

Resveratrol protects mice from paraquat-induced lung injury: The important role of SIRT1 and NRF2 antioxidant pathways

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Abstract. Sirtuin 1 (SIRT1) acts via the deacetylation of a number of crucial transcription factors and has been implicated in various biological processes, including oxidative stress. Previous studies have indicated that nuclear factor, erythroid 2-like 2 (NRF2) is an effective target of antioxidant therapy for paraquat (PQ) poisoning. However, the association between SIRT1 and NRF2 and their effects in PQ-induced oxidative stress remains to be elucidated. The current study demonstrated that PQ exposure upregulated the expression of SIRT1 and NRF2 following 6- and 24-h exposure in the lungs of mice. However, long-term exposure to PQ significantly decreased the expression of SIRT1 and NRF2. Resveratrol is a SIRT1 activator, and strongly enhanced SIRT1 expression and attenuated the lung injury resulting from PQ exposure in the current study. Additionally, treatment with resveratrol upregulated the expression of NRF2 and glutathione, increased the activity of heme oxygenase-1, superoxide dismutase and catalase, but depleted the expression of malondialdehyde. The present results demonstrated that resveratrol reduced PQ-induced oxidative stress and lung injury, potentially through the positive feedback signaling loop between SIRT1 and NRF2.

Introduction

Paraquat (1,1'-dimethyl-4,4'-bipyridinium dichloride; PQ) is an effective, widely used, nonselective herbicide. It is toxic to humans and is associated with high mortality, mainly as a consequence of respiratory failure (1). Upon entering the cell, PQ undergoes cyclic single-electron reduction/oxidation through

its quaternary ammonium nitrogen atoms and bipyridyl ring, producing reactive oxygen species (ROS) and PQ radicals (2). Redox cycling is considered to serve an important role in the initiation of the lung damage and fibrosis resulting from PQ exposure (2,3). In view of this, antioxidant therapy is considered an important strategy for the treatment of PQ poisoning (2,3).

Sirtuin 1 (SIRT1) is an NAD⁺-dependent deacetylase (4). SIRT1 regulates a wide variety of cellular processes by deacetylating histones and numerous other crucial transcription factors, including factors in control of ROS production (5). The nuclear factor, erythroid 2-like 2 (NRF2) transcription factor is a member of the cap 'n' collar basic-region leucine zipper transcription factors, and is considered an effective target for antioxidant therapy for PQ poisoning (3,6). Previous studies have demonstrated that the SIRT1-mediated deacetylation of NRF2 terminated the transcription of antioxidant genes (7). By contrast, SIRT1 is known to protect cells from oxidative stress injury, and silencing its activity results in decreased NRF2 expression levels (8,9). However, the association between SIRT1 and NRF2 and their effect in PQ-induced oxidative stress remains unclear.

Resveratrol (3,4',5-trihydroxystilbene; Res) is a natural polyphenol present in grapes, berries, peanuts and other plants (10). Previous studies have reported the beneficial effects of Res in numerous diseases, including cancer, cardiovascular diseases, ischemic injuries and acute poisoning (11-13). This wide range of biological effects may be explained in part by the antioxidant properties of Res, and the activation and expression of SIRT1 is postulated to be a key event in the pathophysiology of Res (10,11). Based on these data, the current study investigated the effects of Res on PQ-induced oxidative stress and lung injury. Furthermore, the potential roles of SIRT1 and NRF2 in this process were also illustrated.

Materials and methods

Animals and reagents. Male Institute of Cancer Research mice (age, 6-8 weeks; weight, 18-20 g) were provided by the Animal Experimental Center of Wenzhou Medical University (Wenzhou, China). The study was approved by the Laboratory Animal Ethics Committee of Wenzhou Medical University & Laboratory Animal Centre of Wenzhou Medical University (Wenzhou, China). Animals were housed in a

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room with a 12-h light/dark cycle and allowed free access to tap water and standard laboratory food. The PQ and Res used in the experiment were purchased from Sigma-Aldrich (St. Louis, MO, USA). Rabbit anti-mouse SIRT1 monoclonal antibody (cat. no. 3931) and rabbit anti-mouse Nrf2 polyclonal antibody (cat. no. ab31163) were obtained from Cell Signaling Technology, Inc. (Beverly, MA, USA) and Abcam (Cambridge, MA, USA), respectively. Polyclonal mouse anti- β actin antibody (cat. no. BS1002) was purchased from Bioworld Technology (St. Louis Park, MN, USA). Formaldehyde, Hematoxylin and Eosin (H&E) Staining kit, agarose gel, SDS-PAGE and Nuclear and Cytoplasmic Protein Extraction kit (no. P0028) were purchased from Beyotime Institute of Biotechnology (Haimen, China)

Experimental design. The dose- and time-dependent effects of Res on SIRT1 expression and lung injury after PQ exposure were examined by two independent experiments. A) Mice were randomly divided into four groups as follows: i) The control group, mice received saline solution by intraperitoneal (i.p.) injection (n=6); ii) The Res group, mice received Res (30 mg/kg i.p.; n=6); iii) The PQ group, mice received PQ (20 mg/kg i.p.; n=6); iv) The PQ + Res group, mice received PQ (20 mg/kg i.p.) and Res (15, 30 or 60 mg/kg i.p.; n=6 for each condition). PQ was dissolved in saline solution and injected intraperitoneally in a single toxic dose of 20 mg/kg of body weight based on preliminary experiments. Mice were anesthetized with 50 mg/kg pentobarbital (Hanlim Pharm Co., Ltd., Seoul, Korea). Pulmonary samples were collected at 24 h subsequent to PQ injection. B) Mice were randomly divided into three groups as follows: i) Control group (n=18); ii) The PQ group, mice received PQ (20 mg/kg i.p.; n=18); iii) The PQ + Res group, mice received PQ (20 mg/kg i.p.) and Res (30 mg/kg i.p.; n=18). Pulmonary samples were collected at 6, 24 and 72 h subsequent to PQ exposure, and tissues from the same mice were used for histopathological, PCR and western blot analyses.

Histopathological assessment of pulmonary tissue. The lower lobes of the right lungs of six mice pre group were removed and then transferred to 4% formaldehyde for 24 h. The lungs were embedded in paraffin (Leica Microsystems, Wetzlar, Germany), and 2-3 butterfly-shaped sections of 4- μ m thickness were cut per sample using a microtome (Leica RM2016; Leica Microsystems) and placed on glass microscope slides stained with hematoxylin and eosin (H&E) for histopathological analysis. Lung histopathology images were acquired using a microscope (Nikon Eclipse 80i; Nikon, Tokyo, Japan). The severity of lung injury was determined by a histopathologist blinded to the protocol design.

Wet/dry (W/D) weight ratio. To quantify the magnitude of pulmonary edema, the lung W/D weight ratio was evaluated. The middle lobe of right lung of six animals was excised and the wet weight was determined, following which the lung was placed in a drying oven at 60°C for 72 h to stabilize the dry weight. The ratio of W/D was calculated.

RNA isolation and reverse transcription-semiquantitative polymerase chain reaction (RT-sqPCR). The total RNA of the upper right lung lobe of six mice per group was extracted using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham,

MA USA). The cDNA was prepared from 2 μ g RNA using a PCR master mix (Promega Corp., Madison, WI, USA). The primers used were as follows: SIRT1, F 5'-ACGCTGTGGCAG ATTGTTATTA-3' and R 5'-TTGAAGAATGGTCTTGGG TCTT-3'; NRF2, F 5'-ATTCTTTCAGCAGCATCCTCTC-3' and R 5'-ACACTTCCAG GGGCACTATCTA-3', resulting in PCR products of 278 and 403 bp, respectively. The primers for mouse β -actin were F 5'-ATATCGCTGCGCTGGTTCGTC-3' and R 5'-AGGATGGCGTGAGGGAGAGC-3', resulting in a PCR product of 517 bp. Amplified fragments of expected size were analyzed using a 2% agarose gel and images were captured under ultraviolet (UV) light (302 nm) (Tianneng Co., Shanghai, China). Gels were imaged using the ChemiDoc™ MP Imaging System (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and quantified by densitometry using Quantity One 4.52 software (Bio-Rad Laboratories, Inc.). Data are presented as fold changes in gene expression normalized to β -actin.

Western blot analysis. The upper parts of the left lung from six animals per group were harvested and homogenized immediately. Total proteins were extracted from the lungs using the Nuclear and Cytoplasmic Protein Extraction kit according to the manufacturer's protocol. Equal amounts of total proteins (40 μ g) per lane were separated by 8% SDS-PAGE (80 V, 30 min; 120 V, 90 min) and then transferred to polyvinylidene fluoride membranes (Solarbio, Beijing, China). Membranes were incubated with rabbit anti-mouse SIRT1 monoclonal antibody (1:1,000 dilution) or rabbit anti-mouse Nrf2 polyclonal antibody (1:500 dilution) at 4°C for 24 h and washed with Tris-buffered saline containing Tween 20 (Solarbio). Subsequent to washing, samples were incubated with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G secondary antibody (cat. no. sc-2030; 1:2,000 dilution; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) for 1 h at room temperature and visualized using Western Blotting Luminol reagent (cat. no. sc-2048; Santa Cruz Biotechnology, Inc.). Images of the blots were captured using the ChemiDoc™ MP Imaging System and quantified by densitometry using Quantity One 4.52 software.

Enzyme-linked immunosorbent assay (ELISA). The heme oxygenase-1 (HO-1) activity in samples of the lower part of the left lung of six mice was measured by commercially available ELISA kits (Westang Biotechnology Co., Shanghai, China). ELISA was performed following the protocols provided by the manufacturer.

Detection of superoxide dismutase (SOD) and catalase (CAT) activity and protein levels of glutathione (GSH) and malondialdehyde (MDA). The MDA and GSH contents in samples of the lower part of the left lung of six mice were detected according to the instructions of the MDA and GSH assay kits purchased from Nanjing Jiancheng Bioengineering Research Institute (Nanjing, China). The SOD and CAT activities were detected according to the instructions of the SOD and CAT assay kits (Nanjing Keygen Biotech Co., Ltd., Nanjing, China).

Statistical analysis. All data are described as the mean \pm standard deviation and analyzed using SPSS 19.0 software (International Business Machines, Armonk, NY, USA). The differences between groups were analyzed by one-way analysis

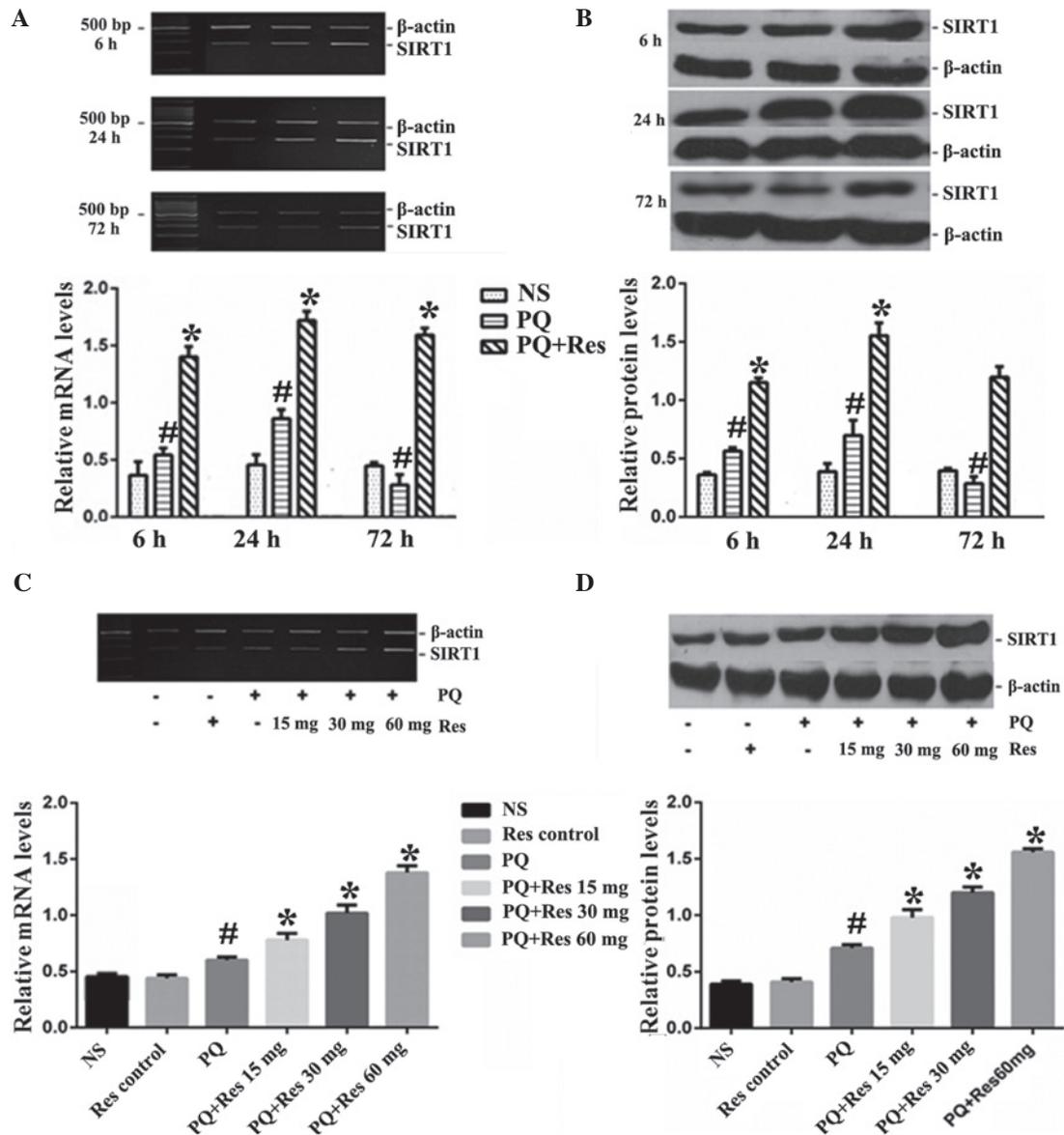


Figure 1. Res upregulated the expression of SIRT1 in PQ-induced mouse lung tissue. (A) mRNA and (B) protein levels of SIRT1 following 6-, 24- and 72-h PQ exposure. (C) mRNA and (D) protein levels of SIRT1 following 15, 30 and 60 mg Res treatment. Data are presented as the mean \pm standard deviation (n=6). #P<0.05 vs. the NS group, *P<0.05 vs. the PQ group. Res, resveratrol; SIRT1, sirtuin 1; PQ, paraquat; sqPCR, semiquantitative polymerase chain reaction; NS, normal saline.

of variance. P<0.05 was considered to indicate a statistically significant difference.

Results

Res upregulated the expression of SIRT1 in PQ-exposed lung tissue. To assess the potential role of SIRT1 in PQ-induced lung injury, the SIRT1 levels in lung tissue prior and following Res injection were determined. As demonstrated in Fig. 1A and B, the expression of SIRT1 mRNA and protein levels were elevated at 6 and 24 h after PQ exposure compared with the saline group (P<0.05). At 72 h, the SIRT1 mRNA and protein levels were significantly decreased compared with the saline group (P<0.05). Additionally, administration of 30 mg/kg Res significantly increased the mRNA and protein levels of SIRT1 at all time points compared with the PQ group (P<0.05). Administration of 15, 30 or 60 mg/kg

Res upregulated SIRT1 expression levels in the lung at 24 h compared with the PQ group (P<0.05; Fig. 1C and D).

Res attenuates PQ-induced lung injury in mice.

Histopathological and W/D weight ratio analyses were performed to determine the effect of Res on PQ-induced lung injury. Compared with the control group, PQ administration caused marked lung hemorrhage, edema, alveolar septal thickening, influx of inflammatory cells and fibrin deposition (Fig. 2A). In the PQ+Res group, similar changes were identified, however to a lesser degree. Additionally, the W/D weight ratios of the lung tissues were significantly increased following PQ administration compared with the ratios in the NS group (P<0.05; Fig. 2B). In the PQ+Res group, the lung W/D weight ratio was significantly lower than that in the PQ group at 24 and 72 h subsequent to PQ exposure (P<0.05; Fig. 2B).

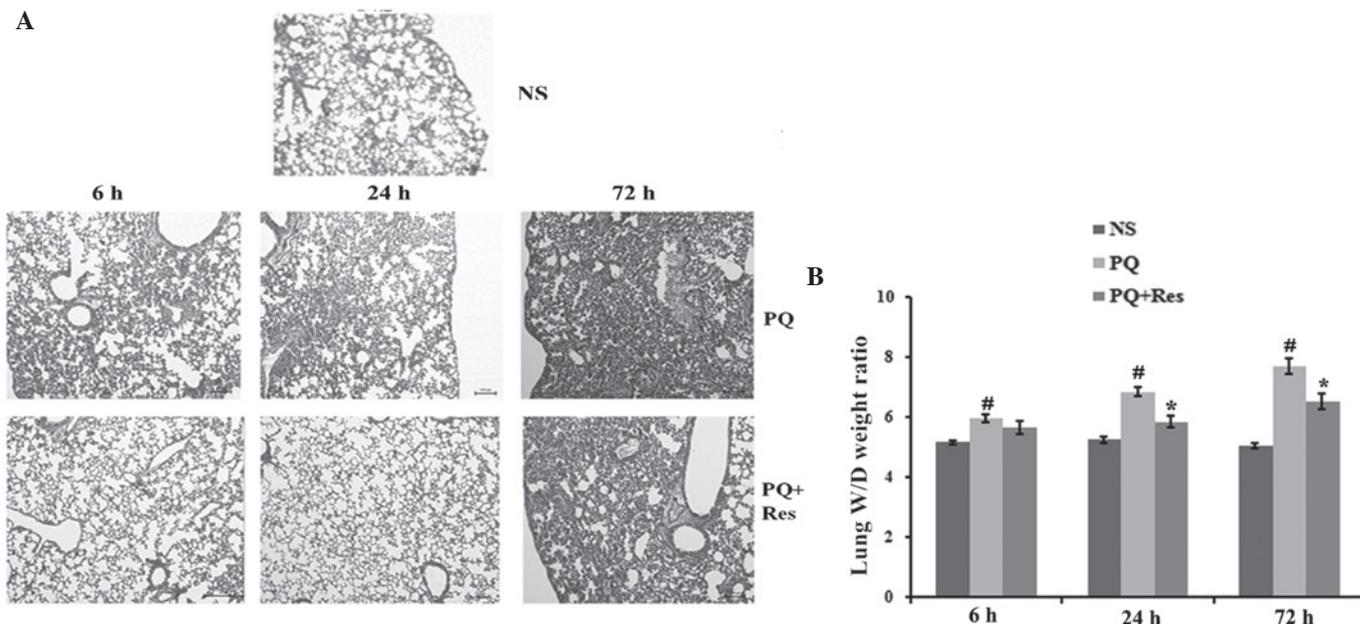


Figure 2. Res attenuates PQ-induced lung injury in mice. (A) Histological examination of mouse lung tissue was performed at 6, 24 and 72 h post-PQ treatment. Tissue was fixed and stained with hematoxylin and eosin (magnification, x100). In the he NS group, parenchyma and lung airways are normal, whereas the PQ groups show marked lung hemorrhage, edema, alveolar septal thickening, influx of inflammatory cells and fibrin deposition. These changes were ameliorated by Res treatment. (B) The lung W/D ratio was determined at 6, 24 and 72 h after PQ administration. Data are presented as the mean ± standard deviation (n=6). [#]P<0.05 vs. the NS group, ^{*}P<0.05 vs. the PQ group. Res, resveratrol; PQ, paraquat, W/D, wet/dry; NS, normal saline.

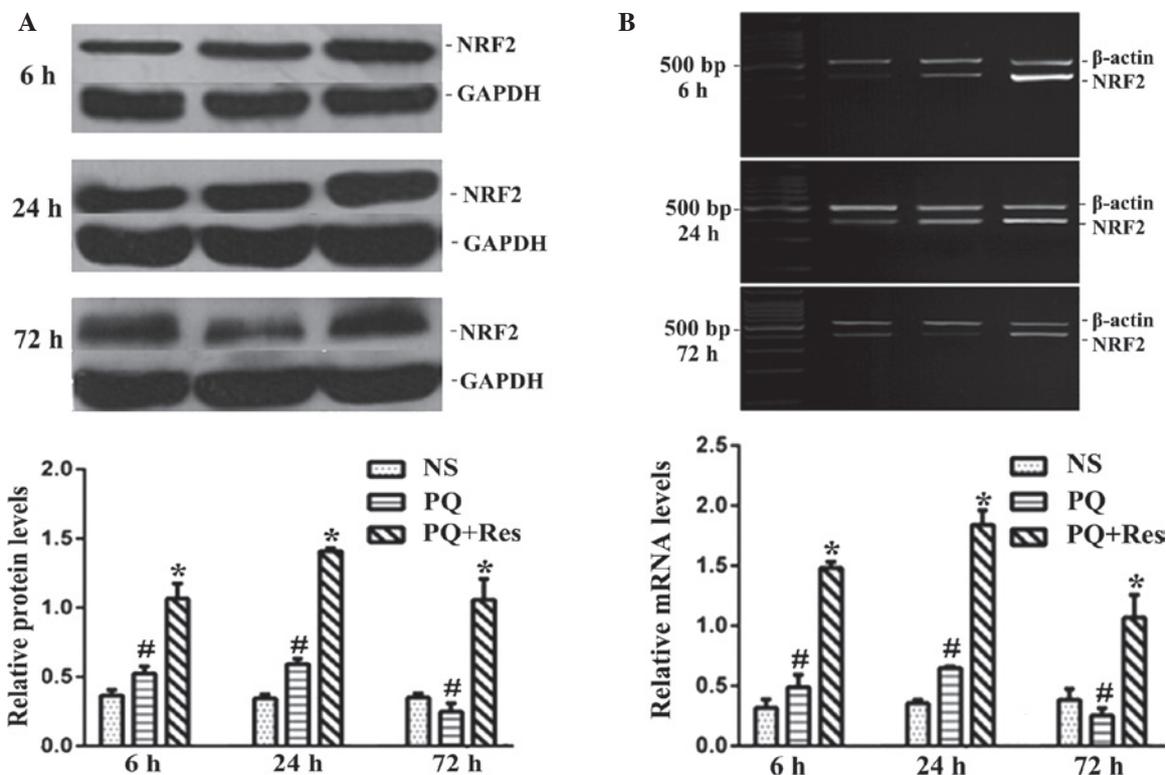


Figure 3. Effects of Res on NRF2 expression levels in mouse lung tissue following PQ exposure. The protein and mRNA expression levels of NRF2 were determined by (A) western blot and (B) semiquantitative polymerase chain reaction analyses, respectively, at 6, 24 and 72 h after PQ administration. Data are presented as the mean ± standard deviation (n=6). [#]P<0.05 vs. the NS group, ^{*}P<0.05 vs. the PQ group. Res, resveratrol; NRF2, nuclear factor, erythroid 2-like 2; PQ, paraquat, NS, normal saline.

Effects of Res on NRF2 expression in the lung following PQ exposure. To investigate whether the protective role of Res was mediated through NRF2, the NRF2 expression levels in the

mouse lung tissues were determined. NRF2 protein and mRNA expression levels were significantly elevated at 6 and 24 h after PQ administration but were decreased at 72 h, compared

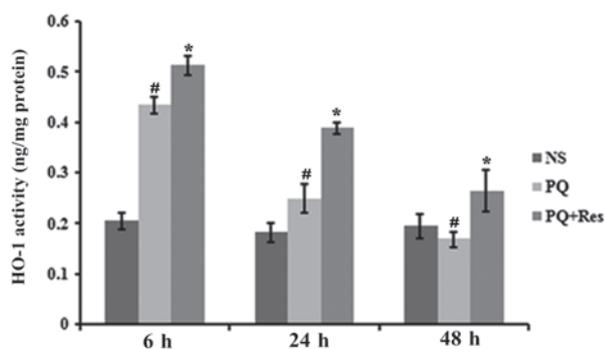


Figure 4. Effects of Res on HO-1 activity in mouse lung tissue after PQ exposure. The activity of HO-1 was determined by ELISA at 6, 24 and 72 h after PQ administration. Data are presented as the mean \pm standard deviation (n=6). [#]P<0.05 vs. the NS group, ^{*}P<0.05 vs. the PQ group. Res, resveratrol; HO-1, heme oxygenase-1; PQ, paraquat; NS, normal saline.

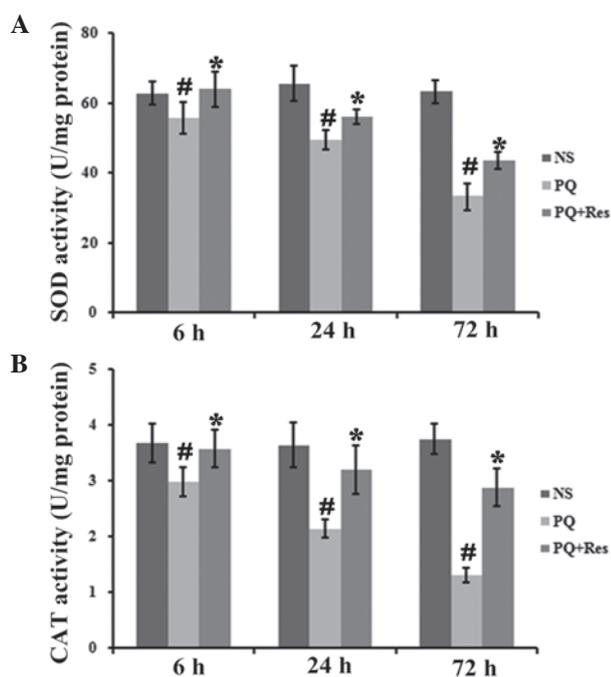


Figure 5. Effects of Res and PQ on SOD and CAT activity in mouse lung tissues. The activity of (A) SOD and (B) CAT were determined at 6, 24 and 72 h after PQ administration. Data are presented as the mean \pm standard deviation (n=6). [#]P<0.05 vs. the NS group, ^{*}P<0.05 vs. the PQ group. Res, resveratrol; PQ, paraquat; SOD, superoxide dismutase; CAT, catalase; NS, normal saline.

with the NS group (P<0.05; Fig. 3). However, Res administration significantly increased NRF2 protein expression levels compared with the PQ group at all time points (P<0.05; Fig. 3).

Effects of Res on HO-1 activity in mouse lung tissue following PQ exposure. Compared with the NS group, HO-1 activity in the lung tissue was upregulated markedly at 6 and 24 h after PQ administration, but was decreased at 72 h (P<0.05; Fig. 4). In the PQ+Res group, HO-1 activity was upregulated compared with that in the PQ group (P<0.05; Fig. 4).

Effects of Res and PQ on SOD and CAT activity levels in mouse lung tissues. SOD and CAT activity levels in the mouse lung tissue were measured. Compared with the NS group, the PQ

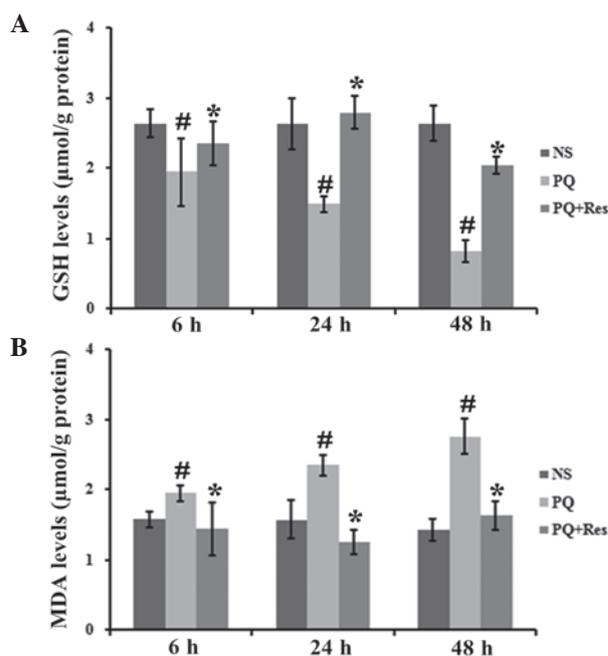


Figure 6. Effects of Res and PQ on (A) GSH and (B) MDA levels in mouse lung tissue. Levels of GSH and MDA were determined at 6, 24 and 72 h after PQ administration. Data are presented as the mean \pm standard deviation (n=6). [#]P<0.05 vs. the NS group, ^{*}P<0.05 vs. the PQ group. Res, resveratrol; PQ, paraquat; GSH, glutathione; MDA, malondialdehyde; NS, normal saline.

group exhibited a significant decrease in SOD and CAT activity levels at all time points (P<0.05; Fig. 5). Following Res administration, SOD and CAT activity levels significantly increased in the lung tissue, compared with the NS group (P<0.05; Fig. 5).

Effects of Res and PQ on GSH and MDA levels in mouse lung tissues. Following PQ administration, GSH protein levels were decreased in the lung tissue compared with the NS group (P<0.05; Fig. 6A). The GSH protein levels were significantly increased in the PQ+Res group compared with those in the PQ group (P<0.05; Fig. 6A). By contrast, PQ administration led to an increase in MDA activity levels compared with the NS group (P<0.05; Fig. 6B). Res administration decreased MDA activity levels compared with those in the PQ group (P<0.05; Fig. 6B).

Discussion

The lung is targeted in PQ poisoning through the pulmonary polyamine uptake system that recruits PQ. This results in a 6-10-fold increase in the lung PQ levels compared with plasma levels. Based on its induction of redox cycling, PQ in the lung results in oxidative stress-associated cell death and lung injury (14,15).

Sirtuins are a unique class of type III histone deacetylases with seven isoforms, SIRT1-7 (16). SIRT1 is the best characterized member among the mammalian sirtuins (16). Previous studies have revealed a crosstalk between the SIRT1 expression levels and oxidative stress (5,17). Oxidative stress can inhibit the SIRT1 expression, and the overexpression of SIRT1 has been demonstrated to inhibit apoptosis induced by oxidative stress (18,19). The current study demonstrated increased SIRT1 expression levels in mouse lung tissue at 6 and 24 h after PQ

administration. However, long-term exposure to PQ significantly decreased the expression of SIRT1. Treatment with Res, a known allosteric activator of SIRT1, upregulated the expression of SIRT1, accompanied by decreased oxidative stress and lung injury. This demonstrated that the SIRT1 agonist in Res treatment has a protective role in PQ-induced lung injury by decreasing oxidative stress.

A major mechanism in the cellular defense against oxidative stress is the activation of the NRF2 antioxidant response element signaling pathway (20). Previous studies have demonstrated that NRF2 is important in PQ-induced lung injury; PQ can inhibit the NRF2 expression *in vitro* and *in vivo* (6,21). Additionally, overexpression of NRF2 can ameliorate PQ-induced lung injury and cell apoptosis (21,22). The function of the NRF2-antioxidant pathway is controlled by multiple factors, including the acetylation-deacetylation of NRF2. Kawai *et al* (7) demonstrated that SIRT1-mediated deacetylation of the NRF2 protein terminated the transcription of antioxidant genes *in vitro*. However, other studies have demonstrated that SIRT1 overexpression significantly promoted the nuclear accumulation, DNA binding and transcriptional activity of NRF2 and NRF2-mediated gene expression (8,9,23). In the current study, an increase in the SIRT1 expression levels by Res was associated with a high level of NRF2. In addition, the HO-1, SOD and CAT activity and the levels of GSH were upregulated following Res administration. Indeed, previous studies indicated that the acetylation of NRF2 can reduce NRF2 stability and impaired antioxidant defenses (24). Therefore, as a protein deacetylase, SIRT1 may be important for NRF2 protein stability and expression.

Taken together, the results of the current study demonstrated that Res, an SIRT1 activator, can reduce PQ-induced lung injury by regulating SIRT1 expression in combination with the NRF2 antioxidant pathway. The present study indicated that SIRT1 and NRF2 serve a critical role in PQ-induced lung injury. However, the mechanism of regulation of NRF2 expression levels by SIRT1 requires further investigation.

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