ORIGINAL ARTICLE

Simvastatin improves survival and reduces leukocyte recruitment and hepatocyte apoptosis in endotoxin-induced liver injury

ABSTRAKT

BACKGROUND. Endotoxemia provokes excessive host response to bacteria or microbial compounds, resulting in systemic inflammation, organ injury and mortality.

Aim: This study examined the effects of simvastatin on survival and liver injury in endotoxic shock in a rat model of endotoxemia.

METHODS. Male Wistar rats were injected intraperitoneally with *E.coli*-lipopolysaccharide (LPS) and the medial lethal dose (LD_{50}) was determined. Simvastatin was given orally (5-40 mg/kg body weight) for five days prior to either a single LD_{50} dose of LPS or 2, 2.5 or 3x LD_{50} . Liver damage was assessed by histological examination and expressed as a tissue damaged score (TDS). Another group of rats was treated with simvastatin and then challenged with LPS to determine the degree of apoptosis in hepatocytes, liver immune cells, and cleaved caspase-3 activity after 24h of endotoxemia.

RESULTS. Endotoxemia caused substantial mortality with associated leukocyte infiltration, liver injury (TDS = 3.67, SD = 0.55), as well as notable apoptosis of hepatocytes and resident liver macrophages. Simvastatin, in a dose-dependent manner significantly reduced LPS-induced mortality, hepatocellular damage (TDS = 1.5, SD = 0.55), inflammatory infiltration, and drug markedly decreased apoptosis and expression of cleaved caspase-3.

CONCLUSIONS. Simvastatin improves survival in endotoxic shock and prevents endotoxemic liver injury by inhibiting leukocyte infiltration and hepatocellular apoptosis. These results suggest that simvastatin could be used to prevent endotoxemia-associated liver dysfunction by simvastatin.

KEY WORDS

simvastatin, liver injury, endotoxin, inflammation, apoptosis

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Sepsis is a leading cause of death among critically ill patients. Despite extensive investigation over the past three decades, the incidence of sepsis and sepsis-related deaths is increasing.¹ Endotoxin or lipopolysaccharide (LPS), the dominant portion of the outer membrane of Gram-negative bacteria, activates the inflammatory cells and increases production of pro-inflammatory cytokines including tumor necrosis factor alpha (TNF- α , and other factors, such as nitric oxide (NO)).²⁻⁴ Excessive production of pro-inflammatory mediators, by the host in response to LPS challenge results in systemic inflammation, tissue injury and organ failure, events that are strongly associated with septic shock.⁵ The relative severity of sepsis depends upon

the balance between pro-inflammatory and anti-inflammatory states. Liver failure is an insidious problem for critically ill patients. Numerous studies suggest that hepatic infiltration of leukocytes as a cause of endotoxemic liver damage.⁶⁻⁷ The recruitment process of leukocytes in venules is a multistep process, involving adhesion of rolling leukocytes via a selectin-mediated mechanism,^{8,9} although leukocyte trapping in hepatic sinusoids occurs without of this interaction.¹⁰ Contact between the lymphocyte function antigen-1 (LFA-1) and a family members of adhesive molecules expressed on endothelial cells firmly establishes leukocyte adhesion in the liver microvascular endothelium in endotoxemic mice.¹¹ Transendothelial migration and tis-

sue accumulation of leukocytes in the liver depend upon the formation of chemokines in hepatocytes. Once established within the extravascular parenchyma, these accumulated leukocytes promote hepatocellular apoptosis, resulting ultimately in liver failure.

Statins, or 3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors have had a major impact on healthcare by decreasing cardiovascular events. The efficacy of statins has been attributed primarily to their lipid-lowering properties. However, a growing body of evidence highlights statin actions independent of its lipid-lowering properties.^{12, 13} These include anti-inflammatory and antioxidant effects. As a result, recently issued, a guideline recommends that patients with diabetes and cardiovascular disease should initiate statin therapy regardless of baseline LDL cholesterol levels.¹⁴ Retrospective and prospective observational studies indicate that that statin treatment reduces the incidence and mortality of sepsis, although not all investigators agree.¹⁵ Prospective clinical trials are currently evaluating the safety and efficacy of statins in septic patients. Along with these observational studies in humans,¹⁵ mouse models of sepsis and endotoxemia also indicate the protective actions of statins, which reportedly reduce mortality, preserve cardiac function, ameliorate inflammation and improve bacterial clearance.^{5, 16} It is not yet known if these beneficial effects will obtain as well in endotoxemic liver injury.

We used a well-known rat model for endotoxemia to determine if oral simvastatin, could improve survival in endotoxic shock and prevent endotoxin-induced leukocyte recruitment, hepatocellular apoptosis and liver injury.

Materials and Methods

Animals. Experiments were performed on male Wistar rats, 6 - 8 weeks old (180 to 220 g body weight - b.w) bred at the Farm for Experimental Animals, Military Medical Academy, Belgrade, Serbia, and kept in the animal unit 7 days before the experiment. They were housed in plastic cages, under standard laboratory conditions (21 - 22°C, 12 h light/dark cycle, 30 - 70% relative humidity). They were supplied with commercial food and tap water *ad libitum*. The animals were deprived of food 18 - 20 h before beginning of experiments with free access to tap water. Experimental groups consisted of six animals each. The study protocol was based on the Guidelines for Animal Study no. 282-12/2002 (Ethics Committee of the Military Medical Academy, Belgrade, Serbia).

Pharmacological Interventions. Simvastatin (Krka, Novo Mesto, Slovenia) was dissolved in 0.5% methylcellulose (Sigma, Taufkirchen, Germany), as 10 or 20 mg/mL stocks. Endotoxin from *E. coli* serotype 0127:B8 (Sigma Aldrich, Germany) was injected intraperitoneally (i.p.) after dilution with sterile pyrogen-free physiologic saline solution, in a volume of 1 mL/kg.

Endotoxin-induced lethality in rats. The animals were divided into three groups ($n=6$ per group), given saline orally (p.o.) and challenged i.p with one of the three doses of LPS (10, 20, 30 mg/kg b.w.). The lethality and changes in body temperature were then monitored over the next 7 days. Mortality resulting from LPS was recorded and the median lethal dose (LD_{50}) of LPS i.p. was calculated.¹⁷

Protective effects of of simvastatin on mortality induced by lethal dose of endotoxin. To establish the effect of short-term pretreatment of simvastatin on the survival of rats injected with LD_{50} of LPS, the animals were divided into four groups ($n=6$ per group); simvastatin was given p.o. in one of the four doses (5, 10, 20 and 40 mg/kg b.w, respectively) per day for 5 days. One and a half h after the last dose of simvastatin the single LD_{50} of LPS was injected i.p. Control group was treated with saline for 5 days, before a single LD_{50} of LPS was injected. The rates of survival of animals and changes in body temperature were monitored for a period of 7 days.

The doses of simvastatin in our study were comparable to those that had previously been used in rat/murine studies *in vivo* (typically 10 - 100 mg/kg/day); however, they were higher than those used in humans because of a significant up-regulation (3- to 8-fold) of HMG-CoA reductase induced by statin treatment in rodents.¹⁸ We also investigated protective efficacy of the drug on survival rates. Simvastatin (5 - 40 mg/kg) was administered p.o. for five days prior to a single absolute lethal dose of LPS (2x, 2.5x or 3x LD_{50} of LPS). The Litchfield&Wilcoxon procedure¹⁷ was used to calculate LD_{50} dose of LPS in the simvastatin pretreated group.

The protection index (PI) was calculated as a ratio of LD_{50} of LPS in simvastatin pretreated group to LD_{50} of LPS. We calculated the effective dose (ED_{50}) of orally administered simvastatin by the same procedure.¹⁷ The ED_{50} is defined as the amount of drug that produces a therapeutic response in 50% of treated subjects.

Histopathological examination. Tissue sections from rat livers were stained with haematoxylin and eosin (H&E). Random fields from each specimen were magnified 20x and viewed with an Olympus-2 microscope (Tokyo, Japan).

Semiquantitative analysis. The type, degree and severity of tissue lesions along with the number of inflammatory cells were assessed in tissue samples from each animal and they were counted in six separate visual fields under 40x magnification. The severity of liver lesions (tissue damage score or TDS) was determined according to a 5-point semi-quantitative scale based on the number of inflammatory cells, haemorrhages, edema, and the number of foci involved. Grade "0" indicated normal findings, while grade "5" indicated pronounced plasmolysis and cariolytic, along with massive hemorrhagic foci and polymorphonuclear cell (PMNC) infiltration.

Immunohistochemical determination of apoptosis-regulating Cleaved Caspase-3. Liver samples from control and treated groups were compared 24h after the animals were treated. The control group was treated with a single LD₅₀ dose of LPS; the treatment group received simvastatin (20 mg/kg) for five days prior to administration of a single LD₅₀ dose of LPS. Paraffin-embedded sections of liver tissue were stained with a polyclonal rabbit antibody to cleaved caspase-3 (Thermo Scientific Fischer Ab-4, RB 1197-R7, USA), according to the manufacturer's instructions. Diaminobenzidine tetrahydrochloride was used to develop the antigen-antibody complex, and all slides were counterstained with H&E, dehydrated, and mounted. Appropriate positive controls were processed in parallel. For each liver section, random visual fields (100x or 200x) were evaluated, and immunopositive cells were assessed for antibody staining (negative, weak, moderate, strong) as a reflection of caspase-3 expression.¹⁹

Statistical analysis. All data are reported as the mean $\pm$ standard deviation (SD), and groups were compared by non-parametric statistical tests (t-test, Mann-Whitney, Kruskal-Wallis rank test). The median lethal dose of endotoxin (LD₅₀ of LPS), the effective dose 50% (ED₅₀) of simvastatin and the protective index were calculated by the Lichfield and Wilcoxon procedure,¹⁷ and 95% confidence intervals were derived. Differences with values of $P < 0.05$ were considered significant.

Results

Determination of LD₅₀ of LPS and protective dose of simvastatin. Table 1 shows that LPS increased lethality of rats in a dose-dependent manner. The LD₅₀ of i.p. LPS was calculated to be 22.15 (95% CI 16.5-29.1) mg/kg.

40 mg/kg of simvastatin ($p = 0.04$). All three doses of LPS induced significant hypothermia ($< 35^\circ$) compared to baseline body temperature within 6 h after challenge ($p < 0.05$). These findings closely predicted lethality within the first 24 hours. Body temperature returned to normal after 24 hours in animals that survived. We did not observe significant changes in body temperature in rats treated with 20 and 40 mg/kg of simvastatin. Simvastatin alone had no effect on body temperature (data not shown).

Protection index and anti-inflammatory effective dose of simvastatin in endotoxic shock. We established the protective efficacy of orally administered 20 mg/kg simvastatin against lethal doses of LPS (2x, 2.5x, 3x LD₅₀). Simvastatin improved survival up to 67% ($p < 0.05$) in animals treated with 2x LD₅₀ of LPS (Table 2). We calculated that the LD₅₀ of LPS increased significantly to 46, 34 (37, 86-56, 74) mg/kg in the simvastatin pretreatment group and determined that the PI = 2.09.

To test the protective effect of statin against a single lethal dose of LPS (2xLD₅₀), we pretreated animals with 5, 10, 20, 40 mg/kg of simvastatin. The highest dose (40 mg/kg) completely prevented mortality (Table 2), while survival obtained with 20 mg/kg of simvastatin was significantly less ($p < 0.05$) (Table 2). The ED₅₀ was 14.14 (22, 41 - 8, 93) mg/kg.

Effects of simvastatin on liver histology in endotoxic shock. Since leukocyte recruitment is a rate-limiting step in endotoxin-induced liver injury, we analyzed hepatic leukocyte infiltration and liver architecture in the randomly selected liver sections. As seen in Figure 1B; a single dose of LD₅₀ of LPS induced severe liver damage characterized

Table 1. Median lethal dose (LD50) of LPS and effect of simvastatin on mortality.

Simvastatin (mg/kg/day p.o.) ^b	LPS (mg/kg, i.p.) ^a	No. of rats (dead/total)	LD ₅₀ of LPS (95% CI) (mg/kg i.p)
None	10	0/6	22.15 (16.5-29.1)
	20	3/6	
	30	4/6	
5	LD ₅₀	3/6	-
10		1/6	
20		0/6	
40		0/6	

^aMale Wistar rats were divided into three groups (n=6 per group), injected i.p. with LPS (10, 20, 30 mg/kg b.w.) i.p. Survival was measured over the 7 days. The dose of LPS that was lethal to 50% of the rats (LD₅₀) was 22.15 mg/kg (95% CI 16.5-29.1). Rats were divided into three groups (n=6 per group) and treated orally with simvastatin (5, 10, 20, or 40 mg/kg/day) for 5 days and then with a single i.p dose of LPS (LD₅₀). Survival of animals was measured over 7 days.

Various doses of simvastatin given prior to LPS challenge resulted in dose-dependent increase in survival. Table 1 shows that complete protection was achieved with 20 and

by sinusoidal hyperemia, vasodilatation and prominent perivascular accumulation of PMNCs. Also, it can be seen a few foci of subcapsular hepatocytes necrosis with PMNC

Table 2. Simvastatin improves survival in rats challenged with lethal doses of LPS. The LD₅₀ of LPS in simvastatin group and protective index (PI) for oral simvastatin (20 mg/kg) was established by a five-day pretreatment regimen. One set of rats was divided into three groups (n=6 per group); the animals were challenged i.p with a single lethal dose of LPS (2x, 2,5x or 3x LD₅₀).

Simvastatin (mg/kg/day, p.o.)	LPS (mg/kg, i.p.)	No. of rats (dead/total)	LD ₅₀ of LPS (95% CI) (mg/kg, i.p.)	PI / ED ₅₀ (95% CI) (mg/kg, p.o.)
20	2 x LD ₅₀	2/6	46, 34 ^a (37, 86 - 56, 74)	2.09 ^a
	2,5 x LD ₅₀	4/6		
	3 x LD ₅₀	6/6		
5	2 x LD ₅₀	6/6	-	14.14 (22, 41 - 8,93) ^b
10		6/6		
20		2/6		
40		0/6		

^a Next, to determine effective dose (ED₅₀) of orally administered simvastatin, another set of animals was divided into four groups (n=6 per group) and simvastatin was given in doses of 5, 10, 20 or 40 mg/kg/day for five days prior to LPS challenge (2x LD₅₀). ^b Survival rate in both sets of animals was assessed over 7 days. The Lichfield & Wilcoxon procedure was used to calculate the LD₅₀ of LPS in simvastatin group, PI and ED₅₀ of simvastatin.

infiltration. Most of the hepatocytes (> 50% cells) showed pronounced vacuolisation of cytoplasm and nucleoplasm, and pycnotic nuclei. Severity of liver injury induced by LD₅₀ of LPS is estimated as TDS of 3.67 (SD = 0.55) (Table 3).

Pretreatment with 20 mg/kg of simvastatin significantly inhibited these pathological changes concomitant with reduction of inflammatory infiltration. (Figure 1C). We noted increased mitotic activity of hepatocytes (mostly with pathological nuclei), vasodilatation and decreased liver damage in sections from animal pretreated with 40 mg/kg of simvastatin. The reduction of inflammatory infiltrate was approximately 50% greater than with 20 mg/kg of simvastatin, suggesting that the protective and anti-inflammatory effects are dose-dependent. Simvastatin (40 mg/kg) significantly decreased TDS compared to that of the untreated group ($p < 0.001$) (Table 3).

Immunohistochemical detection of cleaved caspase-3. Because hepatocellular apoptosis is a hallmark of endotoxin-induced liver damage, we assessed liver cell apoptosis in stained tissue sections and measured cleaved caspase-3 immunohistochemically. As expected, we found that the calculated LPS LD₅₀ increased apoptosis of centrilobular hepatocytes, which can be identified by moderate cytoplasmic staining (Figure 2A and 2B). Moderate nuclear and/or cytoplasmic staining of cleaved caspase-3 was also detected in resident liver macrophages located within the sinusoidal spaces (Kupffer cells). Notably, administration of simvastatin decreased endotoxin-induced apoptosis of macrophages as well as hepatocytic cell death by attenuating expression of cleaved caspase-3. (Figure 2C), which is stained immunohistochemically for cleaved caspase-3, shows only weakly stained immune cells and negative or unstained hepatocytes.

Table 3. Effects of simvastatin on the tissue damage score (TDS) in LPS induced liver injury. Effects of oral simvastatin pretreatment (10, 20 and 40 mg/kg) on TDS were evaluated 7 days after a single dose of LPS (LD₅₀) and compared to TDS in group treated with LPS alone or control group. The TDS was determined in six randomly selected visual fields (40x) from tissue sections of each liver. TDS was graded on a scale of 0–5, based on the amount of inflammatory cells, hemorrhages and oedema as well as the number of foci involved.

Drugs	TDS (6 liver samples x 6 sections)					Values are mean (SD)
	0	1	2	3	4	
Control ¹	36	0	0	0	0	0
LD ₅₀ LPS	0	0	0	13	23	3.67 (0.55) ²
Simvastatin, 10 mg/kg	0	0	12	24	0	2.67 (0.52) ³
Simvastatin, 20 mg/kg	0	12	14	10	0	2.00 (0.89) ^{3,4}
Simvastatin, 40 mg/kg	0	15	21	0	0	1.5 (0.55) ⁴

§ ¹Saline (1 mL/kg). The differences in TDS between groups were statistically analyzed using the Kruskal–Wallis rank test and results were expressed in X (SD) ² $p < 0.001$ versus control, ³ $p < 0.05$ versus control, ⁴ $p < 0.001$ versus LD₅₀ LPS.

Figure 1. Simvastatin protects against pathological changes in the liver induced by LPS.

Representative section from each group of six rats (haematoxylin and eosin staining, magnification 20x). **A** = Control (untreated) rat. **B** = LPS challenged rat (LD_{50}) showing sinusoidal hyperemia, prominent infiltration of PNMC, local necrotic foci, with moderate disorganization of liver architecture. **C** = Pretreatment with simvastatin (20 mg/kg, orally) before the single LD_{50} of LPS; note the significant reduction in inflammatory infiltrates compared to **B**.

Figure 2. Immunohistochemical detection of liver cell apoptosis in endotoxic shock.

A. Cytoplasmic staining for cleaved caspase-3 in hepatocytes from rats treated with single LD_{50} of LPS (x10 magnification); **B.** Nuclear and/or cytoplasmic staining for cleaved caspase-3 in Kupffer cells from rats treated with single LD_{50} of LPS (x20 magnification). **C.** Weak staining of Kupffer cells and unstained hepatocytes from rats pretreated with simvastatin 20 mg/kg before the single LD_{50} of LPS (x20 magnification).

Discussion

We find that orally administered simvastatin, in doses comparable to those used in clinical practice, significantly improves survival in a rat model of endotoxemic shock and protects the animals from endotoxin-induced liver injury. Pretreatment with simvastatin markedly reduced intrahepatic infiltration of leukocytes as well as hepatocellular apoptosis in endotoxemic rats.

Statins have potent anti-inflammatory actions that appear to be independent of their effect on cholesterol metabolism.^{11,13,15,16} These drugs are reported to decrease sepsis-induced mortality, although the protective mechanisms remain elusive.^{20,21} Several studies in a murine model of endotoxic shock suggest that simvastatin improves survival and protects against endotoxin-induced multiple organ injury, including kidney and liver failure.^{5,7,22} Various studies confirm that statins suppress release of pro-inflammatory cytokines (TNF- α , IL-1, IL-6),^{4,22} stimulate production of anti-inflammatory biomarkers (IL-10, NO)^{5,22} and decrease markers of organ injury associated with endotoxic shock.^{5,7,22}

Current research indicates that statins may protect against endotoxin-induced hepatotoxicity via a HMG-CoA reductase-dependent mechanism. For example, Slotta et al.⁷ found that co-administration of mevalonate with simvastatin almost completely reversed the protective effect of the statin on endotoxin-induced liver injury, suggesting participation of HMG-CoA reductase-dependent pathways. HMG-CoA reductase-independent effects of statins on endotoxemic liver injury remain to be documented.

Leukocyte recruitment and extravascular accumulation are key components in both host-defense reactions and in organ injury. Because leukocyte recruitment is an early and rate-limiting step in endotoxin-induced liver injury,^{8,9} we examined the effect of simvastatin on intrahepatic infiltration of leukocytes in animals challenged with sublethal and lethal doses of endotoxin. The results, expressed as TDS, indicate that simvastatin significantly reduced endotoxin-provoked infiltration of leukocytes and deterioration of normal structure in a dose-related manner. This observation extends previous studies that reported attenuation by statins of leukocyte infiltration into the brain,²³ retina,²³ heart²², and synovium.²⁴ The data further indicate that leukocyte accumulation in the liver responds to treatment with statins. These drugs inhibit LFA-1, a key adhesion molecule that influences endotoxin-induced leukocyte recruitment and liver damage by binding to a regulatory site on LFA-1.²⁵ Moreover, statins also inhibit a number of critical points in endotoxin-induced leukocyte recruitment and tissue injury. These include the expression of specific adhesion molecules, such as P-selectin⁸ and intercellular adhesion molecule-1,⁹ as well as generation of hepatic chemokines.¹¹

Hepatocyte apoptosis is a prominent and important feature in endotoxemic liver injury, and over-production of TNF- α appears to trigger the extrinsic pathway of apoptosis.²⁶ These findings are in accord with our previous study,⁴ where we found increased production of TNF- α in endotoxemic rats. Hepatocyte apoptosis may be an end-stage of endotoxin-induced liver injury, but it may also signal early changes in endotoxemic hepatotoxicity. Fouzi and associates²⁷ found that activation of a membrane receptor Fas (from superfamily of TNF receptors) causes apoptosis and generates chemokines and inflammation in the liver. Furthermore, endotoxin may trigger apoptosis even before the onset of leukocyte recruitment.²⁸

In contrast, inhibition of chemokines and specific adhesion molecules expression decreases endotoxin-induced apoptosis via attenuation of PMNC infiltration. Together, these findings indicate that endotoxin-induced liver injury involves a complex interplay between inflammation, necrosis and apoptosis. In this escalating cascade, necrotic and apoptotic cells also enhance recruitment of leukocytes, while, in turn, accumulating inflammatory cells reinforce further hepatocellular apoptosis.⁸

In this study, we noted a significant increase in hepatocytes with characteristic nuclear morphological hallmarks of programmed cell death, such as nuclear pyknosis and karyorrhexis. These findings mirrored the increase in caspase-3 activity in the liver. Simvastatin treatment markedly reduced endotoxin-induced increases in caspase-3 activity and apoptosis of liver hepatocytes and macrophages. Slotta et al.⁷ reported that anti-apoptotic activity of simvastatin was reversed by co-injection of mevalonate. Their results suggest that the protective action of the statin depends upon HMG-CoA reductase regulation of programmed cell death in endotoxemic liver injury.

While we used endotoxin (LPS) to initiate acute, severe inflammation and endotoxic or septic shock, our rat model lacks an infection site commonly seen in clinical cases of endotoxic shock; it uses just one of many ways to activate the immune system. Our technique caused an acute, short-lived activation, not the extended inflammatory state where pro-inflammatory cytokines persist in circulation, as occurs with multiple organ injury. The animal species and the endotoxin dose (rodents are resistant to endotoxin and thus require higher doses than humans), as well as the dose and duration of simvastatin treatment, could also affect the results.²⁹

Nonetheless, for this particular model, oral simvastatin exhibits protective and anti-inflammatory effects. It inhibits endotoxin-provoked inflammatory infiltration and deterioration of the liver, including apoptosis of hepatocytes and macrophages, suggesting that therapy with simvastatin or other agents that directly target HMG-CoA reductase could protect the liver against inflammation.

No potential conflict of interest relevant to this article was reported.

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