

Modulatory effect of silymarin on pulmonary vascular dysfunction through HIF-1 α -iNOS following rat lung ischemia-reperfusion injury

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Abstract. Silymarin is a traditional therapeutic used to protect the liver, acting to oppose lipid peroxidation, to enhance liver regeneration and functioning as an antioxidant. However, the effects of silymarin on pulmonary vascular dysfunction have not been investigated. In the present study, the modulatory effects of silymarin on pulmonary vascular dysfunction and the underlying mechanisms behind this were investigated in a lung ischemia-reperfusion (I/R) injury rat model. Male Sprague Dawley rats were randomly divided into 3 groups, including: i) A control group (n=10); ii) an I/R group (n=10); and iii) a silymarin-treated group (n=10). All experimental rats received 250 mg/kg/day of silymarin for 8 days. Silymarin was demonstrated to markedly improve lung I/R-induced pulmonary vascular dysfunction and lung moisture. Following silymarin treatment, inflammation and oxidative stress in the lung I/R-injury rats were demonstrably suppressed. Treatment with silymarin also inhibited the activation of caspase-3 and -9, and hypoxia inducible factor-1 α (HIF-1 α) and inducible nitric oxide synthase (iNOS) protein expression in the lung I/R-injury rats. Silymarin was concluded to impact upon pulmonary vascular dysfunction through the HIF-1 α -iNOS pathway in the lung I/R injury rat model.

Introduction

Lung ischemic-reperfusion (I/R) injury typically occurs as a result of lung transplantation, pulmonary thromboembolism

resection, pulmonary embolism following thrombolysis treatment and numerous other types of extracorporeal circulation cardiac surgery; I/R is an early post-operative complication, and an important cause of mortality (1). Lung transplantation is the last-resort treatment option for patients with end-stage lung disease, but following lung transplantation, the patient 1-year survival rate is only ~70%; patients tend to succumb to post-operative mortality within 30 days of surgery (2). Graft failure following transplantation can cause lung I/R injury, including injury to the pulmonary vascular endothelium, resulting in marked lung damage (3).

The complex etiology of pulmonary vascular dysfunction has not been fully elucidated; previous studies have associated this disease with oxidative damage caused by oxygen free radicals, calcium overload and neutrophil recruitment caused by excessive inflammation. These physiological effects may produce a greater number of oxygen free radicals than are removed, causing an exacerbation of lung I/R injury (4). A previous study associated hypoxia inducible factor-1 α (HIF-1 α) and inducible nitric oxide synthase (iNOS) with pulmonary hypertension; furthermore, structural damage to the pulmonary vascular endothelium was also considered to be an initial contributor to pulmonary vascular dysfunction (5).

Silymarin is an active element extracted from *Silybum marianum*, by 4 isomers of the original ketone group dihydrogen color, including *Silybum marianum*, has the main pharmacological effects (6). Silymarin can eliminate reactive oxygen species, protect against lipid peroxidation, maintain cell membrane fluidity, protect liver cell membranes and promote liver cell repair and regeneration, which all contribute to combat liver fibrosis (7). The present study aimed to investigate the effects of silymarin on pulmonary vascular dysfunction in a lung I/R injury rat model and assess its underlying molecular mechanism of action.

Materials and methods

Animals. All experimental procedures conformed to the Guide for the Care and Use of Laboratory Animals (Shandong University, Shandong, China) and were approved by the Institutional Ethics Committee of Shandong University. Male

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Sprague Dawley (SD) rats (180-220 g) were obtained from the Laboratory Animal Center of Shandong University, and were housed in units maintained at appropriate environmental conditions, with access to food and water *ad libitum*.

Lung I/R injury. SD rats were administered 0.1 mg/kg/min of pentobarbital by intraperitoneal injection. Following endotracheal intubation, mechanical ventilation with 100% oxygen was applied, with a tidal volume of 10 ml/kg (70 breaths/min). A midline thoracotomy was performed under sterile conditions, the pericardium was opened, and then the left pulmonary artery was ligated using an 8-0 prolene suture and was secured with polyethylene tubing without damaging the vessel. After 1 h of ischemia, the left pulmonary artery occlusion was cleared and the artery was reperfused with blood. The chest cavity was then closed, and SD rats were restored to normal respiration. Following 24 h of reperfusion, the rats were sacrificed by decollation following anesthetization.

Experimental design. SD rats were randomly divided into 3 groups, as follows: i) Control group rats (n=10), administered saline solution; ii) I/R group rats (n=10), receiving the aforementioned lung I/R injury and administered saline solution; iii) silymarin group rats (n=10), receiving lung I/R injury and administered 250 mg/kg/day of silymarin (Sigma-Aldrich, St. Louis, MO, USA) for 8 days (8).

Vascular reactivity. Vascular reactivity was recorded with a myograph, as previously described (9). Briefly, the pulmonary arteries were dissected from the rats and the connective tissue was removed. The pulmonary arteries and surrounding endothelium were maintained in Krebs solution and a myograph was used to estimate the tension in relation to internal circumference. The functional integrity of the pulmonary artery tissue segments was confirmed using 60 mmol/l KCl after 60 min of stabilization. The endothelium function was evaluated by measuring the relaxant response of segments that had been contracted with 1 μ mol/l phenylephrine (Wuhan Xinxin beauty Biotechnology, Co., Ltd., Wuhan, China) to 1 μ mol/l acetylcholine (Wuhan Dong Kangyuan Technology, Co., Ltd., Wuhan, China).

Mass calculations. Lung tissue samples were weighed to calculate wet mass; they were then placed in a drying oven at 80°C for 12-36 h and the corresponding dry weight was also recorded. Lung moisture was calculated as the wet/dry weight ratio.

Western blotting. Following treatment with silymarin, lung tissue samples were homogenized with radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology, Jiangsu, China). The homogenized samples were centrifuged at 1,200 x g for 10 min at 4°C. The total protein in each supernatant was quantified using a bicinchoninic acid assay (Beyotime Institute of Biotechnology). Equal protein was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes at 100 V for 1-2 h. The membranes were blocked with phosphate-buffered saline containing 5% skimmed milk powder for 1-2 h at room temperature to prevent non-specific binding. The membranes

were incubated with primary antibodies against HIF-1 α (1:1,000; C-19; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), iNOS (1:1,000; sc-49058; Santa Cruz Biotechnology, Inc.) and β -actin (1:500; D110007; Sangon Biotech Co. Ltd., Shanghai, China) overnight at 4°C. The membranes were incubated with secondary antibodies (1:5,000, BestBio Inc, Shanghai, China) for 2-3 h, then developed using chemiluminescence detection (BestBio Inc.). Relative band density was measured using Multi-Analyst software (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Inflammatory cytokine levels. The supernatant from centrifuged, homogenized lung tissue samples was acquired and the serum levels of nuclear factor- κ B (NF- κ B), tumor necrosis factor- α (TNF- α), interleukin (IL)-1 β and IL-6 were analyzed using ELISA kits (KF008, R019 and H002, respectively; Nanjing Jiancheng Bioengineering Institute, Nanjing, China), according to the manufacturer's protocols.

Oxidative stress. The supernatant from centrifuged, homogenized lung tissue samples was acquired and the serum superoxide dismutase (SOD) and malondialdehyde (MDA) levels were analyzed using ELISA kits (A001-3 and A003-1, respectively; Nanjing Jiancheng Bioengineering Institute), according to the manufacturer's protocols.

Caspase-9 and caspase-3 levels. The supernatant from centrifuged, homogenized lung tissue samples was acquired and the total protein was quantified using the bicinchoninic acid assay. Equal protein (30 μ g) across samples was incubated with reaction buffer (Ac-DEVD-pNA to detect caspase-3 or Ac-LEHD-pNA to detect caspase-9) at 37°C for 2 h in the dark, and caspase-3 and -9 levels were measured at an absorbance of 405 nm.

Statistical analysis. Data are presented as the mean \pm standard error of the mean. Statistical analyses, including a one-way analysis of variance with Student's t-test post-hoc analysis, were conducted using SPSS 15.0 statistical software (SPSS, Inc., Chicago, IL, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Silymarin improves I/R-induced pulmonary vascular dysfunction. The chemical structure of silymarin is indicated in Fig. 1 (Sigma-Aldrich Chemie GmbH, Munich, Germany). To determine whether silymarin affects lung I/R-induced pulmonary vascular dysfunction in rats, vascular reactivity was evaluated following reperfusion. Compared with the control group, the relaxant response was effectively decreased in lung I/R-injured rats ($P < 0.01$; Fig. 2). However, treatment with silymarin improved the relaxant response in lung I/R-injured rats, when compared with that of the saline-treated lung I/R-injured group ($P < 0.01$; Fig. 2).

Silymarin reduces I/R-induced lung moisture. The effects of silymarin on lung moisture following I/R injury were also evaluated. As indicated in Fig. 3, lung moisture was significantly higher in the I/R-injured group than the control

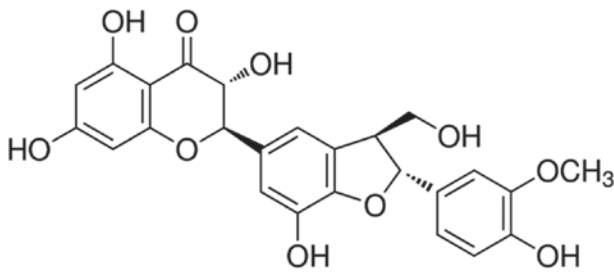
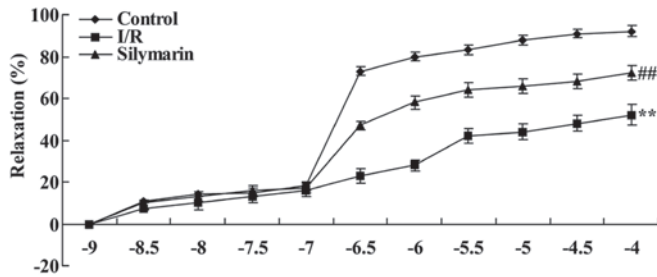
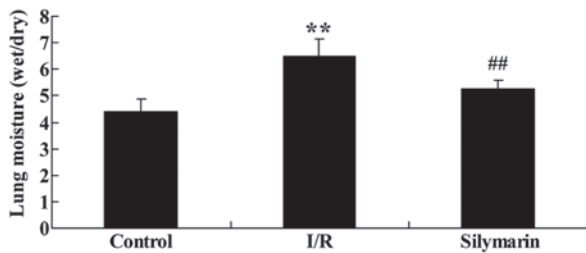


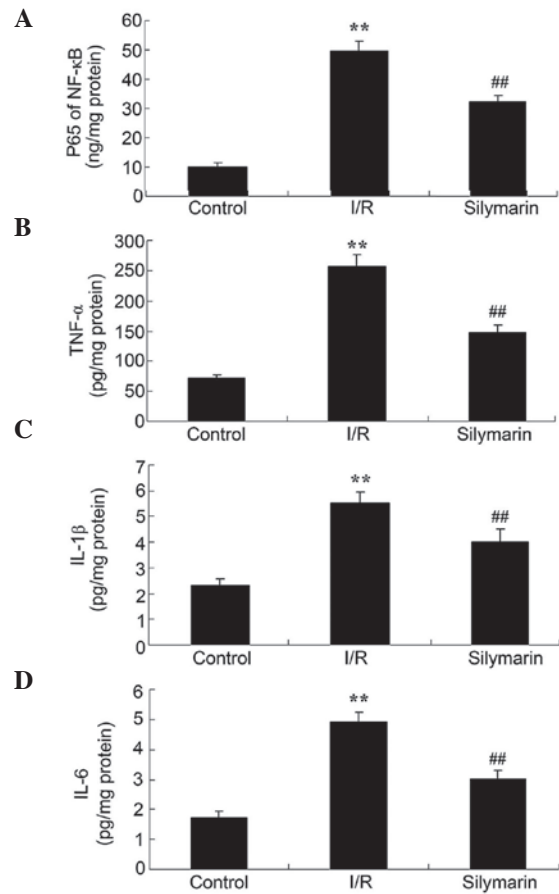
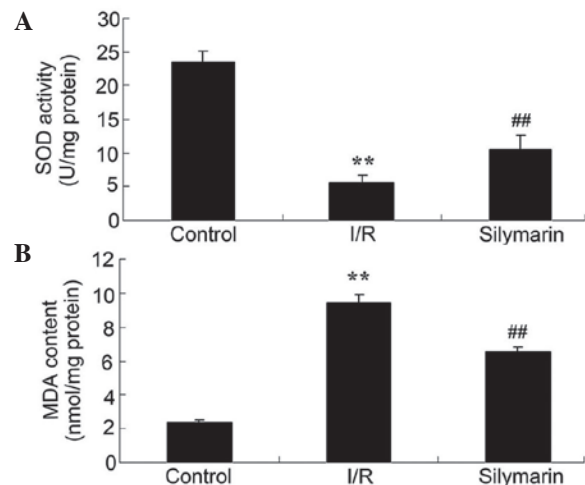
Figure 1. Chemical structure of silymarin.

Figure 2. Silymarin increases I/R-induced pulmonary vascular dysfunction. ** $P < 0.01$ compared with the control group; ## $P < 0.01$ compared with the I/R group. I/R, ischemia/reperfusion.Figure 3. Silymarin reduces I/R-induced lung moisture. ** $P < 0.01$ compared with the control group; ## $P < 0.01$ compared with the I/R group. I/R, ischemia/reperfusion.

group ($P < 0.01$), but treatment with silymarin markedly reduced lung moisture compared with the I/R-injured group ($P < 0.01$).

Silymarin reduces I/R-induced inflammation of the lungs. In order to investigate the inflammatory effects of silymarin, the serum NF- κ B, TNF- α , IL-1 β and IL-6 protein levels were analyzed following reperfusion. These inflammatory factors were significantly increased in I/R-injured rats compared with the control group ($P < 0.01$; Fig. 4). However, protein levels of these inflammatory factors were significantly reduced following silymarin treatment of I/R-injured rats ($P < 0.01$; Fig. 4).

Silymarin increases SOD and reduces MDA levels in rat lungs following I/R injury. The anti-oxidative effects of silymarin were also examined, using the serum SOD and MDA levels. Serum SOD levels were significantly decreased and serum MDA levels were significantly increased in the lungs of rats following I/R injury, as compared with the control group ($P < 0.01$; Fig. 5). However, serum levels of SOD were

Figure 4. Silymarin reduces I/R-induced inflammation in the lungs, as evaluated by serum levels of (A) NF- κ B, (B) TNF- α , (C) IL-1 β and (D) IL-6. ** $P < 0.01$ compared with the control group; ## $P < 0.01$ compared with the I/R group. I/R, ischemia/reperfusion; NF- κ B, nuclear factor κ B; TNF- α , tumor necrosis factor- α ; IL, interleukin.Figure 5. Silymarin increases (A) SOD and reduces (B) MDA levels in the lungs of rats following I/R injury. ** $P < 0.01$ compared with the control group; ## $P < 0.01$ compared with the I/R group. I/R, ischemic/reperfusion; SOD, superoxide dismutase; MDA, malondialdehyde.

significantly increased and serum levels of MDA were significantly decreased in the lungs of silymarin-treated rats, as compared with the I/R-injured group ($P < 0.01$; Fig. 5).

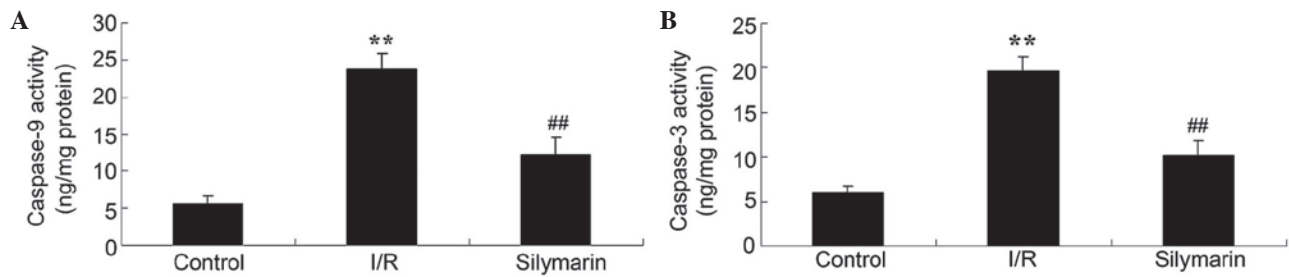


Figure 6. Silymarin prevents I/R-induced cell apoptosis in the lungs, as measured by (A) caspase-9 and (B) caspase-3 levels. ** $P < 0.01$ compared with the control group; ## $P < 0.01$ compared with the I/R group. I/R, ischemia/reperfusion.

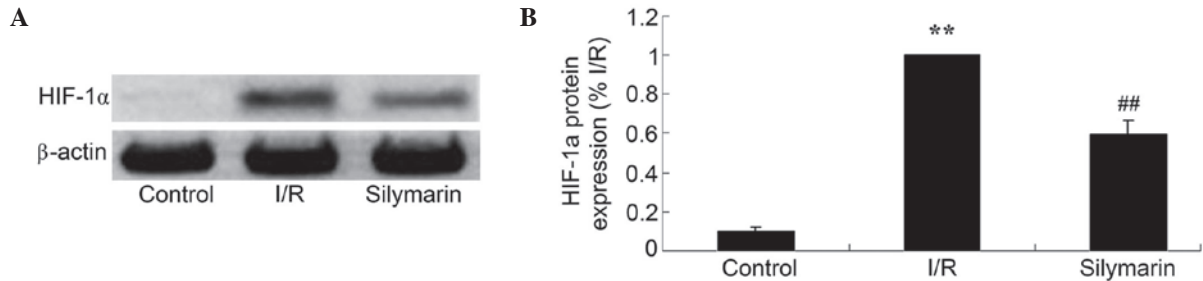


Figure 7. Silymarin reduces I/R-induced HIF-1 α expression in the lungs, evaluated using (A) western blotting assays and (B) statistical analysis of HIF-1 α expression levels. ** $P < 0.01$ compared with the control group; ## $P < 0.01$ compared with the I/R group. HIF-1 α , hypoxia inducible factor-1 α ; I/R, ischemia/reperfusion.

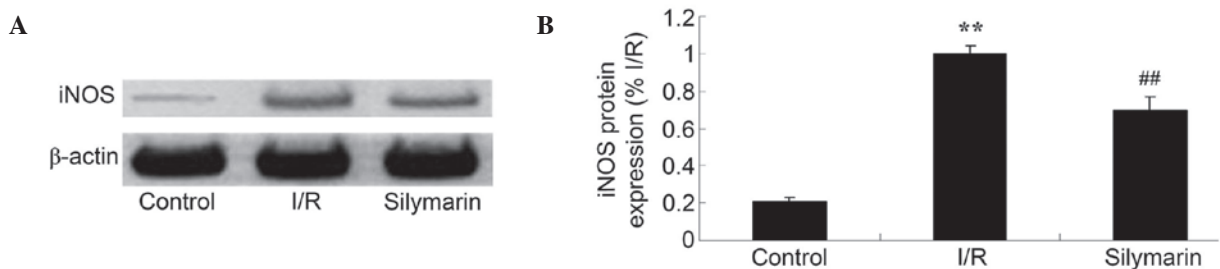


Figure 8. Silymarin attenuates I/R-induced iNOS expression in the lungs, as evaluated using (A) western blotting assays and (B) statistical analysis of iNOS expression levels. ** $P < 0.01$ compared with the control group; ## $P < 0.01$ compared with the I/R group. iNOS, inducible nitric oxide synthase; I/R, ischemia/reperfusion.

Silymarin ameliorates I/R-induced cell apoptosis in the lungs. In order to elucidate the effect of silymarin on cell apoptosis following lung I/R, the levels of caspase-3 and caspase-9 were investigated following reperfusion. Lung I/R injury markedly increased the caspase-3 and -9 levels compared with the control group ($P < 0.01$; Fig. 6). However, treatment with silymarin markedly decreased caspase-3 and -9 levels compared with the I/R-injured group ($P < 0.01$; Fig. 6).

Silymarin attenuates I/R-induced HIF-1 α expression in the lungs. In order to reveal the effects of silymarin on HIF-1 α expression, HIF-1 α protein expression was analyzed using western blotting. HIF-1 α protein levels were increased subsequent to I/R injury compared with the control group ($P < 0.01$; Fig. 7). As demonstrated in Fig. 7, HIF-1 α protein levels were decreased following silymarin treatment of lung I/R-injured rats ($P < 0.01$; Fig. 7).

Silymarin reduces I/R-induced iNOS expression in the lungs. The effects of silymarin treatment on iNOS expression were examined using western blotting; this revealed that the protein expression level of iNOS was increased following I/R injury compared with the control group ($P < 0.01$; Fig. 8). However, silymarin treatment decreased iNOS expression compared with that in lung I/R-injured rats ($P < 0.01$; Fig. 8).

Discussion

Lung I/R injury refers to restore blood flow perfusion, on the basis of ischemia of lung tissue damage is aggravating pathological state. In numerous situations known to cause lung I/R injury, including thrombolysis for pulmonary embolism, interventional therapy and lung transplantation, the clinical focus is the prevention of lung I/R injury (10). The major pathological changes following I/R injury are as follows: i) Intimal hyperplasia of the pulmonary artery, increased elastic fibers

and collagen matrix, hardening of blood vessels and increased resistance of the blood vessels; ii) hyperplasia and hypertrophy of the smooth muscle cell membrane, leading to intermembrane hypertrophy; and iii) loss of the muscular layer of the pulmonary arteriole (11). The present study demonstrated that silymarin significantly reduced lung I/R-induced pulmonary vascular dysfunction and lung moisture. Concordantly, Demir *et al* (12) revealed that silymarin attenuates mesenteric I/R injury, and Turgut *et al* (13) reported that silymarin protects against kidney I/R injury. The use of silymarin may therefore be considered a novel therapeutic strategy for protection against lung I/R injury.

Animal and clinical trials have confirmed that the inflammatory response is focal to continuing tissue damage following cerebral ischemia, and this is observed to a greater degree following lung I/R injury (14,15). Reduction in the inflammatory response is therefore an important strategy to attenuate lung I/R injury. NF- κ B is an important signal transduction molecule in the inflammatory response, with a central role in the inflammatory response to lung I/R injury (16,17). Activation of the NF- κ B pathway induces the expression of adhesion molecules and chemokines, and the expression of pro-inflammatory factors; this response leads to leukocyte adhesion, aggregation and infiltration in the ischemic region, aggravating lung I/R injury (18). In the present study, silymarin significantly reduced the serum NF- κ B, TNF- α , IL-1 β and IL-6 levels in the lungs of I/R-injured rats. A previous study demonstrated that silymarin suppressed inflammation in mycobacterial adjuvant-induced arthritis (19) and in the skin of SENCAR mice (20). Silymarin may therefore represent a potent agent for protection against the lung I/R-induced inflammatory response.

Lung I/R injury is a significant cause of post-operative acute lung injury and multiple organ failure. Oxidative stress is central to I/R injury; a previous study reported that the majority of lung tissue cells generate damaging reactive oxygen species, and that the abundant capillaries and fragile wall structure of the lungs makes them vulnerable to oxidative stress (21). MDA is a terminal product of lipid peroxidation, such that its expression levels reflect the degree of lipid peroxidation damage (22). Furthermore, SOD and catalase are important antioxidant enzymes of the cell; the levels of which reflect the protective ability of lung tissue cells (23). Therefore, oxidative stress may be considered important in the process of lung I/R injury (22). The data from the present study demonstrated that silymarin significantly increased the serum levels of SOD in rats with lung I/R injuries. A previous study by Clichici *et al* (24) reported that silymarin inhibited the progression of fibrosis through suppression of oxidative stress, and Moshtaghion *et al* (25) revealed that silymarin prevented varicocele-induced damage through an increase in MDA expression. The beneficial effects of silymarin may therefore be attributed to anti-oxidative effects.

HIF-1 α is a transcription factor involved in altering cellular oxygen concentration and regulation of gene expression (26). The lung I/R injury-induced HIF-1 α expression observed in the present study likely exacerbated the injury of lung tissue cells, which is consistent with the results of a previous study (27). HIF-1 α promotion of lung tissue cell injury may be associated with hypoxia; the mitochondria of I/R-conditioned cells

produce large amounts of reactive oxygen species, in addition to the hydroxide produced during I/R (28). A previous study in a rat model of pulmonary hypertension reported increased HIF-1 α expression in the pulmonary artery intima, raising the level of downstream iNOS expression; this resulted in the proliferation of vascular endothelial cells within the pulmonary artery intima and concomitant structural damage to the pulmonary vascular endothelium, thereby promoting angiogenesis and pulmonary vascular remodeling (29). This prior study indicated that, at the protein and gene level, HIF-1 α and iNOS may contribute to hypoxia, causing lung I/R injury (4). In the present study, silymarin significantly reduced caspase-3 and -9, HIF-1 α and iNOS expression levels in rats following lung I/R injury. Similarly, Li *et al* (30) previously reported that silymarin reduced the UV-irradiated caspase-3 and -9 activities in A375-S2 cells, and Atawia *et al* (31) demonstrated that silymarin reduced the production of inflammatory mediators by downregulation of HIF-1 α , iNOS and NF- κ B. Kim *et al* (32) demonstrated that silymarin inhibits NO and iNOS production in pancreatic β cells. Therefore, the regulatory effect of silymarin against lung I/R injury may be associated with the caspase/HIF-1 α /iNOS pathway.

In conclusion, the present study demonstrated that silymarin treatment was able to improve pulmonary vascular dysfunction following lung I/R injury via the HIF-1 α -iNOS pathway. Further studies are required in order to elucidate the protective effects of silymarin against lung I/R injury.

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