Original Article
The anti-inflammatory effects of simvastatin in a rat model of smoke inhalation lung injury

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Abstract: To observe the protective effect of simvastatin on early smoke inhalation lung injury in rats, 54 healthy, adult Sprague-Dawley rats were randomly divided into normal, saline, and simvastatin groups. Following exposure to the smoke, the rats received simvastatin 50 mg/kg once daily or normal saline by gastric lavage; the normal group received no treatment or smoke exposure. At 6, 24 and 48 h after the perfusion, arterial blood serum and bronchoalveolar lavage fluid were collected for the measurement of the TNF-α and IL-6 levels, while the TNF-α, IL-6, and NF-κB protein expressions was evaluated in the lung tissue. A histological analysis was also performed in the lung tissue 48 h after the perfusion. The smoke inhalation lung injury caused a significant increase in the TNF-α and IL-6 levels in the serum and bronchoalveolar lavage fluid at all time points. However, these levels were significantly lower in the simvastatin group compared with the saline group at 24 and 48 h, but not at 6 h. The TNF-α, IL-6, and NF-κB expressions also significantly increased following the smoke exposure, but were significantly decreased at 24 and 48 h, but not at 6 h, by simvastatin. A histological analysis showed that the exposure to the smoke caused obvious hyperemia and hemorrhage, alveolar structure destruction, alveolar septum thickening and inflammatory cell infiltration in the mesenchyme of the lung tissue in the saline group. However, these pathological changes were significantly reduced by the simvastatin treatment. Simvastatin may therefore protect against early smoke inhalation lung injury in rats by reducing the serum and lung tissue levels of TNF-α, IL-6, and NF-κB.

Keywords: Smoke inhalation injury, TNF-α, IL-6, NF-κB, simvastatin, rats

Introduction

Smoke inhalation lung injury refers to a series of pathophysiological changes to the respiratory system such as inflammatory cell infiltration, thickening of the alveolar wall, pulmonary edema and alveolar protein exudation, which can be caused by heat and/or smoke. Lung damage associated with smoke inhalation following a fire, for example, causes a high death rate. Smoke inhalation acute lung injury remains a difficult condition to treat among victims of fires [1].

In recent years, studies have shown that, in addition to their lipid-lowering function, statins exert a strong anti-inflammatory effect and can reduce the extent of acute lung injury [5-7]. It has been reported that simvastatin, for example, can block the secretion and functional expressions of several inflammatory factors and cytokines such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and NF-κB. By reducing the serum expression of these inflammatory mediators, simvastatin may inhibit local and systemic inflammatory responses [2, 3]. However, the effects of simvastatin on smoke-induced lung injury remain unclear, and its capacity to reduce the expressions of TNF-α, IL-6, and NF-κB in serum, bronchoalveolar lavage fluid and lung tissue have not been reported. The objective of this study was therefore to examine the protective effect of simvastatin on early smoke inhalation lung injury in rats.

Materials and methods

Materials

Experimental animals: Fifty-four male and female, healthy adult Sprague-Dawley rats, weighing 200-250 g, were purchased from the

Table 1. Serum TNF-α levels in each group at different time points (pg/ml, $\bar{x} \pm s$)

	6 h	24 h	48 h
Normal group	105.21 ± 11.17	93.46 ± 9.45	110.32 ± 10.81
Saline group	135.38 ± 7.42[a]	194.50 ± 10.13[a]	147.00 ± 9.36[a]
Simvastatin group	137.28 ± 4.98[a]	166.29 ± 4.44[a,b]	129.11 ± 4.24[a,b]

Note: [a]P<0.05 compared with the normal group; [b]P<0.05 compared with the saline group.

Table 2. BALF TNF-α levels in each group at different time points (pg/ml, $\bar{x} \pm s$)

	6 h	24 h	48 h
Normal group	60.59 ± 1.93	62.09 ± 2.89	56.38 ± 3.23
Saline group	70.98 ± 1.40[a]	127.32 ± 3.64[a]	92.46 ± 1.74[a]
Simvastatin group	67.94 ± 1.74[a]	107.07 ± 2.72[a,b]	72.90 ± 1.66[a,b]

Note: [a]P<0.05 compared with the normal group; [b]P<0.05 compared with the saline group.

Animal Experiment Center of Zhengzhou University.

Drugs and reagents: Simvastatin tablets (Zhejiang Jingxin Pharmaceutical Co., Ltd.), rat TNF-α ELISA kits (Shenzhen Dakewe Biotech Co., Ltd.), rat IL-6 ELISA kits (Wuhan Boster Biological Technology Co., Ltd.), rabbit anti-TNF-α antibody (Wuhan Boster Biological Technology Co., Ltd.), rabbit anti-rm. IL-6 antibody (Wuhan Boster Biological Technology Co., Ltd.) NF-κB polyclonal antibody (Shanghai Bioleaf Biotech Co., Ltd.), loading buffer (Beijing Com-Win Biotech Co., Ltd.), and an SDS-PAGE gel kit (Beijing ComWin Biotech Co., Ltd.) were obtained.

Methods

Experimental grouping: The rats were randomly divided into three groups (18 rats per group): the normal group, the saline group, and the simvastatin group. The normal group received no treatment or smoke injury and was used as a baseline control, while a smoke inhalation lung injury model was established in the saline and simvastatin groups as described previously [4]. At 30 min after the smoke inhalation injury, the experimental group received simvastatin at 50 mg/kg per day by gastric perfusion. The saline group was perfused with a corresponding volume of normal saline.

Specimen collection and processing: All the rats were anesthetized with 350 mg/kg of 10% chloral hydrate at 6, 24, and 48 h after perfusion (6 rats per time point). Blood was collected from the heart following the exposure of the chest cavity. It was placed at room temperature for 2 h and then centrifuged at 1,500 rpm for 10 min. The resulting supernatant was stored at -20°C until it was used for ELISA according to the kit's instructions. A tissue forceps blunt dissection was used to completely expose and ligate the main bronchus of the right lung. A polyvinyl chloride tube 5 cm in length with an outer diameter 2 mm was used for the endotracheal intubation and slow perfusion of the left with 2 ml of sterile normal saline to collect bronchoalveolar lavage fluid (BALF). This process was performed three times, with a recovery rate >75%. The BALF was centrifuged at 1,500 rpm for 10 min at 4°C and the supernatant used for ELISA. The upper lobe tissue of the right lung was excised and placed in a cryo-preservation tube for storage at -80°C. The protein was subsequently extracted and stored at -20°C and western blotting was performed. The lower lobe tissue from the right lung was excised and fixed in a 10% formalin solution for 24 h, and then it was cut into slices for hematoxylin and eosin (HE) staining.

Statistical processing: SPSS v. 17.0 statistical software was used for the analysis. The data were expressed as the means ± standard deviation ($\bar{x} \pm s$), and a one-way analysis of variance (ANOVA) was used for the comparisons. Values of *P*<0.05 were considered statistically significant.

Results

Decreased TNF-α in serum and BALF following simvastatin treatment

Following the smoke inhalation injury, the TNF-α levels in the serum and BALF were significantly increased in the simvastatin and saline groups compared with the normal group at each time point (P<0.05). However, the TNF-α levels in serum and BALF were significantly decreased in the simvastatin group compared with the saline group at 24 and 48 h, but not at 6 h (P<0.05) (**Tables 1** and **2**).

Table 3. Serum IL-6 levels in each group at different time points (pg/ml, $\overline{x} \pm s$)

	6 h	24 h	48 h
Normal group	37.97 ± 13.78	41.34 ± 9.59	43.19 ± 12.40
Saline group	143.46 ± 40.02[a]	390.30 ±39.35[a]	289.93 ± 42.71[a]
Simvastatin group	147.07 ± 20.60[a]	279.41 ± 34.41[a,b]	110.85 ± 27.01[a,b]

Note: [a]P<0.05 compared with the normal group; [b]P<0.05 compared with the saline group.

Table 4. BALF IL-6 levels in each group at different time points (pg/ml, $\overline{x} \pm s$)

	6 h	24 h	48 h
Normal group	25.16 ± 2.60	26.01 ± 6.10	32.29 ± 7.02
Saline group	75.13 ± 7.36[a]	322.03 ± 11.46[a]	179.54 ± 12.42[a]
Simvastatin group	74.87 ± 7.36[a]	267.55 ± 7.65[a,b]	103.28 ± 6.70[a,b]

Note: [a]P<0.05 compared with the normal group; [b]P<0.05 compared with the saline group.

Decreased IL-6 in serum and BALF following simvastatin treatment

Following the smoke inhalation injury, the IL-6 levels in the serum and BALF were significantly increased in the simvastatin and saline groups compared with the normal group at each time point (P<0.05). However, the IL-6 levels in the serum and BALF were significantly decreased in the simvastatin group compared with the saline group at 24 and 48 h, but not at 6 h (P<0.05) (**Tables 3** and **4**).

Simvastatin mitigated the increase in protein expressions of TNF-α, IL-6, and NF-κB in lung tissue following smoke exposure

Western blotting showed that the protein expressions of TNF-α, IL-6 and NF-κB were significantly increased in the saline and simvastatin groups compared with the normal group (P<0.05); however, the TNF-α, IL-6 and NF-κB expressions in the simvastatin group were significantly decreased compared with the saline group at 24 and 48 h, but not at 6 h (P<0.05) (**Figures 1**-**3**).

Effect of simvastatin on lung tissue pathology 48 h after perfusion

At 48 h after perfusion, the alveolar spaces of rats in the normal group were clear, intact and clean, without any asymmetry or swelling, and there was no aggregation of inflammatory cells in the mesenchyme (**Figure 4**). However, obvious hyperemia and hemorrhage, alveolar structure destruction, alveolar septum thickening, and infiltration of inflammatory cells in the mesenchyme were observed in lung tissue of rats in the saline group (**Figure 5**). Alveolar wall thickening, alveolar destruction, and interstitial inflammatory cell infiltration were significantly less in the lung tissue from the rats in the simvastatin group than they were in the saline group (**Figure 6**).

Discussion

Inhalation lung injury refers to the thermal and chemical damage to the respiratory tract caused by the heat and/or smoke inhalation. The pathological mechanisms of this type of injury typically involve a series of pathophysiological changes such as inflammatory cell infiltration, thickening of the alveolar wall, pulmonary edema and alveolar protein exudation. These changes after smoke inhalation injury may be the result of the release of a variety of cytokines and inflammatory mediators (e.g. TNF-α and IL-6) by macrophages and neutrophils, leading to an inflammatory cascade which results in lung injury via increased vascular permeability, pulmonary edema formation, and airway obstruction. Damage arising from smoke inhalation after a fire significantly increases mortality and is among the most difficult burn injuries to treat. Clinically, the treatment of smoke inhalation lung injury includes respiratory support, bronchoalveolar lavage, and the administration of drugs, including antibiotics, expectorants, anticoagulants and bronchodilators. The complexity of this type of injury and the high incidence of death, combined with an intensive focus of treatment outcomes, means that the mechanism behind the progression of smoke-induced acute lung injury can be overlooked. Therefore, appropriate treatment to suppress the inflammatory response process can, to some extent, reduce lung inflammation and improve the lung injury, thus improving a patient's prognosis.

As the first generation of statins, the lipid-lowering function and anti-inflammatory effects of simvastatin are well-established [5-7]. Sim-
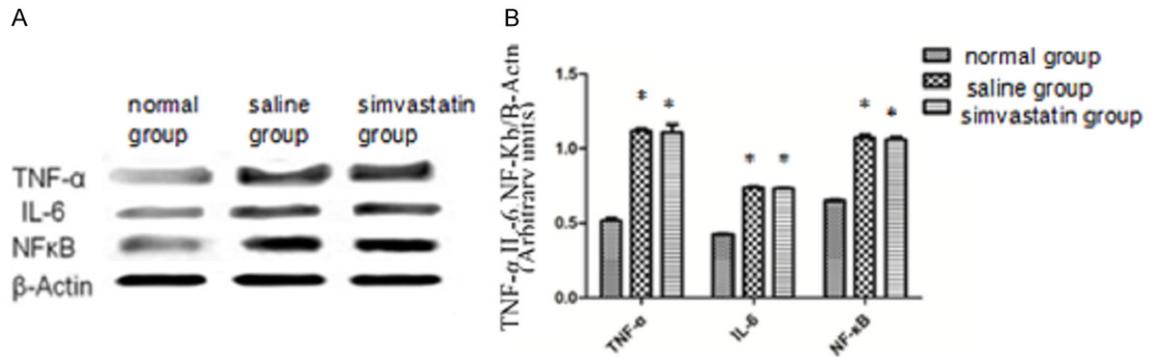
Figure 1. The protein expressions of TNF-α, IL-6, and NF-κB in each group at 6 h after perfusion. (A) Western blot image and (B) densitometry analysis. *: P<0.05 compared with the normal group.
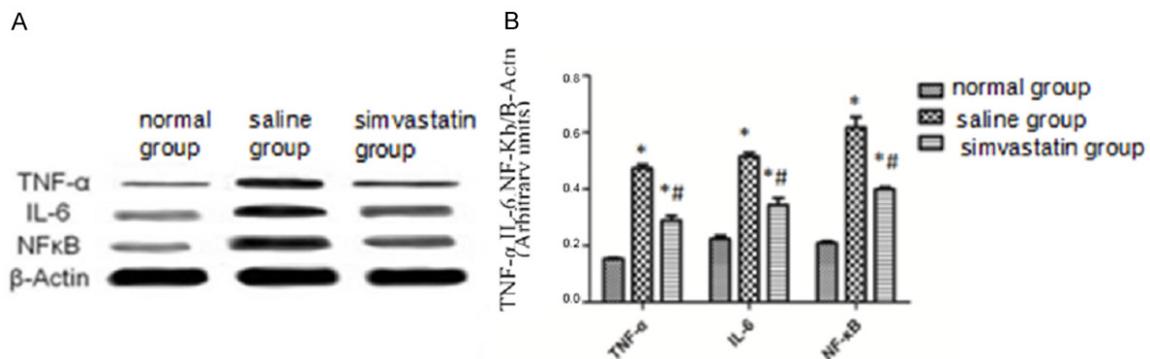

Figure 2. The protein expressions of TNF-α, IL-6, and NF-κB of each group at 24 h after perfusion. (A) Western blot image and (B) densitometry analysis. *: P<0.05 compared with the normal group; #: P<0.05 compared with the saline group.
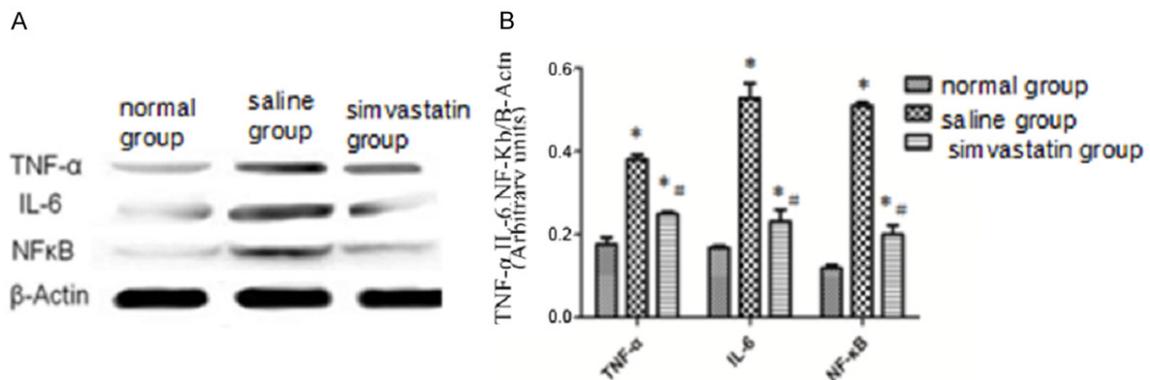

Figure 3. The protein expressions of TNF-α, IL-6, and NF-κB of each group at 48 h after perfusion. (A) Western blot image and (B) densitometry analysis. *: P<0.05 compared with the normal group; #: P<0.05 as compared with the saline group.

vastatin has also been shown to decrease the expression of TNF-α and IL-6 at the mRNA expression and protein levels, thereby preventing monocyte-mediated inflammatory injury effects [11]. In ischemia-reperfusion-induced lung inju- ry, it has also been reported to inhibit NF-κB expression, thus reducing inflammatory cell in- filtration and protecting against lung injury [8]. Although simvastatin is an approved drug for dyslipidemia, its mechanisms of action with re-
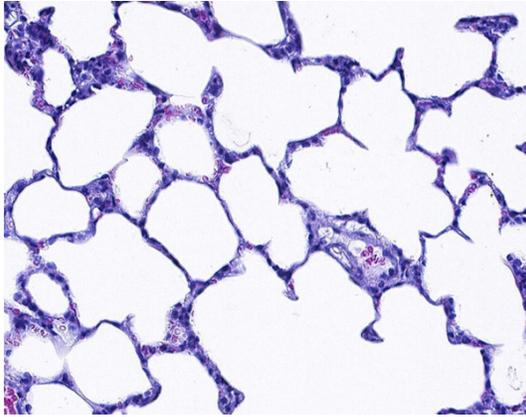
Figure 4. Clear, intact, and clean alveolar spaces, without asymmetry or swelling, and an absence interstitial inflammatory cell infiltration in the lung tissue of mice in the normal group (HE staining ×200).
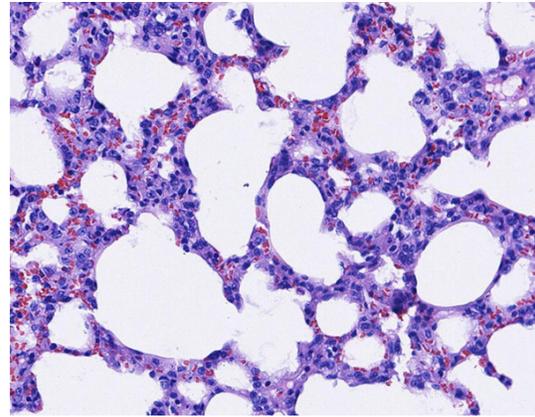

Figure 6. A reduction in alveolar wall thickening, alveolar destruction and interstitial inflammatory cell infiltration in the lung tissue of rats in the simvastatin group (HE staining ×200).
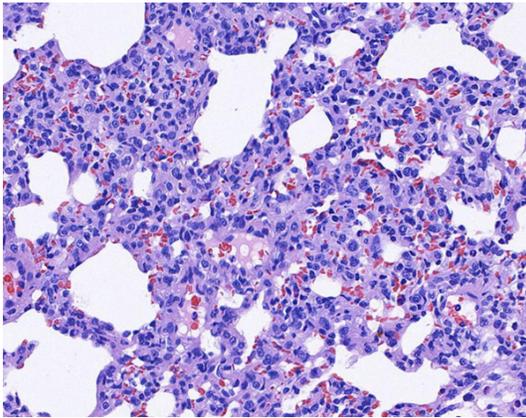

Figure 5. Alveolar structure destruction, alveolar septum thickening, and the infiltration of inflammatory cells in the mesenchyme in lung tissue from rats in the saline group (HE staining ×200).

spect to alternative therapeutic effects remain unclear. This study was designed to determine whether simvastatin could reduce lung injury by decreasing the expressions of TNF-α, IL-6, and NF-κB in the serum, BALF, and lung tissue in a model of smoke inhalation acute lung injury.

The results showed that the levels of TNF-α and IL-6 in the simvastatin- and saline-treated groups after smoke inhalation injury increased compared with the normal group. However, TNF-α and IL-6 levels in the serum and BALF at 24 and 48 h were significantly lower in the simvastatin group than they were in the saline group. Furthermore, the protein expressions of TNF-α and IL-6 in the lung tissue after perfusion were significantly less in the simvastatin

group than they were in the saline group at 24 and 48 h, but not at 6 h. We speculate that simvastatin may take a certain amount of time to affect the expression of the inflammatory cytokines TNF-α and IL-6, vascular permeability, and lung injury, and to play a protective role in lung injury. A pulmonary pathological analysis also confirmed that less exudation, bleeding, alveolar structure destruction, and inflammatory cell infiltration were observed in the simvastatin group than in the saline group. TNF-α is a key endogenous mediator produced early after smoke inhalation injury, primarily by macrophages, and it induces alveolar epithelial cells to produce other pro-inflammatory cytokines such as IL-6 [9], thus destroying the vascular endothelial cells in the lungs. They can induce the formation and release of phospholipase A2, which directly damages the pulmonary surfactant and intima of pulmonary capillaries, increases the permeability of blood vessels, and eventually leads to lung injury and pulmonary edema [10]. Meanwhile, IL-6 is considered the most important cytokine in the inflammatory phase and can negatively regulate the immune system-neuroendocrine axis by stimulating the pituitary gland to produce adrenocorticotropic hormones [11], leading to the production of protein by hepatocytes during the acute phase of inflammation. It also plays an important role in inducing the migration of eosinophils to the site of inflammation [12, 13]. A previous study has shown that simvastatin can decrease the TNF-α and IL-6 levels by downregulating the expressions of their mRNA and

protein, thus preventing monocyte-mediated inflammatory injury [14].

Following the establishment of the rat model of smoke inhalation injury, the NF-κB protein expression in the lung tissue was increased, indicating the involvement of NF-κB in the inflammatory response that causes a series of pathophysiological changes, including the destruction of lung structure and the functional impairment of the lung. Compared with the saline group, the expression of NF-κB in the lung tissue of the simvastatin group was significantly decreased at 24 and 48 h after perfusion. We suggest that simvastatin inhibits the activation of NF-κB and inflammation and improves lung injury, consistent with previous findings related to TNF-α, IL-6, and lung pathology. Previous studies have found that, upon the occurrence of a smoke inhalation injury, the lung cells can produce a variety of pro-inflammatory cytokines as well as NF-κB in a non-activated state in the cytoplasm, which subsequently migrates to the nucleus following polysaccharide receptor interaction, where it promotes the transcription of pro-inflammatory mediators related to acute inflammatory responses, such as interleukin IL-8, macrophage inflammatory protein-1, IL-6 and TNF-α [15]. Furthermore, in experimental in vivo and in vitro models of several pulmonary diseases, such as acute lung injury, systemic inflammatory response syndrome, vesicular fibrosis and asthma, certain pathways have been shown to involve NF-κB-mediated inflammatory processes [16]. NF-κB is an important promoter of inflammation in lung injuries induced by smoke inhalation, as it can upregulate the expression of pro-inflammatory cytokines such as TNF-α to cause a cascade effect, thus inhibiting the expression of NF-κB while reducing the expression of TNF-α [17]. In an animal model of ischemia-reperfusion-induced lung injury, simvastatin reduces inflammatory cell infiltration by inhibiting the expression of NF-κB, thus protecting against lung injury [8]. The results of the present study showed that, to a certain extent, simvastatin can reduce smoke-induced lung injury in rats by decreasing the expressions of TNF-α, IL-6, and NF-κB in the serum, BALF and lung tissue. However, several mechanisms may be involved in smoke inhalation lung injury, and simvastatin has only been shown to inhibit cytokine activity and NF-κB.

In summary, simvastatin exerts a protective effect in acute lung injury by regulating the expressions of TNF-α, IL-6, and NF-κB, but the mechanisms involved in this type of injury and the precise role of simvastatin require further study.

Acknowledgements

Disclosure of conflict of interest

None.

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