

Original Article

Effects of simvastatin on the expression of inducible NOS in acute lung injury in septic rats

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Abstract: Background: The available evidence suggests that simvastatin plays a beneficial role in lung injury. In addition, statins have been shown to inhibit the activity of inducible nitric oxide synthase (iNOS). The aim of the present study was to investigate the effects of simvastatin on iNOS expression based on a lipopolysaccharide (LPS)-induced septic rat model. Methods: Thirty-six rats were randomly divided into 3 groups (control group, sepsis group and simvastatin group). A rat model of sepsis was established with LPS. The simvastatin group was pre-treated with simvastatin, whereas the control and sepsis groups were treated with saline before LPS treatment. LPS was injected into the rats in the simvastatin and sepsis groups, while as a negative control, the control group received saline alone. The oxygenation index, expression levels of iNOS and IL-6, and pathological integral of lung injury were analyzed to evaluate the effect of simvastatin on septic rats. Results: Compared with the septic group, significant decreases in the oxygenation index and expression level of iNOS were observed in the simvastatin group. Furthermore, simvastatin treatment resulted in a significant decrease in iNOS levels and the pathological integral of lung injury score in septic rats. Conclusion: Simvastatin can relieve acute lung injury induced by sepsis in rats. Decreasing iNOS levels may contribute to the protective role of simvastatin in lung injury.

Keywords: Simvastatin, inducible nitric oxide synthase, lung injury, septic rats

Introduction

Sepsis is the systemic inflammatory response syndrome caused by infection by pathogens, including bacteria, fungi, parasites and viruses, among others. The pathophysiologic features of sepsis result from the comprehensive effects of microbes and their products, including the lipopolysaccharide (LPS) produced by Gram-negative bacteria, the peptidoglycan of Gram-positive bacterium, acidic compounds and the peptides found in fungal cell walls [1]. These products can activate cytokines, complement, the coagulation system, plasmakinin, endorphins, and the sympathetic nervous system, among others. Subsequently, various bioactive substances are generated and interact with one another. The target organs of the pathophysiological effects are endothelial cells and the microcirculatory system [2, 3].

The pathogenesis of sepsis and acute lung injury/acute respiratory distress syndrome are

closely linked [4]. In clinical statistics, lung injury occurs in 63%~100% patients with multiple organ dysfunction syndrome (MODS). A large portion of these patients initially develop acute respiratory distress syndrome, followed by the dysfunction of other organs in a successive manner and, finally, MODS [5].

Sepsis can lead to acute lung injury/acute respiratory distress syndrome (ALI/ARDS) when uncontrolled inflammation occurs, and the imbalance of the systemic inflammatory response and compensatory anti-inflammatory response is the central mechanism. A large number of inflammatory mediators, including TNF- α , INF, IL-1, IL-6, IL-8, phospholipase A2, platelet activating factor (PAF), nitric oxide (NO) and lipid metabolites, are released into the blood circulation during sepsis, stimulating inflammatory cell recruitment and activation in the lung. Furthermore, cytokines, chemokines and oxyradicals are produced, all of which can augment the inflammation and create a "waterfall-like chain

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reaction” of inflammatory effects [6]. While endogenous anti-inflammatory mediators, including IL-4, IL-10, IL-13, and IL-17, cannot counteract the inflammatory mediators, the imbalance between the pro-inflammatory and anti-inflammatory mediators released into the blood causes a mixed anti-inflammatory response syndrome, which enhances anticoagulant activity, fibrinolysis and oxyradical production, reduces the levels of pulmonary surfactant (PS) and the expression of the transcription factor NF- κ B, and causes gene defects. Eventually, pulmonary capillary endothelial cell and alveolar epithelial cell injury occur, alveolar capillary membrane permeability to water and protein increase, a fluid exchange barrier develops between pulmonary vessels and the interstitium, and osmotic edema of the alveolar and pulmonary interstitium are observed [7].

Statins are hydroxymethylglutaryl coenzyme A reductase (HMG-CoA) inhibitors. Their main role is to disturb the metabolic process of cholesterol. Statins can be hydrolyzed into β -hydroxy acid metabolite in vivo, which can competitively inhibit enzyme functions required for endogenous cholesterol synthesis by HMG-CoA reductase, obstructing the generation of HMG-CoA from mevalonate (cholesterol precursor) and diminishing the biosynthesis of endogenous cholesterol, which reduces the synthesis of low-density lipoprotein (LDL-C). Furthermore, statins reduce blood lipid levels [8].

In many clinical studies, we have observed that statins have significant functions in improving the prognosis of sepsis and ALI/ARDS while protecting organ function. Statins can ameliorate endothelial function by increasing levels of endothelial nitric oxide synthase (eNOS) and the activity of nitric oxide (NO). Statins can inhibit the activity of inducible nitric oxide synthase (iNOS) and increase the expression of eNOS, and they can also prolong the half-life of eNOS and restore the balance between eNOS and iNOS. Ultimately statins can protect endothelial cells and facilitate the recovery of endothelial function, playing an important role in the prevention and treatment of sepsis [1].

In the present study, we treated LPS-induced septic rats with simvastatin and detected the expression of iNOS to assess the protective effects in the lungs of septic rats. Furthermore

we evaluated the effect of simvastatin administration on the expression level of iNOS and IL-6.

Materials and methods

Experimental design

A total of 36 SD rats that were SPF grade and weighed from 180~220 g were obtained from the laboratory of Sun Yet-sun University. The rats were numbered by hair dyeing and randomly allocated to one of three groups: simvastatin group, sepsis group and control group. The simvastatin group was pretreated with simvastatin 40 mg·kg⁻¹·day⁻¹ in 1 ml of distilled water for 4 days. The control group and sepsis group received the same volume of normal saline. This was administered via an orogastric tube that was inserted each day, and the final dose was administered 3 hours before the experiment.

First, the simvastatin and sepsis groups received an intraperitoneal injection of LPS 5 mg·kg⁻¹, and the control group received the same dose of normal saline. Two and 6 hours after the injection, 6 rats were anesthetized and sacrificed to determine the oxygenation index and expression levels of iNOS and IL-6.

Oxygenation index analysis

Arterial blood samples (1 ml) were drawn from the aorta to determine the oxygenation index. The right middle lobe was removed without bronchial tissue, and the inferior lobe of the right lung was fixed with 10% formaldehyde for 24 hours and then sliced into paraffin sections and stained with H.E. to observe the pathological changes under light microscopy. Tissue from the left lung (2 mg) was removed to assess the expression of iNOS and IL-6 and to evaluate lung injury.

Enzyme-linked immunosorbent assay (ELISA)

iNOS and IL-6 levels were measured by ELISA using the R&D System ELISA kit according to the instructions. Samples seeded in 96-well plates (100 μ L/well) were incubated with anti-iNOS or IL-6 antibody (CST, USA) for 1 hour at 37 °C. The supernatant was removed, and the plates were washed 3 times with PBS. After a 30-minute incubation at 37 °C with streptavidin-HRP, the plates were again washed 3 times

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Table 1. Comparison of the oxygenation index

Group	n	Oxygenation Index ($\bar{x} \pm s$, %)	
		2 h	6 h
Control	6	404.0 \pm 35.4	393.7 \pm 40.1
Sepsis	6	292.1 \pm 41.9 $\Delta\Delta$	323.0 \pm 44.7 $\Delta\Delta$
Simvastatin	6	353.2 \pm 38.7*	388.1 \pm 29.4*

Compared with the control group, " $\Delta\Delta$ " $P < 0.01$. Compared with the control group, "*" $P < 0.05$.

and incubated with TMB substrate at 37°C. After a 12-minute incubation, stop solution was added to the plates (100 μ L/well). The absorbance was measured at 450 nm, and the value was recorded.

Pathological integral of lung injury

The left upper lung tissue was assessed by a pathologist who was blinded to the source of the HE-stained lung tissue slide. Quantitative analyses of alveolar collapse, perivascular hemorrhage, alveolar hemorrhage, perivascular edema, vascular congestion, alveolar polymorph nuclear leukocytes, alveolar edema and macrophages were performed according to a previous report [9]. Each item was scored as follow, 0 = normal; 1 = mild; 2 = moderate; 3 = severe. Lung injury was calculated according to the total score in each group.

Statistical analysis

All of the data are expressed as the mean \pm SD. Multiple comparisons were performed using one-way analysis of variance (ANOVA). The LSD t-test was applied to compare means between groups. The level of significance was set at $P < 0.05$.

Results

Simvastatin decreases the oxygenation index in LPS-induced septic rats

After administration of LPS, compared with the control rats, the septic rats exhibited reduced activity, were shaggy and listless, and some individuals had diarrhea, with the observed symptoms increasing over time. The symptoms of the rats in the simvastatin group were similar to those in the sepsis group but slightly less severe.

Compared with the control group, the oxygenation index of the rats in the sepsis group decreased significantly at 2 and 6 hours after

modeling ($P < 0.01$); the index at 2 hours achieved the diagnostic criteria of ALI. Compared with the sepsis group, the oxygenation index in the simvastatin group at 2 and 6 hours after modeling increased ($P < 0.05$) but did not reach the diagnostic criteria of ALI (**Table 1**).

Simvastatin decreases iNOS levels in LPS-induced septic rats

Compared with the control group, the content of iNOS in the group with sepsis was significantly elevated at 2 and 6 hours after modeling ($P < 0.01$). Compared with the group with sepsis, the content of iNOS in the simvastatin group decreased significantly at 6 hours after modeling ($P < 0.05$) (**Table 2**).

Simvastatin decreases iNOS levels in LPS-induced septic rats

Compared with the control group, the levels of IL-6 in the septic group were significantly elevated at 2 and 6 hours after modeling ($P < 0.01$). Compared with the septic group, the levels of IL-6 in the simvastatin group decreased at 2 and 6 hours after modeling, and the decline at 6 hours was statistically significant ($P < 0.05$) (**Table 3**).

Pathological integral of lung injury decreases in the simvastatin treatment group

The pathological integral of lung injury was conducted to evaluate the role of simvastatin in acute lung injury based on a rat model of sepsis. As shown in the image of hematoxylin-eosin (HE)-stained tissue (**Figure 1**) and based on the lung injury score table (**Table 4**), in comparison to the control group, the integral of lung injury in the septic group rose significantly at 2 and 6 hours ($P < 0.05$). Compared with the septic group, the integral determined for the treatment group declined at 2 and 6 hours, and the decline at 6 hours was statistically significant ($P < 0.05$). This result suggests that simvastatin may play an important role in protecting the lung against injury.

Discussion

Acute lung injury often occurs initially in patients with sepsis-induced MODS. The mortality rate of ALI/ARDS is 50%~60%, and death due to ARDS is often attributed to sepsis and MODS rather than to primary respiratory failure. In

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Table 2. Comparison of iNOS Levels

Group	Dose (mg/kg)	n	iNOS ($\bar{x} \pm s$, U/ml)	
			2 h	6 h
Control	-	6	188.6 \pm 17.4	180.5 \pm 29.4
Sepsis	-	6	344.4 \pm 98.6 $\Delta\Delta$	279.5 \pm 57.3 $\Delta\Delta$
Simvastatin	40	6	349.9 \pm 117.3	219.5 \pm 44.2*

Compared with the control group, " $\Delta\Delta$ " $P < 0.01$. Compared with the septic group, "*" $P < 0.05$.

Table 3. Comparison of IL-6 levels in the lung tissue

Group	Dose (mg/kg)	n	IL-6 ($\bar{x} \pm s$, pg/ml)	
			2 h	6 h
Control	-	6	1.38 \pm 0.20	1.56 \pm 0.27
Sepsis	-	6	2.53 \pm 0.87 $\Delta\Delta$	2.19 \pm 0.46 $\Delta\Delta$
Simvastatin	40	6	2.06 \pm 0.56	1.65 \pm 0.20*

Compared with the control group, " $\Delta\Delta$ " $P < 0.01$. Compared with the septic group, "*" $P < 0.05$.

contrast, the mortality rate of sepsis-induced ARDS reaches from 70%~90%. Thus, proactive, effective treatment of sepsis may help to reduce the mortality rate associated with ALI/ARDS. Reducing the occurrence of ALI and halting the progression from ALI to ARDS are very important for reducing the incidence of ARDS and the mortality resulting from sepsis [10].

Nitric oxide is an important informational molecule. Nitric oxide synthase is a key enzyme in the synthesis of NO. NOS comprise three subtypes: nNOS, eNOS, and iNOS. eNOS is expressed in vascular endothelial cells in normal tissues. Under physiological conditions, small amounts of NO are synthesized to maintain angiogenesis, sphincter relaxation and other physiological functions [11]. eNOS expression reveals the integrity of the endothelial function because it catalyzes the formation of small amounts of NO, which can prevent thrombosis and maintain the structure and integrity of the vascular endothelial cells. In diseases such as sepsis, the expression of eNOS is down-regulated, and therefore, eNOS-derived nitric oxide levels decrease; endothelial dysfunction and symptoms of vasculitis can occur in clinical practice.

As a non-calcium-dependent protease, iNOS is widely distributed in brain tissue, macrophages, endothelial cells, and the peripheral nervous system. Following stimulation by a variety of

inflammatory mediators in sepsis and in other conditions, iNOS is activated and abundantly expressed, in contrast to its inactive state under physiological conditions. iNOS can catalyze the synthesis of high levels of NO (levels approximately one thousand times those stimulated by eNOS).

A large amount of NO is synthesized as a consequence of the expression of iNOS, and the high NO concentrations become a double-edged sword that determines the direction of disease progression. The high concentration of NO plays a direct bactericidal effect. However, it can also relax the blood vessels and reduce blood pressure, leading to the extravasation of plasma through the activated vascular endothelial cells, stimulating the systematic inflammatory response as the main inflammatory mediator and ultimately exacerbating hypotension, leading to shock. During the process of sepsis, when the reserved amounts of L-arginine in the body are insufficient, the uncoupled NOS will produce O_2^- but also generate ONOO $^-$ after reacting with NO. Subsequently, a large number of free radicals are able to cause direct damage to tissues. The results also show that selective inhibition of iNOS may reduce mortality in rats with endotoxemia [12].

Many studies have focused on the balance between iNOS and eNOS. eNOS can synthesize NO levels of 10^{-12} g (picograms), while iNOS can synthesize NO levels of 10^{-9} g (nanograms). This huge gap in NO production is important for the progression of disease, and therefore, an equilibrium between eNOS and iNOS expression may be necessary to maintain the physiological function of blood vessels. Sepsis can abrogate the balance between eNOS and iNOS, resulting in severe hypotension by injuring the blood vessel endothelium, which can cause MODS and even death. A restoration of the balance between iNOS and eNOS by inhibiting the expression of iNOS and prolonging the half-life of eNOS can recover the function of endothelial cells, which may play an important role in the prevention and treatment of sepsis.

In the present study, in comparison to the control group, the levels of iNOS in the group with sepsis increased significantly at 2 and 6 hours, suggesting that iNOS plays a role in aggravating and worsening disease in sepsis. The iNOS decreased significantly at 6 hours in both the

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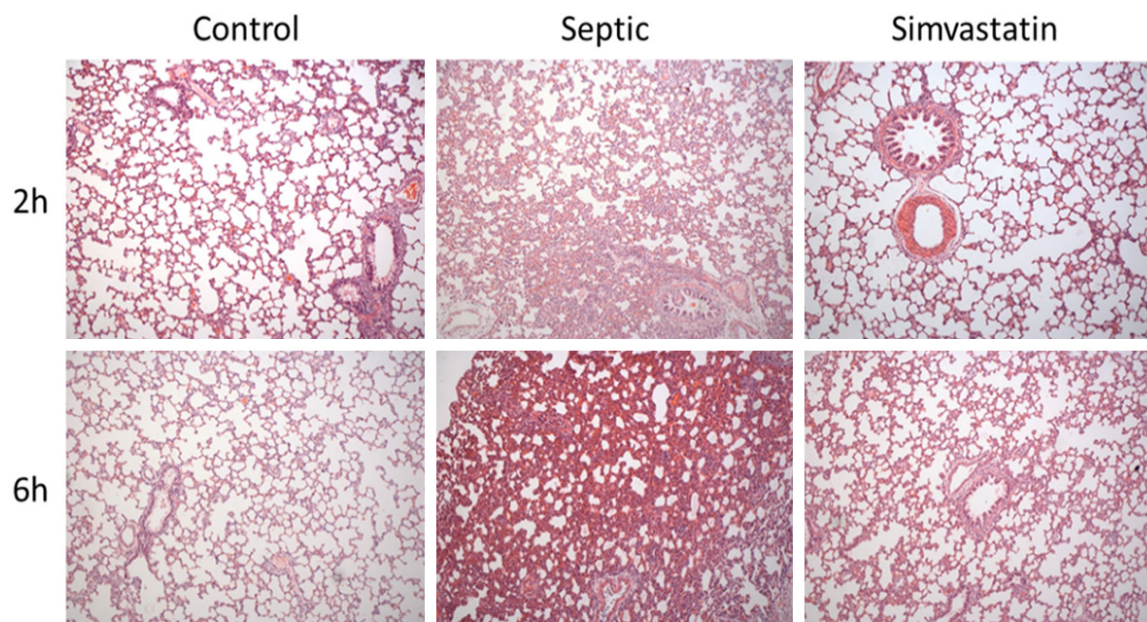


Figure 1. Pathological integral of lung injury in each group. The pathological integral of lung injury decreased in the simvastatin treatment group. Simvastatin: rats that were pre-treated with simvastatin and then administered LPS. Septic: rats that received saline treatment followed by LPS administration. Control: rats that were pre-treated with saline as a control and then administered saline treatment.

Table 4. Pathological integral of lung injury

Time point	Group	n	value ($\bar{x} \pm s$)
2 h	Control	6	1.33±1.03
	Sepsis	6	3.17±2.71
	Simvastatin	6	2.33±2.25
6 h	Control	6	1.67±0.82
	Sepsis	6	6.50±0.55**
	Simvastatin	6	3.67±2.66#

**vs. control, $P < 0.01$. #vs. sepsis, $P < 0.05$.

simvastatin and the sepsis group, indicating that simvastatin could inhibit the expression of iNOS in rats with sepsis. In addition, compared with the sepsis group, the content of IL-6 in the simvastatin group decreased to some extent, especially at 6 hours, which demonstrated a statistically significant decrease. In contrast, the oxygenation index in the treatment group was significantly increased. All of these findings suggest that simvastatin may play a role in protecting the lung by inhibiting the expression of iNOS in rats with sepsis, reducing lung injury, inhibiting the release of inflammatory cytokines, reducing the expression of IL-6, and ultimately improving the oxygen supply in the tissue.

In summary, the balance between eNOS and iNOS in sepsis represents an opportunity to

solve the complex phenomena leading to inflammation. Statins may protect cells and cellular structures and improve the prognosis in sepsis by correcting imbalances in iNOS and eNOS.

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Disclosure of conflict of interest

None.

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References

- [1] Siempos II, Maniatis NA, Kopterides P, Magkou C, Glynos C, Roussos C, Armaganidis A. Pre-treatment with atorvastatin attenuates lung injury caused by high-stretch mechanical ven-

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- tilation in an isolated rabbit lung model. *Crit Care Med* 2010; 38: 1321-1328.
- [2] Almeida JL, Sampietre SN, Mendonca Coelho AM, Trindade Molan NA, Machado MC, Je MDC, Jukemura J. Statin pretreatment in experimental acute pancreatitis. *JOP* 2008; 9: 431-439.
- [3] Ince AT, Baysal B. Pathophysiology, classification and available guidelines of acute pancreatitis. *Turk J Gastroenterol* 2014; 25: 351-357.
- [4] Guisasola MC, Dulin E, Almendral J, Garcia-Barreno P. Reduction of heat shock protein antibody levels by statin therapy. *Lipids* 2009; 44: 317-324.
- [5] Huang PM, Lin TH, Tsai PR, Ko WJ. Intrapleural Steroid Instillation For Multiple Organ Failure with Acute Respiratory Distress Syndrome. *Shock* 2013; 40: 392-397.
- [6] Li GF, Tong X, Luan T, Zang B. [The effects of postconditioning with propofol on Toll-like receptor 4 expression in the lung tissue of rat with acute lung injury]. *Zhongguo Wei Zhong Bing Ji Jiu Yi Xue* 2012; 24: 620-623.
- [7] Delsesto D, Opal SM. Future perspectives on regulating pro-and anti-inflammatory responses in sepsis. *Contrib Microbiol* 2011; 17: 137-156.
- [8] Wang HE, Griffin R, Shapiro NI, Howard G, Saford MM. Chronic statin use and long-term rates of sepsis: a population-based cohort study. *J Intens Care Med* 2014; [Epub ahead of print].
- [9] Christaki E, Anyfanti P, Opal SM. Immunomodulatory therapy for sepsis: an update. *Exp Rev Anti-Infect Ther* 2011; 9: 1013-1033.
- [10] Ortolan LS, Sercundes MK, Barboza R, Debone D, Murillo O, Hagen SC, Russo M, D' Império Lima MR, Alvarez JM, Amaku M, Marinho CR, Epiphanio S. Predictive criteria to study the pathogenesis of malaria-associated ALI/ARDS in mice. *Medi Inflamm* 2014; 2014: 872464.
- [11] Wang Q. Intestinal permeability is reduced and IL-10 levels are increased in septic IL-6 knock-out mice. *Am J Physiol Regul Integr Comp Physiol* 2001; 281: R1013.
- [12] Zhang J, Cheng X, Liao YH, Lu B, Yang Y, Li B, Ge H, Wang M, Liu Y, Guo Z, Zhang L. Simvastatin regulates myocardial cytokine expression and improves ventricular remodeling in rats after acute myocardial infarction. *Cardiovasc Drugs Ther* 2005; 19: 13-21.