Lipoproteins in innate immunity
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Citation for published version (APA):
Levels, J. H. M. (2002). Lipoproteins in innate immunity

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CHAPTER VIII

Alterations in lipoprotein homeostasis during experimental and clinical sepsis in primates

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(Submitted)
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Abstract

Cell wall constituents of Gram-negative or Gram-positive bacteria such as lipopolysaccharides (LPS) and lipoteichoic acids (LTA) respectively, are potent endotoxins that initiate inflammatory responses in sepsis. Lipoproteins, in particular high-density lipoprotein (HDL), are able to bind and neutralize endotoxin and peptidoglycans. During the acute phase response that is initiated in sepsis, dramatic changes in lipid metabolism occur that are predicted to affect the ability of lipoproteins to scavenge bacterial toxins, but the pathophysiological processes responsible for these changes have not been completely elucidated. Sequential changes in lipid binding proteins and in lipoprotein composition in two experimental models (lethal bacteremia in baboons and low-dose endotoxemia in humans) as well as patients with severe sepsis were studied. In addition, the effect of reconstituted HDL (rHDL) administration on lipid homeostasis in a human endotoxemia model was investigated.

Decreases in lipoprotein concentrations and changes in composition accompanied by an increase in phase marker proteins were similar in all clinical and experimental settings, except for the triglyceride concentrations, which increased in baboons and decreased in human endotoxemia and sepsis. rHDL infusion did not alter the lipid changes during low-dose endotoxemia. However, while infusion of rHDL caused long-lasting increases of circulating HDL cholesterol, a high initial turnover of phosphatidylcholine was observed, indicative of extensive remodeling of the rHDL particle. 2-D electrophoresis of the HDL composition of the baboons showed marked differences between normal HDL and acute phase HDL. In the clinical sepsis model, strong negative correlations between the lipid transfer proteins, LCAT and CETP, and CRP were observed whereas PLTP activity showed remarkably positive correlations between LBP and CRP levels.
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Introduction

Systemic inflammatory responses in patients with bacterial sepsis are characterized by cytokine-mediated alterations in the composition of plasma proteins, known as the acute-phase response (APR). More recently, it has been recognized that during infection and inflammation in animal models and in humans a variety of profound alterations in the lipid metabolism occur (1), (2), (3), (4), including a reduction of the plasma concentration of total cholesterol (TC), HDL cholesterol, LDL cholesterol, and phospholipids (PL) (5), (6), (7), (8), (9), (10). Alterations of triglyceride concentrations are dependent on the species studied and the type of infection (chronic or acute) (11). The changes of the plasma lipid composition affect the host immune response, because lipoproteins are able to bind several molecules that activate toll-like receptors through pathogen-associated molecular patterns, including endotoxin and peptidoglycans (12), (13), and generally serve as scavenger pathways for such molecules (14), (15), (16). Several lipid-transporting proteins, including cholesterol ester transfer protein (CETP) and phospholipid transfer protein (PLTP) modify the lipid composition of lipoproteins. These proteins display marked structural homology to endotoxin binding proteins such as lipopolysaccharide binding protein (LBP) and bactericidal permeability/increasing protein (BPI) (17). LBP is an acute phase protein (18) that plays a central role in the activation of the cellular response to endotoxin by presenting LPS monomers to membrane-bound CD14 (mCD14) on monocytes and macrophages (19), (20). However, LBP can also present a complex of LPS and soluble CD14 to HDL (21). Hence, LBP and possibly PLTP function as molecules that transfer LPS to lipoproteins (22), (17).

Pre-incubation of endotoxin with various lipoproteins, protected rodents against endotoxin-induced death (23), (24), (25), (26), (27). In addition, specific lipid ligands such as apo E showed a similar protective effect (28), and several studies have reported that administration of reconstituted HDL (rHDL) has anti-inflammatory effects in experimental endotoxemia in animal models and in healthy volunteers (29), (30), (31), (32).

Hence, the plasma concentration of lipoproteins is affected by the innate host immune response to bacterial sepsis, but a precise sequential characterization of these changes in primates and humans is lacking. We therefore set up this study to register sequential changes in time during the development of the acute phase and subsequent recovery. We report changes in
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lipid and apolipoprotein composition of the main lipoproteins during two experimental sepsis models in primates, i.e. baboons who are lethally challenged with E. coli. and healthy human volunteers challenged with LPS with or without rHDL treatment. Further, we describe the sequential changes of the plasma concentrations of the lipoproteins as well as three lipid-transport proteins (LCAT, CETP, and PLTP) during severe sepsis.

Materials and Methods

Experimental endotoxia in baboons

Ten juvenile baboons (Papio Anubis/Cynocephalus) were challenged with a lethal 2-hour infusion of Escherichia coli (about 4 X 10^11 organisms/kg) as part of a study to investigate the therapeutic efficacy of antithrombin III treatment (33). The experiments were approved by the institutional Review Board of the Oklahoma Medical Research Foundation (Oklahoma City, OK, USA) for animal studies. The animals were fasted overnight and were given water ad libitum. The preparation and administration of the E. coli was performed as described (33). In brief, baboons were given gentamycin as a 60-minute infusion (9 mg/kg, intravenously) 2 hours after the start and as a 30-minute infusion (4.5 mg/kg) on 6 and 9 hours after the start of the experiment. Subsequently, gentamycin was given intramuscularly twice daily. During the whole experiment, all animals were sedated with ketamine hydrochloride (14 mg/kg, intramuscularly on the morning of the study) and anesthetized with sodium pentobarbital (2 mg/kg) via a percutaneous catheter positioned in the cephalic vein. EDTA samples were drawn (final concentration of 10 mM) at time points t = 0 and at t = 0.5, 2, 4, 6, and 12 hours relative to E. coli administration.

Experimental endotoxia in humans

Serial blood samples were obtained during a double-blind, cross-over, randomized, placebo-controlled study to investigate the effect of rHDL on endotoxin-induced cytokine release, coagulation, fibrinolysis and platelet activation (29, 30). Eight healthy male volunteers (mean age = 24, range 20 to 28 years), with normal medical history, physical and routine laboratory examination, chest X-ray and electrocardiogram participated in the study after obtaining a written informed consent. The volunteers did not smoke, used any medication or had any febrile illness in the month preceding the study. Each participant was studied on two occasions, separated by a washout period of six weeks. On one occasion, the subject was challenged with endotoxin in combination with placebo, on the other in combination with rHDL. rHDL (ZLB Central Laboratory, Bern, Switzerland) was supplied as a pyrogen-free, virus-inactivated lyophilized product with 91 % apo A-I purity. The appropriate amount of rHDL, in a solution containing 2 % protein and 10 % saccharose, was aspirated into dark-colored Amberlite syringes (Plastipak, Becton-Dickinson, Mountain View, CA) and administered as a four-hour infusion through an intravenous line (40 mg/kg). The placebo solution consisted of isotonic saline and was administered in an identical way. The Escherichia coli endotoxin preparation used in this study, lot EC-6 (D. Hochstein, Bureau of Biologics, Food and Drug Administration, Bethesda, MD) was
administered over one minute in an ante-cubical vein of the contra-lateral arm at a dose of 4 ng/kg. 3.5 hours after the initiation of the placebo or rHDL infusion. The study was performed at a special research unit under continuous supervision of at least two physicians with emergency and resuscitation equipment immediately available. Blood samples were collected at -3.5, 0, 1, 2, 3, 4, 6, 8 and 24 hours relative to LPS challenge. The research and ethical committees of the Academic Medical Center approved the studies; written informed consent was obtained from all participants or their legal representatives (septic patients) or from all volunteers.

Clinical sepsis

Twenty consecutive patients with the clinical diagnosis of Gram-negative sepsis admitted at the Intensive Care Unit at the Academic Medical Center were enrolled in the study. The criteria for the diagnosis for sepsis were defined as described previously (34), (35), (36) and had to be met within 24 hours before enrollment. Baseline blood samples of patients with clinical sepsis and samples of all suspected foci of infection were obtained for culture. The Apache-II score assessed the severity of the illness. From six additional patients blood was collected at several time points (t= 0, 2, 4, 6, 12 hours), at day 1, day 2, day 3 and day 7 following admission to the intensive care unit. Plasma lipid parameters, lipid transfer-protein parameters as well as plasma acute phase plasma markers were determined at these time points. Patients were not eligible if they were less than 18 years of age, if they had undergone organ transplantation; if there was an uncontrolled hemorrhage; if there was a cardiogenic shock: or if the primary acute underlying condition was burn injury.

Blood sampling and handling

Blood from baboons was obtained via the aseptically cannulated femoral vein in tubes containing EDTA and plasma was prepared by centrifugation (2000 g) at room temperature. From the human volunteers challenged with endotoxin and/or rHDL, EDTA blood was collected by separate veni-punctures. Blood for lipoproteins measurements from septic patients was collected on admission to the Intensive Care Unit. For lipoprotein measurements, blood was collected in non-additive or EDTA (10 mM final) containing vacutainer tubes (Becton Dickinson, Mountain View, CA). For the APTT analysis, citrated blood was collected. Serum or plasma was stored at -80 °C until batch wise assessment.

Assays

Cholesterol concentrations in the main lipoprotein classes (VLDL, LDL and HDL) in baboons and in the six critically ill patients with clinical sepsis were determined using high performance gel filtration chromatography (HPGC). The system contained a PU-980 ternary pump with an LG-980-02 linear degasser, FP-920 fluorescence and UV-975 UV/VIS detectors (Jasco, Tokyo, Japan). An extra P-50 pump (Pharmacia Biotech, Uppsala, Sweden) was used for in-line cholesterol PAP enzymatic reagent (Biomerieux, Marcy l'Etoile, France) addition at 0.1 ml/min. Plasma lipoprotein separations were performed with a Superose 6 HR 10/30 column (Pharmacia Biotech, Uppsala Sweden) with TBS pH 7.4 containing 0.005 % (v/v) Tween-20, as eluent at a flow rate of 0.31 ml/min. Computer analysis of the chromatograms for quantification of the lipoproteins.
was carried out using Borwin Chromatographic software, version 1.23 (JMBS Developments, Le Fontanil, France).

Total cholesterol and triglycerides were determined using PAP 250 cholesterol and PAP 250 triglyceride enzymatic methods (Biomerieux, Le Fontanille, France). Commercially available lipid plasma standards (low, medium and high) were used for quantitative analysis (SKZL, Nijmegen, the Netherlands) for TC, TG and HPGC separated lipoproteins. Cholate levels were determined by a colorimetric assay (Merck).

In the human endotoxemia study and in the clinical sepsis study. HDL cholesterol levels were determined by measuring cholesterol in the supernatant after precipitation of the apolipoprotein B (apo B) containing lipoproteins (VLDL, LDL and Lp(a)). LDL cholesterol levels could then be calculated from total plasma cholesterol, TG and HDL cholesterol levels by the Freidewald formula (37).

Apolipoprotein A-I (apo A-I) and apo B were either determined by nephelometric immunochemistry (Beckman, USA) or by rocket immuno-electrophoresis (SEBIA, Fontanille, France) depending on the available plasma volume. No differences in measured concentrations were observed between the two methods.

Other assays

The CRP ELISA was from Kordia (Leiden, the Netherlands), the LBP ELISA from Hycult (Netherlands), but in the human experimental endotoxemia study LBP was measured with an ELISA obtained from XOMA (Berkely, CA, USA). Interleukin-6, Interleukin-8 and TNF were determined by ELISA from Pelikine (CLB, Amsterdam). CETP and PLTP assays were performed as described previously (38). CETP and PLTP activities were expressed as percentage of normal human reference plasma, which was set at 100 % (equivalent to 215.6 nmol/ml per h for CETP and 13.9 µmol/ml per h for PLTP-activity). LCAT activity was determined by measuring the formation of radiolabelled cholesteryl ester after addition of 10 or 20 µl human plasma to excess heat-inactivated plasma containing [1H] cholesteryl (Amersham Pharmacia Biotech, Roosendaal, The Netherlands) (39). LCAT activities were expressed as percentage of normal human reference plasma. Activated partial thromboplastin time (APTT) analysis was carried out according to standard procedures.

Isolation of lipoproteins Lipoprotein fractions were isolated from 100 µl plasma samples by size-exclusion chromatography using a Superose 6 HR 10/30 (Pharmacia Biotech, Uppsala, Sweden) column at a flow rate of 0.31 ml/min with inline fluorescence and UV detection. VLDL, LDL and HDL containing plasma fractions were collected and concentrated with Centricon-100 concentrator filters to a final volume of 100 µl. Samples were processed immediately or frozen in liquid nitrogen and stored at -80 °C.

2-D electrophoresis of HDL

A plasma sample containing 300 µg of protein was pre-treated with a solution containing 9 M Urea, 2 % (w/v) CHAPS, 40 mM Tris, 2.3% (w/v) DTT and 2 % (w/v) IPG buffer in a final volume of 250 µl by incubation for one hour at room temperature before sample application onto
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Immobilin dry strips (Amersham Pharmacia Biotech). Passive rehydration was allowed to proceed overnight at room temperature.

The first dimension was run for 35000 Vh using a Multiphor II electrophoresis unit and subsequently the strips were equilibrated with 1% (w/v) DTT for 15 minutes at room temperature followed by a second equilibration step with 2.5% (w/v) iodoacetamide without DTT in SDS equilibration buffer (50 mM Tris, 6 M Urea, 30% (w/v) glycerol and 2% (w/v) SDS). Finally, the strips were loaded onto 12% (w/v) SDS polyacrylamide gels and subjected to electrophoresis for one hour at 20 mA/gel and subsequently 3 to 4 hours at 60 mA/gel in a Hoefer electrophoresis system (Pharmacia Biotech, Uppsala, Sweden). Gels were fixed, stained by a modified silver stain protocol (40) and dried between cellophane sheets.

Determination of the phospholipid composition of lipoproteins

100 μl of the isolated lipoprotein fraction was extracted with 1.0 ml chloroform : methanol : 2.4 N HCl (ratio 1.5:1:1.5). Subsequently, the mixture was sonificated for 10 minutes at 4 °C and phospholipids were separated from proteins with phase-sep silicon treated paper (Whatmann, Maidstone, Kent, UK). After three washes with chloroform : methanol (ratio 1:3), the samples were dried under continuous nitrogen flow. The residue was dissolved in 200 μl CHCl3/CH3OH and sonificated for 1 minute and subsequently volumes of 5 and 10 μl were applied on the HPTLC plate. Chromatography was performed as described previously (41).

Statistical analysis

Values are given as mean ± SEM. In the baboon study, changes in time were tested by analysis of variance (one way ANOVA). In the experimental endotoxemia model differences between placebo and rHDL treatment periods in the human endotoxemia model were tested by analysis of variance (ANOVA) for repeated measures. Changes of parameters in time were tested using one-way ANOVA. In the clinical sepsis study, two sample comparisons between survivors and non-survivors were performed using the Mann-Whitney test. Correlations were calculated by MANOVA controlling for repeated measures in time in different subjects. For comparison of survivors and non-survivors between day 1 and day 7, the last value carried forward technique was applied. All statistics were performed using SPSS (version 10.1 Chicago, IL) for Windows. A two-sided value of p < 0.05 was considered to be significantly different.
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Fig. 1. CRP levels in baboons during development of the acute phase reaction. Depicted are the means ± SEM. *P < 0.05 for t=12 hours compared to t=0.

Results
Plasma lipid changes during experimental endotoxemia in baboons

Administration of *E. coli* to baboons caused an acute phase reaction in all baboons as confirmed by increases of plasma CRP (+34%, Figure 1) and LBP (+105%, table 1) concentrations. Immediately following the start of the *E. coli* administration significant changes in lipid homeostasis were observed (table 1). Cholesterol concentrations showed a linear decrease (from mean level of 2.20 ± 0.34 mM (t=0) to 1.61 ± 0.20 mM (t=4) and 0.86 ± 0.25 mM (t=12)). To get more insight in the cholesterol distribution among the main lipoprotein classes during the development of sepsis we determined total cholesterol concentrations in VLDL, LDL and HDL by HPGC analysis. In contrast to humans, where LDL is the main carrier of free cholesterol and cholesterol esters, in baboons, most cholesterol was found in HDL (1.19 ± 0.26 mM at t=0) and lower levels were found in LDL (0.66 ± 0.20mM) and VLDL (0.33 ±0.11mM). During the development of the acute phase, a persistent decrease of HDL- and LDL-cholesterol was seen (Figure 2B) and within 6 hours, approximately a 50% decrease of the HDL cholesterol(0.65±0.43 mM) and LDL-cholesterol (0.34 ± 0.11) were observed.
Fig. 2. Changes in total plasma cholesterol (A), total cholesterol in the main lipoprotein classes (B) and total plasma triglycerides (C) in baboons during the development of the acute phase reaction. All data are presented as mean ± SEM. Statistically significant difference with ANOVA analysis for repeated measures was determined as indicated in the graphs. Statistically significant differences between t=0 and other time points were: * p < 0.001 and # p<0.05.
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Fig. 3. Determination of apo A-I and apo B during the acute phase response in baboons. Data are presented as mean ± 1 SEM. Statistically significant difference with ANOVA analysis for repeated measures was determined as indicated in the graphs. Statistically significant differences between t=0 and other time points were: * p < 0.001 and # p<0.05.

However, the VLDL-cholesterol increased during the two-hour E. coli administration, remained elevated until 6 hours after the start of the experiment and only returned to baseline at t=12h. Triglyceride levels in baboons were significant increased (figure 2C).

Figures 3A and 3B, the changes in apolipoprotein A-I (apo A-I) and apolipoprotein B (apo B) concentrations are depicted. During the two-hour E. coli administration, no significant changes in concentrations of apo A-I and apo B were observed. However at later time points a significant decrease in apo A-I and apo B levels was observed.

2-D electrophoresis of HDL containing plasma fractions

The HDL fractions were isolated from pooled baboon plasma samples (equivalent volumes of plasma from 10 baboons) obtained at t=0 and 12 hours and was subjected to two-dimensional electrophoresis. Proteins were separated by iso-electric focusing in the first dimension and with SDS-PAGE in the second dimension (Figure 4). Significant changes occurred between these time points. The septic phenotype was associated with novel protein spots on the gel.
Fig. 4. Two-dimensional gel electrophoresis of HDL at t=0 just before and t=12 hours after the start of *E. coli* administration to baboons. Equal plasma volumes of 10 baboons were pooled and the HDL containing plasma fraction was isolated with size exclusion chromatography. A total of 300 µg/ml protein was used for 2D electrophoresis. The following spots were identified either by MALDI-TOF mass spectrometry or by comparison with known 2D gels from human plasma extracted from the SWISS protein data bank: 1: apo C-II/C-III apo A-II cluster, 2:apo A-I, 3: Haptoglobin, 4: Hemoglobin β chain, 5: apo A-IV subunit, 6a: IgG light chain, 6b IgG intermediate chain, 6c: IgG heavy chain, 7:Apo E, 8: Fibrinogen, 9: apo J, 10: complement factor 4a and 4b, 11: apo D.

while other proteins disappeared. Some of the spots were identified by MALDI-TOF analysis, while the protein spots that could not be identified, assumptions were made based on the literature and/or online human databases. We observed a lower abundance of the apo C-II/III and the apo A-II cluster in the t=12 gel. A higher abundance of apo A-IV, apo E, apo J, fibrinogen and haptoglobin was observed at t=12 compared to t=0. However also a number of sepsis associated protein spots could not be identified (Figure 4).

**Human endotoxemia, clinical symptoms and vital signs**

Endotoxin administration to human volunteers elicited clinical symptoms as reported previously (6). Briefly, flu-like symptoms were observed including headache, chills, nausea, vomiting, myalgia and backache. All volunteers were symptom-free within 24 hours following endotoxin challenge. rHDL importantly reduced LPS-induced clinical symptoms, cytokine release and activation of coagulation and fibrinolysis as reported elsewhere (29), (30).
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Infusion of rHDL did not cause any side effects or changes in routine laboratory parameters.

Plasma lipid changes during experimental endotoxemia in humans

Administration of LPS caused a decrease in total cholesterol levels from baseline levels of 4.48 ± 0.38 mM to 4.06 ± 0.26 mM at t = 6 h (p < 0.001) and a decrease in HDL cholesterol levels from 1.12 ± 0.07 mM at t = -3.5 h to 1.03 ± 0.08 at t = 2 h (p = 0.1). Further, a decrease in triglyceride concentrations from 1.31 ± 0.13 mM at t = -3.5 h to 0.93 ± 0.09 at t = 6 h (p = 0.001) were observed.

Following rHDL treatment, total cholesterol levels increased from 4.47 ± 0.42 mM at t = -3.5 h to 5.15 ± 0.37 mM at t = 1 h, returning to baseline levels 4 hours after LPS challenge (p<0.001 versus placebo). HDL cholesterol concentrations also increased from 1.16 ± 0.06 mM at t = -3.5 h to 1.64 ± 0.07 mM at t = 8 h. HDL cholesterol levels remained elevated until the end of the study period. 1.48 ± 0.05 mM at t = 24 h (p < 0.001 versus placebo). During endotoxemia, plasma apo A-I concentrations decreased from baseline levels of 1.42 ± 0.08 g/l at t = -3.5 h to 1.32 ± 0.09 g/l at t = 6 h (p < 0.001 in time). rHDL treatment increased Apo A-I concentrations from 1.45 ± 0.08 at t = -3.5 h to 2.39 ± 0.14 at t = 1 h. Apo A-I levels remained elevated until the end of the study period. 1.8 ± 0.04 g/l at t = 24 h (p < 0.001 versus placebo). LDL cholesterol levels decreased from 2.76 ± 0.29 mM at t = -3.5 h to 2.54 ± 0.22 mM at t = 4 h (p < 0.001), and were not affected by rHDL treatment. The plasma concentration of cholate, an rHDL constitute, was significantly elevated in the rHDL treated group compared to the placebo group (p<0.001). At t=0 just after the end of the rHDL infusion a peak level of 30.7 ± 1.9 μM was reached which rapidly decreased to 5.0 ± 0.9 μM at t=2. Baseline levels of cholate stayed in the rHDL treated group higher than in the placebo group till t=24 (p<0.001).

Phospholipid distribution during rHDL and/or endotoxin administration

The phosphatidylcholine (PC), lyso-phosphatidylcholine (L-PC) (figure 6), phosphatidyl-ethanolamine (PE) and sphingomyelin (SM) (figure 7) content of HDL, LDL and VLDL was measured in the endotoxin-challenged volunteers using high performance thin layer chromatography (HPTLC).
**Phospholipid composition in HDL**

Low dose endotoxemia did not alter PC and L-PC concentrations. The L-PC levels in the endotoxin treated group showed some minor alterations upon LPS administration and no significant changes in SM levels in the lipoproteins occurred (figure 7) with the exception of a slight increase in PE levels.
Fig. 6. The phospholipid changes in the human endotoxemia model. Profiles of phosphatidyl choline (PC), lyso PC in HDL, LDL and VLDL respectively observed during LPS (placebo) or rHDL/LPS (rHDL) administration. Data are presented as mean ± 1 SEM. The p values indicate the significant differences between placebo and the rHDL group. * Asterisk indicates the statistically differences compared to t = -3.5 hours.
Infusion of rHDL caused a transient increase of PC content from 221 ± 46 μM at t=-3.5 to 557 ± 11 μM at t = 2 (p<0.001) and a transiently increased L-PC from 43 ± 11 μM at t=-3.5 to 284 ± 99 μM at t=0 (p<0.05 compared to t=-3.5). Subsequently, L-PC returned back to baseline levels at t=4 hours.

During rHDL treatment a long-lasting increase of SM was seen (from 38 ± 7 μM at t=-3.5 to 63 ±7 μM at t=4 and 50 ± 6 μM at t=24). PE levels in the rHDL group showed a continuous increase upon endotoxin administration from 3.7 ± 1.0 at t=0 μM to 10.3 ± 2.3 μM at t=24 (p<0.05 compared to t=0).

**PC and L-PC in VLDL and LDL** LPS administration caused no significant changes of the PC and L-PC content of LDL or VLDL. However, rHDL infusion markedly increased the LDL L-PC and VLDL PC content (Figure 6).
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Clinical sepsis

Patients.

A total of 26 patients with a clinical diagnosis of Gram-negative sepsis were included (35). A proven gram-negative focus was found in 22 out of the 26 patients. 8 patients had Gram-negative bacteremia, and in 7 patients a positive Gram-positive bacteria were cultured from infectious foci. From the 26 patients 15 patients survived and 11 died within one month after the inclusion in the study. Age (60 ± 2.6 versus 59.8 ± 3.6 years), APACHE scores (25.2 ± 3.0 versus 28.8 ± 2.9), sex, heart rate (129 ± 7 versus 133 ± 7 beats/min), and mean arterial pressure (62.4 ± 4.6 versus 55.3 ± 3.8 mm Hg) at baseline did not differ between survivors and non-survivors.

Inflammatory mediators.

Consistent with the clinical diagnosis, baseline circulating TNF-α, IL-6, IL-8, LBP and CRP levels in septic patients were elevated, and no significant differences were detected between survivors and non-survivors. However, during the one-week follow-up period LBP, IL-6, IL-8 and CRP concentrations decreased in the survivors whereas in the non-survivors no significant change in time was found (Table 3).

Lipid changes.

The septic patients had a marked reduction of plasma cholesterol concentrations (1.52 ± 0.14 mM, normal range: 2.0 to 5.2 mM) (Table 2, day 1) that increased in time. As shown in table 3, total cholesterol levels (Day 1) in survivors were significantly higher in comparison to non-survivors (1.74 ± 0.19 mM 1.23 ± 0.20 mM, p<0.03). LDL cholesterol levels were also decreased (0.77 ± 0.09 mM) as compared to normal levels (2.0 to 2.5 mM) and were significantly higher in survivors compared to non-survivors (0.95 ± 0.12 mM to 0.53 ±0.10 mM (p<0.03)) at baseline (table 3).

At inclusion in the study, total triglycerides (TG), free cholesterol (FC), VLDL, and HDL cholesterol, phospholipids, apo A-I, apo B were all well below the normal range (Table 2). Survivors were characterized by higher TG, apo A-I, and apo B and lower free cholesterol (FC), VLDL cholesterol, HDL cholesterol and phospholipids compared to non-survivors. In time, overall TG, TC, HDL cholesterol, phospholipids and apo A-I all increased (Table 2).
### Table 2. The overall mean ± 1 SEM concentrations of the plasma parameters in time during clinical sepsis in humans

<table>
<thead>
<tr>
<th>Parameter (units) normal value</th>
<th>n</th>
<th>day 1</th>
<th>n</th>
<th>Day 2</th>
<th>n</th>
<th>day 3</th>
<th>n</th>
<th>day 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>TG (mM)</td>
<td>26</td>
<td>1.30 ± 0.19</td>
<td>4</td>
<td>1.27 ± 0.20</td>
<td>3</td>
<td>1.16 ± 0.65</td>
<td>3</td>
<td>2.22 ± 1.35</td>
</tr>
<tr>
<td>TC (mM)</td>
<td>26</td>
<td>1.52 ± 0.14</td>
<td>4</td>
<td>1.60 ± 0.27</td>
<td>3</td>
<td>1.37 ± 0.18</td>
<td>3</td>
<td>2.50 ± 0.49</td>
</tr>
<tr>
<td>FC (mM)</td>
<td>6</td>
<td>0.92 ± 0.13</td>
<td>4</td>
<td>0.99 ± 0.25</td>
<td>3</td>
<td>0.79 ± 0.17</td>
<td>3</td>
<td>1.08 ± 0.11</td>
</tr>
<tr>
<td>VLDL (chol) (mM)</td>
<td>6</td>
<td>0.68 ± 0.22</td>
<td>4</td>
<td>0.74 ± 0.39</td>
<td>3</td>
<td>0.42 ± 0.33</td>
<td>3</td>
<td>0.46 ± 0.42</td>
</tr>
<tr>
<td>LDL (chol) (mM)</td>
<td>26</td>
<td>0.77 ± 0.11</td>
<td>4</td>
<td>0.52 ± 0.33</td>
<td>3</td>
<td>0.71 ± 0.36</td>
<td>3</td>
<td>0.59 ± 0.75</td>
</tr>
<tr>
<td>HDL (chol) (mM)</td>
<td>26</td>
<td>0.25 ± 0.07</td>
<td>4</td>
<td>0.45 ± 0.09</td>
<td>3</td>
<td>0.23 ± 0.09</td>
<td>3</td>
<td>0.47 ± 0.24</td>
</tr>
<tr>
<td>Apo A-I (mg/L) 1000-2000</td>
<td>26</td>
<td>274 ± 49</td>
<td>4</td>
<td>317 ± 85</td>
<td>3</td>
<td>249 ± 87</td>
<td>3</td>
<td>516 ± 72</td>
</tr>
<tr>
<td>Apo B (mg/L) 500-1100</td>
<td>19</td>
<td>459 ± 77</td>
<td>4</td>
<td>305 ± 88</td>
<td>3</td>
<td>302 ± 70</td>
<td>3</td>
<td>578 ± 106</td>
</tr>
<tr>
<td>LCAT (% of normal) 100</td>
<td>6</td>
<td>22 ± 5.7</td>
<td>4</td>
<td>20.3 ± 7.7</td>
<td>3</td>
<td>17 ± 5.8</td>
<td>3</td>
<td>37 ± 7.2</td>
</tr>
<tr>
<td>CETP (mg/L) 1.8-2.0</td>
<td>6</td>
<td>0.77 ± 0.18</td>
<td>4</td>
<td>0.59 ± 0.15</td>
<td>3</td>
<td>0.40 ± 0.23</td>
<td>3</td>
<td>0.85 ± 0.46</td>
</tr>
<tr>
<td>PLTP (% of normal) 100</td>
<td>6</td>
<td>206 ± 35</td>
<td>4</td>
<td>224 ± 47</td>
<td>3</td>
<td>163 ± 47</td>
<td>3</td>
<td>194 ± 48</td>
</tr>
<tr>
<td>LBP (mg/L) &lt;2</td>
<td>22</td>
<td>79 ± 13</td>
<td>4</td>
<td>27 ± 4</td>
<td>3</td>
<td>19 ± 1</td>
<td>3</td>
<td>10 ± 1</td>
</tr>
<tr>
<td>IL-6 (ng/L)</td>
<td>0</td>
<td>2896 ± 912</td>
<td>4</td>
<td>629 ± 346</td>
<td>3</td>
<td>460 ± 385</td>
<td>3</td>
<td>12 ± 1</td>
</tr>
<tr>
<td>IL-8 (ng/L)</td>
<td>0</td>
<td>958 ± 323</td>
<td>4</td>
<td>374 ± 104</td>
<td>3</td>
<td>225 ± 155</td>
<td>2</td>
<td>79 ± 48</td>
</tr>
<tr>
<td>CRP (mg/L) &lt; 1.0</td>
<td>6</td>
<td>149 ± 32</td>
<td>3</td>
<td>220 ± 34</td>
<td>3</td>
<td>160 ± 26</td>
<td>3</td>
<td>40 ± 17</td>
</tr>
<tr>
<td>APTT (seconds) 25-33</td>
<td>6</td>
<td>42.4 ± 2.3</td>
<td>4</td>
<td>39.8 ± 3.4</td>
<td>3</td>
<td>42.2 ± 4.5</td>
<td>3</td>
<td>35.8 ± 7.9</td>
</tr>
<tr>
<td>TNF (ng/L)</td>
<td>0</td>
<td>179 ± 103</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### Table 3

The mean ± 1 SEM concentrations of the plasma parameters during acute phase in all patients at day 1 and in survivors and non-survivors at day 1 and day 7. Last measured data of non-survivors were carried forward to day-7 and compared with the survival data on day 7.

<table>
<thead>
<tr>
<th>Parameter (units)</th>
<th>normal value</th>
<th>n</th>
<th>Day 1 Overall</th>
<th>n</th>
<th>Day 1 Survivors</th>
<th>n</th>
<th>Day 1 Non-Surv.</th>
<th>n</th>
<th>Day 7 Survivors</th>
<th>n</th>
<th>Day 7 Non-Surv.</th>
</tr>
</thead>
<tbody>
<tr>
<td>TG (mM)</td>
<td>0.8 - 2.0</td>
<td>26</td>
<td>1.30 ± 0.19</td>
<td>15</td>
<td>1.40 ± 0.23</td>
<td>11</td>
<td>1.18 ± 0.32</td>
<td>3</td>
<td>2.22 ± 1.35</td>
<td>3</td>
<td>1.92 ± 0.28</td>
</tr>
<tr>
<td>TC (mM)</td>
<td>2.0 - 5.2</td>
<td>26</td>
<td>1.52 ± 0.14</td>
<td>15</td>
<td>1.74 ± 0.19</td>
<td>11</td>
<td>1.23 ± 0.15</td>
<td>3</td>
<td>2.50 ± 0.49</td>
<td>3</td>
<td>1.08 ± 0.10</td>
</tr>
<tr>
<td>TC (mM)</td>
<td>1.0 - 1.5</td>
<td>6</td>
<td>0.92 ± 0.13</td>
<td>3</td>
<td>0.72 ± 0.04</td>
<td>3</td>
<td>1.10 ± 0.22</td>
<td>3</td>
<td>1.08 ± 0.11</td>
<td>3</td>
<td>1.18 ± 0.29</td>
</tr>
<tr>
<td>LDL (cholesterol)</td>
<td>0.8 - 1.0</td>
<td>6</td>
<td>0.68 ± 0.22</td>
<td>3</td>
<td>0.34 ± 0.16</td>
<td>3</td>
<td>1.02 ± 0.31</td>
<td>3</td>
<td>0.46 ± 0.42</td>
<td>3</td>
<td>1.17 ± 0.42</td>
</tr>
<tr>
<td>HDL (cholesterol)</td>
<td>2.0 - 4.0</td>
<td>26</td>
<td>0.77 ± 0.11</td>
<td>15</td>
<td>0.95 ± 0.12</td>
<td>11</td>
<td>0.53 ± 0.10</td>
<td>3</td>
<td>1.59 ± 0.76</td>
<td>3</td>
<td>0.37 ± 0.26</td>
</tr>
<tr>
<td>Apo A-1 (mg/L)</td>
<td>1000 - 2000</td>
<td>26</td>
<td>274 ± 49</td>
<td>15</td>
<td>327 ± 67</td>
<td>11</td>
<td>202 ± 78</td>
<td>3</td>
<td>516 ± 72</td>
<td>3</td>
<td>428 ± 142</td>
</tr>
<tr>
<td>Apo B (mg/L)</td>
<td>500 - 1100</td>
<td>19</td>
<td>459 ± 77</td>
<td>8</td>
<td>584 ± 112</td>
<td>11</td>
<td>359 ± 98</td>
<td>3</td>
<td>577 ± 106</td>
<td>3</td>
<td>336 ± 58</td>
</tr>
<tr>
<td>LCAT activity (%)</td>
<td>&lt; 100</td>
<td>6</td>
<td>22 ± 5.7</td>
<td>3</td>
<td>21 ± 10.2</td>
<td>3</td>
<td>25 ± 7.4</td>
<td>3</td>
<td>37 ± 7.2</td>
<td>3</td>
<td>23 ± 7.7</td>
</tr>
<tr>
<td>CETP (mg/L)</td>
<td>1.8 - 2.0</td>
<td>6</td>
<td>0.77 ± 0.18</td>
<td>3</td>
<td>0.66 ± 0.28</td>
<td>3</td>
<td>0.87 ± 0.25</td>
<td>3</td>
<td>0.85 ± 0.46</td>
<td>3</td>
<td>0.82 ± 0.26</td>
</tr>
<tr>
<td>PLTP (mg/L)</td>
<td>&lt; 100</td>
<td>6</td>
<td>206 ± 35</td>
<td>3</td>
<td>201 ± 62</td>
<td>3</td>
<td>210 ± 49</td>
<td>3</td>
<td>194 ± 48</td>
<td>3</td>
<td>236 ± 49</td>
</tr>
<tr>
<td>IL-6 (ng/L)</td>
<td>&lt; 100</td>
<td>25</td>
<td>289 ± 912</td>
<td>14</td>
<td>2950 ± 1415</td>
<td>11</td>
<td>2827 ± 1107</td>
<td>3</td>
<td>12 ± 1.1</td>
<td>3</td>
<td>1260 ± 519</td>
</tr>
<tr>
<td>IL-8 (ng/L)</td>
<td>&lt; 100</td>
<td>24</td>
<td>958 ± 323</td>
<td>14</td>
<td>977 ± 488</td>
<td>10</td>
<td>931 ± 398</td>
<td>3</td>
<td>79 ± 48</td>
<td>3</td>
<td>717 ± 136</td>
</tr>
<tr>
<td>CRP (mg/L)</td>
<td>&lt; 1.0</td>
<td>6</td>
<td>1.49 ± 32</td>
<td>3</td>
<td>170 ± 49</td>
<td>3</td>
<td>130 ± 48</td>
<td>3</td>
<td>41 ± 17</td>
<td>3</td>
<td>151 ± 44</td>
</tr>
<tr>
<td>APT1 (seconds)</td>
<td>25 - 33</td>
<td>6</td>
<td>42.4 ± 2.3</td>
<td>3</td>
<td>39.4 ± 3.1</td>
<td>3</td>
<td>45.5 ± 2.7</td>
<td>3</td>
<td>35.8 ± 7.9</td>
<td>3</td>
<td>42.9 ± 4.3</td>
</tr>
<tr>
<td>TNF (ng/L)</td>
<td>&lt; 100</td>
<td>19</td>
<td>179 ± 103</td>
<td>11</td>
<td>96 ± 36</td>
<td>7</td>
<td>294 ± 242</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
Alterations in lipid homeostasis

The apo B levels positively correlated with its most abundant fat constituents TG, TC, LDL cholesterol, PC, whereas apo A-I positively correlated with TC, PC and HDL cholesterol (table 4). The lipid transport proteins CETP mass and LCAT activity showed positive correlations with apo A-I, apo B, TC and HDL cholesterol. However, PLTP activity was negatively correlated with HDL cholesterol (table 4) and positive correlated with LBP, CRP and IL-6 (table 5). All lipid components were strongly negatively correlated with CRP and LBP levels.

Lipid transport proteins and the acute phase markers

The total circulating concentrations of LCAT and CETP were dramatically reduced in septic patients, whereas PLTP activity was increased (Table 2). In time, LCAT and CETP remained well below and PLTP above normal levels, respectively, and at study entry the concentrations of these proteins did not differ between survivors and non-survivors (table 3).

Table 4. Correlations (r) among the plasma parameters of the septic patients which had a statistical significance of P <0.05 (n=40).

<table>
<thead>
<tr>
<th></th>
<th>TG</th>
<th>TC</th>
<th>Apo A-I</th>
<th>Apo B</th>
<th>LDL</th>
<th>HDL</th>
<th>LBP</th>
<th>CRP</th>
</tr>
</thead>
<tbody>
<tr>
<td>TG</td>
<td>1</td>
<td>.81</td>
<td>--</td>
<td>.74</td>
<td>.68</td>
<td>--</td>
<td>-.42</td>
<td>--</td>
</tr>
<tr>
<td>TC</td>
<td>1</td>
<td>.84</td>
<td>.87</td>
<td>.84</td>
<td>--</td>
<td>-.57</td>
<td>-.64</td>
<td></td>
</tr>
<tr>
<td>Apo A-I</td>
<td>.81</td>
<td>.76</td>
<td>.88</td>
<td>--</td>
<td>--</td>
<td>-.72</td>
<td>-.63</td>
<td></td>
</tr>
<tr>
<td>Apo B</td>
<td>1</td>
<td></td>
<td>.80</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td></td>
</tr>
<tr>
<td>PC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PLTP</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>-.78</td>
<td>.42</td>
<td>.76</td>
</tr>
<tr>
<td>CETP</td>
<td>--</td>
<td>.53</td>
<td>.61</td>
<td>.65</td>
<td>--</td>
<td>-.36</td>
<td>-.46</td>
<td></td>
</tr>
<tr>
<td>LCAT</td>
<td>--</td>
<td>.84</td>
<td>.87</td>
<td>.85</td>
<td>--</td>
<td>-.70</td>
<td>-.85</td>
<td></td>
</tr>
<tr>
<td>LBP</td>
<td>-.57</td>
<td>--</td>
<td>-.72</td>
<td>--</td>
<td>--</td>
<td>1</td>
<td>.83</td>
<td></td>
</tr>
<tr>
<td>CRP</td>
<td>-.80</td>
<td>-.63</td>
<td>-.54</td>
<td>-.46</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

All calculated correlations were corrected for repeated measures and shared time points of the individual subjects using MANOVA. -- = no significant correlation found. TG: Triglycerides. TG: total cholesterol. PC: phosphatidyl-choline. LDL: LDL cholesterol. HDL: HDL cholesterol.

The lipid transport proteins CETP and LCAT were negatively correlated with the acute phase markers LBP and CRP, whereas for PLTP activity a positive correlation with CRP, LBP and IL-6 was demonstrated (table 5).
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Table 5. Correlations (r) among the inflammation and lipid transport specific plasma parameters which have a statistically significance of P <0.05 (n=40).

<table>
<thead>
<tr>
<th></th>
<th>PLTP</th>
<th>CETP</th>
<th>LCAT</th>
<th>LBP</th>
<th>CRP</th>
<th>IL-6</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLTP</td>
<td>1</td>
<td>--</td>
<td>--</td>
<td>.42</td>
<td>.76</td>
<td>.73</td>
</tr>
<tr>
<td>CETP</td>
<td>1</td>
<td>.70</td>
<td>-.36</td>
<td>-.46</td>
<td>--</td>
<td></td>
</tr>
<tr>
<td>LCAT</td>
<td>1</td>
<td></td>
<td>-.70</td>
<td>-.85</td>
<td>--</td>
<td></td>
</tr>
<tr>
<td>LBP</td>
<td>1</td>
<td></td>
<td></td>
<td>.83</td>
<td>--</td>
<td></td>
</tr>
<tr>
<td>CRP</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td>--</td>
<td></td>
</tr>
<tr>
<td>IL-6</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

All correlations were corrected for repeated measures and shared time points of the individual subjects with MANOVA. -- = no significant correlation found.

Discussion

This study is the first to report sequential changes of lipoprotein concentration, their lipid content, and lipid binding proteins in experimental and clinical endotoxemia and sepsis. In the first model, intravenous E. coli was administrated to baboons and lipid core and lipid surface parameters were measured. The protein composition of HDL was studied before and 12 hours after E.coli administration. In the second model, we investigated the lipid changes during low-dose endotoxemia and the effects of rHDL administration in this setting. Studying the sequential alterations in lipid profiles and lipid binding proteins during severe sepsis in time corroborated the results of these two experimental studies. The acute phase reaction in the baboons resulted in immediate changes in lipid core parameters, i.e. a TG increase, and a TC. HDL and LDL cholesterol decrease. However, VLDL cholesterol levels increased during the first 5-6 hours after E. coli administration, possible as a consequence of the increased number of particles which are synthesized during the acute phase reaction.

Apo A-I and apo B, the main apolipoproteins of HDL and LDL respectively, showed a different behavior than the lipid core constituents. A clear decrease in concentration of the apolipoproteins was observed but this lagged significantly behind the decrease of the lipids. Hence, the initial response to bacteremia is a release of cholesterol from HDL and LDL, whereas the concentration of lipoprotein particles decreases at a later stage, either because of enhanced clearing or reduced synthesis. Two-dimensional electrophoresis of
HDL isolated from the baboons showed marked sepsis-induced changes in protein composition. Identification of the protein spots by mass spectrometry (42) or comparison of plasma 2D gels from the SWISS 2D data bank revealed that HDL-associated apo E, apo J and haptoglobin increased during the acute phase, which is consistent previous reports on acute phase HDL (43), (44). Apo E has a protective effect against LPS in the circulation (45), (46), (28) whereas the function of apo J is not clear.

Upon endotoxin administration to healthy volunteers, a similar behavior of TC and apo A-I concentrations was observed. but in contrast to the baboons, decreased TG levels were observed upon LPS administration in both placebo and rHDL treated endotoxemic volunteers. Upon LPS administration, TG levels in both groups decreased but fully recovered within 24 hours. This is in contrast to other studies in rodents and rabbits, that generally reported TG increases (47), (48), (49). It is possible that the more severe inflammatory stimuli the these latter studies was responsible for these differences(1). We conclude that TG level changes in the acute phase reaction as initiated by LPS administration are species dependent even in primates. rHDL treatment increased the absolute TC, HDL cholesterol and apo A-I levels, but did not prevent the typical acute phase lipid alterations.

We next investigated changes in phospholipid composition of the lipoproteins. Phosphatidyl choline (PC) levels rapidly increased in HDL and to a lesser extent in LDL and VLDL during rHDL administration. Compared to the steady increase of apo A-I and HDL cholesterol levels following rHDL administration, rHDL-derived PC was rapidly exchanged to LDL and VLDL and possibly to cell membranes. We assume that this exchange is even accelerated due to increased PLTP activity (50), (this study) and elevated LBP (29) concentration. We consider this rapid and dynamic exchange of PC amongst the lipoproteins of particular importance, because it is known that endotoxin is similarly transported by lipid binding proteins. Indeed, we have previously reported that endotoxin, after initial binding to HDL is redistributed to LDL, in a LBP and PLTP-dependent manner (this thesis). We speculate that endotoxin is subsequently transported to the liver by LDL to removed from the organism be excretion in the bile. In accordance with this hypothesis, experimental bile duct ligation caused hepatic accumulation of endotoxin, leading to increased hepatic cytokine production and increased mortality following rHDL infusion (51).
Apart from a rapid PC turnover, rHDL infusion caused a remarkable increase of the HDL and LDL L-PC content, and an increase of the HDL, but not the LDL sphingomyelin (SM) and phosphatidyl-ethanolamine (PE) content. rHDL phospholipid analysis revealed that less than 3% of the rHDL was L-PC and that SM and PE were not present, excluding the possibility that these lipids were derived from rHDL. Most likely this phenomenon is explained by transport of L-PC, SM and PE from the peripheral tissues to HDL as a consequence of the conversion of rHDL to mature HDL particles.

We finally investigated whether the changes observed in the two experimental models were relevant for clinical sepsis. In patients with severe sepsis, changes in lipid core and lipid ligands were comparable to the observations made in the experimental setting. In accordance with earlier observations, low total cholesterol, triglycerides, VLDL, LDL and HDL cholesterol, Apo A-I and apo B levels, combined with high LBP, CRP, IL-6 and IL-8 levels were observed (11) Surprisingly, although LBP is known to be associated with HDL, in severe sepsis the LBP plasma concentration did not correlate with apo A-I or other HDL constituents (12). We did find a significant correlation of plasma LBP with the apoB concentration, which may suggest that in sepsis LDL rather than HDL is the main LBP carrier.

We found that patients with severe sepsis have very low plasma concentrations of the lipid transport proteins CETP and LCAT, which is probably, a result of decreased synthesis (52) that was sustained for at least a week. In contrast, PLTP activity was increased, and during the study period these activities did not differ between survivors and non-survivors. In a single patient we found remarkably elevated PLTP activity (237 %) 5 weeks after inclusion in the study, whereas CETP mass (0.92 mg/L) and LCAT activity (48%) were still depressed and the acute phase markers, CRP, LBP and IL-6, had normalized. In our study positive correlations of PLTP activity with CRP but also with IL-6 and LBP were found, which is in concordance with previous reports (50), (53). We hypothesize that these changes are part of the trigger to suppress reversed cholesterol transport and the enhancement of the synthesis of pre-beta HDL in an attempt to normalize the HDL levels (54), (55).

In summary we found profound changes in lipid homeostasis in experimental endotoxemia in humans and bacteremia in baboons, that, with exception of the TG levels that were species dependent, were markedly similar. These changes are relevant for clinical sepsis, which was characterized by the same changes of
Alterations in lipid homeostasis

Alterations in lipid homeostasis involve changes in lipoprotein concentration and composition. These changes are in large part mediated by altered concentrations of LBP and altered activity of PLTP, both correlating to the magnitude of the acute phase response. rHDL administration did not alter these changes but resulted in a marked dynamic exchange of PC towards LDL and VLDL. Because endotoxin is transported between lipoproteins by the same lipid binding proteins, this mechanism may explain the protective effects of rHDL in models of experimental endotoxemia.

References


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