International Immunopharmacology 17 (2013) 57–64

Contents lists available at SciVerse ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Redox markers and inflammation are differentially affected by atorvastatin, pravastatin or simvastatin administered before endotoxin-induced acute lung injury

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ARTICLE INFO

Article history:
Received 30 December 2012
Received in revised form 3 May 2013
Accepted 20 May 2013
Available online 6 June 2013

Keywords:
Atorvastatin
Pravastatin
Simvastatin
Acute lung injury
Inflammation
Oxidative stress

ABSTRACT

Statins are standard therapy for the treatment of lipid disorders, and the field of redox biology accepts that statins have antioxidant properties. Our aim in this report was to consider the pleiotropic effects of atorvastatin, pravastatin and simvastatin administered prior to endotoxin-induced acute lung injury. Male mice were divided into 5 groups and intraperitoneally injected with LPS (10 mg/kg), LPS plus atorvastatin (10 mg/kg/day; A + LPS group), LPS plus pravastatin (5 mg/kg/day; P + LPS group) or LPS plus simvastatin (20 mg/kg/day; S + LPS group). The control group received saline. All mice were sacrificed one day later. There were fewer leukocytes in the P + LPS and S + LPS groups than in the LPS group. MCP-1 cytokine levels were lower in the P + LPS group, while IL-6 levels were lower in the P + LPS and S + LPS groups. TNF-α was lower in all statin-treated groups. Levels of redox markers (superoxide dismutase and catalase) were lower in the A + LPS group ($p < 0.01$). The extent of lipid peroxidation (malondialdehyde and hydroperoxides) was reduced in all statin-treated groups ($p < 0.05$). Myeloperoxidase was lower in the P + LPS group ($p < 0.01$). Elastance levels were significantly greater in the LPS group compared to the statin groups. Our results suggest that atorvastatin and pravastatin but not simvastatin exhibit anti-inflammatory and antioxidant activity in endotoxin-induced acute lung injury.

1. Introduction

Acute lung injury and its most severe manifestation, acute respiratory distress syndrome, is a clinical syndrome defined by acute respiratory hypoxemia, bilateral pulmonary infiltrates consistent with edema and normal cardiac filling pressure [4]. Acute lung injury and acute respiratory distress syndrome are characterized by the leakage of a fluid rich in protein within the interstitium and alveolar space, an extensive release of cytokines and neutrophil migration [5]. A common cause of acute lung injury is systemic inflammatory response syndrome. Systemic inflammatory response syndrome is an imbalance of the immune response leading to the systemic release of proinflammatory cytokines, chemokines and vasoactive amines [6]. Lipopolysaccharide (LPS) has been implicated as an important inducer of lung injury and endotoxemia and is therefore used to induce acute lung injury in animal models [7,8]. The lungs are particularly vulnerable to inflammatory lesions because mediators are released into the circulation, and lungs receive the entire cardiac output. The accumulation of activated neutrophils in the lung is an initial step in the pulmonary inflammation that leads to acute lung injury and histological damage [9,10]. Neutrophils mediate lung injury by several mechanisms, including the release of reactive oxygen and nitrogen species, the production of cytokines and growth factors that can amplify the inflammatory response and the release of proteolytic enzymes [11,12].

Statins, which are inhibitors of the enzyme 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, are a class of drugs used to lower cholesterol levels in blood [13]. Recent reviews have identified pleiotropic properties of statins that go beyond their known lipid-lowering abilities, such as anti-inflammatory [14,15] and antioxidant [16,17] activities. It is speculated that the antioxidant effects of statins relate to their ability to inhibit the isoprenoid compounds produced by the mevalonate pathway and their ability to inhibit the activation of nicotinamide adenine dinucleotide phosphate [18]. In this report,

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http://dx.doi.org/10.1016/j.intimp.2013.05.016

our aim was to study the possible pleiotropic action of atorvastatin, pravastatin and simvastatin against lung oxidative stimulation caused by LPS. The method of choice in this study was i.p. administration of LPS to create a model of secondary acute lung injury, where the stimulus originated outside of the lung and affected this organ by a systemic route. In addition to redox markers, we analyzed inflammation and pulmonary function.

2. Materials and methods

2.1. Animals

Male C57BL/6 mice, 8 to 10 weeks old, were bred and maintained under standard conditions in the animal facility of the Institute of Biomedical Science, Federal University of Rio de Janeiro (Rio de Janeiro, Brazil). All of the procedures were in accordance with international guidelines and Brazilian law (the "Arouca" Law) for the use of animals (Law 11,794 from 10/08/2008), and this study received prior approval from the animal ethics committee of the Federal University of Rio de Janeiro (DFBCICB046). During the experiment, the animals were maintained under controlled temperature and humidity (21 ± 2 °C, 50 ± 10%, respectively) and were subjected to 12 h light/dark cycles. During the experimental procedures, the animals received standard chow and water ad libitum. This experimental design was repeated twice (20 mice per group).

2.2. Experimental design

All biochemical reagents were purchased from Sigma (Saint Louis, MO, USA) unless otherwise specified. Mice received atorvastatin (10 mg/kg/day i.p.; [19]), pravastatin (5 mg/kg/day i.p.; [20]), simvastatin (20 mg/kg/day i.p.; [21]) or saline (i.p.) for three days. On the third day, mice received LPS (10 mg/kg i.p.). The control group received saline (i.p.). These doses were sufficient to fully inhibit HMG Co-A reductase according to cited references. One day after LPS or saline injection, the mice were killed. Mice were randomly selected for biochemical (n = 10 per group) or histological (n = 5 per group) analyses. This experimental design was repeated for pulmonary mechanics (n = 5 each group, see Section 2.8). The groups were Control, LPS, A + LPS (atorvastatin), P + LPS (pravastatin) and S + LPS (simvastatin). A previous experiment (n = 5 per group) was performed with only statins and saline (i.p.), and no histological changes were observed (data not shown). Additionally, serum alanine aminotransferase or glutamic pyruvic transaminase activity was determined using standard spectrophotometric procedures according to the manufacturer's protocol (Katal Biotechnology, Belo Horizonte, Minas Gerais, Brazil), as a control for liver toxicity following statin treatment (Fig. 1).

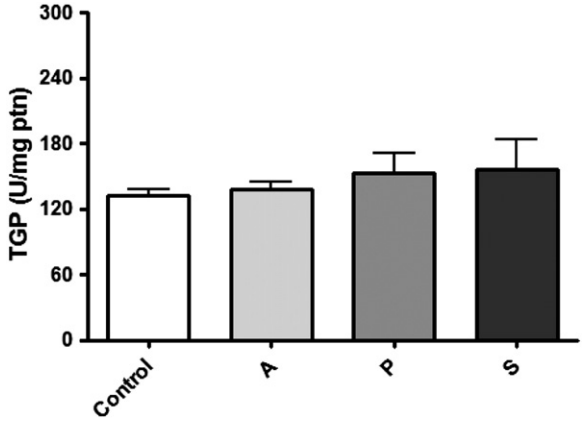

Fig. 1. Serum glutamic pyruvic transaminase (GPT) activity as a control for liver toxicity following statin treatment. Data are the means ± SEM (n = 5 for each group).

2.3. Bronchoalveolar lavage

The lung air spaces were washed three times with buffered saline solution (500 μL) for a final bronchoalveolar lavage (BAL) fluid volume of 1.2–1.5 mL. The collected BAL fluid was stored on ice. The total number of cells in the BAL fluid was determined using a Neubauer chamber. After BAL, the lungs (n = 10 per group) were removed immediately, homogenized on ice with 10% (w/v) 0.1 M potassium phosphate buffer (pH 7.4) using a tissue homogenizer (Nova técnica homogenizer model NT136, Campinas, São Paulo, Brazil) and centrifuged at 800 g for 5 min. The supernatants were stored at −20 °C for biochemical analysis. The protein concentrations in the lung homogenate samples were determined by the Bradford method [22].

2.4. Histopathology

Twenty-four hours after LPS administration, a group of mice (n = 5 per group) was killed. After a midline thoracotomy, the trachea was cannulated, and the lungs were fixed by the instillation of 0.5 mL of buffered formalin (10%) at a pressure of 18-22 cmH_2O for 1–2 min. The trachea was then ligated, and the lungs were immersed in fixative solution for 48 h. The organs were embedded in paraffin, sliced (5 μm) and stained with H&E. Polymorphonuclear (PMN) and mononuclear (MN) cells in alveoli were counted by morphometry at 1000× magnification across 10 random non-coincident microscopic fields in two sections per animal. Acute lung injury was scored in ten fields from non-identified sections of each group according to the following four criteria: alveolar congestion, hemorrhage, the infiltration of leukocytes in lung tissue, and the thickness of the alveolar wall/hyaline membrane formation [1]. Each criterion was graded according to a 5-point scale: 0, minimal (little) damage; 1, mild damage; 2, moderate damage; 3, severe damage; and 4, maximal damage. Two investigators performed all of the measurements by counting blinded sections.

2.5. Redox markers

Superoxide dismutase (SOD) activity was assayed by measuring the inhibition of adrenaline auto-oxidation by absorbance at 480 nm [23]. Catalase (CAT) activity was measured by the rate of decrease in hydrogen peroxide concentration at 240 nm [24]. As an index of lipid peroxidation, we used the thiobarbituric acid-reactive substances (TBARS) method for analyzing malondialdehyde products during an acid-heating reaction, as previously described by Draper and co-workers [25]. Briefly, samples from lung homogenates were mixed with 1 mL of 10% trichloroacetic acid and 1 mL of 0.67% thiobarbituric acid; the samples were then heated in a boiling water bath for 30 min. TBARS levels were determined by absorbance at 532 nm and expressed as malondialdehyde equivalents (nmol/mg protein). Lipid hydroperoxide content was assayed with xylenol orange [26]. Briefly, aliquots of 10–25 μL of the homogenates were incubated at room temperature for 30 min in a medium containing 0.25 mM $FeSO_4$, 25 mM H_2SO_4, and 0.1 mM xylenol orange. Blanks contained all components without supernatant. The final sample volume was 1.5 mL. After incubation, the absorbance at 580 nm was measured. Carbonyl groups of proteins were determined as previously described [27]. The tissue samples were mixed with 0.25 mL of 40% trichloroacetic acid (TCA) (final TCA concentration 20%) and centrifuged (5000 g, 5 min, 20 °C). The protein carbonyl content was measured in the resulting pellets by reaction with 10 mM 2,4-dinitrophenylhydrazine, leading to the formation of dinitrophenylhydrazones. Myeloperoxidase activity was measured using hydrogen peroxide, HTAB, and TMB. Initially, 100 μL of each BAL sample was centrifuged with 900 μL of HTAB at 14,000 g for 15 min. The supernatant (75 μL) was incubated with 5 μL of TMB for 5 min at 37 °C. The mixture was then incubated with 50 μL of hydrogen peroxide for 10 min at 37 °C, after which 125 μL of sodium acetate buffer was added. The reaction absorbance was measured at

630 nm [28]. The concentration of myeloperoxidase (MPO) in the samples was determined using a standard curve established using purified MPO. Nitrite, a byproduct of NO metabolism, was measured using the Griess reaction [29]. BAL samples were reacted with 50 μL of 1% sulfanilamide solution for 10 min and mixed with 50 μL of 0.1% naphthyl ethylenediamine solution. The formation of the stable azo compound with a purple color was measured spectrophotometrically by absorbance at 540 nm. The method was standardized with known concentrations of nitrite. Lactate dehydrogenase (LDH) activity in BAL was determined by monitoring the LDH-catalyzed oxidation of pyruvate coupled with the reduction of NAD using a commercial kit from Katal Biotechnology and following the manufacturer's instructions.

2.6. ELISA

Samples of BAL from mice were used for the quantification of MCP-1, IL-6 and TNF-α, which were measured using ELISA kits (R&D, Minneapolis, MN, USA).

2.7. RNA extraction and quantitative real-time PCR

Lung tissue fragments were collected, snap frozen, and stored at −80 °C in RNAlater (Qiagen, Valencia, CA, USA). Total RNA was isolated using the RNeasy tissue kit (Qiagen). Single-stranded cDNA was synthesized using the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. Quantitative real-time PCR was performed using a 7300 real-time PCR System (Applied Biosystems), and the threshold cycle numbers were determined using the RQ Study Software (Applied Biosystems). The reactions were performed in triplicate, and the threshold cycle numbers were averaged. The 50 μL reaction mixture was prepared as follows: 25 μL of Platinum SYBR Green Quantitative PCR SuperMix-UDG (Invitrogen Life Technologies, Alameda, CA, USA), 10 μmol/L of each primer (Table 1) and 10 μL of cDNA (100 ng). The reaction was performed with a preliminary uracil–DNA glycosylase treatment for 2 min at 50 °C and a denaturation step for 2 min at 95 °C, followed by 45 cycles of denaturation at 95 °C for 15 s, annealing for 15 s, and primer extension at 72 °C for 15 s. This process was followed by melting point analysis of the double-stranded amplicons, consisting of 40 cycles of 1 °C decrements (15 s each) from 95 °C. The first derivative of this plot, d*F*/d*T*, is the rate of change of fluorescence in the reaction, and a significant change in fluorescence accompanies the melting curve of the double-stranded PCR products. A plot of –dF/dT vs. temperature displays these changes as distinct peaks. Thus, SOD, CAT, MCP-1 and IL-6 expression were examined and normalized to a constitutive gene (HPRT-1), and the relative fold induction was calculated according to the formula $2^{(-\Delta\Delta Ct)}$ [30].

Table 1
The primers used in quantitative real-time PCR.

Gene	Primer	Sequence (5'→3')
SOD	Sense	TCAATGGTGGGGGACATATT
	Antisense	GCTTGATAGCCTCCAGCAAC
CAT	Sense	CCTCGTTCAGGATGTGGTTT
	Antisense	TCTGGTGATATCGTGGGTGA
MCP-1	Sense	ATTCTCCACACCCTGTTTCG
	Antisense	GATTCCTGGAAGGTGGTCAA
IL-6	Sense	CCGGAGAGGAGACTTCACAG
	Antisense	TCCACGATTTCCCAGAGAAC
HRPT-1	Sense	GCTACAGCTTCACCACCACA
	Antisense	TCTCCAGGGAGGAAGAGGAT

SOD, superoxide dismutase; CAT, catalase; MCP-1, monocyte chemotactic protein-1; IL-6, interleukin 6; HRPT-1 — hypoxanthine phosphoribosyl-transferase 1.

2.8. Pulmonary mechanics

Mice were sedated with diazepam (1 mg, i.p.), anesthetized with pentobarbital sodium (20 mg/kg BW, i.p.), tracheotomized, and a snugly fitting cannula (0.8 mm i.d.) was introduced into the trachea. The animals were then paralyzed with pancuronium bromide (0.1 mg/kg, i.v.) and mechanically ventilated with a constant-flow ventilator (Samay VR15, Universidad de la Republica, Montevideo, Uruguay) with a respiratory frequency of 100 breaths/min, tidal volume of 0.2 mL, flow of 1 mL/s, and positive end-expiratory pressure of 2 cmH_2O. The anterior chest wall was then surgically removed.

A pneumotachograph (15 mm i.d., length 4.2 cm, distance between side ports = 2.1 cm) [31] was connected to the tracheal cannula for the measurements of airflow (V'). Lung volume (V_T) was measured by flow signal integration. The pressure gradient across the pneumotachograph was determined using a Validyne MP45-2 differential pressure transducer (Engineering Corp., Northridge, CA, USA). The flow resistance of the equipment (R_{eq}), tracheal cannula included, was constant up to flow rates of 26 mL/s and was equal to 0.12 $cmH_2O\ mL^{-1}\ s$. Equipment resistive pressure ($=R_{eq} \cdot V'$) was subtracted from pulmonary resistive pressure so that the results represent intrinsic values. Tracheal pressure was measured with a Validyne MP-45 differential pressure transducer (Engineering Corp. Northridge, CA, USA). All signals were conditioned and amplified in a Beckman type R Dynograph (Schiller Park, IL, USA). Flow and pressure signals were passed through 8-pole Bessel low-pass filters (902LPF, Frequency Devices, Haverhill, MA, USA) with the corner requency set at 100 Hz, sampled at 200 Hz with a 12-bit analog-to-digital converter (DT2801A, Data Translation, Marlboro, MA, USA), and stored on a microcomputer. All data were collected using LABDAT software (RHT-InfoData Inc., Montreal, QC, Canada).

Lung resistive (ΔP1) and viscoelastic/inhomogeneous (ΔP2) pressures, total resistive pressure drop (ΔPtot = ΔP1 + ΔP2), static elastance (Est), and viscoelastic component of elastance (ΔE) were measured by the end-inflation occlusion method [32,33]. Briefly, after end-inspiratory occlusion, there is an initial rapid drop in transpulmonary pressure (ΔP1) from the pre-occlusion value down to an inflection point (Pi), followed by a slow pressure decay (ΔP2) until a plateau is reached. This plateau corresponds to the elastic recoil pressure of the lung (Pel). ΔP1 selectively reflects airway resistance in normal animals and humans and ΔP2 reflects stress relaxation, or viscoelastic properties of the lung, together with a small contribution of time constant inequalities at the peripheral airspaces [34]. Lung static elastance (Est) was calculated by dividing Pel by the tidal volume. ΔE was calculated as the difference between static and dynamic elastances and reflects the viscoelastic component of elastance.

2.9. Statistical analyses

All data are presented as the means ± standard error of the means, and after testing normality by the Kolmogorov–Smirnov test, the significance of differences was analyzed by one-way ANOVA followed by Tukey's post-hoc test, with $p < 0.05$. The unique exception was the data from the inflammatory score, which was tested by the Kruskal–Wallis test followed by Dunn's multiple comparison tests ($p < 0.05$). The software GraphPad Prism 5 was used for statistical analysis (GraphPad Prism version 5.0, San Diego, CA, USA).

3. Results

3.1. Effects of statins on lung histology

Histological analysis was scored (Table 2) and revealed that control mice had intact alveolar septa and normal alveoli (Fig. 2). The LPS group showed areas of alveolar septal edema, congestion and

Table 2
The effects of statins on the inflammatory scores of mouse lungs.

Groups	Alveolar congestion	Hemorrhage	Infiltration of leukocytes	Alveolar wall thickness
Control	0	0	0.4 ± 0.16	0.2 ± 0.13
LPS	3.7 ± 0.33***	2.6 ± 0.26***	4.3 ± 0.15***	2.9 ± 0.17***
A + LPS	0.5 ± 0.16###	0.9 ± 0.17#	1.0 ± 0.21###	0.9 ± 0.27##
P + LPS	0.8 ± 0.24##	1.1 ± 0.23	1.7 ± 0.21#	0.7 ± 0.21##
S + LPS	0.9 ± 0.23#	0.5 ± 0.16###	2.5 ± 0.34	1.8 ± 0.41

Acute lung injury was scored in ten fields from non-identified sections of each group according to the following four criteria: alveolar congestion, hemorrhage, infiltration of leukocytes in lung tissue, and thickness of the alveolar wall/hyaline membrane formation. Each criterion was graded according to a 5-point scale: 0, minimal (little) damage; 1, mild damage; 2, moderate damage; 3, severe damage; and 4, maximal damage. The statistical analyses were performed with the Kruskal–Wallis test followed by Dunn's multiple comparison test, with a significance level of 5%. *** $p < 0.001$ compared with the control group. # $p < 0.05$, ## $p < 0.01$ and ### $p < 0.001$ when compared with the LPS group.

hemorrhage without parenchymal collapse. Many inflammatory cells were observed in the alveoli (Fig. 2b). The A + LPS group showed areas of alveolar septal edema, but to a lesser extent than the LPS group, and some degree of bleeding without congestion and without parenchymal collapse. Few inflammatory cells were observed in the alveoli (Fig. 2c). The P + LPS group also showed areas of alveolar septal edema, again to a lesser extent than the LPS group, some degree of bleeding, and no congestion or collapse of the parenchyma. Inflammatory cells were again observed in the alveoli (Fig. 2d). The S + LPS group showed small areas of alveolar septal edema compared to the LPS group but did not show hemorrhage, congestion or collapse of the parenchyma. Few inflammatory cells were observed in the alveoli (Fig. 2e). Among the groups treated with statins, the most normal histology was observed in mice pretreated with pravastatin followed by pretreatment with atorvastatin. The simvastatin group, despite having a better histology than the LPS group, showed the weakest protective effect.

3.2. The effects of statins on inflammatory markers

Inflammatory markers are shown in Table 3. Bronchoalveolar lavage was used to evaluate the alveolar leukocyte count as an independent measure of alveolar inflammation. In animals injected with LPS, total leukocytes increased 83% compared with control animals ($p < 0.01$). Pre-treatment with pravastatin reduced the number of leukocytes by 44% ($p < 0.001$), and pre-treatment with simvastatin reduced the number of leukocytes by 39% ($p < 0.05$) compared to the LPS group. We did not observe a significant reduction in leukocyte numbers in the group treated with atorvastatin. Polymorphonuclear (PMN) and mononuclear (MN) cells in alveoli were counted in lung

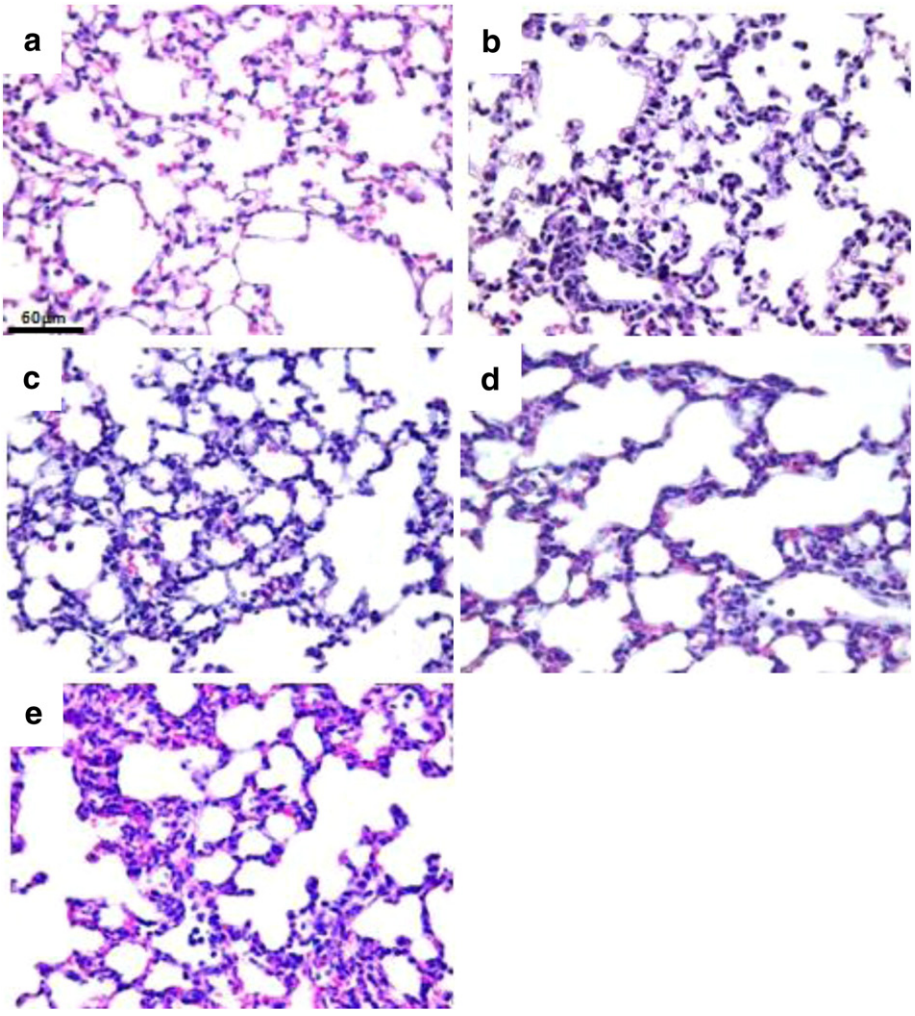

Fig. 2. Lung parenchyma HE-stained images. a) control group with intact alveolar septa and normal alveoli; b) LPS group with areas of alveolar septal edema, congestion and hemorrhage without parenchymal collapse; c) A + LPS with areas of alveolar septal edema and some degree of bleeding, without congestion and parenchymal collapse; d) P + LPS group with areas of alveolar septal edema and some degree of bleeding, without congestion and collapse of the parenchyma; e) S + LPS group with small areas of alveolar septal edema compared to the LPS group and without hemorrhage, congestion or collapse of the parenchyma.

Table 3
The effects of statins on inflammatory markers.

Groups	Control	LPS	A + LPS	P + LPS	S + LPS
Leukocytes ($\times 10^5$/mL)	3.40 ± 0.17	6.24 ± 0.65**	5.37 ± 0.49	3.52 ± 0.27##	3.85 ± 0.52#
MCP-1 (ng/mL)	0.26 ± 0.04	2.57 ± 0.39***	1.80 ± 0.16	0.69 ± 0.09##	3.17 ± 0.45
MCP-1 mRNA (fold)	0.69 ± 0.21	2.12 ± 0.52**	0.88 ± 0.30##	1.22 ± 0.53	0.93 ± 0.43#
IL-6 (ng/mL)	5.47 ± 0.41	9.76 ± 1.27**	8.75 ± 0.44	5.10 ± 0.66##	5.86 ± 0.68#
IL-6 mRNA (fold)	0.56 ± 0.14	1.52 ± 0.21*	0.66 ± 0.23##	0.76 ± 0.12#	0.51 ± 0.16##
TNF-α (ng/mL)	6.01 ± 0.46	13.67 ± 0.95***	8.86 ± 0.66##	7.27 ± 0.67###	6.04 ± 0.42###
TNF-α (fold)	0.52 ± 0.21	1.08 ± 0.21*	0.62 ± 0.33#	0.77 ± 0.24	0.87 ± 0.33

The data are expressed as the means ± SEM. MCP-1, IL-6 and TNF-α mRNA expression were examined, and the relative fold induction was normalized to a constitutive gene (HPRT-1). The statistical analyses were performed using a one-way ANOVA followed by Tukey's post-hoc test with a significance level of 5%. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared with the control group. # $p < 0.05$, ## $p < 0.01$ and ### $p < 0.001$ when compared with the LPS group. ELISA was performed on BAL samples ($n = 10$). qPCR was performed on lung homogenates ($n = 5$).

sections with a 100x objective lens (Fig. 3). The number of MN cells in the LPS group was 90% higher than that in the control group. The number of MN cells was reduced in the P + LPS (52%) group compared to the LPS group. The number of PMN cells in the LPS group was 20 times higher than that in the control group (Fig. 3). The number of PMN cells was reduced in the A + LPS (24%), P + LPS (46%) and S + LPS (31%) groups when compared with the LPS group. When we examined the amount of MCP-1 in the lung after LPS treatment, we observed an approximate 10-fold increase ($p < 0.001$), while we observed an almost 2-fold increase for IL-6 and TNF-α ($p < 0.01$ and $p < 0.001$, respectively) compared to the control group. Pre-treatment with atorvastatin reduced the amount of TNF-α by 34% ($p < 0.01$) compared to the LPS group. Pre-treatment with pravastatin reduced the amount of MCP-1 by 74% ($p < 0.01$), while the reductions observed for IL-6 and TNF-α were 48% ($p < 0.01$) and 47% ($p < 0.001$), respectively, compared to the LPS group. Pre-treatment with simvastatin reduced the amount of IL-6 and TNF-α by 41% ($p < 0.05$) and 55% ($p < 0.001$), respectively, compared to the LPS group. MCP-1 gene expression increased 207% ($p < 0.01$), while IL-6 and TNF-α increased by 171% ($p < 0.05$) and two-fold ($p < 0.05$), respectively, compared to the control group. Atorvastatin and simvastatin show a 59% ($p < 0.05$) and 57% ($p < 0.01$) reduction in MCP-1 gene expression, respectively, when compared to LPS. Atorvastatin, pravastatin and simvastatin also reduced the gene expression of IL-6 by 57% ($p < 0.01$), 50% ($p < 0.05$) and 67% ($p < 0.01$), respectively, when compared to LPS. Only atorvastatin reduced the expression of the TNF-α gene by 42% ($p < 0.05$) when compared to LPS.

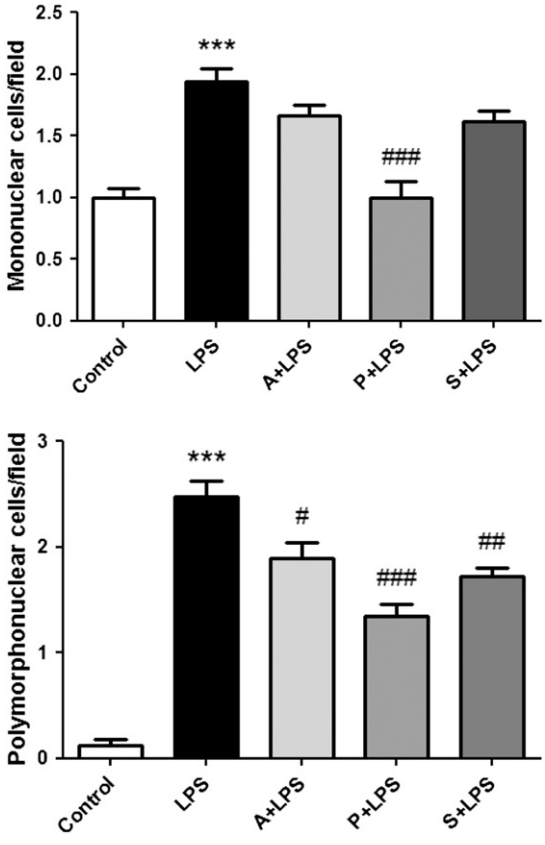

Fig. 3. Morphometry was performed by counting mononuclear (MN) and polymorphonuclear (PMN) cells/field in two different sections. Two investigators counted blinded sections. The number of MN cells in the LPS group was 90% higher than that in the control group. The number of MN cells was reduced in the P + LPS (52%) group when compared with the LPS group. The number of PMN cells in the LPS group was 20 times greater than that in the control group. The number of PMN cells was reduced in the A + LPS (24%), P + LPS (46%) and S + LPS (31%) groups when compared to the LPS group. The data are expressed as the means ± SEM. The statistical analyses were performed by one way ANOVA followed by Tukey's post-hoc test, with a significance level of 5%. *** $p < 0.001$ compared with the control group. # $p < 0.05$, ## $p < 0.01$ and ### $p < 0.001$ when compared with the LPS group. ($n = 5$ per group).

3.3. The effects of statins on redox markers

Redox markers are shown in Table 4. The activity of SOD increased in the LPS group by 83% ($p < 0.01$) when compared to the control group. Only treatment with atorvastatin significantly reduced (40%, $p < 0.05$) SOD activity compared to the LPS group. We observed an approximately 7-fold ($p < 0.01$) increase in the expression of the superoxide dismutase (SOD) gene in the group injected with LPS compared to the control group. However, none of the statins reduced the expression of SOD compared to the LPS group. The activity of CAT increased in the LPS group by 60% ($p < 0.001$) compared to the control group. Only pre-treatment with atorvastatin reduced (41%, $p < 0.01$) CAT activity compared with LPS. With regard to catalase (CAT) gene expression, we observed an increase of approximately 7-fold ($p < 0.05$) in the LPS group over the control group. Pre-treatment with pravastatin and atorvastatin reduced CAT gene expression by 77% ($p < 0.01$) and 53% ($p < 0.05$), respectively, compared to the LPS group. Malondialdehyde was increased by 80% ($p < 0.01$) in the LPS group compared to the control group. Pre-treatment with atorvastatin, pravastatin and simvastatin reduced the levels of malondialdehyde by up to 41% ($p < 0.05$, $p < 0.05$ and $p < 0.01$, respectively) compared with the LPS group. Hydroperoxide levels increased in the LPS group by 81% ($p < 0.01$) compared with the control group. Pre-treatment with atorvastatin, pravastatin and simvastatin reduced these levels by up to 38% ($p < 0.01$, $p < 0.05$ and $p < 0.01$, respectively) compared with the LPS group. We also observed an increase of 145% ($p < 0.05$) in the levels of protein carbonylation for the LPS group compared to the control group. None of the statins reduced protein carbonylation, although atorvastatin had a tendency to decrease carbonylation (34%) compared to the LPS group. The MPO activity in the BAL was used as a marker of acute lung injury related to the accumulation of neutrophils. We observed that mice injected with LPS showed an increase in MPO activity of

Table 4
Effects of statins on redox markers.

Groups	Control	LPS	A + LPS	P + LPS	S + LPS
SOD (U/mg protein)	1.80 ± 0.11	3.34 ± 0.11**	2.04 ± 0.13##	3.27 ± 0.29	3.22 ± 0.25
SOD mRNA (fold)	0.80 ± 0.22	5.31 ± 0.39**	5.18 ± 1.51	4.30 ± 1.72	4.91 ± 1.46
CAT (U/mg protein)	7.81 ± 0.30	12.56 ± 0.67***	7.48 ± 0.85##	11.54 ± 0.81	12.22 ± 0.81
CAT mRNA (fold)	0.16 ± 0.02	0.93 ± 0.47*	0.22 ± 0.06##	0.44 ± 0.15#	0.63 ± 0.18
MDA (nM/mg protein)	8.26 ± 0.96	14.87 ± 0.92**	10.12 ± 0.61#	9.70 ± 0.80#	8.83 ± 0.95##
LOOH (nM/mg protein)	0.027 ± 0.002	0.049 ± 0.004**	0.029 ± 0.002##	0.030 ± 0.002#	0.027 ± 0.001##
Carbonyl (nM/mg protein)	0.011 ± 0.001	0.027 ± 0.002*	0.018 ± 0.002	0.048 ± 0.007	0.052 ± 0.004
MPO (mU/mg protein)	6.43 ± 0.86	23.95 ± 3.09***	18.85 ± 0.79	13.67 ± 0.99##	25.63 ± 1.89
Nitrite (mM/mg protein)	7.95 ± 0.94	12.94 ± 0.78*	14.83 ± 1.76	12.50 ± 0.95	18.70 ± 1.60
LDH (U/mg protein)	74.48 ± 29.98	301.80 ± 45.58**	187.70 ± 19.02	199.60 ± 35.78	357.55 ± 67.77

SOD, superoxide dismutase; CAT, catalase; MDA, malondialdehyde equivalents; LOOH, hydroperoxides; MPO, myeloperoxidase; LDH, lactate dehydrogenase. The data are expressed as the means ± SEM. SOD and CAT mRNA expressions were examined, normalized and the relative fold induction was calculated to a constitutive gene (HRTP-1). The statistical analyses were performed by one way ANOVA followed by Tukey's post-hoc test, with a significance level of 5%. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared with the control group. # $p < 0.05$ and ## $p < 0.01$ when compared with the LPS group. MPO, nitrite and LDH were measured in BAL; other markers were measured in lung homogenates. N = 10 for all analyses except for qPCR, which was performed in lung homogenates with n = 5.

283% ($p < 0.001$) compared with the control group. Pre-treatment with pravastatin reduced MPO activity by 44% ($p < 0.01$) compared to the LPS group. There was no significant difference in MPO activity compared to the other statins. The amount of nitrite in the BAL was analyzed here as an indirect indication of nitric oxide. We observed a 63% increase ($p < 0.05$) in nitrite levels in the LPS group compared to the control group. None of the statins reduced nitrite levels compared with LPS. The activity of lactate dehydrogenase (LDH) was higher (300%, $p < 0.01$) in the LPS group than in the control group. However, none of the statins significantly reduced the activity of this enzyme, despite the fact that atorvastatin (35%) and pravastatin (34%) presented a downward trend compared to the LPS group.

3.4. Effects of statins on pulmonary function

Respiratory function parameters were determined 24 hours after injection of LPS or vehicle (Table 5). We observed that the LPS group showed an increase in static elastance ($p < 0.01$), total airway resistance ($p < 0.05$) and total pressure of the respiratory system ($p < 0.05$) compared with the control group. Treatment with atorvastatin ($p < 0.05$) and pravastatin ($p < 0.01$) reduced static elastance compared to the LPS group, while pre-treatment with simvastatin had no effect. Total airway resistance and total pressure of the respiratory system were not altered by pre-treatment with statins compared to the LPS group, although pre-treatment with atorvastatin and to a lesser extent with pravastatin shows a tendency to decrease these parameters.

4. Discussion

Lipopolysaccharide is a constituent of the cell wall of gram negative bacteria, contributing to inflammation and systemic toxicity [35]. Experimental models using endotoxins such as LPS have been used to study acute lung injury [36]. Endotoxemia induced by intraperitoneal administration of LPS leads to lung injury and neutrophil activation, and this situation causes free radical production [4]. The redox imbalance caused by an increase in free radicals and a reduction in the antioxidant system may cause tissue damage [37]. According to our study, statins possess pleiotropic effects beyond their lipid-lowering properties, such as antioxidant and anti-inflammatory activity.

In the present study, we observed an increase in leukocytes in the bronchoalveolar lavage fluid of the LPS group compared to the control, and this increase was reduced by pre-treatment with pravastatin and simvastatin but not atorvastatin. We also observed the increased levels of MCP-1, IL-6 and TNF-α in the same groups. Pravastatin reduced the levels of both MCP-1 and IL-6 but only induced changes in IL-6, while not affecting MCP-1 or TNF-α expression. Simvastatin reduced the levels of IL-6 and also reduced the gene expression of MCP-1 and IL-6; these data are in concordance with Jacobson et al. [38]. Atorvastatin did not cause changes in the levels of these cytokines but reduced their expression, with the exception of TNF-α. Interestingly all statins reduced TNF-α levels. Thus, statins exhibit anti-inflammatory actions, possibly by influencing endothelial cells through the increased expression of β4 integrin [39], the inhibition of endothelial activation, i.e., the inhibition of NFκB activation [40], the inhibition of ICAM-1 expression [41], or the inhibition of cytokine (IL-6) and chemokine (MCP-1) production to prevent leukocyte adhesion to the endothelium [42]. The increased gene expression of MCP-1 and IL-6 as well as the increased levels of these inflammatory mediators are found in acute coronary syndrome [43]. It should be noted that the key hallmark of acute lung injury is damage to the blood barrier (pulmonary vascular endothelium and alveolar basement membrane), where neutrophils adhere to the wall of injured endothelium and migrate into the airspace [44]. Pravastatin, which has microvascular benefits and anti-inflammatory effects, has been shown to reduce leukocyte adhesion and macromolecular extravasation in the rat mesentery 2–4 h after LPS-induced endotoxemia [45]. Pruefer and coworkers published a paper demonstrating the effect of simvastatin on the interaction between leukocytes and endothelial cells by intravital microscopy in vivo. They concluded that simvastatin inhibited leukocyte rolling, adhesion and transmigration in acute inflammatory states and that this effect could be related to the downregulation of P-selectin expression on endothelial cells [46]. We found no reduction in the level of BAL leukocytes in the atorvastatin-treated

Table 5
Effects of statins on pulmonary function.

Groups	Control	LPS	A + LPS	P + LPS	S + LPS
Est,L (cm H2O/mL)	24.70 ± 1.62	34.56 ± 1.84**	26.11 ± 1.23#	23.46 ± 1.37##	33.12 ± 1.32
Rtot,L-(cm H2O/mL)	0.67 ± 0.04	0.90 ± 0.04*	0.69 ± 0.06	0.80 ± 0.09	0.84 ± 0.06
DPtot,L (cm H2O/mL)	0.65 ± 0.04	0.90 ± 0.04*	0.69 ± 0.06	0.64 ± 0.08	0.91 ± 0.03

Est,L — static elastance; Rtot,L — total resistance of the airways; DPtot,L — total pressure of the respiratory system. The data are expressed as the means ± SEM. The statistical analyses were performed by one way ANOVA followed by Tukey's post-hoc test, with a significance level of 5%. * $p < 0.05$ and ** $p < 0.01$ compared with the control group. # $p < 0.05$ and ## $p < 0.01$ when compared with the LPS group. n = 5 for all.

group, although the literature shows the opposite [47]; we did observe fewer polymorphonuclear cells in lung sections. In another study, performed by Gaugler and colleagues with cultured endothelial cells from the lungs of humans, pravastatin (1-1000 μM final concentration) was administered to limit the inflammatory response and endothelial thrombosis after irradiation. The researchers observed an inhibition of the excessive production of MCP-1, IL-6 and IL-8 [48]. Different families of statins may have different biochemical functions even within the same class, and there is a dose-response effect that can differentiate the therapeutic potential of different statins [49]. One example is the work of Iwata and colleagues, who used two types of statins, a lipophilic (pitavastatin) and a hydrophilic (pravastatin) statin, and examined the effects of these statins on cytokine production in human bronchial epithelial cells stimulated by LPS [50]. The expression of IL-6 and IL-8 were significantly inhibited by both statins, and this inhibition was removed when mevalonate was added, suggesting that their anti-inflammatory effects may be connected to the mevalonic cascade. In a study of lung injury due to ischemia and reperfusion, Wu and co-workers observed damage to the alveolar cell type II and consequently impaired lung repair of alveolar cell transdifferentiation in alveolar cell type I. They used *in vitro* human ATII (A549) and mouse (MLE-12) cells, which were subjected to hypoxia and re-oxygenation. Pre-treatment with simvastatin reduced the apoptosis of these cells and the increased proliferation and expression of surfactant proteins. In the experimental model of ischemia/reperfusion, rats were treated with simvastatin and also showed increased proliferation of ATII cells in vivo [2]. Finally, a recent study from Wu and co-workers investigated the effect of simvastatin *in vitro* on alveolar macrophages from human volunteers. The cells were incubated with lipoteichoic acid, a component of the cell wall of gram-positive bacteria (Staphylococcus aureus) and treated with simvastatin. The results showed that ATL induced a potent pro-inflammatory and pro-apoptotic state, and simvastatin exerted anti-inflammatory effects by mediating the inhibition of NFκB activation and cytokine expression in human alveolar macrophages [3].

We observed increased MPO in the LPS group compared to the control group. This finding suggests that the observed accumulation of leukocytes in BAL fluid was most likely derived primarily from neutrophils. In support of this hypothesis, Suda et al. found abundant BAL neutrophils after endotoxin-induced acute lung injury [51]. Pre-treatment with pravastatin caused a significant reduction of MPO. A previous study reported the protective effects of pravastatin in acute lung injury induced by LPS with effects similar to those observed here. The group treated with pravastatin showed a significant reduction not only in lung vascular leakage induced by LPS but also in cellular infiltration in the lung tissue, including a reduction in MPO activity [52]. In our study, we did not observe any change in this marker from prior administration of atorvastatin or simvastatin. Contrasting results were observed by Grommes et al. [53] with respect to the simvastatin treatment, which may be explained by the duration of treatment and the administration route.

Leukocyte activation can increase the production and release of free radicals, causing redox imbalance in the lung. If the antioxidant system of the lung does not provide adequate protection, oxidative stress conditions occur with possible damage to tissues. Thus, we investigated the activity and expression of the antioxidant enzymes SOD and CAT as well as markers of oxidative damage (malondialdehyde, carbonyl, hydroperoxides and lactate dehydrogenase) in lung homogenate. The group that was stimulated with LPS showed increased expression of SOD and CAT. No difference in SOD gene expression was observed in groups treated with statins. However, we observed a reduction in CAT in groups treated with atorvastatin or pravastatin but not simvastatin. All statins reduced MDA levels (marker of lipid peroxidation) with simvastatin having the strongest activity; these data are in accordance with those of Altintas et al. [54]. Only the group treated with atorvastatin exhibited decreased SOD and CAT activity as well as reduced oxidative damage, as determined by hydroperoxide levels. None of the statins reduced lactate dehydrogenase levels, perhaps because this enzyme has an extremely short half-life. Finally, nitrite was not altered with any statin treatment compared to the LPS group. Thus, the pharmacological properties of each statin may produce different antioxidant activities. The duration of pretreatment can also affect each drug's antioxidant properties. We suggest that the oxidative stress observed in this study is partially due to the increased number of leukocytes in the LPS group. Therefore, a reduction in leukocytes could directly cause a reduction in redox markers. A survey of 114 patients with dyslipidemia who were treated with statins was performed to evaluate the activities of antioxidant enzymes (CAT, SOD and GPx) in erythrocytes. After treatment, which lasted from 4 to 12 weeks, there was a significant increase in antioxidant enzyme activity in patients who received atorvastatin and simvastatin. Therapy with pravastatin only impacted CAT activity; there were no effects on GPx and SOD [55]. We did not observe the same results in our study: only the group receiving atorvastatin showed a decrease in the activity of these enzymes when compared with LPS.

Endotoxin-induced LPA is associated with changes in respiratory function, indicated by reduced dynamic compliance and increased lung resistance. Mechanical changes have been attributed to pulmonary edema followed by a deficit in gas exchange and airway constriction [56]. Our findings show that the groups treated with pravastatin and atorvastatin reduced lung elasticity compared to the LPS group, and there was a trend toward a reduction in airway resistance in the atorvastatin group and in respiratory system pressure for the pravastatin group. No positive results were observed with simvastatin. Interestingly, a previous report in patients pre-treated with simvastatin with acute lung injury and under mechanical ventilation did not show any effect on pulmonary function [57].

The pravastatin group achieved better results than the atorvastatin group with regard to inflammation markers (leukocyte counts, the production of MCP-1, IL-6 and myeloperoxidase). Moreover, the histopathological profile with pravastatin was better than the atorvastatin group when both were compared to LPS. In this study, we found that atorvastatin exhibited the best results in terms of enhanced antioxidant enzyme (SOD and CAT) activity in addition to a reduction in markers of oxidative damage (MDA and hydroperoxide). We suggest that the mechanism by which atorvastatin can influence lung elasticity is by reducing alveolar edema, given atorvastatin's capacity to act as an antioxidant, but more research is needed to strengthen this suggestion. The differences between classes of statins and their pharmacokinetic characteristics, such as liver metabolism and half-life associated with the dose and duration of drug use, are features that should be taken into account in future studies.

In this study, we used 3 different statins: atorvastatin, which is lipophilic and synthetic; pravastatin, which is hydrophilic and fermentation derived and simvastatin, which is lipophilic and fermentation derived. Neither the lipophilicity nor the origin of the statins was associated with oxidative stress or the reduction of inflammation in the acute lung injury groups.

In conclusion, we suggest, after histological, biochemical and functional analysis of our endotoxin-induced acute lung injury model, that pravastatin is the best anti-inflammatory therapy, while atorvastatin is the best antioxidant therapy. Simvastatin showed the least pleiotropic activities.

Acknowledgements

The authors thank all undergraduate and graduate students from LABCOM (ICB-UFRJ) involved with biochemical analyses in this study. The authors declare no conflicts of interest.

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