

# Short-term simvastatin treatment has no effect on plasma cytokine response in a human *in vivo* model of low-grade inflammation

C. Erikstrup,\* H. Ullum\*<sup>†</sup> and B. K. Pedersen\*

\*Centre of Inflammation and Metabolism, Department of Infectious Diseases and The Copenhagen Muscle Research Centre, Rigshospitalet and Faculty of Health Sciences, University of Copenhagen, Copenhagen, Denmark, and <sup>†</sup>Department of Clinical Immunology, Rigshospitalet, Copenhagen, Denmark

Accepted for publication 13 January 2006

Correspondence: Christian Erikstrup MD, The Department of Infectious Diseases M7641, Rigshospitalet, Blegdamsvej 9, 2100 Copenhagen, Denmark.

E-mail: erikstrup@dadlnet.dk

## Introduction

Statins or hydroxymethylglutaryl coenzyme A reductase inhibitors reduce plasma cholesterol levels and are used widely in the treatment of patients with cardiovascular disease. Statins are generally prescribed for their low density lipoprotein (LDL) reducing effect, but several studies have recently emphasized statins as potential modifiers of the inflammatory process [1–5]. During an inflammatory process the first cytokine to be produced is tumour necrosis factor (TNF)- $\alpha$ , which subsequently induces the production of interleukin (IL)-1 and IL-6. TNF- $\alpha$  and IL-6 production has been found to be attenuated in endotoxin-stimulated monocyte and whole-blood cultures after *in vivo* treatment with statins [6,7]. In clinical trials, statins have been reported to lower plasma C-reactive protein (CRP) [2,3,8] and to reduce circulating levels of TNF- $\alpha$  and IL-1 $\beta$  [1,9]. Statin treatment has been suggested recently to have a potential preventive effect on sepsis development [10]. Statins have been shown to possess anti-inflammatory effects via inhibition of the isoprenylation of small guanosine triphosphatases (GTPases) [11–14]. Furthermore, Weitz-Schmidt *et al.* reported that

## Summary

Statins reduce plasma cholesterol, but clinical trials and *in vitro* studies indicate that they might also possess anti-inflammatory properties. The effect of simvastatin on circulating cytokines and leucocytes was evaluated in a human *in vivo* model of low-grade inflammation. Thirty young healthy male participants received an injection of the bacterial cell wall product endotoxin (0.06 ng/kg) to induce systemic inflammation. Participants were then randomized into a control and a simvastatin group. The simvastatin group received simvastatin 20 mg daily for 14 days. All participants returned after 14 days to receive a second endotoxin injection. Plasma concentrations of tumour necrosis factor (TNF)- $\alpha$ , interleukin (IL)-6 and IL-1 receptor antagonist were measured by enzyme-linked immunosorbent assay (ELISA) before and hourly for 6 hours after endotoxin administration. Plasma cytokines as well as total leucocyte counts increased in all participants upon endotoxin challenge but were not affected by simvastatin treatment. Tolerance to endotoxin was observed in both groups after 14 days. Short-term treatment with simvastatin (20 mg/day) did not influence circulating cytokine levels during endotoxaemia in this human *in vivo* study.

**Keywords:** cytokines, inflammation, endotoxin, *in vivo*, statins

the anti-inflammatory effects of simvastatin and lovastatin might be mediated by inhibition of the leucocyte function antigen 1, independent from inhibition of the co-enzyme A reductase [4].

The purpose of the present study was to evaluate the anti-inflammatory effects of simvastatin in a human experimental *in vivo* model of endotoxaemia. Endotoxin or lipopolysaccharide (LPS) is a product of the Gram-negative bacterial cell wall. Upon introduction in the bloodstream endotoxin binds to LPS-binding protein and this complex binds to CD14 on monocytes. CD14 does not have an intracellular domain but signals through Toll-like receptor 4, leading eventually to activation of the transcription factor NF- $\kappa$ B and to production of TNF- $\alpha$ , IL-1 and IL-6 [15,16]. Endotoxin administration to humans is a well-established model of systemic inflammation [17–19], which has been modified recently to study low-grade inflammation [20,21]. The low dose of endotoxin elicits an acute mild systemic inflammation with a significant and reproducible cytokine and leucocyte response, with normalization of levels within 24 h. In the present study we hypothesized that simvastatin-treated participants would

produce an attenuated *in vivo* cytokine response compared to the control group.

## Materials and methods

### Participants

Thirty young healthy males (median age: 24 years; range: 21–35) were recruited for the study. Participants had body mass indexes between 22 and 26. Standard biochemical markers including haematological values, leucocyte counts, electrolytes, hepatic and renal values were measured before inclusion. One participant from the control group was excluded from the study because of a high level of alkaline phosphatase, which interfered with enzyme-linked immunosorbent assay (ELISA) measurements. None of the participants had experienced symptoms of infection in the fortnight preceding the study. The Scientific-Ethical Committee of Copenhagen and Frederiksberg Municipalities approved the study (KF 01–144/98 with amendment: KF 11–128/03), and oral and written informed consent was obtained from each volunteer.

### Study design

On day 0 of the study, the participants reported to the clinic after an overnight fast. A peripheral catheter was inserted into an antecubital vein. After baseline samples were drawn the participants received a bolus of *Escherichia coli* endotoxin (endotoxin, *E. coli*, lot G2 B274, United States Pharmacopoeial Convention, Rockville, MD, USA) intravenously at a dose of 0.06 ng/kg. The participants were then kept resting under medical surveillance and were allowed to drink only water during the next 6 hours. Blood was drawn hourly for 6 h after the endotoxin administration.

Between 5 and 6 h after the endotoxin administration the participants were assigned randomly to a control group ( $n = 14$ ) or a simvastatin group ( $n = 15$ ). The participants in the simvastatin group were to receive simvastatin 20 mg (Nycomed, Roskilde, Denmark) orally each night for 14 days.

On day 14 all the participants received a second dose of endotoxin and samples were drawn as on day 0 (Fig. 1).

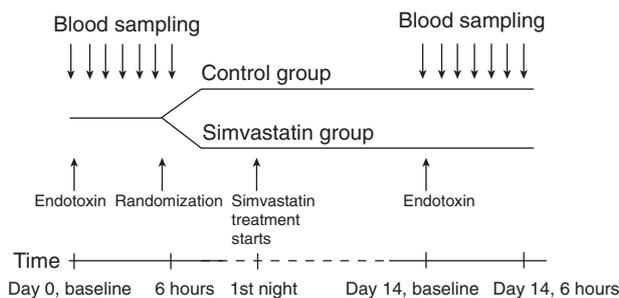


Fig. 1. Diagram of study design.

Symptoms reported by the participants were limited to light tiredness lasting for a few hours after the endotoxin injections.

Because of the LDL-lowering properties of simvastatin, a potential impact on glucose and lipid metabolism during endotoxaemia was assessed by measurement of glucose and insulin as well as cholesterol, triglycerides (TG) and free fatty acids (FFA).

### Measurements

#### Plasma separation

Blood was drawn into ethylenediamine tetraacetic acid (EDTA)-containing tubes and centrifuged immediately. Plasma was separated and stored at  $-80^{\circ}\text{C}$  until analysis.

#### Serum separation

Blood was drawn into uncoated tubes, allowed to clot, then centrifuged and stored at  $-20^{\circ}\text{C}$  until analysis.

#### Cytokines and C-reactive protein (CRP)

Plasma levels of TNF- $\alpha$ , IL-6 and IL-1 receptor antagonist (IL-1ra) were measured by ELISA (R&D Systems, Minneapolis, MN, USA). Serum levels of CRP were assessed by ELISA (Alpha Diagnostic, San Antonio, TX, USA). All ELISA measurements were run in duplicate, and mean concentrations were calculated.

#### Insulin

Plasma insulin levels were determined by ELISA (DakoCytomation, Glostrup, Denmark).

#### FFA

Plasma levels of FFA were measured using an automatic analyser (Cobas Fara, Roche, Basel, Switzerland).

#### Standard biochemical parameters

Lipid profile (total cholesterol, LDL, high-density lipoprotein (HDL) and TG), haematological parameters, leucocyte counts, electrolytes, glucose, hepatic and renal parameters were assessed through standard laboratory procedures (Modular Roche, Basel, Switzerland and Sysmex, Kobe, Japan).

### Statistics

All statistical analyses were made using SAS 8.2 (SAS Institute Inc., Cary, NC, USA). Log-transformed values were used when appropriate to approximate normal distribution. The

term 'baseline' is defined as the time just before the participant received the endotoxin injection. Therefore, by definition there is a baseline on the first as well as the second visit.

The change of a parameter over time during endotoxin influence was evaluated by the paired *t*-test on baseline *versus* peak or nadir values.

The study design allowed us to calculate the change of a parameter between the 2 days of endotoxin challenge for each participant to minimize the interpersonal variation. For parameters not fluctuating during the 6-hour time-span (metabolic parameters and CRP) the change between visits, designated  $\Delta_{0-14 \text{ days}}\text{-baseline}$ , was calculated as the baseline value on the second visit minus the baseline value on the first visit. Plasma cytokines and leucocyte subsets fluctuated during the 6-hour time span. For these parameters the individual response to endotoxin on each visit was estimated as an area under the curve (AUC). The areas were divided subsequently by 6 hours; the AUC may then be interpreted as a weighted average of the concentration of the parameter during the 6-hour period, with interpretable confidence intervals. Individual differences in AUCs between visits were calculated as  $\Delta_{0-14 \text{ days}}\text{-AUC}$  as described above (AUC second visit minus AUC first visit).

Values of  $\Delta_{0-14 \text{ days}}\text{-baseline}$  and  $\Delta_{0-14 \text{ days}}\text{-AUC}$  were compared between groups by Student's *t*-test. The change within groups between the two visits was quantified by the paired *t*-test on values of  $\Delta_{0-14 \text{ days}}\text{-baseline}$  and  $\Delta_{0-14 \text{ days}}\text{-AUC}$ .

Multivariate linear regression analyses were performed with plasma cytokine AUCs as response variables and lipid concentrations as predictors.

Normality was checked graphically. Data are presented as means with confidence intervals (CI). Data from regressions are presented as regression coefficients (RC) with CI. All confidence intervals reported are 95% CI. Values for  $P < 0.05$  were considered significant.

## Results

### Plasma cholesterol

All simvastatin-treated participants had lower LDL levels on the second visit compared to the first visit. The simvastatin-treated group experienced a reduction in LDL levels from 2.25 mmol/l to 1.31 mmol/l between days 0 and 14 ( $P < 0.0001$ ). There was no difference in LDL levels between

**Table 1.**  $\Delta_{0-14 \text{ days}}\text{-baseline}$  has been calculated as value on the second occasion minus value on the first occasion. Results are means or geometric means with 95% confidence limits, *P*-values from *t*-tests.

	Time 0 – before endotoxin administration			
	1st occasion		Delta-value	<i>P</i> -value
Total cholesterol mmol/l				
Control	3.85	(3.59–4.10)	0.18	(0.05 to 0.30)
Treatment	3.79	(3.40–4.18)	– 0.87	(–1.14 to –0.60)
LDL mmol/l				
Control	2.49	(2.22–2.77)	0.05	(–0.13 to 0.23)
Treatment	2.25	(1.89–2.60)	– 0.93	(–1.19 to –0.67)
HDL mmol/l				
Control	1.24	(1.07–1.41)	0.00	(–0.07 to 0.07)
Treatment	1.46	(1.31–1.62)	0.01	(–0.08 to 0.09)
CRP pg/ml*				
Control	152	(69–336)	2.11	(1.14 to 3.93)
Treatment	226	(128–398)	1.18	(0.55 to 2.55)
Glucose mmol/ml				
Control	4.59	(4.23–4.95)	0.00	(–0.26 to 0.26)
Treatment	4.63	(4.37–4.88)	0.19	(–0.13 to 0.50)
Insulin pg/ml				
Control	46.7	(35.4–57.9)	– 1.3	(–12.3 to 9.7)
Treatment	43.1	(34.2–52.0)	7.7	(–11.1 to 26.5)
FFA $\mu$ mol/ml				
Control	519	(389–648)	– 32	(–200 to 136)
Treatment	608	(494–723)	– 147	(–298 to 4)
Triglycerides mmol/ml				
Control	1.14	(0.92–1.37)	0.01	(–0.36 to 0.38)
Treatment	0.99	(0.68–1.29)	– 0.14	(–0.41 to 0.13)

\*The log-transformed values of C-reactive protein (CRP) were back-transformed and here the  $\Delta_{0-14 \text{ days}}\text{-values}$  estimate the ratio of second-occasion response/first-occasion response. LDL: low-density lipoprotein; HDL: high-density lipoprotein; CRP: C-reactive protein; FFA: free fatty acids.

visits among controls. When comparing  $\Delta_{0-14}$ -baseline levels of LDL between groups, the simvastatin group experienced an LDL reduction ( $P < 0.0001$ ).  $\Delta_{0-14}$ -baseline levels of HDL, glucose, insulin, FFA, TG and CRP did not differ between groups (Table 1).

### Plasma cytokines and inflammatory markers

Figure 2a depicts the TNF- $\alpha$  levels during days 0 and 14 in the two groups. Plasma TNF- $\alpha$  increased twofold and peaked at 2 h after endotoxin administration ( $P < 0.0001$ ). When comparing the groups we found no effect of simvastatin. This was evident when we compared  $\Delta_{0-14}$ -baseline and  $\Delta_{0-14}$ -AUC of plasma TNF- $\alpha$  between groups ( $P = 0.61$  and  $P = 0.89$  in unpaired *t*-tests).

Plasma IL-6 levels rose significantly ( $P < 0.0001$ ), with peak value at 3 h after endotoxin administration (Fig. 2b). Simvastatin had no effect on the level of circulating IL-6: there was no difference between groups in  $\Delta_{0-14}$ -baseline levels of IL-6 and no difference in  $\Delta_{0-14}$ -AUC.

IL-1ra was measured at baseline, 4 and 6 h, as a pilot study in our group had shown IL-1ra to peak 4 h after low-dose endotoxin (K. S. Krabbe, personal communication). Simvastatin did not influence  $\Delta_{0-14}$ -values of either IL-1ra at baseline or IL-1ra AUC (Fig. 2c).

CRP was measured at 0 and 6 h. There was no difference in  $\Delta_{0-14}$ -values of CRP between groups at baseline or 6 h after endotoxin administration (Table 1).

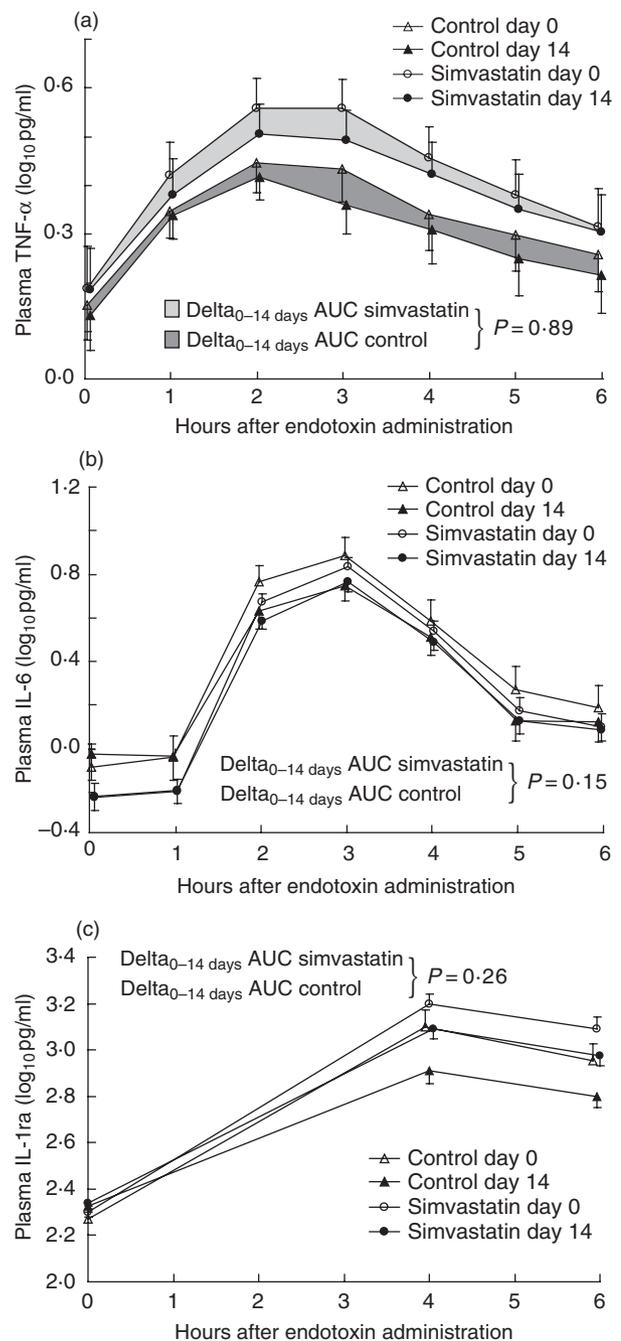
### Leucocyte subsets

When challenged by endotoxin all participants experienced a decline in total leucocyte count within the first few hours followed by a significant rise ( $P < 0.0001$ ) around 4 h after the bolus of endotoxin (Fig. 3a). Simvastatin did not change the levels of circulating leucocytes when the groups were compared by *t*-test on  $\Delta_{0-14}$ -AUC. The rise in leucocytes was due primarily to neutrophils peaking at 4 h. As with total leucocyte count, no difference in  $\Delta_{0-14}$ -values of AUC between groups was revealed (Fig. 3b). Lymphocytes decreased ( $P < 0.0001$ ) during the endotoxin influence to a nadir at 4 h, with no difference in  $\Delta_{0-14}$ -AUC between groups (Fig. 3c). Monocytes decreased significantly ( $P < 0.0001$ ) and reached a nadir at 2 h followed by a rise ( $P < 0.0001$ ) with a peak at 5 h (Fig. 3d). The magnitude of these responses was not influenced by simvastatin.

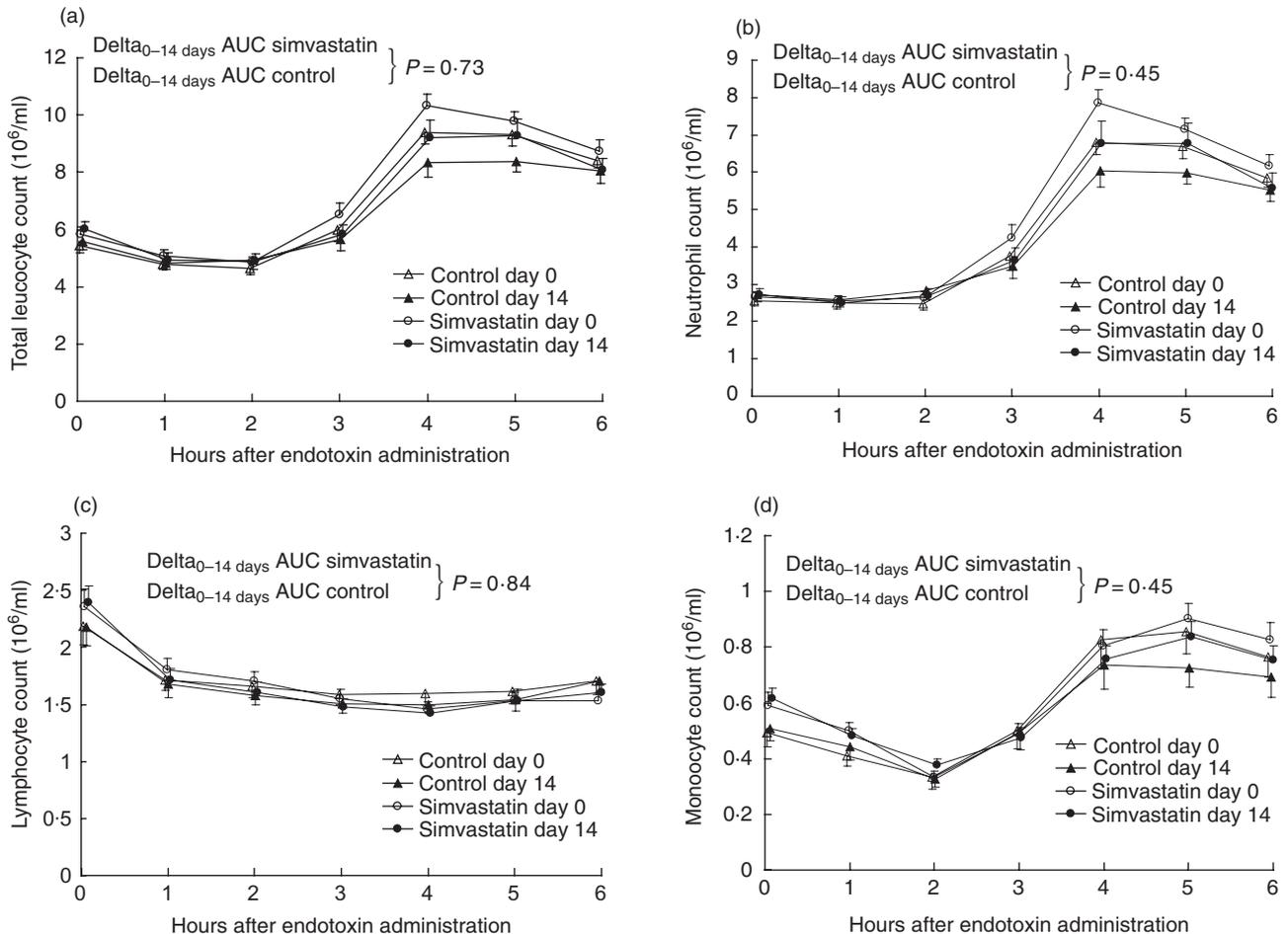
In multivariate regression analyses at day 0 lipid levels (LDL, HDL, TG and FFA) did not predict plasma TNF- $\alpha$ , IL-6 and IL-1ra responses (AUCs) (data not shown).

### Tolerance

We observed that plasma cytokine levels as well as levels of circulating total leucocytes were attenuated on the second visit compared to the first visit and this was confirmed by the



**Fig. 2.** Log<sub>10</sub>-transformed mean values of plasma cytokine concentrations plus or minus standard error on both occasions in both groups. There were no differences between groups in any of the cytokines. Below are the statistics for each cytokine. Delta-values (calculated as value on the second occasion minus value on the first occasion) of areas under the curve (AUC) have been back-transformed, thereby representing ratios of second-occasion AUC/first-occasion AUC, with 95% confidence limits. (a) Tumour necrosis factor (TNF)- $\alpha$ : treatment group: second-occasion AUC/first-occasion AUC: 0.91 (0.84–0.98); control group: 0.91 (0.81–1.03). (b) Interleukin (IL)-6: treatment group: second-occasion AUC/first-occasion AUC: 0.88 (0.76–1.02); control group: 0.77 (0.68–0.87). (c) IL-1ra: treatment group: second-occasion AUC/first-occasion AUC: 0.80 (0.68–0.94); control group: 0.69 (0.54–0.87).



**Fig. 3.** Leucocyte subsets: numbers in response to endotoxin on both experimental days. Values are means plus or minus standard error. There were no differences between groups in any subset. Below are the statistics for each subset. Delta-values are calculated as area under the curve (AUC) on the second occasion minus AUC on the first occasion. Results are means with 95% confidence limits. (a) Total leucocyte count: treatment group:  $\text{delta}_{0-14 \text{ days}}\text{-AUC}$ :  $-0.45 \text{ } 10^6/\text{ml}$  ( $-0.89$  to  $-0.02$ ); control group:  $-0.36 \text{ } 10^6/\text{ml}$  ( $-0.69$  to  $-0.03$ ). (b) Neutrophil count: treatment group:  $\text{delta}_{0-14 \text{ days}}\text{-AUC}$ :  $-0.39 \text{ } 10^6/\text{ml}$  ( $-0.72$  to  $-0.06$ ); control group:  $-0.23 \text{ } 10^6/\text{ml}$  ( $-0.52$  to  $-0.05$ ). (c) Lymphocyte count: treatment group:  $\text{delta}_{0-14 \text{ days}}\text{-AUC}$ :  $-0.04 \text{ } 10^6/\text{ml}$  ( $-0.17$  to  $0.09$ ); control group:  $-0.06 \text{ } 10^6/\text{ml}$  ( $-0.22$  to  $0.10$ ). (d) Monocyte count: treatment group:  $\text{delta}_{0-14 \text{ days}}\text{-AUC}$ :  $-0.02 \text{ } 10^6/\text{ml}$  ( $-0.06$  to  $0.02$ ); control group:  $-0.04 \text{ } 10^6/\text{ml}$  ( $-0.07$  to  $-0.01$ ).

paired *t*-test on  $\text{delta}_{0-14 \text{ days}}\text{-AUC}$  with the groups combined (TNF- $\alpha$ :  $P < 0.01$ , IL-6:  $P < 0.001$ , IL-1ra:  $P < 0.0001$ , total leucocyte count:  $P < 0.01$ ).

## Discussion

In this human *in vivo* model of low-grade inflammation we observed no effect of simvastatin treatment on baseline or endotoxin-induced changes in plasma levels of TNF- $\alpha$ , IL-6, IL-1ra, CRP or leucocyte subpopulations.

In clinical trials statins have been shown to reduce CRP [8,22,23], also on a short time-scale similar to ours [2,24], and some trials have shown attenuation of circulating cytokine levels [1,9]. According to the confidence limits of the comparison of  $\text{delta}_{0-14 \text{ days}}$ -values of AUC between groups, our model would have been able to determine a 15–20% differ-

ence in the cytokine responses between groups. In a recent study, Steiner *et al.* found an anti-inflammatory effect after 4 days of simvastatin treatment in a human model of endotoxaemia, measured by monocyte tissue factor, high sensitivity CRP and monocyte chemoattractant protein 1 [25]. They used a dose of simvastatin of 80 mg per day, and the dose of endotoxin was 2 ng/kg. We chose to use simvastatin 20 mg per day, a dose often prescribed clinically, and endotoxin 0.06 ng/kg was employed to mimic a low-grade inflammation as found in patients with the metabolic syndrome. We used a pretreatment endotoxin infusion allowing us to calculate delta-values, enhancing the strength of the study. Other trials have demonstrated statins to decrease blood levels of inflammatory markers in patients with low-grade systemic inflammation such as hypercholesterolaemic patients [9,26]. Our study does not confirm this finding. Neverthe-

less, as we have assessed potential effects on only circulating parameters, we cannot exclude that statins might possess anti-inflammatory properties at the tissue or endothelial level. Given that the atherosclerotic process is characterized by inflammation, one alternative explanation could be that statins inhibit the vascular inflammation and hence offer protection against the resulting systemic low-grade inflammation. However, there are several limitations to our experimental design: we chose endotoxin as an inductor of inflammation, but this is just one of many ways to activate the immune system, and it must be emphasized that we induced only an acute, short-lived activation and not a chronic inflammatory state which is observed among relevant patients. The dose of endotoxin, as well as the dose and duration of simvastatin treatment, could also inflict results.

An unexpected secondary finding of our study is the development of tolerance to endotoxin observed in both groups. Peak values as well as AUCs of TNF- $\alpha$ , IL-6 IL-1ra and leucocyte counts were lower on the second visit when compared to the first visit in both groups. Several studies on endotoxin tolerance have been performed on both animals and humans [27–29], but to our knowledge endotoxin tolerance has not been described as late as 2 weeks after a previous challenge.

In conclusion, circulating levels of cytokines are not attenuated by simvastatin (20 mg/day) in this human *in vivo* model of low-grade inflammation. Therefore, this study does not support a universal anti-inflammatory role of statins in healthy participants.

## Acknowledgements

The volunteers are thanked for their participation. Ruth Rousing and Hanne Willumsen are acknowledged for their excellent technical assistance. We gratefully acknowledge: The Centre of Inflammation and Metabolism (Danish National Research Foundation DG 02-512-555); The Copenhagen Hospital Corporation; The Danish National Research Foundation (504-14); The Commission of the European Communities (LSHM-CT-2004-005272 EXGENESIS). Christian Erikstrup was employed by a grant from The Faculty of Health Sciences of the University of Copenhagen in Denmark.

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