



# Methane Inhalation Protects Against Lung Ischemia-Reperfusion Injury in Rats by Regulating Pulmonary Surfactant *via* the Nrf2 Pathway

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Methane (CH<sub>4</sub>) exerted protective effects against lung ischemia-reperfusion (I/R) injury, but the mechanism remains unclear, especially the role of pulmonary surfactant. Therefore, this study aimed to explore the effects of CH<sub>4</sub> inhalation on pulmonary surfactant in rat lung I/R injury and to elucidate the mechanism. Rats were randomly divided into three groups ( $n = 6$ ): the sham, I/R control, and I/R CH<sub>4</sub> groups. In the sham group, only thoracotomy was performed on the rats. In the I/R control and I/R CH<sub>4</sub> groups, the rats underwent left hilum occlusion for 90 min, followed by reperfusion for 180 min and ventilation with O<sub>2</sub> or 2.5% CH<sub>4</sub>, respectively. Compared with those of the sham group, the levels of large surfactant aggregates (LAs) in pulmonary surfactant, lung compliance, oxygenation decreased, the small surfactant aggregates (SAs), inflammatory response, oxidative stress injury, and cell apoptosis increased in the control group ( $P < 0.05$ ). Compared to the control treatment, CH<sub>4</sub> increased LA ( $0.42 \pm 0.06$  vs.  $0.31 \pm 0.09$  mg/kg), oxygenation ( $201 \pm 11$  vs.  $151 \pm 14$  mmHg), and lung compliance ( $16.8 \pm 1.0$  vs.  $11.5 \pm 1.3$  ml/kg), as well as total antioxidant capacity and Nrf2 protein expression and decreased the inflammatory response and number of apoptotic cells ( $P < 0.05$ ). In conclusion, CH<sub>4</sub> inhalation decreased oxidative stress injury, inflammatory response, and cell apoptosis, and improved lung function through Nrf2-mediated pulmonary surfactant regulation in rat lung I/R injury.

**Keywords:** methane, lung ischemia-reperfusion injury, pulmonary surfactant, lung function, Nrf2

## INTRODUCTION

Lung ischemia-reperfusion (I/R) injury can occur during many clinical events, such as lung transplantation, cardiopulmonary bypass, pulmonary embolism, single-lung ventilation, resuscitation for circulatory arrest, and shock, and can contribute to severe organ failure and increased mortality in patients (Tejwani et al., 2016; Saito et al., 2019). Lung I/R injury is a complex pathological process that includes oxidative stress injury, the inflammation response, and cell apoptosis and is characterized by poor oxygenation, tissue edema, and neutrophil infiltration (den Hengst et al., 2010). Many measures have been applied, such as ischemic preconditioning, protective drug injection, gene therapy, and protective gas inhalation (Meng et al., 2016a;

Jiang et al., 2019). Unfortunately, these methods still cannot effectively control lung I/R injury. Thus, a novel protective strategy needs to be explored.

Oxidative stress injury is an important factor contributing to lung I/R injury, leading to the intracellular generation of reactive oxygen species (ROS) (Boros and Keppeler, 2019). The accelerated generation of ROS is a major cause of cell apoptosis and inflammation and exacerbates lung injury during the ischemia and reperfusion phase (Yang et al., 2019). Nuclear factor E2-related factor 2 (Nrf2), a redox-sensitive transcription factor, is commonly believed to play a key role in antioxidant processes (Meng et al., 2017). In our previous study, we found that Nrf2 exerted antioxidant effects against lung I/R injury in a rat lung transplantation model (Meng et al., 2017). Thus, the Nrf2 pathway may be a crucial mechanism in lung I/R injury.

Pulmonary surfactant consists of phospholipids and surfactant proteins (Yang et al., 2020). Pulmonary surfactant can stabilize the alveoli at the end of exhalation and prevent collapse by maintaining alveolar surface tension (Topercerova et al., 2019). Pulmonary surfactant can regulate pulmonary inflammation and cell apoptosis via IL-8 (Yang et al., 2020), and is affected by oxygen free radicals (Mathew and Sarada, 2018). Thus, pulmonary surfactant may play an important role in lung I/R injury. In addition, a study found that the Nrf2 pathway was closely related to changes in pulmonary surfactant expression in A549 cells (Mathew and Sarada, 2018). Thus, during lung I/R injury, Nrf2-mediated pulmonary surfactant regulation may play a role.

Methane (CH<sub>4</sub>), the simplest alkane and the most abundant organic gas in the atmosphere, is commonly used as a type of fuel (Liu et al., 2012). Although CH<sub>4</sub> is widely considered a biologically inactive gas, several researchers have focused on its biological characteristics, and several studies have demonstrated the therapeutic effects of CH<sub>4</sub> in many organs associated with I/R injury via anti-oxidant, anti-inflammatory, and anti-apoptotic properties (Li et al., 2017). However, the effects of CH<sub>4</sub> on pulmonary surfactant and the specific mechanism remain unclear. Therefore, this study aimed to observe the effects of CH<sub>4</sub> inhalation on pulmonary surfactant and lung injury induced by I/R in rats and to explore the potential role of the Nrf2 pathway.

## MATERIALS AND METHODS

### Animals

Adult male Wistar rats weighing 250–280 g were purchased from the Experiment Center of the Affiliated Hospital of Qingdao University. The animals were housed at cage feeding racks in a temperature-controlled room with free access to a standard diet and water for 1 week before the experiment. The Ethics Committee of the Affiliated Hospital of Qingdao University approved all experiments and procedures in this study (No. AHQU20180702).

### Lung I/R Injury Model

The rats were anesthetized by an intraperitoneal injection of sodium pentobarbital (60 mg/kg) and intubated with a tracheal

cannula. Then, the rats were ventilated with a tidal volume of 8 ml/kg at a respiratory rate of 50 breaths/min. The femoral artery and vein were cannulated for blood pressure monitoring and drug administration, respectively. After a left lateral thoracotomy, the left lung hilum was clamped with a non-clash microclip at the end of expiration 5 min after the administration of heparin (50 IU/animal). The tidal volume was reduced to 6 ml/kg during clamping. After a 90-min ischemic period, the microclip was removed for 180 min of reperfusion. Then, the tidal volume was adjusted to 8 ml/kg. During the experiment, sodium pentobarbital and rocuronium bromide were used to maintain anesthesia and muscle relaxation. The breath rate was adjusted to maintain an arterial carbon dioxide tension (PaCO<sub>2</sub>) of 35–45 mmHg, and the body temperature was maintained between 37.5 and 38.5°C by a heating blanket.

### Experimental Protocol

The rats were randomly divided into three groups ( $n = 6$ ): the sham group, the I/R control group, and the I/R CH<sub>4</sub> group. In the sham group, the rats underwent thoracotomy and were ventilated with 40% O<sub>2</sub> + 60% N<sub>2</sub> without ischemia and reperfusion. The rats in the I/R control group underwent I/R and were ventilated with 40% O<sub>2</sub> + 60% N<sub>2</sub>. The rats in the I/R CH<sub>4</sub> group underwent I/R and were ventilated with 2.5% CH<sub>4</sub> + 40% O<sub>2</sub> + 57.5% N<sub>2</sub> beginning 10 min before the end of the 90-min ischemic period until 60 min after the beginning of the 180-min reperfusion period. Then the rats in the I/R CH<sub>4</sub> group were ventilated with 40% O<sub>2</sub> + 60% N<sub>2</sub> (Figure 1).

### Blood Gas Analysis

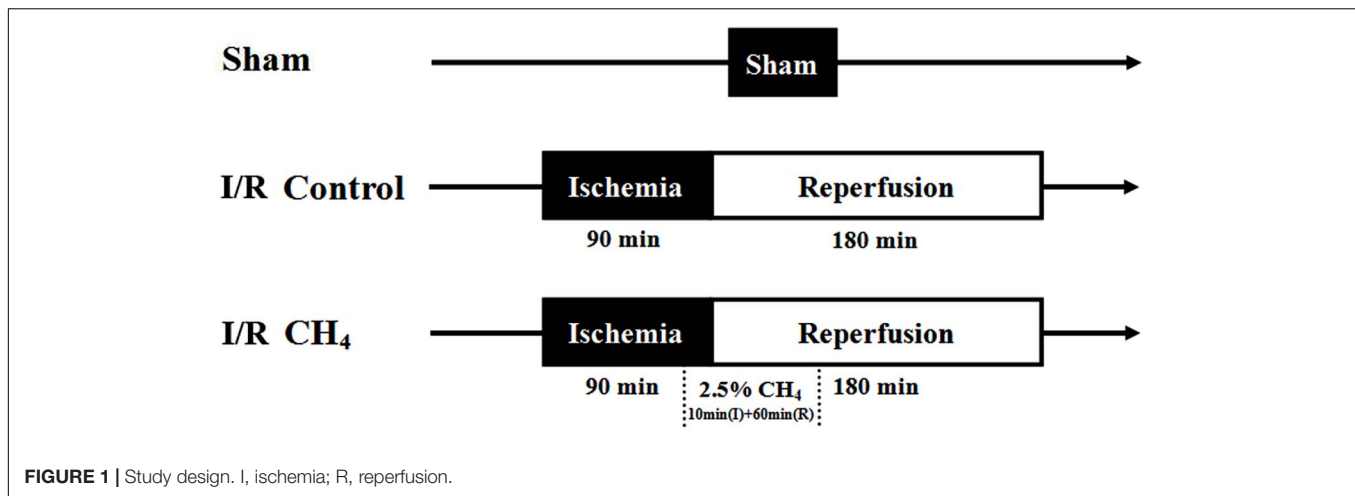
Arterial blood gas analysis was performed at baseline (3 min after ventilation), at 60 min after ischemia, and at 60, 120, and 180 min after reperfusion and were recorded as T0–T4, respectively. At the end of the experiment, blood from the left pulmonary vein was collected for blood gas analysis (Rapid Lab 248, Bayer, Medfield, United States).

### Determination of the Lung Static Pressure-Volume (P-V) Curve

After 180 min of reperfusion, the rats were euthanized via exsanguination. Median sternotomies were performed immediately, and both right and left lungs were isolated with a tracheal cannula. Then, the right lung hilum was ligated. The tracheal cannula was connected to an apparatus to measure the static P-V curve of the left lung. Airway pressure was increased to 30 cm H<sub>2</sub>O before being decreased to 0 cm H<sub>2</sub>O in stepwise intervals of 5 cm. After 1 min of stabilization, the lung volume was recorded through gas compression in the apparatus (Meng et al., 2016b).

### Detection of Pulmonary Surfactant

After the P-V curve determination, the left lung was infused with 10 ml of cold normal saline three times. Each wash was infused slowly, withdrawn gently, and re-infused after 1 min of stabilization three times (Li et al., 2018). The cumulative volume of bronchoalveolar lavage fluid (BALF) was collected, and 1 ml of



BALF was used to count the number of total cells and neutrophils. The rest of the BALF was centrifuged at 150 g for 10 min, and the supernatant was collected to measure phospholipids. The remaining sample was centrifuged at 40,000 g for 15 min to measure the large surfactant aggregates (LAs) and small surfactant aggregates (SAs). The phosphorus measurement was used to determine the level of phospholipids using a phosphate assay kit (Jiancheng Bio-Technology, Nanjing, China).

### Detection of Inflammatory Indices and Oxidative Stress Parameters

After 180 min of reperfusion, the lower lobe of the left lung was homogenized with cold normal saline to determine myeloperoxidase (MPO) activity using a reagent kit (Beyotime Biotechnology, Shanghai, China). The supernatants were collected to measure the interleukin (IL)-10, IL-1 $\beta$ , and tumor necrosis factor (TNF)- $\alpha$  by enzyme-linked immunosorbent assay kits (R&D Systems, Minneapolis, MN, United States), and the malondialdehyde (MDA) levels, superoxide dismutase (SOD) activity, and total antioxidant capacity (T-AOC) were measured by commercially available kits and a BCA kit (Beyotime Biotechnology, Shanghai, China). The BCA kit was used for total protein detection, which was used to calculate MDA, SOD, and T-AOC. The upper lobe of the left lung was dried at 80°C for 72 h to a constant weight to determine the wet-to-dry weight (W/D) ratio.

### Detection of Histopathology and Cell Apoptosis

The middle lobe of the left lung was used for hematoxylin-eosin (H&E) staining and terminal deoxynucleotidyl transferase dUTP nick end-labeling (TUNEL) staining (Zhongshan Golden Bridge Biotechnology, Beijing, China). The lung injury score (LIS) (Meng et al., 2016a) was evaluated by histopathology based on the following criteria: (1) neutrophil infiltration, (2) airway epithelial cell damage, (3) interstitial edema, (4) hyaline membrane formation, and (5) hemorrhage. Each criteria have five scores as follows: normal = 0, minimal change = 1, mild

change = 2, moderate change = 3, and severe change = 4. The apoptotic index (AI), a measure of the number of positive cells per 100 cells in five random fields of the same section, was evaluated by TUNEL staining (Meng et al., 2017). All sections were examined by a pathologist who was blinded to the study conditions.

### Western Blot Analysis

The lower lobe of the left lung was homogenized in lysis buffer, and the proteins were collected. For the detection of Nrf2 and NF- $\kappa$ B, the cytoplasmic component and nuclear component were isolated by treating a nuclear protein extraction kit (Beyotime Biotechnology, Shanghai, China) and centrifuged at 12,000 g for 10 min at 4°C. After the protein concentration was measured by a BCA kit, 50- $\mu$ g protein samples were separated by SDS-PAGE and transferred onto a PVDF membrane. The membrane was blocked in 5% non-fat dry milk, followed by incubation with pro-caspase-3 (1:8,000, Abcam, United States), cleaved-caspase-3 (1:500, Abcam, United States), pro-caspase-9 (1:800, Abcam, United States), cleaved-caspase-9 (1:1,000, Abcam, United States), Nrf2 (1:5,000, Abcam, United States), and NF- $\kappa$ B (1:3,000, Abcam, United States) primary antibodies overnight at 4°C. After being washed and incubated with secondary antibody (1:5,000, Zhongshan Golden Bridge Biotechnology, Beijing, China) at room temperature for 1 h, the proteins were visualized with the enhanced chemiluminescence reagent (GE Healthcare Bio-Sciences, Pittsburgh, PA, United States), analyzed using ImageJ version 1.61 software (National Institutes of Health, Bethesda, MD, United States) and normalized to  $\beta$ -actin and Lamin B.

### Statistical Analysis

All quantitative data are expressed as the mean values  $\pm$  standard deviations. Differences between groups were assessed by one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test. The repeated data were analyzed through repeated analysis of variance followed by Dunnett's test. The non-parametric method with the Kruskal-Wallis test was used for the analysis of LIS data. A value of  $P < 0.05$  was considered

statistically significant. The statistical analyses were performed with SPSS 20.0 software (SPSS, Chicago, IL).

## RESULTS

### Experiment-Related Data

All rats were operated on successfully in this study. After the microclip was removed, the chest was closed. Thus, the total time the chest was open was  $91.6 \pm 0.8$  min in the sham group,  $92.2 \pm 1.2$  min in the I/R control group, and  $92.5 \pm 1.0$  min in the I/R CH<sub>4</sub> group, and the differences were not statistically significant. Additionally, the mean arterial pressure (MAP) and heart rate (HR) were stable among the three groups. The baseline data in all the groups showed no significant differences.

### CH<sub>4</sub> Inhalation Improved Lung Oxygenation Functions

At the T0 time point, the oxygenation index [the partial pressure of arterial oxygen (PaO<sub>2</sub>)/fraction of inspired oxygen (FiO<sub>2</sub>)] was not significantly different among the three groups. At the T1 time point, the oxygenation index was lower in the I/R control and I/R CH<sub>4</sub> groups than in the sham group ( $P < 0.05$ ). Compared with that of the sham group, the oxygenation index decreased significantly in the I/R control group and was significantly higher in the I/R CH<sub>4</sub> group than in the control group at T2–T4 ( $P < 0.05$ ). The base excess (BE) and pH values exhibited similar trends as the oxygenation index (Table 1). Additionally, there was also a similar trend in the analysis of blood from the left pulmonary vein as the relative indices shown above (Table 2).

### CH<sub>4</sub> Inhalation Maintained Lung Tissue Structure

Compared with those of the sham group, more neutrophil infiltration, interstitial edema, and hemorrhage were observed in the I/R control group. Compared with those of the I/R control group, the neutrophil infiltration, interstitial edema, and

**TABLE 2 |** The indices of blood gas analysis from pulmonary vein (mean  $\pm$  SD,  $n = 6$ ).

	PvO <sub>2</sub> /FiO <sub>2</sub> (mmHg)	pH value	BE value (mmol/L)	PvCO <sub>2</sub> (mm Hg)
Sham group	246 $\pm$ 9	7.40 $\pm$ 0.02	0.07 $\pm$ 0.02	38.6 $\pm$ 1.6
I/R control group	131 $\pm$ 38*	7.17 $\pm$ 0.04*	-4.27 $\pm$ 0.54*	38.9 $\pm$ 1.2
I/R CH <sub>4</sub> group	188 $\pm$ 17*#	7.33 $\pm$ 0.04*#	-2.14 $\pm$ 0.32*#	38.0 $\pm$ 2.8

PvO<sub>2</sub>/FiO<sub>2</sub>, pulmonary venous oxygen tension (PvO<sub>2</sub>)/fraction of inspired oxygen (FiO<sub>2</sub>); BE, base excess. \* $P < 0.05$  vs. the sham group; # $P < 0.05$  vs. the I/R control group.

hemorrhage decreased in the I/R CH<sub>4</sub> group. Therefore, the neutrophil infiltration LIS in the I/R control group [3.5 (3–4)] was higher than that in the sham group [0.5 (0–1)] ( $P < 0.05$ ) and that in the I/R CH<sub>4</sub> group [2 (1–3)] was lower than that in the I/R control group ( $P < 0.05$ ). The LISs of the other criteria also exhibited similar trends (Figure 2).

### CH<sub>4</sub> Inhalation Regulated Phospholipid and Pulmonary Surfactant Proteins

Compared with those of the sham group, the phospholipid level ( $1.48 \pm 0.21$  mg/kg), LA level ( $0.51 \pm 0.07$  mg/kg), and LA percentage ( $0.55 \pm 0.06$ ) in BALF were decreased and the SA level ( $0.42 \pm 0.07$  mg/kg) was increased in the I/R control group ( $0.63 \pm 0.19$  mg/kg,  $0.31 \pm 0.09$  mg/kg,  $0.31 \pm 0.04$ ,  $0.68 \pm 0.13$  mg/kg) ( $P < 0.05$ ). Compared with those of I/R control group, the phospholipid level ( $1.16 \pm 0.24$  mg/kg), LA level ( $0.42 \pm 0.06$  mg/kg), and LA percentage ( $0.43 \pm 0.04$ ) were increased and the SA level ( $0.55 \pm 0.10$  mg/kg) was decreased in the I/R CH<sub>4</sub> group ( $P < 0.05$ ) (Figure 3).

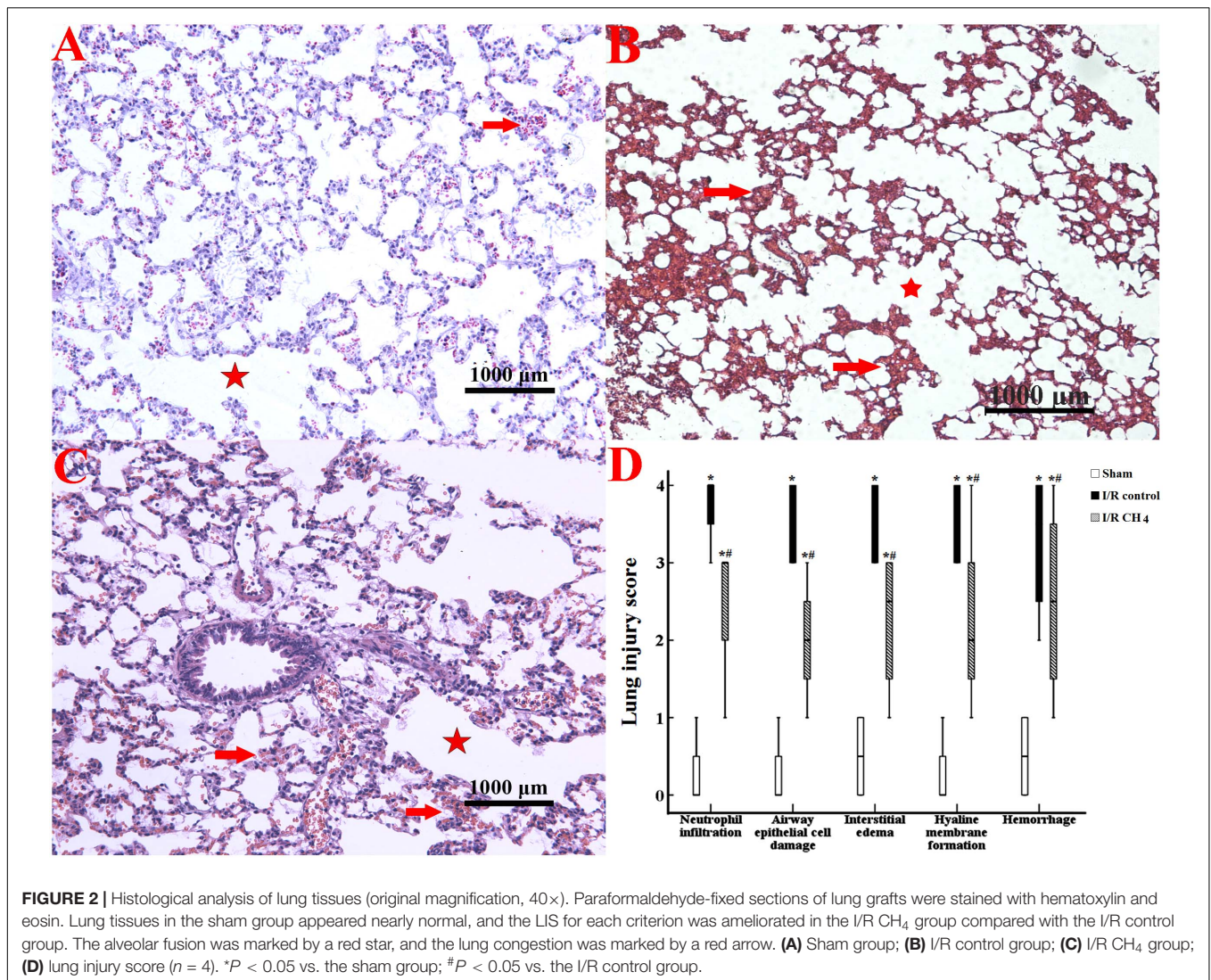
### CH<sub>4</sub> Inhalation Improved Lung Compliance

The P-V curve value of the I/R control group was significantly lower than that of the sham group, and the value of the I/R CH<sub>4</sub> group was markedly higher than that of the I/R control

**TABLE 1 |** The indices of blood gas analysis (mean  $\pm$  SD,  $n = 6$ ).

	Group	T0	T1	T2	T3	T4
PaO <sub>2</sub> /FiO <sub>2</sub> (mm Hg)	Sham group	241 $\pm$ 5	244 $\pm$ 6	242 $\pm$ 4	239 $\pm$ 4	245 $\pm$ 7
	I/R control group	242 $\pm$ 7	219 $\pm$ 14*	199 $\pm$ 11*	176 $\pm$ 18*	151 $\pm$ 14*
	I/R CH <sub>4</sub> group	245 $\pm$ 5	222 $\pm$ 15*	217 $\pm$ 15*#	211 $\pm$ 13*#	201 $\pm$ 11*#
pH value	Sham group	7.39 $\pm$ 0.02	7.39 $\pm$ 0.02	7.39 $\pm$ 0.02	7.40 $\pm$ 0.01	7.40 $\pm$ 0.01
	I/R control group	7.40 $\pm$ 0.02	7.35 $\pm$ 0.03*	7.30 $\pm$ 0.03*	7.26 $\pm$ 0.01*	7.23 $\pm$ 0.03*
	I/R CH <sub>4</sub> group	7.39 $\pm$ 0.01	7.35 $\pm$ 0.02*	7.33 $\pm$ 0.03*#	7.32 $\pm$ 0.02*#	7.31 $\pm$ 0.03*#
BE value (mmol/L)	Sham group	0.07 $\pm$ 0.02	0.08 $\pm$ 0.03	0.08 $\pm$ 0.04	0.08 $\pm$ 0.04	0.08 $\pm$ 0.03
	I/R control group	0.07 $\pm$ 0.04	-1.45 $\pm$ 0.25*	-3.07 $\pm$ 0.09*	-4.06 $\pm$ 0.30*	-4.50 $\pm$ 0.20*
	I/R CH <sub>4</sub> group	0.08 $\pm$ 0.26	-1.46 $\pm$ 0.14*	-2.01 $\pm$ 0.11*#	-2.39 $\pm$ 0.12*#	-2.49 $\pm$ 0.21*#
PaCO <sub>2</sub> (mm Hg)	Sham group	38.8 $\pm$ 1.8	38.9 $\pm$ 2.1	39.2 $\pm$ 1.8	38.9 $\pm$ 1.8	39.0 $\pm$ 1.7
	I/R control group	40.1 $\pm$ 1.2	38.1 $\pm$ 3.0	39.4 $\pm$ 2.2	38.4 $\pm$ 2.7	39.2 $\pm$ 1.6
	I/R CH <sub>4</sub> group	39.2 $\pm$ 1.7	38.2 $\pm$ 1.4	39.1 $\pm$ 1.3	38.7 $\pm$ 1.0	38.1 $\pm$ 1.3

T0–T4 represented the following time points: the baseline, 60 min after ischemia, and 60, 120, and 180 min after reperfusion. PaO<sub>2</sub>/FiO<sub>2</sub>, partial pressure of arterial oxygen (PaO<sub>2</sub>)/fraction of inspired oxygen (FiO<sub>2</sub>); BE, base excess; PaCO<sub>2</sub>, arterial carbon dioxide tension. \* $P < 0.05$  vs. the sham group; # $P < 0.05$  vs. the I/R control group.



group ( $P < 0.05$ ). At a pressure of 30 cm H<sub>2</sub>O, the values in the sham, I/R control, and I/R CH<sub>4</sub> groups were  $18.3 \pm 1.0$  ml/kg,  $11.5 \pm 1.3$  ml/kg, and  $16.8 \pm 1.0$  ml/kg, respectively (Figure 4).

### CH<sub>4</sub> Inhalation Decreased the Lung Inflammatory Response

The W/D ratio in the I/R control group was significantly higher than that in the sham group, and the W/D ratio in the I/R CH<sub>4</sub> group was higher than that in the I/R control group ( $P < 0.05$ ). The total number of cells and neutrophils in BALF exhibited the same trends as the W/D ratio. Additionally, the MPO activity and the levels of IL-1 $\beta$  and TNF- $\alpha$  in the I/R control group increased significantly compared with those of the sham group, and the MPO activity and levels of IL-1 $\beta$  and TNF- $\alpha$  in the I/R CH<sub>4</sub> group decreased significantly compared with those of the I/R control group ( $P < 0.05$ ). IL-10 exhibited the opposite trend as TNF- $\alpha$  (Table 3). Additionally, compared with the sham group, the nucleus NF- $\kappa$ B protein expression in the I/R control group increased significantly, and that of the I/R CH<sub>4</sub> group decreased

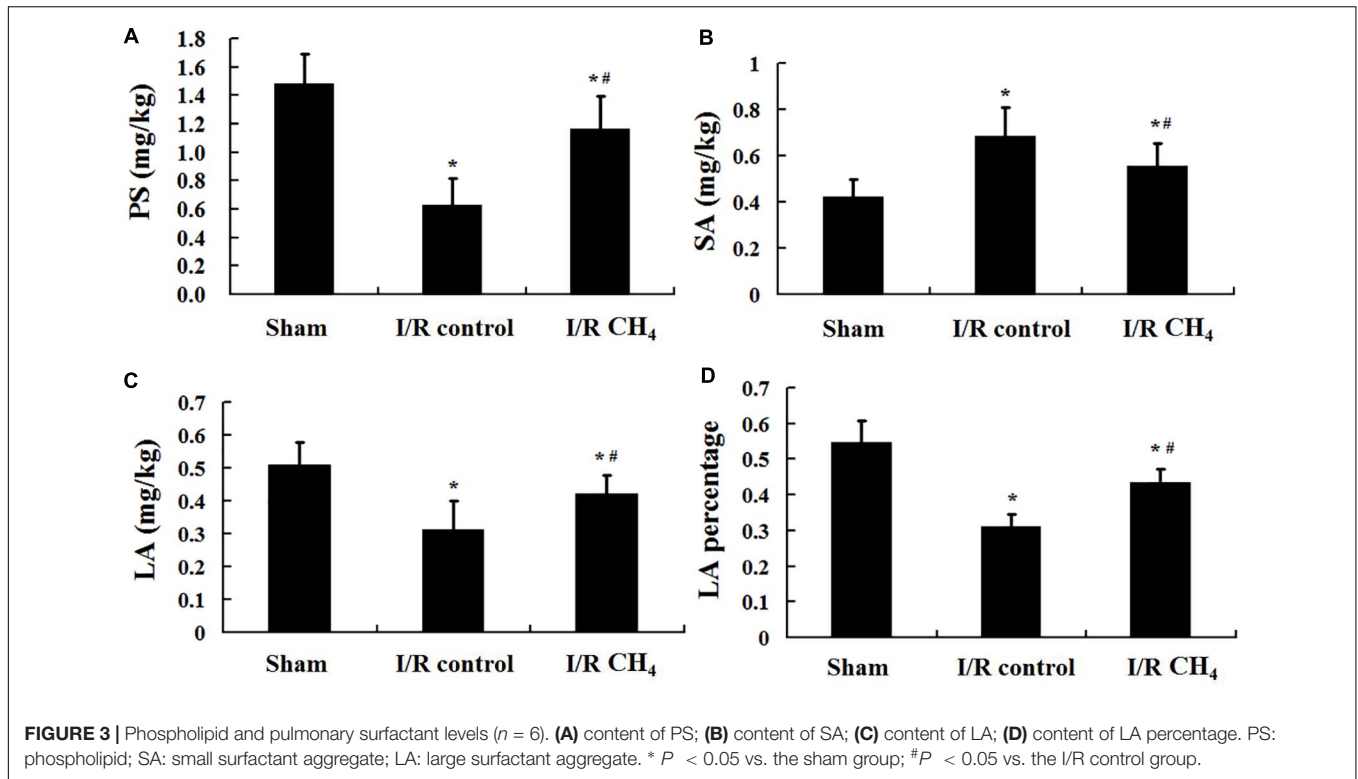
significantly compared with the I/R control group ( $P < 0.05$ ). NF- $\kappa$ B protein expression in the cytosol showed a trend that was contrary to that in the nucleus (Figure 5).

### CH<sub>4</sub> Inhalation Decreased Lung Oxidative Stress Injury

In the I/R control group, the MDA level was significantly higher and the SOD and T-AOC activities were significantly lower than those in the sham group ( $P < 0.05$ ). Compared with those of the I/R control group, the MDA level decreased and the SOD and T-AOC activities increased significantly in the I/R CH<sub>4</sub> group ( $P < 0.05$ ) (Table 4).

### CH<sub>4</sub> Inhalation Decreased Apoptosis in Lung Tissue

Compared with that of the sham group, the number of TUNEL-positive cells in the I/R control group increased significantly and that of the I/R CH<sub>4</sub> group decreased significantly ( $P < 0.05$ ).



Therefore, the AI in the I/R control group ( $44.6 \pm 9.4$ ) was significantly higher than that of the sham group ( $6.4 \pm 2.2$ ), and the AI in the I/R CH<sub>4</sub> group ( $17.4 \pm 3.3$ ) was lower than that of the I/R control group ( $P < 0.05$ ) (Figure 6). The protein expression of cleaved-caspase-3 and cleaved-caspase-9 in the I/R control group were significant higher than that of the sham group, and that of the I/R CH<sub>4</sub> group decreased significantly

( $P < 0.05$ ). Converse trends were observed in the protein expression of pro-caspase-3 and pro-caspase-9 (Figure 7).

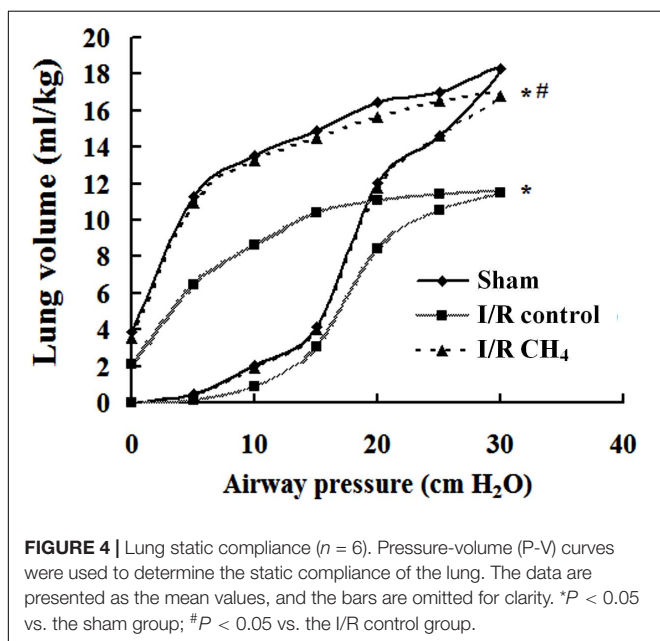
### CH<sub>4</sub> Inhalation Activated the Nrf2 Pathway

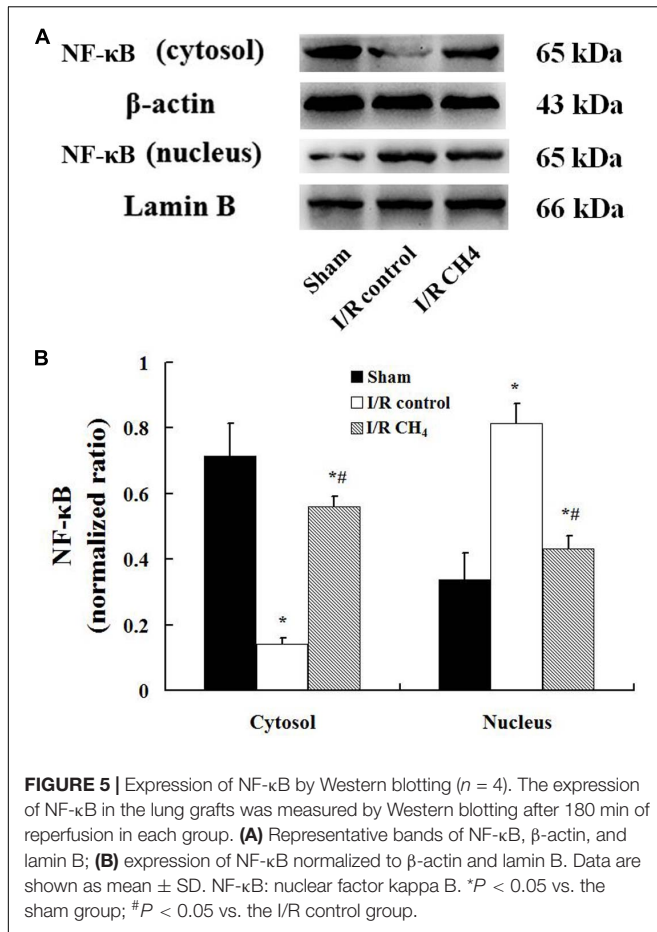
Compared with that of the sham and I/R control groups, the nucleus Nrf2 protein expression in the I/R CH<sub>4</sub> group increased significantly ( $P < 0.05$ ). Additionally, Nrf2 protein expression in the cytosol showed a trend that was contrary to that in the nucleus (Figure 8).

### DISCUSSION

CH<sub>4</sub> is a non-toxic gas that can be produced by bacteria within the human body. CH<sub>4</sub>, similar to nitric oxide, carbon monoxide, and hydrogen sulfide, also plays an important role in the biological systems (Poles et al., 2019). Studies have shown that CH<sub>4</sub> can penetrate membranes and diffuse into the organelles, including the nucleus and mitochondria (Poles et al., 2019). In 2012, Boros et al. (2012) found that CH<sub>4</sub> inhalation decreased intestinal I/R injury. More recently, CH<sub>4</sub> was shown to exert protective effects in the liver, spinal, and lung I/R injury (Ye et al., 2015; Wang et al., 2017, 2020). These results suggest that CH<sub>4</sub> may be a good therapeutic agent.

In this study, CH<sub>4</sub> inhalation decreased MPO activity, the levels of IL-1 $\beta$  and TNF- $\alpha$ , MDA activity, NF- $\kappa$ B protein expression, and cell apoptosis, increased IL-10 levels and SOD and T-AOC activities, maintained lung tissue structure and pulmonary surfactant, and ameliorated oxygenation function and





lung compliance in rats with lung I/R injury. Additionally, CH<sub>4</sub> inhalation also up-regulated the protein expression of Nrf2.

Pulmonary surfactant is synthesized in the endoplasmic reticulum of type II alveolar epithelial cells. Phospholipids play a major role in reducing the alveolar gas-liquid surface tension. The surfactant proteins can be divided into active LAs and inactive SAs according to density, including SP-A, SP-B, and SP-C, which are mainly LAs, and SP-D, which is mainly in SAs. A previous study indicated that LAs were the metabolic precursors of SAs (Baritussio et al., 1984), and the composition and metabolism of pulmonary surfactant were recognized as important factors in lung I/R injury (Novick et al., 1996). In this study, CH<sub>4</sub> inhalation inhibited the decrease in phospholipid level, LA level, and LA percentage induced by I/R, slowed the

**TABLE 4 |** Oxidative stress indices (mean  $\pm$  SD,  $n = 6$ ).

	SOD (U/mg protein)	MDA (nmol/mg protein)	T-AOC (U/mg protein)
Sham group	92.8 $\pm$ 15.3	3.46 $\pm$ 0.36	2.13 $\pm$ 0.24
I/R control group	40.1 $\pm$ 8.6*	9.37 $\pm$ 1.42*	0.94 $\pm$ 0.18*
I/R CH <sub>4</sub> group	74.8 $\pm$ 15.3*#	4.83 $\pm$ 1.21*#	1.51 $\pm$ 0.44*#

SOD, superoxide dismutase; MDA, malonaldehyde; T-AOC, total antioxidative capabilities. \* $P < 0.05$  vs. the sham group; # $P < 0.05$  vs. the I/R control group.

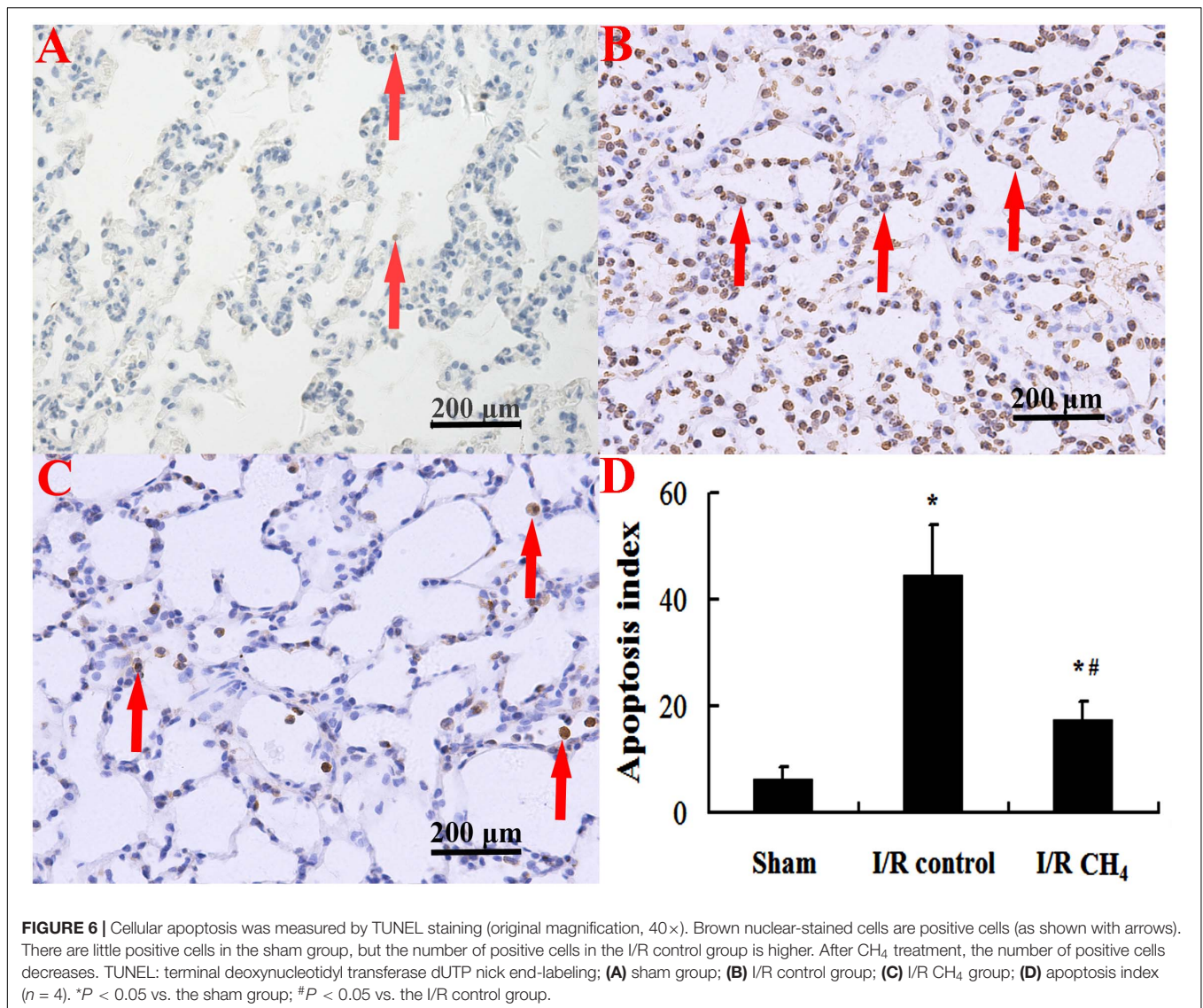
conversion of LA to SA, and improved pulmonary surfactant, lung compliance, lung oxygenation function, and lung tissue structure. This finding also demonstrated that CH<sub>4</sub> had practical effects on improving pulmonary surfactant. In 2002, Maitra et al. (2002) found that the rapid conversion of LA to SA occurred in acute lung injury induced by hyperventilation and lung I/R injury induced by transplantation in dogs. In 2004, Meng et al. (2004) demonstrated that exogenous pulmonary surfactant protected against lung I/R injury in isolated rat lungs and maintained the lung tissue structure. In 2019, decreased levels of phosphatidylcholine and total phospholipids were found in lung injury induced by intestinal I/R in rats (Zhao et al., 2019). These results were similar to those of our study. However, the mechanisms by which CH<sub>4</sub> protects of pulmonary surfactant are still unclear.

Inflammation is recognized as a crucial component of lung I/R injury (den Hengst et al., 2010). It has been proven that CH<sub>4</sub> can alter the inflammatory balance during inflammatory pathology (Boros et al., 2015). TNF- $\alpha$  is a powerful inflammatory mediator that can increase the permeability of alveolar capillaries and exacerbate lung edema (Bai et al., 2013). IL-1 $\beta$  is also a potential mediator that induces the inflammatory response and has similar effects as those of TNF- $\alpha$  (Wu et al., 2013). IL-10 is an anti-inflammatory mediator that inhibits neutrophil activation and the release of pro-inflammatory cytokines (Rojas et al., 2017). In this study, CH<sub>4</sub> inhalation decreased the inflammatory response induced by I/R, as demonstrated by the decreased NF- $\kappa$ B, TNF- $\alpha$ , IL-1 $\beta$ , and MPO levels, and the number of total cells and neutrophils in BALF, increased levels of IL-10, and attenuated lung edema. Many studies had similar results. In models of hepatic I/R injury, spinal cord I/R injury, and myocardial ischemia injury in rats, CH<sub>4</sub> exerted powerful anti-inflammatory effects (Ye et al., 2015; Chen et al., 2016; Wang et al., 2017). Additionally, in lung tissue, the loss of pulmonary surfactant leads to exacerbation of lipopolysaccharide (LPS)-mediated lung inflammation (Arroyo et al., 2019). In 2010,

**TABLE 3 |** W/D ratio and inflammatory mediators (mean  $\pm$  SD,  $n = 6$ ).

	W/D ratio	Cells in BALF (10 <sup>6</sup> /l)	Neutrophils in BALF (%)	MPO (U/g)	IL-1 $\beta$ (pg/mg)	TNF- $\alpha$ (pg/mg)	IL-10 (pg/mg)
Sham group	3.4 $\pm$ 0.2	54.0 $\pm$ 7.9	10.0 $\pm$ 1.1	0.43 $\pm$ 0.23	98.4 $\pm$ 8.6	299.9 $\pm$ 58.5	46.8 $\pm$ 16.9
I/R control group	5.8 $\pm$ 0.7*	199.3 $\pm$ 9.8*	67.4 $\pm$ 7.5*	1.55 $\pm$ 0.38*	334.6 $\pm$ 28.0*	579.5 $\pm$ 54.5*	92.4 $\pm$ 14.1*
I/R CH <sub>4</sub> group	4.4 $\pm$ 0.5*#	166.5 $\pm$ 9.3*#	46.3 $\pm$ 5.4*#	0.86 $\pm$ 0.22*#	267.7 $\pm$ 16.2*#	420.6 $\pm$ 48.7*#	140.0 $\pm$ 27.1*#

W/D ratio, wet weight (W)/dry weight (D) ratio; BALF, bronchoalveolar lavage fluid; MPO, myeloperoxidase; IL-1 $\beta$ , interleukin-1 $\beta$ ; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ . \* $P < 0.05$  vs. the sham group; # $P < 0.05$  vs. the I/R control group.

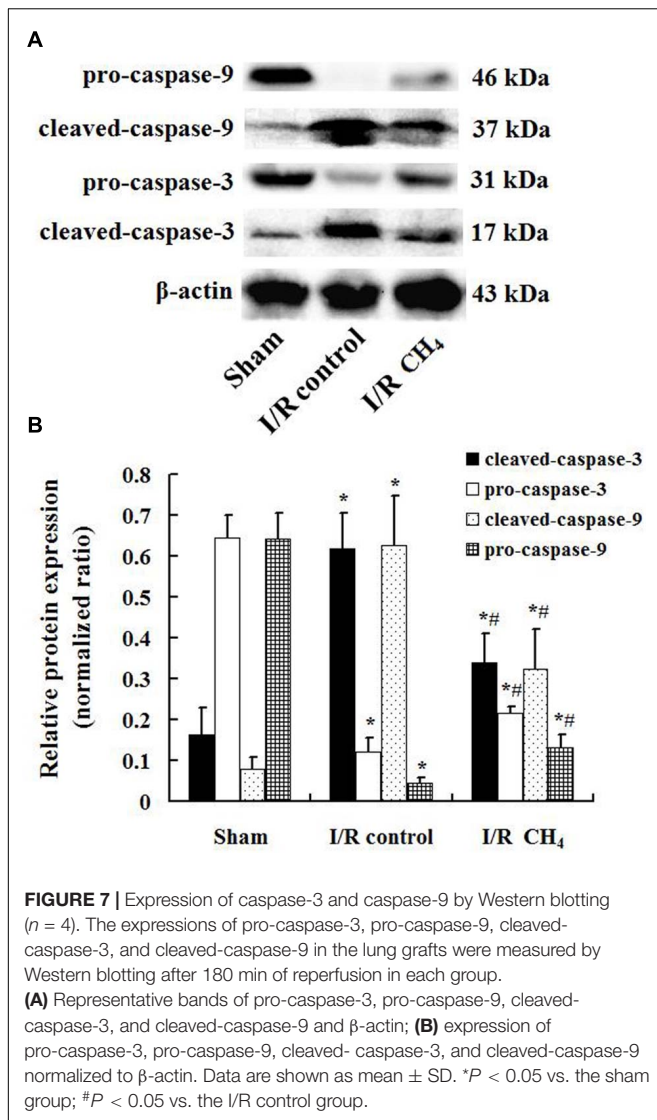


Goto et al. (2010) showed that SP-A decreased the inflammatory response induced by bleomycin-induced acute lung injury via inhibiting inflammatory cytokines, such as TNF- $\alpha$  and IL-1 $\beta$ . Ingenito et al. (2001) showed that the down-regulation of SP-B led to pulmonary dysfunction in a murine model of acute lung injury by regulating inflammatory cytokines, such as TNF- $\alpha$  and IL-1 $\beta$ . In 2020, Yang et al. (2020) showed that inflammatory cytokines such as IL-8 also inhibited SP-A and SP-B and eventually led to lung injury in LPS-induced normal A549 cells. Therefore, CH<sub>4</sub> may affect the inflammatory response in lung I/R injury by regulating pulmonary surfactant.

Oxidative stress injury is the initial mechanism of I/R injury (Lalkovičová and Danielisová, 2016). MDA is one of the most important products of membrane lipid peroxidation, and the degree of membrane system damage can be estimated by the MDA level. SOD is an anti-oxidative metal enzyme that plays an important role in the balance of oxidation and anti-oxidation. T-AOC is an indicator of the total antioxidant level.

In this study, the anti-oxidative indices, including SOD and T-AOC, increased under CH<sub>4</sub> treatment, and the oxidative index MDA decreased. The antioxidant effect of CH<sub>4</sub> has been demonstrated in many studies. Liu et al. (2016) reported that CH<sub>4</sub> attenuated retinal I/R injury by reducing the level of MDA and increasing the level of SOD. The anti-oxidative effect of CH<sub>4</sub> was also observed in the hepatic I/R injury, spinal cord I/R injury, and myocardial ischemia injury, as shown by the decreased MDA and increased SOD (Ye et al., 2015; Chen et al., 2016; Wang et al., 2017). In addition, pulmonary surfactant could also influence the oxidative stress injury. Mathew and Sarada (2018) reported that the composition of pulmonary surfactant changed and returned to homeostatic levels in hypoxic A549 cells, which was related to reinforcing the induction of phase II antioxidant enzymes. Bezerra et al. (2019) found that exogenous pulmonary surfactant attenuated hyperoxia-induced mouse lung injury by decreasing MDA and increasing SOD. Therefore, the anti-oxidative effects of





CH<sub>4</sub> on lung I/R injury may be related to the regulation of pulmonary surfactant.

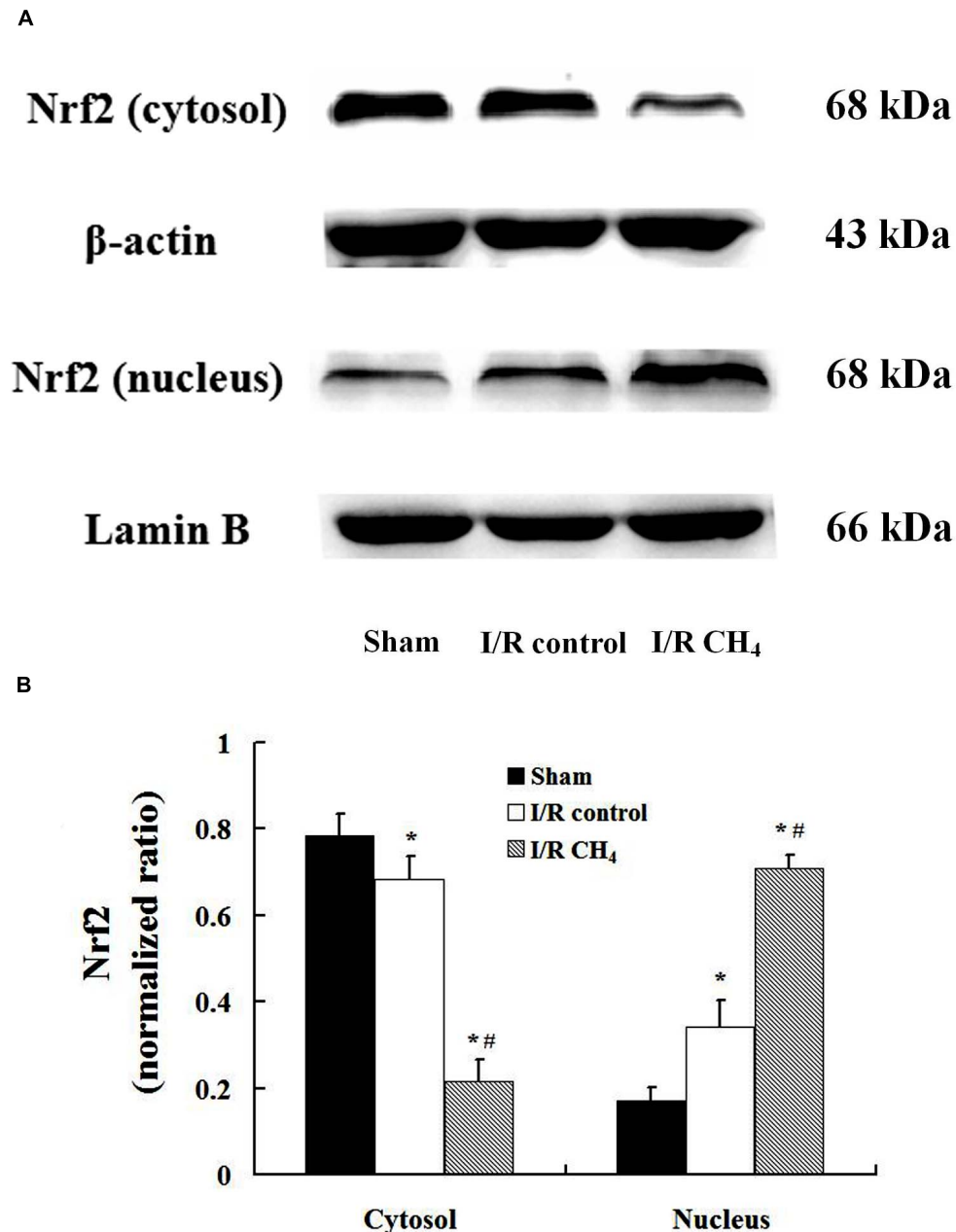
Eventually, the accumulation of inflammatory injury, DNA oxidation, and lipid peroxidation leads to cell apoptosis or death (Aslan et al., 2008). Caspase-9 is an initiator caspase that can be stimulated by many factors and causes a caspase cascade. Caspase-3 is a kind of executioner caspase. The cleaved-caspase-3 and cleaved-caspase-9 are the activated forms. In this study, CH<sub>4</sub> inhalation decreased the number of TUNEL-positive cells and the protein expression of cleaved-caspase-3 and cleaved-caspase-9. Thus, CH<sub>4</sub> showed an anti-apoptotic effect in this study, which has also been observed in previous studies. Song et al. (2015) found that CH<sub>4</sub> decreased the number of TUNEL-positive cells and caspase-3 activity in I/R injury in abdominal skin flaps in rats. In the study by Chen et al. (2016), CH<sub>4</sub> inhibited the increase in TUNEL-positive cells and caspase-3 and caspase-9 protein expression induced by myocardial ischemia. The cleaved-caspase-3 and cleaved-caspase-9 activities

were decreased by CH<sub>4</sub> treatment in spinal cord I/R injury model (Wang et al., 2017). In addition, a strong correlation exists between pulmonary surfactant and apoptosis. Goto et al. (2010) found that TUNEL-positive cells and caspase-3 activity increased a model of bleomycin-induced acute lung injury in SP-A<sup>-/-</sup> mice, and exogenous SP-A inhibited these effects. Yang et al. (2020) showed that silencing SP-A and SP-B resulted in apoptosis by increasing caspase-3 and caspase-9 protein expression in LPS-induced A549 cells. Therefore, CH<sub>4</sub>-mediated inhibition of apoptosis may occur directly through changes of pulmonary surfactant or indirectly through the anti-inflammatory response and anti-oxidative effects.

Presently, the mechanism of CH<sub>4</sub> is still unclear. Zhang et al. (2017) found that CH<sub>4</sub> protected against cerebral I/R injury in rats via a PI3K/Akt/HO-1-dependent anti-oxidative pathway. CH<sub>4</sub> was also shown to protect against liver injury induced by carbon tetrachloride in mice via PI3K/Akt/GSK-3 $\beta$ -mediated anti-inflammatory effects (Yao et al., 2017). Wang et al. (2019) showed that the NF- $\kappa$ B and MAPK pathways are also involved in the protective effects of CH<sub>4</sub> in a mouse model of acetic acid-induced ulcerative colitis. In this study, CH<sub>4</sub> inhalation activated the Nrf2 pathway and protected against lung I/R injury. Wang et al. (2017) also showed that the Nrf2 pathway mediated CH<sub>4</sub>-induced protective effects on spinal cord I/R injury in rats. A previous study reported that Nrf2 not only triggered antioxidants and maintained the balance of lung oxidants and antioxidants, but also altered the production of pulmonary surfactant proteins and maintained surfactant homeostasis (Mathew and Sarada, 2018), which was similar to the results of this study. Therefore, CH<sub>4</sub> inhalation could protect against lung I/R injury via Nrf2-mediated protection of pulmonary surfactant.

In 2020, Wang et al. (2020) reported that intraperitoneal injection of methane-rich saline decreased lung I/R injury in rats and proved the importance of the PI3K-AKT-NF $\kappa$ B signaling pathway in the protective effects of CH<sub>4</sub>. Compared with those of Wang' study (Wang et al., 2020), a different method of CH<sub>4</sub> administration, inhalation, was chosen in this study. Although methane-rich saline is considered safer, inhalation is a more convenient route of administration and acts on the target organ (lung) directly. CH<sub>4</sub> is flammable in a narrow range of concentrations (5–15%) in air (Liu et al., 2012); thus, controlling methane concentration and careful monitoring can completely avoid the risk of explosion. Therefore, the method of CH<sub>4</sub> inhalation is very important and cannot be ignored. Additionally, in this study, we further observed the effect of CH<sub>4</sub> inhalation on pulmonary surfactant, and the results suggest that CH<sub>4</sub> may alleviate lung I/R injury through Nrf2-mediated pulmonary surfactant protection.

This study also has some limitations. First, only 3 h following reperfusion was observed, and a longer period of time may be required to observe the long-term effects of CH<sub>4</sub> inhalation on lung I/R injury. Second, only one CH<sub>4</sub> concentration was used in this study. Different CH<sub>4</sub> concentrations may show different effects, and the optimal CH<sub>4</sub> concentration should be evaluated. Third, different components of pulmonary



**FIGURE 8 |** Expression of Nrf2 by Western blotting ( $n = 4$ ). The expression of Nrf2 in the lung grafts was measured by Western blotting after 180 min of reperfusion in each group. **(A)** Representative bands of Nrf2,  $\beta$ -actin, and lamin B; **(B)** expression of Nrf2 normalized to  $\beta$ -actin and lamin B. Data are shown as mean  $\pm$  SD. Nrf2: nuclear factor E2-related factor. \* $P < 0.05$  vs. the sham group; # $P < 0.05$  vs. the I/R control group.

SP-A, SP-B, SP-C, and SP-D, should be measured so that the effect of CH<sub>4</sub> on pulmonary surfactant would be more specific. Then, the Nrf2 is knocked down/out genetically or by using specific pharmacological inhibitor would further verify the causative role of Nrf2 and this will be studies in the future research. Finally, the CH<sub>4</sub> concentration in blood was not monitored during inhalation. However, the rats did not have any adverse reactions during the experiment. Previous studies also demonstrated that CH<sub>4</sub> inhalation had

no adverse reactions in pigs and rats, and CH<sub>4</sub> in the blood showed rapid metabolism (Mészáros et al., 2017; Bari et al., 2019).

## CONCLUSION

Our results demonstrated that CH<sub>4</sub> inhalation can exert protective effects on lung I/R injury in rats by decreasing

the inflammatory response, inhibiting oxidative stress injury, and attenuating cell apoptosis, which is related to Nrf2-mediated pulmonary surfactant protection. Due to its non-toxic characteristics and membrane permeability, CH<sub>4</sub> will be a new therapeutic gas.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

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## ETHICS STATEMENT

The animal study was reviewed and approved by the Ethics Committee of the Affiliated Hospital of Qingdao University.

## AUTHOR CONTRIBUTIONS

BZ, XT, and CM designed the experiments. BZ, XT, GL, and HZ performed the experiments. CM and XW analyzed the data. YY and JY contributed to the reagents. BZ and CM wrote the manuscript. All authors contributed to the article and approved the submitted version.

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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