part through its activation of TGF-β1/β-Catenin or Snail signaling pathway. By means of the establishment of animal model, the expressions of HIF-1α and fibrosis-related protein at different time point can be detected and processed to explore the relationships between them. Then we explored which fibrosis-related signaling pathway were activated and whether HIF-1α is associated with activated signaling pathway in order to establish the foundation of molecular mechanism of lung fibrosis induced by PQ.

Materials and methods

Chemicals and reagents

PQ solution was purchased from Syngenta Nantong Crop Protection Co. Ltd, China. Antibodies: HIF-1α (BioWorld, USA); TGF-β1 (BioWorld, USA); LOXL2 (Santa Cruz, USA); Snail (Abcam, USA); GSK-3β (Cell Signaling, MA); P-GSK-3β (Cell Signaling, MA); β-Catenin (Santa Cruz, USA); β-actin (Abmart, China).

Animals and treatment

In total, 86 healthy male Spragne-Dawley rats with initial weight of 250 ± 30 g (obtained from Shanghai Laboratory Animal Center; Animal permit number: SYXK (Hu) 2009-0086) were housed individually in rooms maintained at 23 ± 0.5 °C and 50 ± 0.5 humidity using a 12-h light cycle for a week and were given a standard rat chow and water allowed ad libitum. PQ was prepared immediately before administration. These rats were randomly divided into two groups. Rats in the control group ($n = 6$) underwent gavage with 1 mL normal saline, while those in the PQ group ($n = 80$) received intragastric infusion of 20% paraquat solution 50 mg/kg. Rats in PQ group were subsequently divided into 8 subgroups (10 for each subgroup) at different predetermined time points (2, 6, 12, 24, 48, 72, 96 h and 7 d).

However, some poisoned rats in PQ group could not live up to predetermined time points, so only six rats in each PQ subgroup were used for analyses. The current experiment was performed under the guidelines for use of experimental animals with permission of the animal ethical committee of Shanghai Jiao Tong University.

Rats sacrificing and tissue harvesting

At different predetermined time points, the rats were sacrificed after intraperitoneal injection of pentobarbital (50 mg/kg) and adopted blood directly from aorta abdominalis. After exsanguination, the upper lobe of the right lung was harvested and flash frozen in liquid nitrogen for subsequent assays. In order to get histological sections, the left lung was soaked in formalin solution and processed for embedding in paraffin.

Arterial blood gas level

Levels of PaO_2 and $PaCO_2$ were measured by i-STAT 300 (Abbott Laboratories, USA) using CG4 + silicon chips.

Immunofluorescence of HIF-1α

Immunofluorescence analyses were performed as follow. Paraffin sections (4 μm) were deparaffinized and endogenous peroxidase was quenched using 3% hydrogen peroxide diluted in distilled water. After antigen retrieval, the sections were blocked with 5%BSA for 20 min and then exposed to primary antibodies (HIF-1α, 1:200) at 4 °C overnight in a humidified chamber, followed by incubation at room temperature for 30 min with fluorescein-labeled secondary antibody. Then DAPI solution was applied for 5 min for nuclear staining. Each slide (10 random fields on each) was calculated by Image J software to estimate the positive area. The average value of 10 fields was used for this animal.

Western blot

Protein, extracted from the upper lobe of the right lung with RIPA buffer, was centrifuged and quantified using BCA protein assay kits (Bioengineering Institute, China) with bovine serum albumin as the standard. 60 μg of protein from each sample was resolved on 8% SDS-PAGE and transferred to polyvinylidene fluoride membrane which was blocked for 1 h at room temperature. Then the membranes were immunoblotted at 4 °C overnight with primary antibodies (HIF-1α, 1:1500; TGF-β, 1:1500; LOXL2, 1:1500;

Snail, 1:2000; GSK-3β, 1:2000; P-GSK-3β, 1:2000; β-Catenin, 1:2000; β-actin, 1:3000). After incubation with the appropriate HRP-conjugated secondary antibody (Biomart, China), the antigens were detected using ECL (Thermo, USA). All experiments were repeated at least three times. The relative quantities of protein products were determined using Image J software for windows.

Statistical analysis

Data were expressed as mean ± standard deviation (SD). By SPSS 16.0 software, multiple comparisons were initially subjected to one-way analysis of variance (ANOVA) and *t*-test was used for pair-wise comparisons. Pearson coefficient is used for analysis of correlation between the data. $P < 0.05$ was considered the threshold value for statistical significance.

Results

Changes of arterial partial pressure of oxygen and carbon dioxide

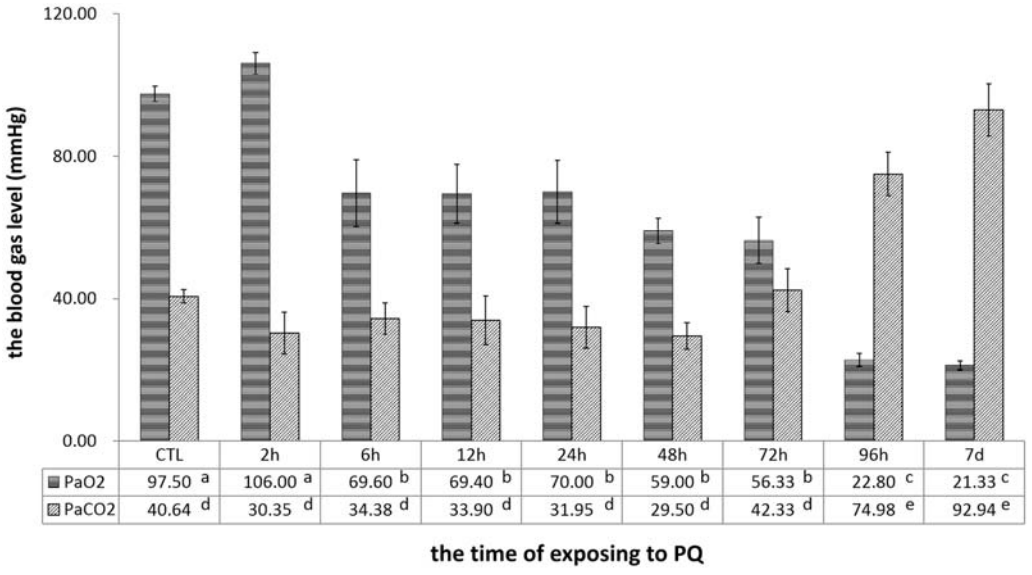
We assessed pulmonary function by the changes of arterial partial pressure of oxygen and carbon dioxide (PaO_2 and $PaCO_2$). During the observed time period, the level of PaO_2 was significantly reduced in the 6 h subgroup (69.60 ± 9.40 mmHg), compared to control group (97.50 ± 2.12 mmHg, $P < 0.01$). Interestingly, between 6 and 72 h subgroups, data showed no significant difference in PaO_2. It means that, at an early stage (between 6 and 48 h), the rats were undergoing relative stable state. On the other hand, the level of $PaCO_2$ started to increase from the 96 h subgroup (110.93 ± 6.16 mmHg, $P < 0.01$). These data demonstrated that hypoxia occurred at early stage (6 h) of PQ-induced ALI and hypercapnia began at 96 h after PQ-poisoning which was mainly due to ventilation dysfunction (Figure 1).

Expression of HIF-1α, β-Catenin, Snail, α-SMA, GSK-3β, and p-GSK-3β

We attempted to determine which pulmonary fibrosis related pathways were activated following PQ poisoning, and whether HIF-1α pathway was associated with the activated pathways. The results of HIF-1α measured by western blot assays exhibited that there was an increase in HIF-1α activity starting from the 2 h subgroup with sustained expression. β-Catenin, Snail and α-SMA, measured by western blot, were increased at 2 h while level of p-GSK-3β was increased at 6 h. And level of GSK-3β showed reduction beginning from 24 h. The level of E-cadherin showed reduction beginning from 48 h and the level of TGF-β1 began to increase at 12 h after the poisoning (Figures 2 and 3). By calculating gray value, HIF-1α was found to be positively correlated with Snail (Pearson coefficient, $r = 0.935$, $p < 0.01$) and β-Catenin (Pearson coefficient, $r = 0.761$, $p < 0.05$).

Immunofluorescence of HIF-1α

Immunofluorescent analysis of HIF-1α revealed partial staining presented at 2 h. In the meanwhile, thickened alveoli septum at 12 h and disorder of alveolar structure beginning at 48 h could also be observed. In order to assess the expression of HIF-1α, immunofluorescent images were calculated by Image J, which indicated the expression of HIF-1α was significantly increased at 2 h (Figure 4).



	CTL	2h	6h	12h	24h	48h	72h	96h	7d
PaO2	97.50 a	106.00 a	69.60 b	69.40 b	70.00 b	59.00 b	56.33 b	22.80 c	21.33 c
PaCO2	40.64 d	30.35 d	34.38 d	33.90 d	31.95 d	29.50 d	42.33 d	74.98 e	92.94 e

Figure 1 Arterial blood gas level on PQ group at different predetermined time points. This table shows the data of PaO_2 and $PaCO_2$ levels at different predetermined time points (CTL, 2, 6, 12, 24, 48, 72, 96 h and 7 d). The statistical data were treated by ANOVA and SNK test. (a–e), Means with the same letter are not significantly different ($P > 0.05$)

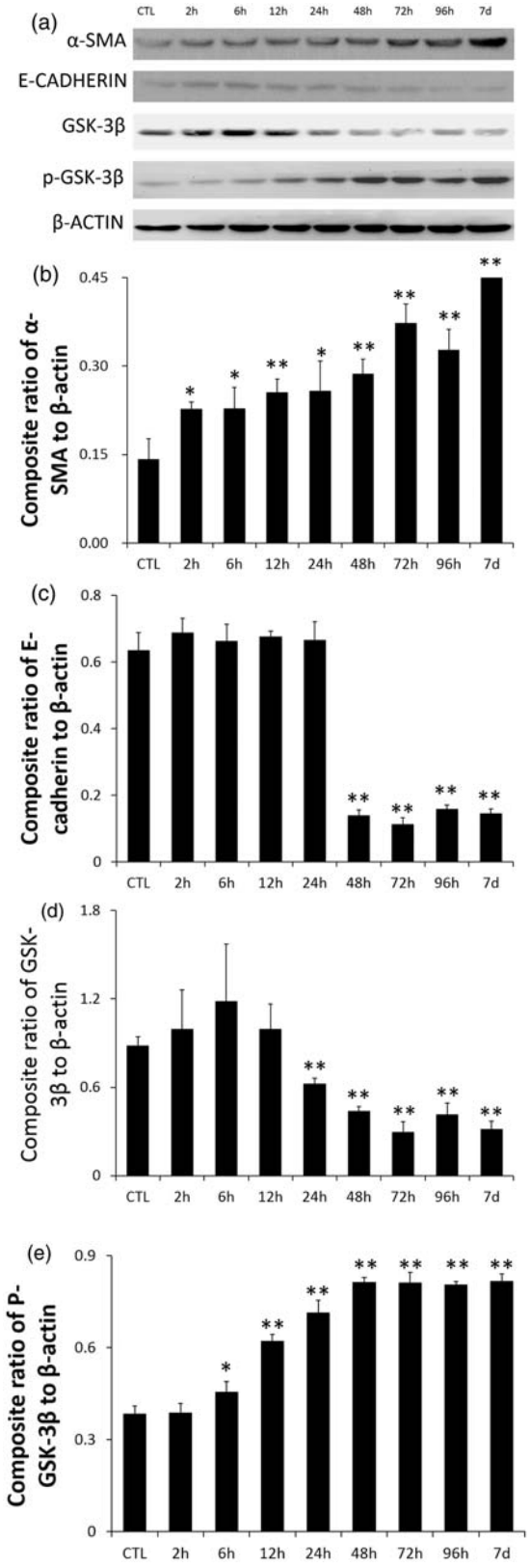


Figure 2 Western blot analysis of α-SMA, E-cadherin, GSK-3β and p-GSK-3β. (a), At different observing point, representative immunoblot of α-SMA, E-cadherin, GSK-3β and p- GSK-3β is shown. (b–e), Western blot analysis of the expression of α-SMA, E-cadherin, GSK-3β and p-GSK-3β in rat lung from each subgroup ($n = 6$) and control group ($n = 6$). β-actin was the loading control. Relative protein expression was determined as the gray value ratio to β-actin and compared with control group. Each bar is the mean ± SD ($n = 6$). *Significantly ($p \leq 0.05$) different from CTL; **Significantly ($p \leq 0.01$) different from CTL

Discussion

During PQ poisoning, the elevation of HIF-1α is not triggered by hypoxia

The lung is considered the primary target organ for PQ toxicity as a consequence of the accumulation of PQ. Hypoxia, resulting from capillary damage and inflammatory effusion, has been considered as an important factor in the lung injure induced by PQ poisoning. In normal conditions, HIF-1α can be activated under hypoxic conditions or in response to certain cytokines. Majority of researches show that, in tissue with normal oxygen level, HIF-1α is rapidly and continuously degraded post-translation due to the existence of dioxygen, which inhibits the dimerization of HIF-1β and subsequent stimulation of the transcription of its target genes.[18]

However, in our experiments, we observed that hypoxia started at 6 h, which was later than the elevation of HIF-1α (2 h). In other words, the initiator of PQ-induced HIF-1α is not hypoxia. These results were consistent with our previous research.[17] It demonstrated that, during the normoxic phase after exposure to PQ, there was an increased expression of HIF-1α. Study reported that blocking HIF-1α degradation by inhibition of prolyl hydroxylases leads to HIF-1α stabilization.[19] Meanwhile, Reactive Oxygen Species generated by the mitochondrial complex III participate in HIF-1α stabilization even under normoxic conditions.[20] These results indicate that during PQ poisoning the elevation of HIF-α is not be triggered by hypoxia-dependent regulation, but appears to be under the influence of other stimuli such as PQ molecule, inflammation, reactive oxygen species or growth factors.[21] It needs to be confirmed by further studies.

EMT participated in the development of lung fibrosis induced by paraquat

It is well described that EMT plays a key role in embryonic development, wound healing, tissue regeneration, organ fibrosis, and cancer progression. EMT is characterized by loss of cellular polarity, intercellular adhesion proteins (e.g. E-cadherin) and transition to a mesenchymal phenotype.[22] In the previous researches,[17,23] we have observed that the exposure of PQ poisoning resulted in an increased expression of mesenchymal marker, such as α-smooth muscle actin (α-SMA), and increase of collagen deposition at early stage (2 h). Moreover, our present data showed a significant decrease in the expression of epithelial marker such as E-cadherin at 48 h, coupled with a significant increase of mesenchymal marker such as α-SMA at 2 h, these data indicated that EMT participated in the development of lung fibrosis induced by paraquat.

Potential relation between HIF-1α and EMT in lung fibrosis induced by paraquat

In order to explore the role of HIF-1α in EMT, we analyzed the expression of several key proteins participated in EMT. TGF-β1 is considered the most important growth factor

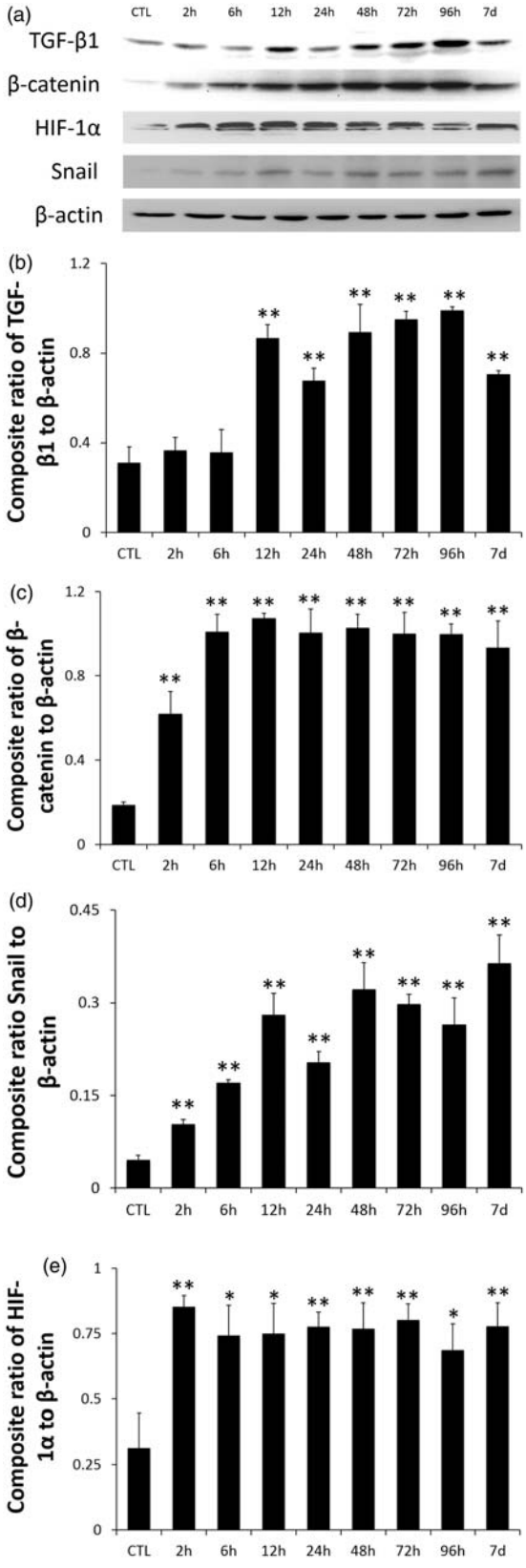


Figure 3 Western blot analysis of TGF-β1, β-Catenin, HIF-1α and Snail. (a), At different predetermined time points, representative immunoblot of TGF-β1, α-SMA, β-Catenin, HIF-1α and Snail is shown. (b–e), Western blot analysis of related protein expression in rat lung from each subgroup ($n = 6$) and control group ($n = 6$). β-actin was the loading control. Relative protein expression was determined as the gray value ratio to β-actin and compared with control group. Each bar is the mean ± SD ($n = 6$). *Significantly ($p \leq 0.05$) different from CTL; **Significantly ($p \leq 0.01$) different from CTL. Correlation is significant at 0.05 level

involved in pulmonary fibrogenesis. According to our results of western blot and immunofluorescence, we observed that the increase of TGF-β1 level was preceded by a rapid raise of HIF-1α. It exhibits that there may be an interaction between HIF-1α and TGF-β1 signaling in PQ-induced fibrosis. Similar to our opinion, a study reported that, in hepatocytes, hypoxia-induced EMT was TGF-β1-dependent and TGF-β1 signaling is the downstream pathway of HIF activation.[16] In contrast, another research reported that, in myocardial fibrosis or cardiomyocyte hypertrophy, HIF-1α played a protective role by suppression of TGF-β1 signaling.[24] So, according to the current literature on the mechanism of fibrosis, the relation between HIF-1α and TGF-β1 is not clear. However, in alveolar epithelial fibrosis induced by PQ, our data demonstrated that HIF-1α participated in TGF-β1 signaling which resulted in EMT.

Snail, the zinc finger transcription factor, is a convergence point in EMT induction and is found to be a downstream transcriptional factor of TGF-β1. Snail genes have been reported to be essential in EMT, which is partly due to the direct repression of E-cadherin transcription and contribution to the expression of mesenchymal phenotype. Meanwhile, Snail genes also act as survival factors and as inducers of cell movement.[6,25] Our data shows a positively correlation between Snail and HIF-1α, suggesting a potential synergistic role for HIF-1α in Snail-induced EMT.

On the other hand, recent report shows that β-Catenin is another potential downstream transcriptional factor of TGF-β1.[26] The activation of Wnt/β-Catenin signaling and downstream target genes have been demonstrated in lungs and AECs in IPF, suggesting a role for Wnt/β-Catenin signaling in EMT.[27] Recent research showed, in LNCaP cell lines, blockage of β-Catenin signaling pathway induced by HIF-1α via shRNA causes reversal of EMT and metastatic phenotypes changes.[28] In our experiments, after PQ poisoning, there was a positive correlation between β-Catenin and HIF-1α, which indicates that there was some interaction between them.

In addition, inactivation of GSK-3β (p-GSK-3β) results in accumulation of β-Catenin and Snail, which has also been strongly implicated in fibrosis.[26,29] Recent discovery of functional cross-regulation between these pathways has shown complex roles of HIF-1α, TGF-β1 and β-Catenin.[30] However, our data showed that the level of GSK-3β decreased much later than the activation of β-Catenin and Snail, suggesting that there was extra-cellular signaling linked to β-Catenin or Snail.

These observations suggested that HIF-1α may regulate β-Catenin or Snail independently from GSK-3β signaling pathway (Figure 5). Recent study has demonstrated that HIF-1α can be activated through the PI3K/Akt pathway in fibrogenesis of human lung fibroblasts.[31] Meanwhile, studies also identified lysysl oxidase (LOX) as a downstream target of HIF-1α, which may functionally interact with transcriptional repressors such as GSK-3β.[11] While in PQ-induced fibrosis, further experiments are needed to confirm our hypothesis.

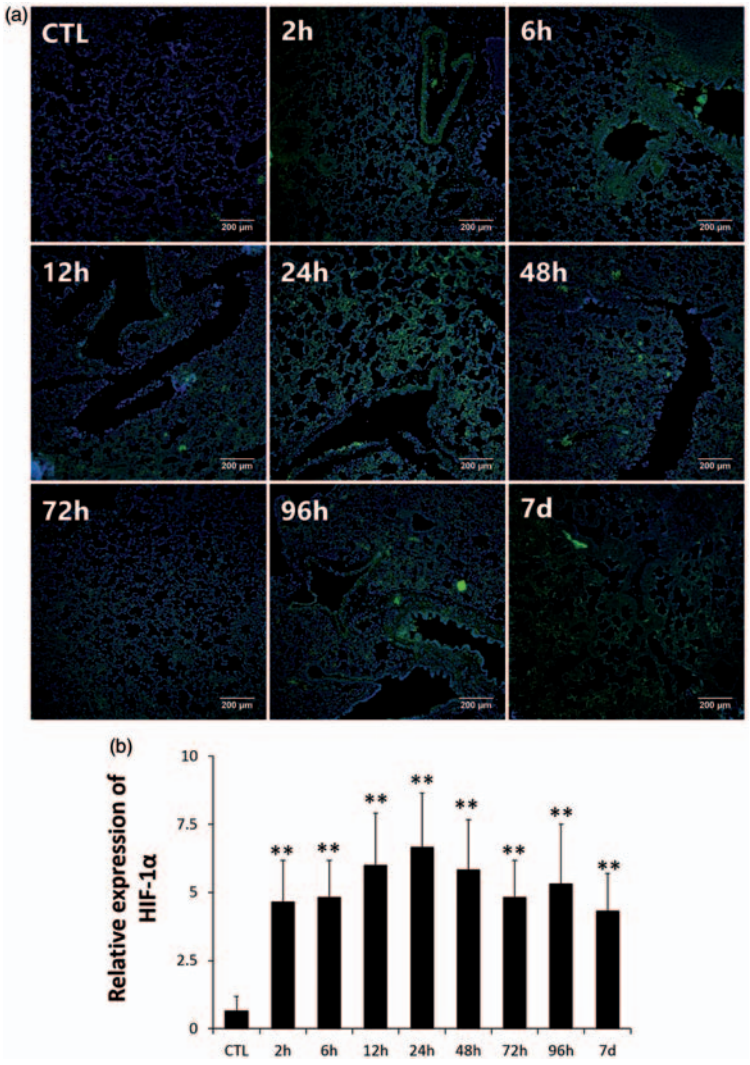


Figure 4 The expression of HIF-1α by immunofluorescent staining (400X). (a), The expression of HIF-1α in rat lung following PQ exposure. Lung sections, prepared for 2, 6, 12, 24, 48, 72, 96 h, and 7 d after exposure to PQ (50 mg/kg) or normal saline control (CTL), were stained by immunofluorescent staining. Representative sections from six rats are shown. (b), Lung sections were calculated for expression of HIF-1α, according to intensity of fluorescence and extent of lesion by Image J. *Significantly ($p \leq 0.05$) different from CTL; **Significantly ($p \leq 0.01$) different from CTL. (A color version of this figure is available in the online journal)

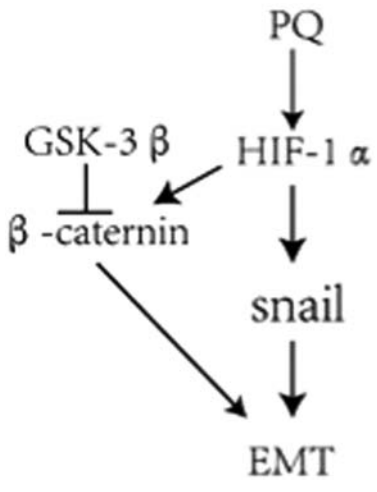


Figure 5 Potential signaling pathways involved in PQ-induced lung fibrosis. PQ may promote the development of lung fibrosis through HIF-1α signaling which may regulate β-Catenin or Snail independently from GSK-3β signaling to induce EMT

After paraquat poisoning, there is a relatively stable stage in pathophysiology

Furthermore, our study also demonstrated that the pulmonary injury, including hypoxia and collagen III deposition induced by PQ poisoning, occurred at very early (between 2 and 6 h).[23] After that, there is a relatively stable period (between 6 and 48 h). During this stage, PaO_2 levels in rats neither exhibited a further decrease, nor was there any significant increase in collagen III deposition, and $PaCO_2$ level. These demonstrated that during the time window (6–48 h) after PQ poisoning in rats, there was a pathophysiological equilibrium without significant organ dysfunction. In addition, during the time window, cells continued to exhibit epithelial-specific morphology and molecular markers (E-cadherin), but showed concomitant expression of mesenchymal marker (α-SMA), suggesting the notion of ''partial EMTs''.[22] This phenotype indicated that epithelial cells under PQ poisoning can advance to various extents of EMT. Since the elevation of HIF-1α does

occur before this time window, possible therapeutic interventions during or before the time window focused on HIF-1α signaling may alleviate EMTs and potentially attenuate organ fibrosis induced by PQ.

Conclusions

According to our data, in PQ poisoning, the elevation of HIF-1α is not triggered by hypoxia-dependent regulation. Meanwhile, EMT is involved in the pathogenesis and development in lung fibrosis induced by paraquat. HIF-1α may regulate β-Catenin or Snail independently from GSK-3β signaling to induce EMT. After paraquat poisoning, there is a relatively stable stage in pathophysiology. during or before this period, therapeutic interventions via HIF-1α signaling may alleviate EMTs and potentially attenuate organ fibrosis induced by PQ.

Author contributions: RW was charged of the design, conceived the study and review of the manuscript; HX and JT performed the experiments and drafted the manuscript. XT and XM made a analysis of the data; SG participated in the manuscript. All authors have read and approved the final manuscript.

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