Sevoflurane inhibits cardiac function in pulmonary fibrosis mice through the TLR4 signaling pathway

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Abstract
Pulmonary fibrosis is often concomitant with myocardial injury. We studied sevoflurane’s effects on cardiac function and the expression of the TLR4/inducible nitric oxide synthase (iNOS) signaling pathway on a pulmonary fibrosis model. C57BL/6J wild-type (WT) and TLR4-deficient (TLR4−/−) mice were randomly divided into a control group and a pulmonary fibrosis group. The model of pulmonary fibrosis was induced by treatment with paraquat (PQ; 20 mg/kg). Four weeks after PQ administration, mice were tested for body weight changes, and histopathology and hydroxyproline in lung. Left ventricular function in each group of mice was measured by echocardiogram before and after sevoflurane inhalation. The expression of TLR4 and iNOS protein were analyzed. Pulmonary fibrosis mice were fed lenalidomide (50 mg/kg/day) for three days and cardiac function was assessed before and after sevoflurane inhalation. WT pulmonary fibrosis mice showed pathological damage and excessive deposition of collagen in the lung and heart. Left ventricular function decreased after four weeks of PQ exposure. TLR4−/− mice were resistant to pulmonary fibrosis like pathological damage and the effect of sevoflurane on heart rate and ejection fraction than that of WT mice. TLR4 and iNOS expression in WT pulmonary fibrosis mice increased significantly after sevoflurane inhalation. Lenalidomide treatment alleviated the effect of sevoflurane on heart rate and ejection fraction in WT pulmonary fibrosis mice. Sevoflurane inhibits cardiac function in pulmonary fibrosis mice through the TLR4/iNOS pathway. Lenalidomide attenuated the sevoflurane’s effect on the cardiac function of mice with pulmonary fibrosis.

Keywords
sevoflurane, cardiac function, pulmonary fibrosis, TLR4

Introduction
Pulmonary fibrosis is an irreversible end stage of lung diseases and carries high disability and mortality.1 From various studies, the probability of pulmonary fibrosis associated with heart disease is in the range of 3–68%.2 Patients with idiopathic pulmonary fibrosis may experience acute deterioration of cardiopulmonary function after surgery.3

Toll-like receptor 4 (TLR4) has been demonstrated to participate in a variety of physiological process such as the immune response, signal transduction, and cell cycle and enzyme regulation.4–6 Endogenous ligands such as endotoxin, hyaluronic acid, fibronectin, and tenasin-C can bind to TLR4 to recruit MyD88 and release nuclear factor-κB (NF-κB) via a series of intracellular reactions.7 Although TLR4 is associated with cardiac dysfunction in sepsis and myocardial infarction, how it is in the cardiac injury of mice with pulmonary fibrosis remains unclear. Sevoflurane has protective effects on myocardial ischemia reperfusion injury;8,9 however, it also can inhibit myocardial contractility.10 Studies have shown that sevoflurane protects
the myocardium from ischemia-reperfusion injury through the TLR4/iNOS signaling pathway.\textsuperscript{11–13} Increasing iNOS can reduce the contractility of myocardium,\textsuperscript{14} tumor necrosis factor-\( \alpha \) (TNF-\( \alpha \)) can cause an inflammatory response and promote myocardial injury, and a TNF-\( \alpha \) inhibitor has been shown to improve cardiac systolic function in obese mice.\textsuperscript{15} However, sevoflurane’s effects on the cardiac function of mice with pulmonary fibrosis remain to be investigated.

This study explored the mechanisms of sevoflurane’s effects on cardiac function in mice with pulmonary fibrosis. We hypothesize that sevoflurane alters TLR4 and iNOS expression and affects the cardiac function of mice with pulmonary fibrosis through the TLR4/iNOS signaling pathway.

### Materials and Methods

#### Animals and pulmonary fibrosis model

Male wild-type (WT) C57BL/6J mice were provided by the Laboratory Animal Center of Central South University (Changsha, China). Male TLR4-deficient C57BL/10JNju (TLR4\(^{-/-}\)) mice were purchased from the Model Animal Research Center of Nanjing University (Nanjing, China). Male WT and TLR4\(^{-/-}\) mice used in the experiments were aged 8–10 weeks and weighed 22–25 g. Mice were fed and housed under controlled temperature (22–25°C), humidity (50–60%), and a 24-h light–dark cycle with access to chow and water ad libitum. All experimental protocols were approved by the Ethics Committee for Animal Research of Central South University. All experimental methods were in accordance with guidelines for treating lab animals.

WT and TLR4\(^{-/-}\) mice were intraperitoneally injected with paraquat (PQ; 20 mg/kg, Sigma, St. Louis, MO, USA) to induce a pulmonary fibrosis model. The control group was treated with 1% dimethyl sulfoxide (DMSO). After three days, HR and EF were measured by echocardiogram before and after inhalation of sevoflurane at T0, T1, and T2.

#### Histopathological study

Mice were euthanized under deep anesthesia and blood was taken from the heart. The lung and heart were then removed. Tissue from the lung and heart, for histopathological analysis, was fixed in 4% paraformaldehyde and tissue for assessment of hydroxyproline content or extraction of protein was frozen in liquid nitrogen and stored at \(-80^\circ\text{C}\).

The lung and heart were embedded in paraffin blocks and cut into 5-\( \mu\)m sections that were stained by hematoxylin and eosin (H&E) or Masson trichrome. Stained sections were assessed using a computerized morphometric system (Qwin, Leica, Wetzlar, Hessen, Germany).

#### Measurement of hydroxyproline content

Hydroxyproline content was measured using a hydroxyproline measurement kit (Jiancheng Company, Nanjing, China) according to the manufacturer’s protocol. Briefly, lung tissues of six mice in each group were weighed and diluted in 1 mL of hydrolysate. Samples were then hydrolyzed at 95°C for 20 min. Reagent I (0.5 mL) was added to a blank tube, standard tube, and sample tube and the mixture was incubated for 10 min; 0.5 mL of Reagent II was added to the above tubes and incubated for 5 min; 0.5 mL of Reagent III was added to the above tubes and incubated at 60°C for 15 min; the mixture was centrifuged at 3500 g for 10 min and the supernatant was collected. Absorbance was measured at 550 nm wavelength using an Infiniti M200 (Tecan Group Ltd., Männedorf, Switzerland).

#### Western blot analysis

Sevoflurane’s effects on TLR4 and iNOS expression in cardiomyocytes in the control and pulmonary fibrosis groups were analyzed by western blot. Total protein extracted from heart tissue was lysed on ice for 10 min by RIPA lysis buffer containing a 1:100 dilution of phenylmethanesulfonyl fluoride (Beyotime, Shanghai, China). The tissues were then sonicated and the crude extracts were centrifuged at 12,000 g for 20 min. The supernatant was diluted 1:10 in loading buffer and then heated at 95°C for 5 min. After electrophoresis, the proteins were transferred to a PVDF membrane and incubated with primary antibodies for TLR4, iNOS, and GAPDH followed by secondary antibodies.

The membranes were visualized using an enhanced chemiluminescence detection system (Tanon, Shanghai, China) according to the manufacturer’s instructions.
10 min at 4°C. The concentration of protein was detected with a BCA Protein Assay Kit (Beyotime, Shanghai, China). Fifty microliters of lysates were loaded onto a 10% SDS-PAGE gel and then transferred to polyvinylidene fluoride membranes (Merck Millipore, Billerica, MA, USA). After membranes were blocked with 5% non-fat milk in Tris-buffered saline plus 0.05% Tween 20 (pH 7.5) for 1 h, they were probed overnight at 4°C with primary rabbit polyclonal anti-TLR4 (1:500; Abcam, Cambridge, MA, USA), rabbit polyclonal anti-iNOS (1:10, Abcam, Cambridge, MA, USA), rabbit monoclonal anti-GAPDH (1:1000, Cell Signal Technology, Danvers, MA, USA). Image Pro Plus 6.0 software was used for densitometry analysis. The protein content was normalized to the standardized GAPDH levels.

Statistical analysis

Statistical analyses were performed with GraphPad Prism 6 software (GraphPad, La Jolla, CA, USA). The values were expressed as means ± standard deviation (SD). Analysis of variance and a Student Newman–Keuls post hoc test were performed to determine the statistical significance ($P < 0.05$) of differences between groups.

Results

Pulmonary fibrosis and myocardial damage

All mice survived the PQ treatment. Compared with saline-treated mice, the PQ administration was associated with a significantly lower body weight in WT mice. Besides, the body weight of PQ-treated WT mice was lower than PQ-treated TLR4$^{-/-}$ mice (Table 1).

There were no significant differences in geometric or functional parameters measured by echocardiography between WT and TLR4$^{-/-}$ mice. After PQ treatment, LVIDd, LVIDs, and HR increased, and EF decreased in WT mice. However, these parameters did not change significantly in TLR4$^{-/-}$ mice. When compared with PQ-treated WT mice, LVIDd, LVIDs, and HR are lower and EF are higher in PQ-treated TLR4$^{-/-}$ mice (Table 1).

Histopathological changes in lung and heart tissue

H&E staining in the lung demonstrated that PQ administration induced inflammatory cell infiltration into the lung when compared with normal saline administration. TLR4$^{-/-}$ mice treated with PQ showed less change in morphology and fewer inflammatory cell infiltration into the lung when compared with PQ-treated WT mice (Fig. 1a–d). Masson staining showed a higher degree of fibrosis in WT mice than in TLR4$^{-/-}$ mice after PQ treatment (Fig. 1e–h). H&E staining in the heart showed that only part of the myocardial interstitial was infiltrated by inflammatory cells in WT mice that developed pulmonary fibrosis; the other three groups were normal (Fig. 1i–l). Masson staining of the right ventricle wall demonstrated that myocardial injury appeared in WT mice exhibiting myocardial interstitial fibrosis (Fig. 1m–p). Therefore, the degree of lung and heart inflammatory infiltration and injury was worse in WT mice than in TLR4$^{-/-}$ mice four weeks after PQ injection.

Measurement of hydroxyproline content

PQ significantly upregulated the hydroxyproline content in WT mice, whereas it did not change the levels in TLR4$^{-/-}$ mice after a 28-day exposure to PQ or saline.

| Parameter          | WT        | WT-PF     | TLR4$^{-/-}$ | TLR4$^{-/-}$/PF |
|--------------------|-----------|-----------|--------------|-----------------|
| BW (g)             | 26.67 ± 0.64 | 24.54 ± 0.80* | 26.02 ± 1.47 | 27.11 ± 1.79** |
| HW (mg)            | 115.71 ± 9.76 | 104.29 ± 7.87 | 118.57 ± 16.76 | 105.71 ± 13.97 |
| LW (mg)            | 168.33 ± 28.28 | 153.33 ± 19.66 | 186.67 ± 17.51 | 171.67 ± 18.35 |
| HW/BW (mg/g)       | 4.38 ± 0.39 | 4.28 ± 0.35 | 4.61 ± 0.59 | 3.93 ± 0.37 |
| LVIDd (cm)         | 0.24 ± 0.030 | 0.31 ± 0.035** | 0.24 ± 0.020 | 0.22 ± 0.014*** |
| LVIDs (cm)         | 0.11 ± 0.036 | 0.17 ± 0.027*** | 0.099 ± 0.021 | 0.080 ± 0.012** *** |
| HR (bpm)           | 547 ± 23 | 611 ± 23** | 564 ± 26 | 578 ± 28* |
| EF (%)             | 92.3 ± 3.5 | 77.2 ± 3.0** | 93.4 ± 2.8 | 93.7 ± 2.0** |

Values are presented as mean ± SD (n = 6 for each group). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, when compared with group WT; *$P < 0.05$, ****$P < 0.001$, when compared with group WT-PF.

BW, body weight; HW, heart weight; LW, lung weight; LVIDd, left ventricular internal dimension diastole; LVIDs, left ventricular internal dimension systole; HR, heart rate; EF, ejection fraction; WT, WT mice after a 28-day exposure to saline; WT-PF, WT mice after a 28-day exposure to paraquat; TLR4$^{-/-}$, TLR4$^{-/-}$ mice after a 28-day exposure to saline; TLR4$^{-/-}$ PF, TLR4$^{-/-}$ mice after a 28-day exposure to paraquat.
mice. When compared with WT mice, the hydroxyproline content of the lung was less in TLR4−/− mice after PQ injection (Table 1).

**Sevoflurane treatment and anti-inflammatory experiment with the TNF-α inhibitor**

After sevoflurane inhalation, HR and EF decreased significantly in WT mice and WT pulmonary fibrosis mice. TLR4−/− mice were more resistant to the effect of sevoflurane on HR and EF than that of WT mice. When compared with WT mice, WT pulmonary fibrosis mice suffered more decline in HR and EF after 2 h of sevoflurane inhalation (HR, WT pulmonary fibrosis mice vs. WT mice: 29.3% vs. 12.2%; EF, WT pulmonary fibrosis mice vs. WT mice: 11.2% vs. 7.2%) (Fig. 2).

Pulmonary fibrosis mice treated with lenalidomide showed a smaller decline in HR (30.5% vs. 22.3%) and had no change in EF after sevoflurane inhalation for 2 h when compared with mice treated with DMSO (Fig. 3).

**Protein expression of TLR4 and iNOS in the heart**

Protein levels of iNOS and TLR4 significantly increased after PQ treatment in WT mice (P < 0.05). After sevoflurane inhalation, iNOS and TLR4 expression were elevated in
both groups. Interestingly, PQ-treated WT mice exhibited an additional iNOS and TLR4 increase from their already higher baseline (Fig. 4).

**Discussion**

In this study, animal models of pulmonary fibrosis were induced by a single intraperitoneal injection of PQ, which is an irreversibly stable model. Fibroblasts are activated and transformed into myofibroblasts under inflammatory stimulation. Myofibroblasts secrete large amounts of collagen and extracellular matrix, leading to pulmonary fibrosis and myocardial injury. The expression of TLR4 was increased in fibrotic tissues; however, the effect of regulating TLR4 expression on lung fibrosis was controversial. In the present study, lung fibrosis was induced by injection of PQ successfully both in WT mice and TLR4−/− mice. We suspect TLR4−/− mice manifest reduced inflammatory infiltration and lung fibrosis after PQ administration based on qualitative histologic analyses, as shown by less histological evidence of inflammatory cellular infiltration and fibrosis, hydroxyproline content in the lung and heart.

In our previous study, left and right ventricular function were altered in rats with pulmonary arterial hypertension. Studies have shown that acute high-dose PQ poisoning caused a decrease in cardiac contractility, an imbalance of intracellular Ca²⁺, apoptosis acceleration, and mitochondrial damage. In the present study, for WT mice after PQ treatment, morphological results showed that only part of the
myocardial interstitial was infiltrated by inflammatory cells and part of the myocardium was injured. Echocardiographic images showed that LV volume increased and EF decreased in PQ-treated WT mice, whereas PQ did not induce obvious alternations in TLR4⁻/⁻ mice. In addition, the protein level of TLR4 significantly increased after PQ treatment in WT mice. This may suggest that TLR4 could be associated with myocardial injury in pulmonary fibrosis mice by modulating the inflammatory response.

Studies have shown that 2.0% sevoflurane anesthesia resulted in apparent changes in microRNA expression in rat lungs, and some of the differentially expressed microRNAs were known to be involved in idiopathic pulmonary fibrosis. Besides, sevoflurane protects the myocardium from ischemia-reperfusion injury through the TLR4 signaling pathway or downstream iNOS. In this study, 2 h after sevoflurane administration, HR and EF decreased in WT pulmonary fibrosis mice, and TLR4 and iNOS expression levels were upregulated, indicating that inhibition of sevoflurane on cardiac function in pulmonary fibrotic mice may be related to TLR4 and iNOS. iNOS was located downstream of the TLR4/MyD88/NF-κB signaling pathway. Upregulation of iNOS may activate protein kinase G and its downstream cGMP, thereby reducing the sensitivity of the myofilament to Ca²⁺ and reducing cardiac contractility. In the present study, WT pulmonary fibrosis mice exhibited a further iNOS and TLR4 increase from their already high baseline after 2 h of sevoflurane inhalation. In addition, WT mice suffered more decline in HR and EF after 2 h of sevoflurane inhalation, while, TLR4⁻/⁻ mice were more resistant to the effect of sevoflurane on HR and EF than that of WT mice. This indicates that TLR4 and its downstream iNOS are related to sevoflurane’s inhibition of cardiac function and that the deficiency of the TLR4 gene attenuated the sevoflurane’s inhibitory effect on cardiac function.

In the TLR4/MyD88/NF-κB signaling pathway, TLR4 upregulates downstream NF-κB expression, thereby promoting the expression of TNF-α. As a pro-inflammatory factor, TNF-α causes inflammatory reactions and promotes myocardial injury. Studies have showed that TNF-α inhibitors such as lenalidomide can improve myocardial contractile dysfunction in obese mice. In acute high-dose PQ exposure, myocardial injury and myocardial contractile dysfunction were associated with TLR4 as well as its downstream cytokine, TNF-α. In this study, WT pulmonary fibrosis mice treated with lenalidomide showed less decline in HR and had no change in EF after 2 h of sevoflurane inhalation. Heart function was significantly optimized in WT pulmonary fibrosis mice by a reduction of the inflammatory response through inhibiting the secretion of TNF-α.

**Study limitations**

In this study, quantification of histological analyses was not acquired. We did not measure systemic blood pressure of the mice except for echocardiography. We also did not investigate the inflammation reaction in a pulmonary fibrosis model. How TLR4 mediates sevoflurane’s inhibition of cardiac function in mice with pulmonary fibrosis still needs to be investigated.

**Conclusion**

Sevoflurane depresses cardiac function in mice with pulmonary fibrosis through the TLR4/iNOS pathway. Lenalidomide can attenuate the effect of sevoflurane on cardiac function in mice with pulmonary fibrosis.

**Conflict of interest**

The author(s) declare that there is no conflict interest.

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