Penehyclidine hydrochloride ameliorates renal ischemia reperfusion-stimulated lung injury in mice by activating Nrf2 signaling

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Abstract

Introduction: Penehyclidine hydrochloride (PHC) is an anticholinergic with anti-inflammatory and anti-oxidation activities. PHC displayed protectivity against renal ischemia reperfusion (RIR) injury. Nevertheless, the precise protectivity of PHC on RIR-induced lung injury remains unknown.

Methods: We examined the effects of PHC on RIR-induced lung injury and investigated the underlying mechanism. We induced RIR in mice and administrated PHC to RIR mice.

Results: PHC greatly downregulated the serum levels of BUN, creatinine, IL-6, NO, malondialdehyde (MDA), and matrix metalloproteinase-2. PHC also ameliorated the lung injury, decreased the MPO activity, and suppressed production of IL-6, TNF-α, IFN-γ, MDA, and O2-, while it promoted production of superoxide dismutase (SOD) and catalase (CAT) in lung. PHC improved the production of Nrf2 and HO-1.

Conclusion: The protectivity of PHC was absent in Nrf2−/− mice. PHC ameliorated RIR-induced lung injury through Nrf2 pathway.

Introduction

Ischemia/reperfusion injury (IR), also called perfusion injury, is the tissue damage caused by restricted blood flow to an organ after ischemia or hypoxemia.1 Renal ischemia reperfusion injury (RIR) is a kidney inflammatory disease with high morality which exceeds 80%.3 Increasing pieces of evidence have proven that kidneys interact with distant organs and RIR has been shown to result in remote organ injury.3,4 It is well-described that there is interaction between the kidneys and lungs. Lung failure is frequently associated with acute kidney injury. Therefore, experimental RIR model has been utilized to develop therapeutic strategy or exploit drugs to treat lung inflammation.

Inflammation is the key factor in the pathophysiology of RIR. RIR leads to the increase of systemic IL-1β, IL-6, and TNF-α cytokines, causing lung alterations. Increased microvascular permeability and neutrophil infiltration are associated with acute lung injury.5,6 Reactive oxygen species (ROS) and oxidative stress also result in the activation of inflammation and dampen lung function. Suppression of inflammation by inhibiting NF-κB and inhibition of oxidative stress have been shown to ameliorate IR-stimulated lung injury, suggesting that preventing inflammation and oxidation may be a useful approach in ameliorating IR-induced lung injury.7,8

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Renal function assessment
To assess renal function, concentration of blood urea nitrogen (BUN) and serum level of creatinine were detected by commercial urea assay kit (Abcam, Shanghai, China) and creatinine assay kit (Abcam) following manufacturer's protocols.

NO, MDA, MPO, SOD, CAT, O2-, and MMP-2 measurement
Twenty-four hours after RIR, mice serum and lung tissues were collected. Concentrations of plasma or lung nitric oxide (NO), malondialdehyde (MDA), matrix metalloproteinase-2 (MMP-2), myeloperoxidase (MPO), superoxide dismutase (SOD), and catalase (CAT) were measured using Nitric Oxide Assay Kit (Abcam), MDA Assay Kit (Abcam), mouse MMP-2 ELISA Kit (R&D Systems, Minneapolis, MN), MPO Activity Assay Kit (Abcam, China), Superoxide Dismutase Activity Assay Kit (R&D Systems), Catalase Activity Assay Kit (Abcam), and O2- Assay Kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) respectively following manufacturer's protocols.

Cytokine measurement
IL-6, TNF-α, and IFN-γ levels in serum or lung tissue homogenates were measured using ELISA kit (Abcam) following the manufacture's protocols.

Real time polymerase chain reaction
Lung RNAs were extracted by NucleoSpin® RNA Plus kit (Takara, Dalian, China). The PrimeScript™ II 1st Strand cDNA Synthesis Kit (Takara) was utilized to obtain cDNA. The real time PCR was performed using TB Green Advantage® qPCR Premix (Takara). The primers used in the present study were:

IL-6 Forward: 5'-TCCAGTTGCTTCTTGGGAC -3' , Reverse: 5'- GTGTAATTAAGCCTCCGACTTG-3' 
TNF-α Forward: 5'-CAT CTT CTC AAA ATT CGA G TG ACA A-3', Reverse: 5'-TGGGAGTGAACAGTGACAGCC-3' 
IFN-γ Forward: 5'-GCCACGGCACAGCTATTTGA-3', Reverse: 5'-TGCTGATGGCCTGATTGTCTT-3' 
Nrf2 Forward: 5'-CTTTAGTACCGCAAGAGGAC-3', Reverse: 5'-AGGCATCTTGTTTGAGGAATGTG-3' 
HO-1 Forward: 5'-ATGACACCAAGGACCAGACG-3', Reverse: 5'-GTGTAAGGACCCATCGAGA-3' 
GAPDH Forward: 5'-TCCACCCACATGGAAGGC-3', Reverse: 5'-GGCATGGACTGTGGTATGA-3'

Western blot
Lung tissues were lysed in RIPA buffer (Invitrogen) for protein extraction. Western blot procedures were performed using standard protocols. Primary antibodies including anti-Nrf2 (Abcam), anti-lamin B1 (Abcam), anti-heme oxygenase 1 (HO-1), and anti-GAPDH (Abcam) were used. Pierce™ ECL Western Blotting Advantage Kit (Thermo Scientific) was used for detection.
Substrate (Thermo Fisher, Waltham, MA) was used for detection of immune-reactive bands. Band intensity was quantitated and analyzed using ImageJ.

**Pulmonary wet/dry (W/D) ratio**
The pulmonary edema was assessed by W/D weight ratio. After weighing, the new lung was dried at 80°C for 24 hours.

**Histology**
Lung samples were fixed in 10% formalin solution (Sigma) and embedded in paraffin. The sections were stained with hematoxylin-eosin (H&E) for lung injury evaluation. Two independent investigators scored the slide and the injury score was calculated using following formula: [(alveolar bleeding points/no. of fields) + 2 × (alveolar infiltration points/no. of fields) + 3 × (hemaleucin points/no. of fields) + (hyperemia of alveolar septum/no. of fields)]/the total count of alveolar as described previously. 20

**Statistical Analysis**
Data were expressed as mean ± SD. One-way or two-way analysis of variance (ANOVA) together with a post hoc test was used to determine statistical difference. When P value was less than 0.05, the difference was considered as statistically significant.

**Results**

**PHC ameliorated RIR-induced injury in mice**
We established the RIR mice model and administrated different amounts of PHC in RIR mice. Serum levels of BUN and creatinine were measured at different time points. As presented in Fig. 1A, RIR mice had increased serum level of BUN with time increase. In contrast, serum BUN level did not change in normal mice (Sham group). Twenty-four hours after RIR, RIR mice had significantly higher serum BUN than normal mice. Administration of PHC decreased the serum BUN of RIR mice and the decreasing was positively correlated to the PHC dose. RIR mice treated with 1 mg/kg PHC had the lowest BUN level in serum at each time point. Twenty-four hours after RIR, 0.1, 0.5, and 1 mg/kg PHC treatment significantly reduced serum BUN level. Similarly, RIR mice had increased serum level of creatinine, which became significant at 24 hours when compared to normal mice (Fig. 1B). Administration of PHC decreased serum level of creatinine and the decreasing was positively correlated to the PHC dose. Together, these results demonstrated that PHC decreased serum level of both BUN and creatinine, indicating PHC ameliorated injury in RIR mice. As 1 mg/kg PHC provided the best protection, we adopt this dose in other experiments.

**PHC inhibited systemic inflammation in RIR-mice**
Next, we evaluated the effects of PHC on inflammation in RIR mice by measuring serum NO, MDA, IL-6, and MMP-2. RIR mice had significantly increased plasma NO (Fig. 2A), MDA (Fig. 2B), IL-6 (Fig. 2C), and MMP-2 (Fig. 2D). Administration of 1 mg/kg PHC significantly decreased plasma NO (Fig. 2A), MDA (Fig. 2B), IL-6 (Fig. 2C), and MMP-2 (Fig. 2D). Collectively, our data demonstrated that PHC inhibited the systemic inflammation in RIR mice.

**PHC ameliorated pulmonary injury in RIR mice**
When compared to normal mice, RIR mice had obvious pulmonary inflammation (Fig. 3A) and significantly increased injury score (Fig. 3B). In contrast, PHC-treated RIR mice had decreased pulmonary inflammation and

![Fig. 1. Penehyclidine hydrochloride ameliorated renal ischemia reperfusion-induced injury in mice. The levels of blood urea nitrogen (A) and serum creatinine (B) were measured 0, 4, 12, and 24 hours after surgery. n = 8 for each group. Data are presented as mean ± SD. ***P < 0.001 compared to sham group, #P < 0.05, ##P < 0.01, and ###P < 0.001 compared to RIR group.](image)

![Fig. 2. Effects of penehyclidine hydrochloride on serum inflammatory molecule levels after renal ischemia reperfusion injury (RIR) in mice. Serum levels of NO (A), MDA (B), IL-6 (C), MMP-2 (D) were measured 24 hours after surgery. n = 6. Data are presented as mean ± SD. **P < 0.01, ***P < 0.001 compared to sham group, #P < 0.05, ##P < 0.01 compared to RIR group.](image)
injury score. Correspondingly, RIR mice had significantly increased lung wet/dry weight ratio (Fig. 3C) and pulmonary MPO activity (Fig. 3D). PHC treatment greatly reduced the lung wet/dry weight ratio and suppressed MPO activity in RIR mice. Taken together, our data demonstrated that PHC ameliorated lung inflammation in RIR mice.

**PHC attenuated pulmonary oxidative stress in RIR mice**

Next the effects of PHC on oxidation in lung of RIR mice were explored. RIR mice had significantly decreased pulmonary SOD (Fig. 4A) and CAT (Fig. 4C), while they had significantly increased pulmonary MDA (Fig. 4B) and O2- (Fig. 4D) when compared to normal mice, indicating strong oxidative stress in lung of RIR mice. In contrast, administration of PHC in RIR mice resulted in significantly increased pulmonary SOD (Fig. 4A) and CAT (Fig. 4C) and decreased pulmonary MDA (Fig. 4B) and O2- (Fig. 4D), suggesting that PHC attenuated the oxidative stress in lung of RIR mice.

**PHC ameliorated pulmonary inflammation in RIR mice**

We further investigated the effects of PHC on inflammation in lung of RIR mice by measuring the IL-6, TNF-α, and IFN-γ levels. When compared to normal mice, RIR mice had significantly increased protein levels of IL-6 (Fig. 5A), TNF-α (Fig. 5B), and IFN-γ (Fig. 5C) in lung. We also identified remarkably increased mRNA levels of IL-6 (Fig. 5D), TNF-α (Fig. 5E), and IFN-γ (Fig. 5F) in lung of RIR mice. Administration of PHC to RIR mice resulted in significantly decreased protein and mRNA levels of IL-6 (Fig. 5A & D), TNF-α (Fig. 5B & E), and IFN-γ (Fig. 5C & F). Collectively, our data showed that PHC ameliorated pulmonary inflammation in RIR mice.

**PHC activated Nrf2/HO-1 pathway**

Nrf2/HO-1 pathway regulated inflammation and oxidative stress. To investigate the effects of PHC on Nrf2/HO-1...
pathway, we monitored the mRNA and protein levels of Nrf2 and HO-1 in lung tissues of RIR mice after PHC treatment. RIR mice had greatly reduced mRNA level (Fig. 6A) as well as protein level (Fig. 6B&C) of Nrf2 in lung when compared to normal mice. In contrast, RIR mice treated with PHC had markedly elevated mRNA and protein level of Nrf2. Similarly, HO-1 mRNA and protein levels were markedly reduced in lung of RIR mice (Fig. 6D-F) while PHC treatment significantly upregulated mRNA and protein levels of HO-1. Together, these results indicated that PHC upregulated Nrf2 and HO-1 in RIR mice.

The protective effects of PHC on lung injury in RIP mice depended on Nrf2

Next, we explored the role of Nrf2 in PHC-mediated protection using Nrf2<sup>−/−</sup> mice. As shown in Fig. 7A, RIR caused significant lung damage in Nrf2<sup>−/−</sup> mice. Administration of PHC did not change the lung injury score in RIR Nrf2<sup>−/−</sup> mice. RIR Nrf2<sup>−/−</sup> mice also had significantly increased pulmonary MPO activity (Fig. 7B), MDA (Fig. 7C), IL-6 (Fig. 7E), and TNF-α (Fig. 7F), and significantly decreased SOD level (Fig. 7D). Administration of PHC did not affect level of MPO, MDA, SOD, IL-6, and TNF-α in RIR Nrf2<sup>−/−</sup> mice. Collectively, our data demonstrated that PHC did not affect lung injury in RIR Nrf2<sup>−/−</sup> mice, indicating that the protective functions of PHC depended on Nrf2.

Discussion

We evaluated the effects of PHC on RIR-induced lung injury. We described that PHC treatment decreased...
RIR-induced pulmonary injury by suppressing both inflammation and oxidative stress. RIR caused significant renal dysfunction as we detected remarkably increased serum levels of BUN and creatinine in our RIR mice. Consistent to a previous study, PHC treatment significantly decreased serum concentrations of BUN and creatinine, indicating protective roles of PHC on RIR-induced renal dysfunction. We demonstrated that PHC attenuated RIR-induced inflammation by decreasing the concentration of inflammatory cytokines including TNF-α, IL-6 in both serum and lung. We also showed PHC prevented RIR-induced oxidative stress by inhibiting the production of MPO, MDA, NO, MMP-2, and O2-, and promoting the production of SOD and CAT. More importantly, we demonstrated that the PHC protected against lung injury through Nrf2/HO-1.

Increased inflammatory cytokine concentration has been implicated in the pathophysiology of RIR. In RIR rat, increased TNF-α was found in heart after renal ischemia. TNF-α is capable of upregulating its own expression and expression of other inflammatory genes. Exposure of tissue to TNF-α results in tissue damage and dysfunction. Blocking TNF-α expression by knocking down TNF-α alleviated severity of lung injury in intestinal ischemia and reperfusion rat. The anti-inflammatory activity of PHC has been well-described. Guo et al showed that PHC decreased TNF-α and IL-6 production in lung and attenuated LPS-induced lung injury in rats. In the present study, we detected significantly increased pro-inflammatory cytokine level in both serum and lung of RIR mice while PHC treatment significantly suppressed the pro-inflammatory cytokines production in RIR mice. MPO is an indirect marker of neutrophil infiltration. Increased MPO activity was detected in lung after RIR, indicating leukocyte infiltration into lung. We detected elevated MPO activity in lung after RIR while PHC treatment reduced MPO activity, suggesting PHC treatment reduced neutrophil infiltration. All these results suggested PHC protected lung from injury after RIR.

Oxidative stress is another contributor to lung injury. During RIR, damaged tissue-produced ROS caused oxidative stress. We detected decreased activity of SOD and CAT in lung of RIR mice while the level of oxidative stress indicators including MDA, NO, and O2 was increased, suggesting an imbalance between oxidant and anti-oxidant. Our finding was consistent with previous findings for which decreased activities of SOD and CAT were detected in liver of RIR mice, implicating oxidative stress in RIR. Our data revealed that PHC enhanced the SOD and CAT and suppressed the production of MDA, NO, and O2, the indicators of oxidative stress. Using a RIR rat model, Wang and colleagues reported that PHC ameliorated renal injury by attenuating oxidative stress. Wang et al also demonstrated that PHC decreased lung tissue content of ROS, MDA, and MPO after lung ischemia-reperfusion. Our and their findings strongly suggested PHC could be used as a novel therapeutic strategy to treat RIR injury.

Nrf2 is a transcriptional factor that regulates the expression of proteins participating in oxidation and inflammation, and balances cellular redox. Nrf2 has been shown to regulate multiple antioxidant proteins including SOD, glutathione peroxidase 1, CAT, glutathione-S-transferase, and HO-1. The inhibition of inflammation by activation of Nrf2 pathway has been described. HO-1 is a protein with anti-inflammatory and anti-oxidant function. Since Nrf2/HO-1 pathway is a key factor in inflammation, activation of Nrf2/HO-1 has been suggested as a potential strategy to treat inflammatory disorders. Our previous study demonstrated that artesunate...
activated HO-1 pathway and suppressed RIR-induced lung inflammation.32 Here we demonstrated that PHC enhanced the expression of both Nrf-2 and HO-1, and activated Nrf-2/HO-1 pathway. PHC activates Nrf2/O-1 pathway in other disease models. Using a rhabdomyolysis model, Zhao and colleagues described that PHC activated Nrf2/HO-1 pathway by promoting Nrf2 and HO-1 expression and ameliorated rhabdomyolysis-induced acute kidney injury.33 These findings strongly suggested that reagents which can activate Nrf2/HO-1 pathway could be good therapeutic candidates to treat inflammatory disease.

There are still several limitations in the present study. Nrf2 functions as a transcription factor to activate HO-1 expression. In the current study, we demonstrated that PHC up-regulated Nrf2 expression. However, we did not fractionate the cells for western blot. Although HO-1 expression reflected the Nrf2 nuclear translocation, it is still useful to provide direct evidence to demonstrate the nuclear translocation of Nrf2 after PHC treatment. To evaluate the kidney injury, we only measured the serum BUN and creatinine levels, which are not precise indicators of the extent and intensity of the damage. It is better to measure the level of kidney injury molecule-1 (KIM-1) and neutrophil gelatinase-associated lipocalin (NGAL), which are new biomarkers of kidney injury.34

Conclusion
We identified the protective effects of PHC in RIR by suppressing inflammation and oxidative stress. PHC induced the expression of Nrf2 and HO-1. Interestingly, the protective effects of PHC depended on Nrf2 as these effects were abolished in the absence of Nrf2. Taken together, we showed that PHC ameliorated RIR-stimulated lung injury through activating Nrf2 signaling.

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None.

Ethical statement
All animal studies were reviewed and approved by the Ethics Committee of Canzhou Central Hospital, China.

Competing interests
None to declare.

Authors’ contributions
QY, ZL, LL, CL, LY, and YC conceived, designed the project, and wrote the paper. All authors approved the final submission.

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