Original Article
Paraquat induces acute respiratory distress syndrome by promoting the lung epithelial-mesenchymal transition

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Abstract: The acute respiratory distress syndrome (ARDS) is an important and common medical emergency. This study aimed to explore the molecular mechanism on how paraquat induced the occurrence of acute respiratory distress syndrome (ARDS). Rats were selected and treated with saline and paraquat for 5 and 21 days, respectively. Lung tissues were selected. Quantitative RT-PCR and western blotting were performed to screen the differentially expressed genes. Masson staining was performed to observe the morphological change of pulmonary fibrosis on lung tissues. Human lung epithelial cell lines HPAEpiC were treated with paraquat, and qRT-PCR was used to determine the changes of growth factor CTGF and TGF-β1, and markers for lung epithelial-mesenchymal transition (EMT) such as E-cadherin, N-cadherin, vimentin and MMP2. HPAEpiC cells were also treated with DMSO, scramble RNA + CTGF, siRNA against TGF-β1 + CTGF. The mRNA levels TGF-β1, Smad2, Samd3, E-cadherin, N-cadherin, vimentin and MMP2 were determined by qRT-PCR, and protein expression of Smad2 and Samd3 were determined by Western blotting. TNF-α, COL1A2, COL3A1, BMP-7, FASLG, TGF-β1 and CTGF were selected as differentially expressed genes. Masson staining showed collagenous fiber in lung tissues increased markedly when treating with paraquat. In HPAEpiC cells, paraquat increased the mRNA levels of CTGF, TGF-β1, E-cadherin, N-cadherin, vimentin and MMP2. CTGF increased the levels of TGF-β1, Smad2, Samd3, E-cadherin, N-cadherin, vimentin and MMP2. Paraquat can induce the occurrence of ARDS by activating TGF-β1/Smad2/3 pathway and promoting lung EMT.

Keywords: Paraquat, acute respiratory distress syndrome, EMT, TGF-β1, CTGF

Introduction

Acute respiratory distress syndrome (ARDS) is an important and common medical emergency and is likely to occur in all hospitals dealing in respiratory care [1]. ARDS is defined as the acute onset of lung injury, bilateral infiltrates on chest radiography, a PaO2/FiO2 ratio of <200, and a pulmonary artery occlusion pressure of ≤18 mmHg [2]. It is characterized by excessive fibro-proliferation, ongoing inflammation, prolonged mechanical ventilation, and a substantial risk of death [3]. The lung inflammation observed in ARDS can be precipitated by diverse disease processes, including both intrapulmonary ones (such as, infection or aspiration) and extrapulmonary ones (such as, shock or extensive trauma) [4]. The acute and persistent inflammation of the lung associated with ARDS often leads to the formation of pulmonary fibrosis, which compromises the structure of alveoli and vasculature and leads to severe functional lung impairment [5]. Pulmonary fibrosis is an inexorably fatal disorder characterized by connective tissue deposition within the terminal air spaces resulting in loss of lung function and eventual respiratory failure [6]. Cytokines are involved in a variety of inflammatory lung diseases, but their pathogenic role and their significance as diagnostic tools are still controversial. Many inflammatory cytokines are involved in the pathogenesis of ARDS, and some have prognostic significance [7]. An important role has been suggested for cytokines that regulate mesenchymal cell proliferation and fibrosis,
Paraquat induces acute respiratory distress syndrome

such as transforming growth factor (TGF)-α and TGF-β, in view of the association between mortality and pulmonary fibrosis in established ARDS [8]. Tumor necrosis factor-α (TNF-α) is secreted by monocytes or macrophages, including those of the alveolar space, after exposure to bacterial lipopolysaccharides and a variety of proinflammatory mediators [9, 10]. Transforming growth factor-β1 (TGF-β1) is a cytokine critically involved in acute lung injury and endothelial cell (EC) barrier dysfunction [11]. TGF-β1 has been specifically implicated in the pathogenesis of pulmonary fibrosis and hepatic fibrosis [12].

Paraquat, a quaternary nitrogen herbicide, is a highly toxic compound for humans and animals and many cases of acute poisoning and death have been reported over the past few decades [13]. It produces degenerative lesions in the lung after systemic administration to man and animals. Paraquat poisoning is characterized by multiorgan failure and pulmonary fibrosis with respiratory failure [14]. Paraquat is widely used in the world, and all treatments for paraquat poisoning have been unsuccessful. Many patients have died of paraquat poisoning in developing countries. Paraquat poisoning was reported to result in ARDS.

In this study, our efforts were aimed at examining and comparing the expression of MMP-1, CCL12, CCL3, ACTA2, COL1A2, COL3A1, IL-1β, TNF-α, INF-γ, BMP7, AKT1, BCL2, FASLG, TIMP1, MMP13, MMP3, TGF-β1, VEGFA, CTGF and NFκB1 in rats injected with paraquat with those with saline. The differential expressed genes were selected and research was performed to explore the molecular mechanism on how paraquat induced ARDS.

Materials and methods

Animal experiments and cell culture

Wistar rats of 120 (approximately 8-11 weeks old) weighing 190 to 305 g were purchased from the Laboratory Animal Center of Jiangsu University, China. 60 of them were intraperitoneally injected with normal saline (20 mg/kg; Beijing Baiao Sentai Biological Technology Co., Ltd. China). Of the 60 rats, half were executed after injection for 5 days and the others for 21 days. The other 60 rats were intraperitoneally injected with paraquat (20 mg/kg; Hubei Shalongda Co., LTD. Hubei, China). 30 rats were executed after injection with paraquat for 5 days and the others for 21 days. All animals were treated according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All animal procedures were conducted after gaining the approval of the animal care committee of Shanghai resident standardization training base.

Human lung epithelial cell lines HPAEpiC were purchased from Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). The cells were routinely cultured in RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin in a humidified cell incubator with an atmosphere of 5% CO₂ at 37°C.

RNA isolation and quantitative RT-PCR

To study the influence of paraquat on the mRNA levels of CCL12, CCL3, ACTA2, COL1A2, COL3A1, IL-1β, TNF-α, INF-γ, BMP7, AKT1, BCL2, FASLG, TIMP1, MMP13, MMP3, TGF-β1, VEGFA, CTGF and NFκB1, lung tissues of the 120 rats were collected. To explore the effect of paraquat on epithelial-mesenchymal transition (EMT), human lung epithelial cell lines HPAEpiC were treated with 100 μmol/L paraquat for 24 h. Then the mRNA levels of CTGF and TGF-β1, and markers of EMT such as E-cadherin, N-cadherin, Vimentin and MMP2 were determined by qRT-PCR. To further explore the influence, human lung epithelial cell lines HPAEpiC were treated with DMSO, scramble RNA + CTGF and 20 nM siRNA against TGF-β1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) + CTGF for 24 h, respectively. Then the mRNA levels of TGF-β1, Smad2, Samd3, E-cadherin, N-cadherin, Vimentin and MMP2 were also determined by qRT-PCR.

For RNA isolation, total RNA was extracted and isolated from tissue samples or cell lines using the TRizol method. Trizol of 1 mL was added and the solution was mixed homogeneously for 10 min. The mixture was then transferred into eppendorf tubes (EP, 1.5 mL) with 200 μL chloroform. After 15 min shake, the EP tubes were centrifuged at 4°C for 15 min (12000×g). The supernatant was transferred into other EP tubes and mixed with isopyknic isopropanol for 15 s. The centrifugation (4°C, 10 min, 12000×g)
Paraquat induces acute respiratory distress syndrome

was carried out again and the supernatant was discarded. The precipitate was washed by 75% ethanole twice and dissolved into 30 μL diethylpyrocarbonate (DEPC) after dried to obtain RNA stock solution. After isolation, the concentration of RNA was assessed using a NanoDrop 1000 spectrophotometer (NanoDrop Technologies, Wilmington, Delaware, USA) and the RAN solution was stored at -80°C for further use.

For qRT-PCR, genes were amplified by specific oligonucleotide primer, and human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as an endogenous control. The detection and quantification contained the following steps: first, reverse transcription was performed at 55°C for 30 min, initial activation for 15 min at 95°C, next 40 cycles of denaturation were conducted at 94°C for 15 s, then annealing for 30 s at 55°C, extension for 30 s at 72°C. The expression level was normalized using U6 small nuclear RNA by the 2^ΔΔCT method. The ΔCT values were normalized to GAPDH level.

Western blot analysis

Lung tissues of rats after treating with paraquat for 21 days were collected, and the protein expression of COCL1A2, COL3A1, BMP7, FASLG, TGF-β1 and CTGF were determined by western blotting. Tissues were washed thrice with PBS and transferred to buffer containing 25 mM HEPES, 2.5 mM EDTA, 0.1% Triton X-100, 1 mM PMSF and 5 μg/mL leupeptin. The mixture was centrifuged at 3000 r/min for 10 min and the supernatant was stored at 4°C. Total protein concentrations were determined with a UV spectrophotometer using a modified Bradford assay (Beckman Coulter, Fullerton, CA, USA). Protein (15 μL) were separated on 5% stacking/15% SDS-polyacrylamide gel electrophoresis gels and transferred to nitrocellulose membranes (Millipore, Billerica, MA), which had been infiltrated by methanol and transfer membrane liquid. The membrane was washed by TBST (50 mM Tris, 150 mM NaCl and 2% Tween-20; pH 7.5) for 3 times and each for 10 min at room temperature, and incubated at 4°C overnight with polyclonal antibody against COCL1A2, COL3A1, BMP7, FASLG, TGF-β1 and CTGF (Santa Cruz, SC12013). Then incubation with the secondary antibody was performed at room temperature for 1 h. Membrane was washed again with TBST and incubated in Super ECL Plus detection reagent (Nanjing KeyGEN Biotech, KGP1123, China), which produced a chemiluminescence signal that was detected by exposure to X-ray film. Images were scanned and analyzed semi-quantitatively using Image (National Institutes of Health, Bethesda, MD) and Image Gauge software (Fujifilm, Tokyo, Japan). The samples were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Enzyme-linked immunosorbent assay

Lung tissues of rats after treatment with paraquat for 21 days were collected, and the protein expression of TNF-α was determined by TNF-α enzyme-linked immunosorbent assay (ELISA) kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Tissues of 100 g after frozen in liquid nitrogen or cell lines was put into homogenizer and 3 times the volume of normal saline was added for ice bath homogenate. The homogenate was centrifuged at 2000 rpm, 4°C for 20 min and the supernatant was collected. After incubated for 1 h at 37°C, the mixture were added with 100 μL streptavidin-horse radish peroxidase (HRP) and incubated for another 30 min. Then, 100 μL stabilized chromogen was put with 100 μL stop buffer and the adsorption value of mixture at 450 nm was read and recorded.

Masson staining

Lung tissues of rats after treating with saline and paraquat for 5 and 21 days were collected and Masson staining was performed to analyze the difference of collagenous fiber. Lung tissues were fixed in 4% paraformaldehyde for 10 min at room temperature and stained for melanin by using a Fontana-Masson staining kit from American Master*Tech Scientific, Inc. (Lodi, CA, USA) according to the manufacturer’s instructions. The paraffin sections dewaxed to water and performed staining. Then samples were stained with regard hematoxylin for 10 min after washed with distilled water. Samples were then stained with Masson ponceaux acid dyeing liquid for 10 min, soaked with 2% glacial acetic acid aqueous solution for 15 s, differentiated with 1% phosphomolybdic acid aqueous solution for 5 min, stained with aniline blue for 5 min and soaked with 0.2% glacial acetic acid aqueous solution for 15 s. As last, samples were sealed with 95% ethanol, anhydrous alcohol, xylene and neutral gum.
Statistical analysis

Statistical analysis was performed by SPSS 16.0 statistical software. All data were expressed as means ± SD from at least three independent experiments. Comparisons between more than two groups were performed by conducting an analysis of variance (one-way ANOVA), and \( P < 0.05 \) was considered statistically significant. For all experiments in this study, the concentration of drugs and the experimental duration were selected based on the pre-experimental results.

Results

Paraquat influenced the levels of COCL1A2 and FASLG

To explore the influence of paraquat on ARDS, the mRNA levels of MMP-1, CCL2, CCL3, ACTA2, COL1A2, COL3A1, IL-1β, TNF-α, INF-γ, BMP-7, AKT1, BCL2, FASLG, TIMP1, MMP13, MMP3, TGF-β1, VEGFA, CTGF and NFκB1 in lung tissues collected from rats treated with saline and paraquat for 5 and 21 days, respectively, were determined. The mRNA level of COL1A2 increased significantly, while the
Paraquat induces acute respiratory distress syndrome

Paraquat accelerates pulmonary fibrosis

As the above results shown, the expression of TNF-α, COL1A2, COL3A1, BMP-7, FASLG, TGF-β1 and CTGF were all markedly increased by paraquat. Moreover, BMP-7 and CTGF were reported to be associated with fibrosis. Therefore, we used Masson staining to observe the changes of lung tissues after treating with paraquat. As shown in Figure 3, collagen fiber in lung tissue increased significantly and appeared broad-bands or flake shape, pulmonary alveoli appeared collapse and fusion, and the structure was disorder. However, there was no or only few filamentous collagenous fiber shown in the control groups of which rats were injected with saline. Therefore, we speculated paraquat can promote pulmonary fibrosis.

CTGF promotes epithelial-mesenchymal transition by TGF-β1

An epithelial-mesenchymal transition (EMT) is a biologic process that allows a polarized epithelial cell, which normally interacts with basement membrane via its basal surface, to undergo multiple biochemical changes that enable it to assume a mesenchymal cell phenotype, which includes enhanced migratory capacity, invasiveness, elevated resistance to apoptosis, and greatly increased production of ECM components [17]. The mRNA levels of CTGF and TGF-β1, and markers of EMT such as E-cadherin, N-cadherin, Vimentin and MMP2 in HPAEpiC cells after treating with paraquat were determined by qRT-PCR. As shown in Figure 4A, the mRNA levels of them all increased significantly after treating with paraquat. To explore...
Paraquat induces acute respiratory distress syndrome

Discussion

Nowadays, the mortality of paraquat poisoning is high and can reaches to 40-50% as reported abroad [18]. When it reaches into human body, the lung, renal, liver and adrenal glands can be harmed [15]. However, the characteristic change of paraquat poisoning is lung injury which appearing the damage of early alveolar epithelial cells, bleeding and edema in alveolar, infiltration on inflammatory cells. In the later, pulmonary fibrosis occurred which is a deviant form of ARDS [19]. Recent years, alveolar macrophages and a variety of cytokines such as TGF-β, TNF-α, PDGF and IL-6 play an important role in exploring the mechanism of pulmonary fibrosis.

In this study, to explore the molecular mechanism about the paraquat resulting in ARDS, Wistar rats were chosen and treated with saline and paraquat for 5 and 21 days, respectively. We used qRT-PCR to determine the mRNA levels of MMP-1, COL12, CCL3, ACTA2, COL1A2, COL3A1, IL-1β, TNF-α, INF-γ, BMP-7, AKT1, BCL2, FASLG, TIMP1, MMP13, MMP3, TGF-β1, VEGFA, CTGF and NFкB1 in lung tissues collected from those rats. The mRNA levels of COCL1A2, COL3A1, TNF-α, BMP-7, FASLG, TGF-β1 and CTGF were significantly increased after treating with paraquat for 21 days. The differences were also verified by western blotting. COCL1A2 is a gene expressing the alpha two chain of type I collagen [20]. During the past two decades, COL1A2 has emerged as an informative model in which to study the general principles that govern the transcriptional control of extracellular matrix deposition in normal and fibrotic conditions [21]. COL3A1 gene can encode the chains of type III procollagen, while Type-III collagen is a fibrous scleroprotein in bone, cartilage, dentin, tendon, bone marrow stroma and other connective tissue [22]. TNF-α is a potent pro-inflammatory and immunomodulatory cytokine implicated in inflammatory conditions such as rheumatoid arthritis, Crohn’s disease, multiple sclerosis and the cachexia associated with cancer or human immunodeficiency virus [23]. In ARDS, the expression of TNF-α was found up-regulated [24]. BMP-7 is a 35-kDa homodimeric protein and a member of the transforming growth factor (TGF)-beta superfamily 1 that counteracts the fibrogenic action of TGF- [25]. Its expression is often downregulated in the fibrotic kidney and supplementation with exogenous BMP-7 suppresses renal fibrosis in experimen-
Paraquat induces acute respiratory distress syndrome

Paraquat induces acute respiratory distress syndrome

Figure 4. Paraquat-induced CTGF promoted lung epithelial-mesenchymal transition by TGF-β1. A: The mRNA levels of CTGF, TGF-β1, E-cadherin, N-cadherin, Vimentin and MMP2 in human lung epithelial cell lines HPAEpiC after treated with saline and paraquat for 24 h. *P<0.05 and **P<0.01, compared with saline group, mRNA level of gene in paraquat group had statistical significance; B: The protein expression of Smad2, p-Smad2, Samd3 and p-Samd3 in human lung epithelial cell lines HPAEpiC after treated with DMSO, Scr + CTGF and siRNA + CTGF for 24 h. C: The mRNA levels of TGF-β1, Smad2, Samd3, E-cadherin, N-cadherin, Vimentin and MMP2 in human lung epithelial cell lines HPAEpiC after treated with DMSO, Scr + CTGF and siRNA + CTGF for 24 h. *P<0.05 and **P<0.01, compared with DMSO group, mRNA level of gene in Scr + CTGF group had statistical significance; *P<0.05 and **P<0.01, compared with Scr + CTGF group, mRNA level of gene in siRNA + CTGF group had statistical significance. Scr + CTGF: Scramble RNA + CTGF; siRNA + CTGF: siRNA against TGF-β1 + CTGF.

FASLG is an important proapoptotic protein that have a significant function in regulating cell growth and apoptosis and play essential roles in many human autoimmune diseases [28]. The binding of FAS by FASLG induces apoptosis [29]. In ARDS patients, the level of FASLG was high [30]. TGF-β1 is a 25-kDa disulfide-linked homodimer, which is involved in cellular growth, differentiation, migration, tissue repair, and immune regulation [31]. In various kinds of experimental models and clinical fibrosis diseases, TGF-β1 continues to rise and the inhibition on its activity can restrain the formation of substrate and regulating fibrosis process [32]. It plays an important role in the fibrosis of tissues and organs and was starting hub for the formation and development of fibrosis [33]. CTGF is a 38-kDa cysteine-rich peptide, whose synthesis and secretion are selectively induced by transforming growth factor beta (TGF-β) in connective tissue cells. It can trigger many of the cellular processes underlying fibrosis, such as cell proliferation, adhesion and migra-
Paraquat induces acute respiratory distress syndrome

tion [34]. CTGF is overexpressed in many fibrotic lesions, including those of the liver [35]. Therefore, we speculated paraquat may influence the pulmonary fibrosis. Masson staining on lung tissues was performed to observe the changes of collagen fiber after rats treated with paraquat. Results showed the collagen fiber increased significantly in paraquat groups compared with the control groups. To explore the mechanism on how CTGF promotes pulmonary fibrosis, the mRNA levels of CTGF, TGF-β1 and markers of EMT such as E-cadherin, N-cadherin, Vimentin and MMP2 were determined by qRT-PCR. Results showed the mRNA levels of CTGF, TGF-β1, N-cadherin, Vimentin and MMP2 increased and the value of E-cadherin decreased significantly after treating with paraquat. Moreover, when human lung epithelial cell lines HPAEpiC were treated with the combining of siRNA against TGF-β1, Smad2, Smad3, N-cadherin, Vimentin and MMP2 decreased and the value of E-cadherin increased significantly. Therefore, we concluded CTGF can promote EMT by increasing the expression of TGF-β1.

In conclusion, paraquat can result in ARDS by inducing the overexpression of CTGF in lung tissues which activating TGF-β1/Smad2/3 pathway, promoting lung epithelial-mesenchymal transition and accelerating pulmonary fibrosis.

Disclosure of conflict of interest

None.

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Paraquat induces acute respiratory distress syndrome


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