Hypoxia-inducible factor-1α regulates epithelial-to-mesenchymal transition in paraquat-induced pulmonary fibrosis by activating lysyl oxidase

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Abstract. Pulmonary fibrosis (PF) is one of the most prevalent causes of death following paraquat (PQ) poisoning. As demonstrated in previous studies by the present authors, epithelial-to-mesenchymal transition (EMT) is associated with PQ-induced PF. In addition, hypoxia-inducible factor-1α (HIF-1α) and lysyl oxidase (LOX) promote EMT following PQ poisoning. However, the association between HIF-1α and LOX-mediated regulation of EMT remains unclear. The present study investigated the association between HIF-1α and LOX with regard to PQ-induced EMT. A549 and RLE-6TN cells were treated with PQ, and HIF-1α and LOX expression was silenced with short interfering RNAs. Changes in the expression of HIF-1α, LOX, β-catenin and EMT-related markers were detected using real-time quantitative polymerase chain reaction, immunofluorescence, and western blotting. HIF-1α and LOX were associated with PQ-induced EMT, and their expression levels were significantly increased (P<0.05). LOX expression was significantly decreased following PQ poisoning when HIF-1α expression was inhibited (P<0.05). However, the level of HIF-1α did not change significantly when LOX was silenced. The expression level of β-catenin and the degree of EMT were significantly decreased following PQ-induced EMT. A549 and RLE-6TN cells were treated with PQ, and HIF-1α and LOX expression was silenced in both cell lines (P<0.05). The association between HIF-1α and LOX in regulating EMT during PQ-induced PF may be unidirectional. HIF-1α may regulate PQ-induced EMT through the LOX/β-catenin pathway.

Introduction

Paraquat (PQ) has been one of the most effective and widely used herbicides over the last few decades, particularly in rural areas of developing countries; however, PQ poisoning has become a serious problem, with reports of mortality >90% (1,2). The primary pathological effects of PQ are observed in the lung, where pulmonary concentrations are 6-10 times higher than in plasma following PQ ingestion (3). Furthermore, PQ accumulates in the lungs as blood levels begin to decrease (3). The rapid accumulation of PQ damages the parenchymal cells in the lung and induces the excessive repair of lung tissues, which results in irreversible and extensive pulmonary fibrosis (PF) (3) and eventually leads to high mortality rates. However, the exact mechanism that leads to toxicity remains unclear, and no specific therapy has been recommended.

Epithelial-to-mesenchymal transition (EMT) occurs in multiple contexts, including embryonic development, tissue fibrosis, and cancer. EMT is defined as the process by which stationary epithelial cells (identified by high levels of E-cadherin and zonula occludens-1, which are markers of epithelial cells) undergo phenotypic changes, including the loss of cell-cell adhesion and apical-basal polarity, and acquire mesenchymal characteristics, including high levels of α-smooth muscle actin (α-SMA) and N-cadherin (markers of mesenchymal cells), that confer migratory capacity (4,5). According to previous findings, EMT has an important role in the development of PF. Alveolar epithelial cells could acquire mesenchymal cell phenotypes through EMT, these cells could then increase the deposition of extracellular matrix and further promote the development of PF (5-7). Furthermore, EMT has been demonstrated to serve an important role in PQ-induced PF in recent studies by the present authors (8,9).

Hypoxia-inducible factor-1α (HIF-1α) has roles in tumorigenesis, inflammation, and cell metabolism in hypoxia, and its expression is correlated with a variety of fibrotic diseases (10,11). HIF-1α has also been demonstrated to induce EMT and contribute to PF (12,13). Previous studies have detected an early increase in HIF-1α expression following PQ poisoning and revealed that HIF-1α modulates EMT in cases of PF (9,14).
Lysyl oxidase (LOX) is a secreted copper-dependent amine oxidase that is important for growth, stabilization, remodeling and repair. Its primary function is to catalyze the covalent cross-linking of collagens and elastin in the extracellular matrix, although it also has intracellular functions (15). LOX participates in various fibrosis processes, such as lung, myocardial and renal fibrosis (16-18). As demonstrated in a previous study by the present authors, LOX promotes EMT in PQ-induced PF (8). LOX was previously considered a critical target of HIF-1α (19); however, HIF-1α and LOX have since been demonstrated to provide bidirectional regulation of colon and ovarian carcinomas (20,21). The potential for dual regulation via HIF-1α and LOX remains controversial, particularly in PQ-induced PF. The present study investigated the association between HIF-1α and LOX with regard to PQ-induced PF.

Materials and methods

Reagents. PQ powder was obtained from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Anti-HIF-1α antibodies (cat. no. BS3514) were purchased from Bioworld Technology, Inc. (St. Louis Park, MN, USA). Anti-LOX (cat. no. ab174316), anti-E-cadherin (cat. no. ab184633) and anti-α-SMA (cat. no. ab7817) primary antibodies were obtained from Abcam (Cambridge, MA, USA). Anti-β-catenin (cat. no. 8480) and anti-GAPDH (cat. no. 5174) antibodies were purchased from Cell Signaling Technology, Inc. (Boston, MA, USA). Horseradish peroxidase-conjugated anti-rabbit immunoglobulin IgG (cat. no. A0208), anti-mouse IgG secondary antibodies (cat. no. A0210), immunofluorescence staining kits with Alexa Fluor 647-labeled goat anti-rabbit immunoglobulin G (cat. no. A0468) and kits with Alexa Fluor 488-labeled goat anti-rabbit IgG (cat. no. A0423) were obtained from Beyotime Institute of Biotechnology (Shanghai, China).

Cell culture. Human lung adenocarcinoma epithelial cells (A549) and rat alveolar type II cells (RLE-6TN) were obtained from the American Type Culture Collection (Manassas, VA, USA). A549 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (HyClone; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and a 1% antibiotic solution (100 U/ml penicillin and 0.1 mg/ml streptomycin). RLE-6TN cells were cultured in DMEM/nutrient mixture F-12 supplemented with 10% FBS and 1% antibiotic solution. Both cell lines were cultured at 37˚C. Cells were washed with PBS, fixed with 4% paraformaldehyde (Sigma-Aldrich; Merck KGaA) for 10 min at RT, permeabilized with 0.5% Triton X100 (Sigma-Aldrich; Merck KGaA) for 10 min and blocked with 5% bovine serum albumin (1 g bovine serum albumin powder and 20 ml Tris-buffered saline; Beyotime Institute of Biotechnology) for 1 h at RT. Subsequently, cells were incubated with anti-LOX (1:100) or anti-HIF-1α (1:50) primary antibodies overnight at 4˚C. Following three washes with TBST, cells were incubated with immunofluorescence staining kits with Alexa Fluor 647-labeled goat anti-rabbit IgG (1:200) and kits with Alexa Fluor 488-labeled goat anti-rabbit IgG (1:500), β-catenin (1:1,000) or GAPDH (1:500) overnight at 4˚C. Membranes were subsequently incubated with horse-radish peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG secondary antibodies (1:2,000; Beyotime Institute of Biotechnology) at RT. Following three washes with TBST, proteins were observed using a highly sensitive enhanced chemiluminescent agent (Thermo Fisher Scientific, Inc.). The band intensity was determined using ImageJ software (version 10.2; National Institutes of Health, Bethesda, MD, USA).

Immunofluorescence staining. Both cell lines were cultured in confocal dishes for 24 h at 37˚C and incubated with PQ for 24 h at 37˚C. Cells were washed with PBS, fixed with 4% paraformaldehyde (Sigma-Aldrich; Merck KGaA) for 10 min at RT, permeabilized with 0.5% Triton X100 (Sigma-Aldrich; Merck KGaA) for 10 min and blocked with 5% bovine serum albumin (1 g bovine serum albumin powder and 20 ml Tris-buffered saline; Beyotime Institute of Biotechnology) for 1 h at RT. Subsequent, cells were incubated with anti-LOX (1:100) or anti-HIF-1α (1:50) primary antibodies overnight at 4˚C. Following three washes with TBST, cells were incubated with immunofluorescence staining kits with Alexa Fluor 647-labeled goat anti-rabbit IgG (1:200) and kits with Alexa Fluor 488-labeled goat anti-rabbit IgG (1:200) for 1.5 h at RT. Nuclei were stained with DAPI (Beyotime Institute of Biotechnology) for 3 min at RT. Fluorescent signals were detected with a laser confocal scanning microscope (Leica TCS SP8; Leica Microsystems GmbH, Wetzlar, Germany) and the cellular morphology was observed with a phase contrast microscope (AMEX1200, Thermo Fisher Scientific, Inc.) was used to observe the change of cellular morphology.

Transient transfection. A549 and RLE-6TN cells were cultured in 6-well culture plates as described above and divided into dimethyl sulfoxide groups (including the control, sicontrol,
siHIF-1α and siLOX groups) and PQ groups (including the control + PQ, sccontrol + PQ, siHIF-1α + PQ and siLOX + PQ groups). HIF-1α and LOX short interfering (si)RNAs and negative control sequences were purchased from Shanghai GenePharma Co., Ltd. (Shanghai, China) and are listed in Table II. For transfection of each siRNA, 4 µl Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) were incubated with 100 pmol siRNA or negative control sequences in 500 µl Opti-MEM medium (Gino Biomedical Technology Co., Ltd., Hangzhou, China) for 20 min at RT. Cells were transfected by replacing the medium with 2 ml Opti-MEM medium containing the siRNA or negative control sequences and Lipofectamine® 2000, and then incubating them at 37˚C in a humidified atmosphere of 5% CO₂ for 6 h. The Opti-MEM medium was then replaced with 2 ml fresh culture medium. Subsequently, the cells in the PQ groups were incubated with PQ for 24 h and the other cells were treated with phosphate buffered saline. The total time from the start of transfection to subsequent experimentation was 48 h.

**Statistical analyses.** Data were analyzed using SPSS (version 16.0; SPSS, Inc., Chicago, IL, USA) and expressed as the mean±standard deviation of triplicate experiments. Comparisons between two groups were performed using a Student's t-test and comparisons of multiple groups were performed using one-way analysis of variance and Dunnett’s post hoc test. P<0.05 was considered to indicate a statistically significant difference.

**Results**

**HIF-1α and LOX may regulate PQ-induced EMT.** PQ treatment induced a significant decrease in E-cadherin expression and significantly increased α-SMA expression as determined by western blotting (Fig. 1A), which confirmed that EMT participated in PQ-induced PF. Protein levels of HIF-1α and LOX were significantly increased in the PQ groups compared with the control groups (Fig. 1B). Based on the immunofluorescence staining, the levels of HIF-1α and LOX were markedly increased with 24 h of treatment with PQ compared with control groups (Fig. 1C). These results suggested that EMT served an important role in PQ-induced PF, and that HIF-1α and LOX may regulate EMT following PQ poisoning.

**HIF-1α may promote EMT by upregulating LOX expression.** The levels of HIF-1α, LOX- and EMT-related markers in PQ-poisoned A549 and RLE-6TN cells were measured following HIF-1α silencing to determine the potential roles of HIF-1α and LOX in PQ-induced EMT. HIF-1α mRNA
LOX promotes PQ-induced EMT independently from HIF-1α. Levels of HIF-1α, LOX and EMT markers in PQ-poisoned cells following LOX silencing were subsequently determined. The level of LOX mRNA was significantly decreased in the siLOX group compared with the sicontrol + PQ group (Fig. 3A). The expression of EMT markers was also reversed following LOX silencing, as α-SMA expression decreased, and E-cadherin increased (Fig. 3B). In addition, phase-contrast microscopy revealed that the morphological changes (the degree of fusiformity was reduced) observed in cells in the PQ groups were alleviated following LOX silencing (Fig. 3C). However, the expression of HIF-1α mRNA was not significantly changed in the siLOX + PQ group compared with the sicontrol + PQ group (Fig. 3D). Levels of HIF-1α protein were also not significantly decreased following LOX expression inhibition (Fig. 3E). These findings suggest that LOX may promote PQ-induced EMT, but it does not regulate HIF-1α expression.

HIF-1α may regulate PQ-induced EMT through the LOX/β-catenin pathway. Changes in the levels of β-catenin were detected following HIF-1α (LOX) inhibition in vitro to further reveal the interactions between HIF-1α, LOX and β-catenin. β-catenin mRNA levels in the PQ groups were significantly decreased following HIF-1α (LOX) silencing (Fig. 4A and B). Similar results were observed for the protein expression of β-catenin (Fig. 4C and D). These findings suggest that HIF-1α may modulate PQ-induced EMT via the LOX/β-catenin pathway.
Discussion

PQ accumulates in the lungs and eventually leads to PF; however, its molecular mechanisms are complex and remain unclear (2-4,7). EMT is known to have an important function in PF (4,7,23). As demonstrated in recent studies by the present authors, EMT occurs in PQ-induced PF and may be modulated by HIF-1α or LOX (8,9). However, the interaction between HIF-1α and LOX remains unclear. Therefore, the association between HIF-1α and LOX was investigated, as was the pathway that regulates PQ-induced EMT. It was demonstrated that HIF-1α may modulate PQ-induced EMT via the LOX/β-catenin pathway.

LOX is a downstream target gene of HIF-1α, and a number of previous gene profiling studies have confirmed that LOX expression is upregulated by HIF-1α (24-27). LOX is also an important regulator of hypoxia-induced tumor progression in a variety of cancers via a HIF-1α-dependent mechanism (19,28). However, the correlation between HIF-1α and LOX in fibrosis remains unexplored. In the present study, HIF-1α and LOX expression were significantly increased in the model of PQ-induced PF. E-cadherin expression was decreased, and α-SMA expression was significantly increased following PQ treatment, which confirmed that HIF-1α, LOX and EMT are associated with PQ-induced PF. Furthermore, HIF-1α silencing downregulated the expression of LOX mRNA and protein. The expression of EMT markers was also reversed following HIF-1α silencing, as α-SMA expression decreased, and E-cadherin expression increased. In addition, changes in cellular morphology were alleviated following HIF-1α silencing, which indicated that the degree of PQ-induced EMT was alleviated following HIF-1α silencing. Therefore, HIF-1α serves an important role

Figure 2. HIF-1α ameliorated the degree of PQ-induced epithelial-to-mesenchymal transition and LOX expression. (A) HIF-1α mRNA levels in HIF-1α-silenced cell lines was detected by RT-qPCR. (B) Protein levels of HIF-1α, E-cadherin, α-SMA and GAPDH were detected by western blotting. GAPDH served as a loading control. (C) Morphological changes were detected using a phase-contrast microscope. Scale bars, 100 µm. (D) The level of LOX mRNA in both HIF-1α-silenced cell lines was detected using RT-qPCR. (E) LOX and GAPDH protein levels were detected by western blotting. *P<0.05 vs. control; #P<0.05 vs. sicontrol; †P<0.05 vs. sicontrol + PQ. HIF-1α, hypoxia-inducible factor-1α; LOX, lysyl oxidase; PQ, paraquat; LOX, lysyl oxidase; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; α-SMA, α-smooth muscle actin; DMSO, dimethyl sulfoxide.
in modulating EMT by activating LOX in PQ-induced PF. This result is consistent with findings from previous studies, which demonstrated that HIF-1α promotes EMT by upregulating LOX expression in ovarian and renal cancers (27,29). It was also confirmed that LOX may be a target of HIF-1α in PQ-induced PF and in tumors by modulating EMT.

In addition to acting as a HIF-1α-responsive gene, LOX may have more complex functions. According to a previous study by Pez et al (21), LOX and HIF-1α act synergistically to promote colon cancer cell proliferation and tumor formation. As previously demonstrated by Ji et al (20), LOX silencing downregulates the protein expression of HIF-1α in epithelial ovarian cancer cells. These findings indicated that LOX and HIF-1α may bidirectionally regulate PQ-induced EMT.

However, in the present study, LOX silencing did not induce changes in the protein and mRNA levels of HIF-1α. However, the expression of EMT markers was ameliorated following LOX silencing. In addition, changes in cellular morphology were alleviated following LOX silencing. Therefore, the degree of PQ-induced EMT was alleviated following LOX silencing in vitro. This finding is consistent with other previously published results (27,29) which reported that LOX inhibition did not prevent HIF-1α upregulation. Furthermore, LOX is only an intermediate signaling molecule that mediates HIF-1α-promoted PQ-induced EMT.

β-catenin is a protein located in cytoplasmic plaques that serves a major role in EMT. β-catenin has been used as a marker of EMT in a number of studies of embryonic development,
According to previous studies, β-catenin is associated with EMT during renal fibrosis (34) and fibrosis in other organs (35,36). In addition, β-catenin participates in the development of PF by transforming A549 cells into fibroblasts (23,37). As demonstrated previously, HIF-1α is positively correlated with β-catenin in rat models, and HIF-1α regulates EMT through the β-catenin pathway (9,38).

β-catenin mRNA and protein levels were significantly decreased when HIF-1α and LOX were silenced in the present study, which suggests that HIF-1α regulates PQ-induced EMT through the LOX/β-catenin pathway.

In conclusion, HIF-1α unidirectionally upregulates LOX expression in PQ-induced EMT. The mechanism may be associated with HIF-1α-induced LOX expression, which subsequently increases β-catenin levels, induces EMT and ultimately leads to the development of PQ-induced PF. Therefore, HIF-1α may be a potential target for restraining the development and exacerbation of PF induced by PQ.
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