Effect of simvastatin on proinflammatory cytokines production during lipopolysaccharide-induced inflammation in rats

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Abstract. The effect of simvastatin applied in a short-term pretreatment on proinflammatory cytokines production in acute systemic inflammation induced by endotoxin – lipopolysaccharide (LPS) in rats was investigated. Both LPS and simvastatin doses were established in separate experiments in which increasing doses of both compounds were given to obtain the LD_{50} LPS and the maximally protective dose of simvastatin against LD_{50} LPS. To determine the anti-inflammatory effect, simvastatin was given orally for 5 days, followed by a single intraperitoneal non-lethal dose of LPS (0.25 LD_{50}). Plasma concentrations of tumor necrosis factor alpha (TNF-α), interleukin (IL)-1β and IL-6 were measured by enzyme-linked immunosorbent assay. The acute i.p. LD_{50} LPS amounted to 22.15 mg/kg. Simvastatin of 20 mg/kg p.o. was maximally protective against LD_{50} LPS, and this dose was used for studying its effects on LPS-induced cytokines production. Cytokines concentrations were significantly increased upon challenge of non-lethal dose of LPS. The peak levels of TNF-α and IL-1β were significantly suppressed by simvastatin, compared to control rats only treated with dimethylsulfoxide before LPS. In contrast, simvastatin did not affect IL-6 levels at all timepoints. Simvastatin pretreatment given orally produced acute anti-inflammatory effects by inhibiting TNF-α and IL-1β, but no IL-6 production.

Key words: Simvastatin — Endotoxin — Inflammation — Cytokines — Rat

Introduction

Several clinical trials showed that statins reduce the risk of cardiovascular events even in the absence of a significant drop of blood cholesterol levels (Ridker et al. 1998; Davignon 2004), suggesting that the benefits of statin therapy may also be ascribed to their action on nonlipid factors involved in inflammation-fibroproliferation, an important feature of atherosclerosis (Marz and Wieland 2000; Bonetti et al. 2003). There is growing evidence that statins have additional anti-inflammatory properties by reducing inflammatory parameters such as C-reactive protein (Albert et al. 2001; Joukhadar et al. 2001), tumor necrosis factor α (TNF-α) and interleukin (IL)-1β in patients with hypercholesterolemia and heart transplant recipients (Holm et al. 2001; Koh et al. 2004), unrelated to their lipid-lowering activity. Moreover, recent studies have shown that prior statins therapy prevented vascular hyporeactivity during acute systemic inflammation in humans (Pleiner et al. 2004) and was associated with a reduction of severe sepsis development (Almog et al. 2004).

Statins are 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors, which reduce low-density lipoprotein (LDL) cholesterol levels by blocking the mevalonate pathway and increase LDL cholesterol receptor expression in the liver. By inhibiting HMG-CoA reductase, statins might directly influence the cellular events other than cholesterol synthesis, because mevalonate, the product of HMG-CoA reductase, is the precursor of not only cholesterol, but also of

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many non-steroidal isoprenoid compounds. The isoprenoids farnesyl pyrophosphate and geranylgeranyl pyrophosphate are known to play an important role in signal transduction pathways by their attachment to signalling proteins, such as Ras and Rho (Kwak et al. 2000; Cordle et al. 2005).

Inhibition of HMG-CoA reductase activity in monocytes (Terkeltaub et al. 1994) and rat mesangial cells (Kim et al. 1995) treated with lipopolysaccharide (LPS) and granulocyte-macrophage-colony stimulating factor reduced the production of IL-8, IL-6, and MCP-1, (monocyte chemotactic protein-1) responsible for leukocyte recruitment at the infection site. Statins have been shown as effective endothelium-protective agents that reduced leukocyte-endothelial cell interactions and improved endothelial function *via* increasing endothelial nitric oxide synthase (eNOS) (Laufs et al. 2002; Pruefer et al. 2002). Recently, using a common model of acute local inflammation (carrageenan-induced rat paw oedema), we also showed that simvastatin administered orally in the single dose produced significant anti-inflammatory activity (footpad swelling inhibition and reduction of polymorphonuclear leukocytes infiltration) comparable to that of indomethacin (Nežić et al. 2009). Study by Wagner et al. (2002) showed that acute pretreatment with atorvastatin inhibits TNF-α plus interferon-γ stimulated transcription factor activation in native endothelial cells *in situ* and the subsequent expression of inducible nitric oxide synthase (iNOS) involved in vascular inflammation and atherosclerosis. Also, cerivastatin administered intraperitoneally (i.p.) significantly improved the survival of mice with LPS-induced sepsis and reduced TNF-α, IL-1β and IL-6 overproduction (Ando et al. 2000; Chaudhry et al. 2008).

In the present study, we investigated if simvastatin, a widely used statin, when given orally to imitate the regular route of statins use in humans, may attenuate systematic inflammation by inhibition of proinflammatory cytokines production. The first part of this study consisted of an experiment examining both lethal and protective doses of LPS and simvastatin, respectively. The second part of the study presented *in vivo* experiments examining anti-inflammatory effects of simvastatin administered in short-term pretreatment prior to non-lethal, but inflammatory dose of LPS, on the pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) production.

Endotoxin or LPS is a component of the outer cell wall of Gram-negative bacteria. Systemic injection of LPS to experimental animals is a widely used *in vivo* model for the study of endotoxic shock and acute systemic inflammation. LPS activates the immune system leading to release of endogenous proinflammatory cytokines such as TNF-α, IL-1β and IL-6 (Ando et al. 2000; Dogan et al. 2002). The important feature of this model is that simvastatin given in short-term treatment does not affect plasma lipid levels (cholesterol-lowering activities takes at least two weeks of therapy), and therefore the results may be interpreted without this confounding variable (Rosenson 1999).

Materials and Methods

Animals

Adult male Wistar rats were obtained from Military Medical Academy Research Laboratories (Belgrade, Serbia) and kept in the animal unit 7 days before the experiment. Animals were given standard laboratory diet and tap water *ad libitum* and housed in an air-conditioned room with an ambient temperature of 22–24°C and a 12 h light/dark cycle (light on at 07:00 a. m.). At the start of the experiment, rats weighing 200–220 g were placed in individual cages. All experimental procedures on animals were conducted in accordance with the Guidelines on human care of experimental animals adopted by the Ethical Committee and other corresponding national legal codes.

Experimental design

Endotoxin-induced lethality in rats

The animals were divided into three groups (n = 6 per group), given saline orally (p.o.) and challenged with one of the three various doses of LPS (10, 20, 30 mg/kg b.w.) given i.p. The lethality was then monitored over the next 7 days. The number of dead rats resulting from LPS was recorded and the median lethal dose (LD_{50}) of LPS (i.p.) was calculated (Litchfield and Wilcoxon 1949).

Protective effect of simvastatin on lethality induced by endotoxin

To determine the effect of short-term pretreatment of simvastatin on the survival of rats injected with LD_{50} of LPS, the animals were divided into three groups (n = 6 per group); simvastatin was given to rats p.o. in one of the three various doses (5, 10, and 20 mg/kg b.w.) per day for 5 days, and 1.5 h after the last dose of simvastatin the single LD_{50} of LPS was injected i.p. The survival of animals was monitored for a period of the next 7 days. Simvastatin alone had no effect on the survival of the animals. The doses of simvastatin used were comparable to those that had previously been used in rat/murine studies *in vivo* (typically 10–100 mg/kg/day) and 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 (Kita et al. 1980; Sparrow et al. 2001; Leung et al. 2003).

Effect of simvastatin on proinflammatory cytokines production

In this experiment, simvastatin was used in a dose of 20 m/kg p.o. (the maximally protective dose of simvastatin against the single LD_{50} of LPS in rats). To induce acute systemic

inflammation and proinflammatory cytokines production, the animals were challenged with a non-lethal dose of endotoxin i.p. (0.25 LD_{50} of LPS).

The rats were randomized into the control and simvastatin-treated experimental groups ($n = 8$ per group). Simvastatin (20 mg/kg) was given p.o. *via* oral gavage for 5 days, and 1.5 h after the last dose of simvastatin, LPS (*Escherichia coli* serotype 0127:B8; Sigma Aldrich, Munich, Germany) at a non-lethal single dose (5.5 mg/kg b.w.) was i.p. administered. The control animals received the same volume of 0.1% dimethylsulfoxide (DMSO) for 5 days, as a vehicle, before LPS injection. The rats were restrained using a restraint tube and blood was collected by tail vein puncture at various time points (0, 60, 90, 120, 180, 240 min) after LPS injection. Blood samples were collected into Eppendorff tubes containing EDTA (ethylenediamine tetraacetic acid) solution and centrifuged (1000 × *g*, 20 min, 4°C). The plasma was stored at –70°C until assay. Plasma levels of TNF-α, IL-1β and IL-6 were determined using rat ELISA (enzyme-linked immunoassay) kits in accordance with to the manufacturer's recommended protocols (R&D Systems, UK). Sensitivity of detection was 20 pg/ml for cytokines.

Drugs

Simvastatin (donated by Krka, Novo Mesto, Slovenia) was dissolved in 0.1% DMSO as a 10 mg/ml stock. LPS from *E. coli* serotype 0127:B8 (Sigma Aldrich) was injected i.p. after dilution with sterile pyrogen-free physiologic saline solution, at an injection volume of 1 ml/kg.

Statistical analysis

The median lethal dose of endotoxin that was lethal to 50% of the rats (LD_{50} of LPS) was calculated by the Lichfield and Wilcoxon procedure (Lichfield and Wilcoxon 1949).

Differences in plasma TNF-α, IL-1β and IL-6 level between the simvastatin treated and the control animals were determined with nonparametric Mann-Whitney U-test and Kruscal-Wallis test. Results were expressed as mean ± SE (standard errors), $p < 0.05$ was considered significant.

Results

Determination of LD_{50} of LPS and protective dose of simvastatin

In Table 1, LPS dose-dependently increased lethality of rats during 7 days after injection is shown. The LD_{50} of LPS administered i.p. was calculated to be 22.15 mg/kg b.w.

The efficacy of various doses of simvastatin administered p.o. in the short-term treatment prior to LD_{50} of LPS challenge resulted in a dose-dependent increase in survival. As shown in Table 1, complete lethality protection was observed at dose of 20 mg/kg b.w. (p.o.) of simvastatin.

Effect of simvastatin on proinflammatory cytokines production

For the purpose of this part of the experiment we used a single dose of LPS (5.5 mg/kg b.w., i.p.) as a non-lethal dose (0.25 LD_{50} of LPS) to induce acute systemic inflammation. The dose of 20 mg/kg b.w. (p.o.) of simvastatin that protected the animals from LPS LD_{50} induced lethality was used to determine the effect of the drug on proinflammatory cytokines production induced by LPS.

All the animals survived this experiment. In addition, simvastatin itself neither increased serum levels of cytokines nor affected the clinical status and the behavior of rats, such as their dietary intake or body weight gain (data not shown).

TNF-α response

The time course of plasma TNF-α level

Intraperitoneal injection of LPS induced a marked increase in plasma TNF-α level. The plasma level increased at 60 min and peaked at 90 min after injection ($p < 0.002$) compared to baseline, with no significant difference in plasma TNF-α levels between these timepoints ($p = 0.52$), and thereafter

Table 1. The effect of simvastatin pretreatment on LPS-induced lethality in rats

Simvastatin pretreatment (mg/kg/day p.o)[b]	LPS (mg/kg i.p)[a]	No. of rats (dead/total)
none	10	0/6
	20	3/6
	30	4/6
5	LD_{50}	3/6
10		1/6
20		0/6

[a] Adult male Wistar rats were divided into three groups ($n = 6$ per group), challenged with one of the various doses of LPS (10, 20, 30 mg/kg b.w.) i.p. Lethality was monitored over the 7 days. The dose of LPS that was lethal to 50% of the rats, i.e. LD_{50} of LPS administered i.p. was calculated to be 22.15 mg/kg b.w. by the Litchfield and Wilcoxon procedure (Litchfield and Wilcoxon 1949). [b] Rats were divided into three groups ($n = 6$) and were given simvastatin p.o. in doses of 5, 10, and 20 mg/kg/day for 5 days, after which the single LD_{50} of LPS was administered i.p. Survival of animals was monitored for a period of the 7 days.

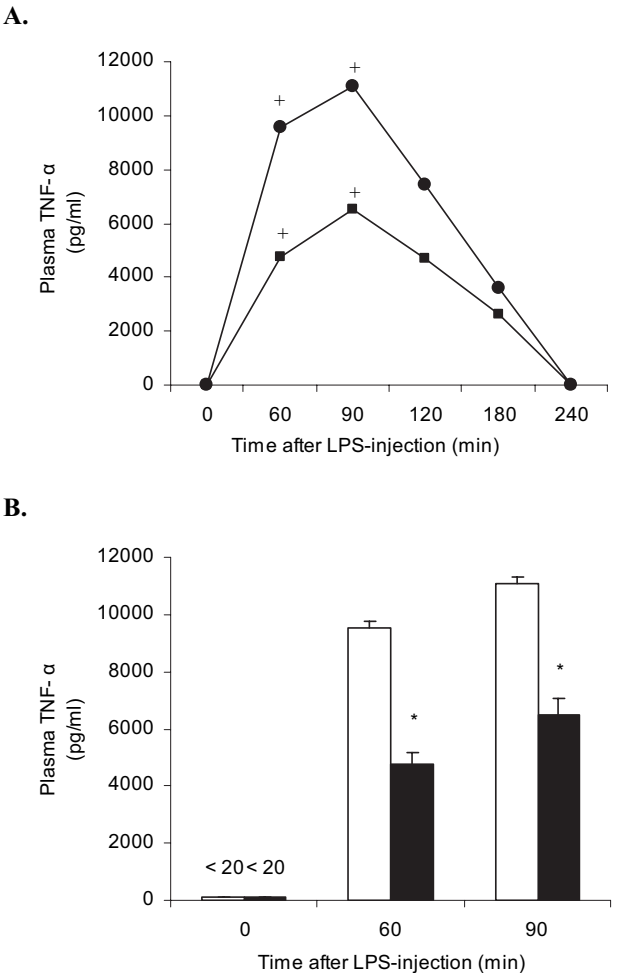

Figure 1. Time course of plasma TNF-α level in control (closed circle, vehicle + LPS) and simvastatin + LPS (closed quadrate)-treated rats. Animals (n = 8 per group) were given simvastatin (20 mg/kg/day p.o) 5 days prior to single dose of LPS (5.5 mg/kg i.p.). Plasma TNF-α was determined at 0, 60, 90, 120, 180 and 240 min after LPS. $^{+}$ $p < 0.002$ compared to baseline (panel A). Maximal inhibitory effect of simvastatin (20 mg/kg, p.o. given 5 days prior to a single dose of LPS of 5.5 mg/kg, i.p.) (black column) was shown at the peak plasma values of TNF-α at 60 and 90 min post LPS injection (values are mean ± S.E.M.). * $p < 0.05$ compared to the control group (white column; vehicle + LPS). The amounts of TNF-α in plasma (baseline) were bellow detection limit (<20 pg/ml) (panel B).

gradually decreased to near basal levels by 240 min (Figure 1, panel A).

The effects of simvastatin on LPS-induced plasma TNF-α elevation

As shown in Figure 1A, in rats pretreated with simvastatin prior to LPS injection, the plasma TNF-α levels significantly increased at 60 min and 90 min, post LPS injection ($p < 0.002$) compared to baseline, with no significant difference in TNF-α plasma levels between these timepoints ($p > 0.05$). *Post hoc* analysis revealed that simvastatin significantly reduced the plasma TNF-α levels compared to the control group, with maximal effects at 60 and 90 min ($p < 0.05$) (Figure 1, panel B).

IL-1β response

The time course of plasma IL-1β level

Plasma IL-1β levels increased significantly with peak value at 120 min after LPS injection ($p < 0.002$), remained elevated at 180 min ($p < 0.002$) and slightly decreased thereafter, but still present after 240 min timepoint (Figure 2, panel A).

The effects of simvastatin on LPS-induced plasma IL-1β elevation

As shown in Figure 2A, in rats pretreated with simvastatin prior to LPS injection, the plasma IL-1β levels significantly increased at 120 min and 180 min post LPS ($p < 0.002$), compared to baseline, with no significant differences in IL-1β levels after 120 min ($p > 0.05$). *Post hoc* analysis revealed that simvastatin significantly reduced the plasma IL-1β levels compared to the control group, with maximal effects at 120 min ($p < 0.01$) and 180 min ($p < 0.05$) (Figure 2, panel B).

IL-6 response

The time course of plasma IL-6 level

Plasma IL-6 levels increased significantly at 120 min ($p < 0.002$), peaked at 180 min and 240 min, after LPS injection ($p < 0.002$), with no significant difference in IL-6 levels between these timepoints, and remained elevated after 240 min (Figure 3, panel A).

The effects of simvastatin on LPS-induced plasma IL-6 elevation

As shown in Figure 3A, in rats pretreated with simvastatin prior to LPS injection, the kinetics of plasma IL-6 levels was similar to that of the control group, and significantly increased compared to baseline ($p < 0.05$). *Post hoc* analysis revealed that simvastatin had no significant effect on the plasma IL-6 levels compared to the control group at all timepoints (Figure 3, panel B).

Discussion

In the present study, we have shown that short-term pretreatment with simvastatin effectively prevented endo-

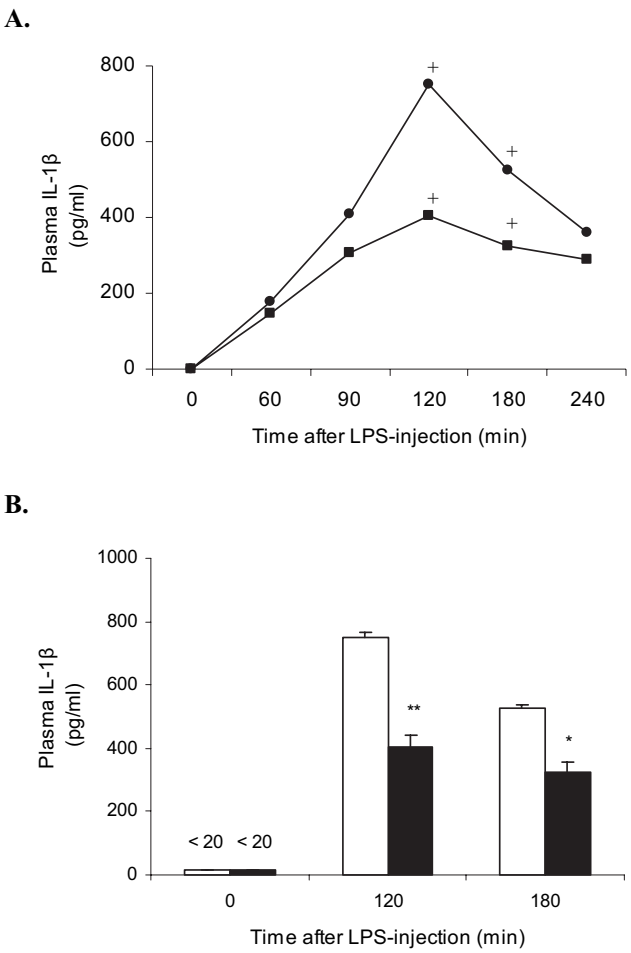

Figure. 2. Time course of plasma IL-1β level in control (closed circle, vehicle + LPS) and simvastatin + LPS (closed quadrate)-treated rats. Animals ($n = 8$ per group) were given simvastatin (20 mg/kg/day p.o) 5 days prior to single dose of LPS (5.5 mg/kg i.p.). Plasma IL-1β was determined at 0, 60, 90, 120, 180 and 240 min after LPS. $^{+}p < 0.002$ compared to baseline (panel A). Maximal inhibitory effect of simvastatin (20 mg/kg p.o. given 5 days prior to a single dose of LPS of 5.5 mg/kg i.p.) (black column) was shown at the peak plasma values of IL-1β at 120 and 180 min post LPS injection (values are mean ± S.E.M.). ** $p < 0.01$; * $p < 0.05$ compared to the control group (white column; vehicle + LPS). The amounts of IL-1β in plasma (baseline) were bellow detection limit (<20 pg/ml) (panel B).

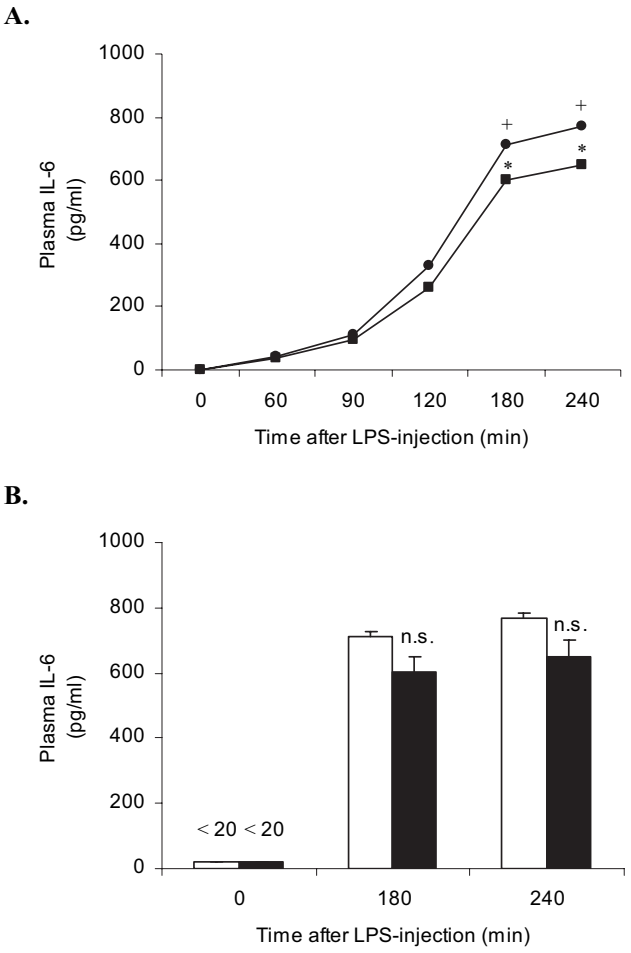

Figure. 3. Time course of plasma IL-6 level in control (closed circle, vehicle + LPS) and simvastatin + LPS (closed quadrate) treated rats. Animals ($n = 8$ per group) were given simvastatin (20 mg/kg/day p.o) 5 days prior to a single dose of LPS (5.5 mg/kg i.p.). Plasma IL-6 was determined at 0, 60, 90, 120, 180 and 240 min after LPS. $^{+}p < 0.002$; * $p < 0.05$ compared to baseline (panel A). Maximal inhibitory effect of simvastatin (20 mg/kg, p.o. given 5 days prior to a single dose of LPS of 5.5 mg/kg i.p.) (black column) was shown at the peak plasma values of IL-6 at 180 and 240 min post LPS injection (values are mean ± S.E.M.). n.s, non significant compared to the control group (white column; vehicle + LPS). The amounts of IL-6 in plasma (baseline) were bellow detection limit (<20 pg/ml) (panel B).

toxin-induced lethality in a dose dependent manner. This is consistent with the results from previous animal studies (Ando et al. 2000; Merx et al. 2004). Also, several clinical studies showed that statin usage was associated with reduced mortality from sepsis and/or bacterial infection in patients with atherosclerosis and present comorbities including diabetes mellitus, chronic renal failure, or a history of infections (Liappis et al. 2001; Almog et al. 2004; Hackam et al. 2006). However, mechanisms by which statins could prevent development of severe sepsis are not completely known yet. Gram negative sepsis is initiated by exposure to the LPS which induces overproduction of proinflammatory cytokines, which in turn up-regulate the expression of iNOS. Large amount of cytokines and nitric oxide contribute to LPS-induced hypotension, multiple organ failure and lethality. Recent study by Yasuda et al. (2006), revealed that short-term pretreatment with simvastatin improved sepsis-induced lethality and acute renal injury,

reversed microvascular perfusion defect with corresponding improvement in tissue oxygenation and also reduced a serum TNF-α. Cerivastatin also acutely protected mice against sepsis related death *via* reduced proinflammatory cytokines production and enhanced bacterial clearance (Chaudhry et al. 2008).

The results of our study demonstrated that a non-lethal dose of LPS challenge results in increased circulating concentrations and similar time courses of proinflammatory cytokines TNF-α, IL-1β and IL-6 as previously described (Ando et al. 2000; Dogan et al. 2002). In this *in vivo* model of acute systemic inflammation, we observed that short-term pretreatment with simvastatin administered orally inhibited LPS-induced secretion of TNF-α and IL-1β, both at their peaked time points following LPS administration. In contrast, simvastatin pretreatment failed to alter LPS-stimulated IL-6 secretion at any of the time points examined. Similar results have been shown in two experimental studies, where simvastatin given orally in lower dose (10 mg/kg) in short-time pretreatment significantly inhibited proinflammatory cytokines production in acute local and systemic inflammation (Diomede et al. 2001; Souza et al. 2006).

In several clinical trials, statins have shown anti-inflammatory effects; also on a short-time scale similar to ours (Holm et al. 2001; Plenge et al. 2002; Steiner et al. 2005). However, our results do not confirm that simvastatin completely modulate proinflammatory cytokines production, as IL-6 levels only tended to be nonsignificantly lower in the simvastatin group. This finding is in line with a previous clinical study that reported slight lowering effect of simvastatin on IL-6 and IL-1β production during endotoxemia (Pleiner et al. 2004). In addition, in a recent study Erikstrup et al. (2006) observed that in *in vivo* model of low-grade inflammation short-term treatment of simvastatin failed to inhibit endotoxin-induced increase in plasma levels of TNF-α, IL-6, IL-1β receptor antagonist, C-reactive protein or leucocytes count. There is a debate regarding the IL-6 response to statin therapy in the literature. Although some studies report a decrease (Rezaie-Majd et al. 2002), others report little (Kinlay et al. 2003) or no effect on IL-6 levels (Jialal et al. 2001). This is probably due to a great circadian variation in IL-6 levels and a high interindividual variability in IL-6 levels, which was also observed after LPS administration (Endler et al. 2004). The molecular mechanisms are unclear and this reflects some of the divergent results reported for statins effects on IL-6 production.

There are several potential mechanisms of acute anti-inflammatory actions of statins in this and similar models of inflammation. Statins, *via i*nhibition of mevalonate pathway, block the synthesis of isoprenoids, geranylgeranyl pyrophosphate and farnesyl pyrophosphate and facilitate accumulation of inactive Rho and Ras in the cytoplasm. Furthermore, statins have also been reported to inhibit nuclear factor kappa B (NF-κB) activation, a transcription factor of a signal transduction pathways that is activated in response to inflammatory stimuli, such as LPS, cytokines (e.g. IL-1β, TNF-α) or trough activated GTP binding proteins (Ras-Rho). In fact, upon stimulation NF-κB enters the nucleus where it can induce the transcription of inflammatory genes and numerous proinflammatory cytokines production (Beutler 2004; Seasholtz and Brown 2004; Abeles and Pillinger 2006). Also, it has been shown that anti-inflammatory effects of statins involves up-regulation of an inhibitor of NF-κB (I κBα), which traps NF-κB in the cytosol (Kleemann et al. 2004). The most frequently proposed model is that statins interrupt the proinflammatory signalling by down-regulation of Rho-related protein activation**,** that, in turn, requires posttranslational modification involving nonsterol mevalonate-derived compounds to be active (Seasholtz and Brown 2004; Abeles and Pillinger 2006). Increasing evidence suggests that statins modulate macrophage functions by inhibiting the activation of inflammatory response genes, such as IL-1β, IL-6, and TNF-α, metalloproteinase (MMP)-2, and MMP-9, and iNOS as a consequence of interference with the sGTP binding proteins/NF-κB transduction pathway (Takemoto and Liao 2001). Recent *in vivo* and *in vitro* studies have also revealed that statins could act in an inflammation *via* mechanisms involving peroxisome proliferator-activated receptor (PPAR) pathway. PPAR is a nuclear receptor, with three isoforms PPAR-α, PPAR-γ and PPAR-δ, which regulate lipid metabolism but also exert pronounced anti-inflammatory activities. It exerts anti-inflammatory activities by negatively interfering with proinflammatory signaling pathways including NF-κB. (Marx et al. 2004; Paumelle et al. 2006). It has been shown that statins, simvastatin and atorvastatin, reduce activity of iNOS, as well as IL-6 and TNF-α levels by increasing PPAR-α and PPAR-γ activities, respectively (Grip et al. 2002; Paumelle et al. 2006).

There are several limitations to our experimental design. We chose endotoxin as an inductor of endotoxic shock and inflammation, but this is just one of many ways to activate the immune system. Clinical endotoxic shock is more complex than can be modeled in these experiments. Patients may be subjected to a large bolus of endotoxin release from an infected site or even a continual release of endotoxin depending on the patient. Also it must be emphasized that we induced only an acute, short-lived activation and not an extended inflammatory state where proinflammatory cytokines circulating levels exist longer. The animal species, the dose of endotoxin, as well as the dose and duration of simvastatin treatment, could also affect the results. Although we have assessed potential effects on survival and only circulating parameters, we cannot exclude that statins might possess anti-inflammatory properties at the tissue or endothelial level.

It is important to emphasize that simvastatin, as shown in our study, could have protective and anti-inflammatory effects when administered orally, which is the route of administration of the drug in clinical practice.

In conclusion, we showed that simvastatin might be useful in prevention of LPS relating severe conditions providing preliminary evidence that circulating levels of proinflammatory cytokines were partly attenuated by simvastatin in an animal *in vivo* model of acute systemic inflammation. However, mechanisms behind the protective and anti-inflammatory properties afforded by short-term treatment of simvastatin and its implications in a clinical setting are not clear enough and warrant further investigation.

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