

Human recombinant apolipoprotein E redirects lipopolysaccharide from Kupffer cells to liver parenchymal cells in rats *In vivo*.

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Research Article

Chylomicrons have been shown to protect mice and rats against a lethal dose of lipopolysaccharide and may serve as a therapeutic means to protect against endotoxemia. However, the requisite of isolation from human lymph hampers pharmaceutical application. Recently, we developed recombinant chylomicrons from commercially available lipids and human recombinant apolipoprotein E. The current study explored the effectiveness of these apoE-enriched emulsions in redirecting LPS from Kupffer cells to liver parenchymal cells. Upon injection into rats, ¹²⁵I-LPS rapidly and specifically associated with the liver (64.3±3.1% of the injected dose) and spleen (4.1±0.7%). The uptake of LPS by the spleen was four- to fivefold reduced upon incubation with the apoE-enriched emulsion or free apoE ($P < 0.0001$), but not with emulsion alone or Lipofundin. Within the liver, ¹²⁵I-LPS mainly associated with Kupffer cells. The uptake by Kupffer cells was eight- to ninefold reduced by the apoE-enriched emulsion or apoE alone ($P < 0.01$), and a 19.6-fold increased uptake ratio by liver parenchymal cells over Kupffer cells was observed. The emulsion without apoE had no effect on the *in vivo* kinetics of LPS. LPS interacted selectively with the apoE moiety of the recombinant chylomicron. Emulsion-associated and free apoE bound approximately two molecules of LPS, possibly by its exposed hydrophilic domain involving arginine residues. We anticipate that the protecting effect of endogenous chylomicrons against [...]

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Human Recombinant Apolipoprotein E Redirects Lipopolysaccharide from Kupffer Cells to Liver Parenchymal Cells in Rats In Vivo

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Abstract

Chylomicrons have been shown to protect mice and rats against a lethal dose of lipopolysaccharide and may serve as a therapeutic means to protect against endotoxemia. However, the requisite of isolation from human lymph hampers pharmaceutical application. Recently, we developed recombinant chylomicrons from commercially available lipids and human recombinant apolipoprotein E. The current study explored the effectiveness of these apoE-enriched emulsions in redirecting LPS from Kupffer cells to liver parenchymal cells. Upon injection into rats, ^{125}I -LPS rapidly and specifically associated with the liver ($64.3 \pm 3.1\%$ of the injected dose) and spleen ($4.1 \pm 0.7\%$). The uptake of LPS by the spleen was four- to fivefold reduced upon incubation with the apoE-enriched emulsion or free apoE ($P < 0.0001$), but not with emulsion alone or Lipofundin. Within the liver, ^{125}I -LPS mainly associated with Kupffer cells. The uptake by Kupffer cells was eight- to ninefold reduced by the apoE-enriched emulsion or apoE alone ($P < 0.01$), and a 19.6-fold increased uptake ratio by liver parenchymal cells over Kupffer cells was observed. The emulsion without apoE had no effect on the in vivo kinetics of LPS. LPS interacted selectively with the apoE moiety of the recombinant chylomicron. Emulsion-associated and free apoE bound approximately two molecules of LPS, possibly by its exposed hydrophilic domain involving arginine residues. We anticipate that the protecting effect of endogenous chylomicrons against LPS-induced endotoxemia may result from the apoE moiety and that human recombinant apoE may serve as a therapeuticum to protect against endotoxemia. (*J. Clin. Invest.* 1997. 99:2438–2445.) Key words: chylomicron • detoxification • emulsion • lipoprotein metabolism • sepsis

Introduction

Sepsis, a syndrome referring to the systemic response to infections, can result in severe hypotension and organ dysfunction. In fact, septic shock is the most common cause of death in intensive care units (1). Most cases of sepsis are caused by

Gram-negative bacteria. LPS, a component of the outer membrane of these bacteria, causes the same clinical features as can be seen in patients with sepsis (2). LPS is mainly cleared from the plasma by the liver upon intravenous injection (3, 4). Within the liver, Kupffer cells are primarily responsible for the uptake of LPS from the blood (5, 6), but endothelial and parenchymal cells also contribute to the clearance of LPS. It has been postulated that within the blood, a complex of LPS and the LPS-binding protein (LBP)¹ is formed that shows a high affinity for CD14 present on macrophages such as Kupffer cells (7). CD14 is involved in intracellular signaling (8), leading to the production of a set of mediators, including IL-1, IL-6, and TNF (9, 10). These mediators are responsible for metabolic and physiological changes that ultimately lead to a pathological situation. Changes in lipid metabolism during sepsis comprise hypertriglyceridemia due to an increased hepatic triglyceride-rich lipoprotein synthesis (11–13), inhibition of adipose tissue lipoprotein lipase activity (14), and a decreased lipoprotein clearance (11, 15, 16).

Lipoproteins such as LDL (17, 18), HDL (18, 19), VLDL, and chylomicrons (18, 20, 21) can bind endotoxin, thereby reducing its toxic properties. It has been postulated that the production of triglyceride-rich lipoproteins contribute to the host's defense mechanism against endotoxin. VLDL and chylomicrons were able to protect mice and rats against a lethal dose of endotoxin (20, 21), and significantly reduced the serum levels of TNF. In rats, triglyceride-rich lipoproteins appeared to redirect LPS to the liver parenchymal cells (21), where LPS was secreted into the bile and could be determined in a deactivated form (22). In this way, Kupffer cells are not activated and the production of cytokines is greatly inhibited.

Therefore, chylomicrons might be used therapeutically to protect against sepsis. The fact that chylomicrons have to be isolated from human lymph, however, hampers their possible pharmaceutical application. Recently, we described the synthesis of recombinant chylomicrons from commercially available lipids and human recombinant apolipoprotein E, which were selectively recognized by and taken up via apoE-specific receptors on liver parenchymal cells (23). The present study investigated the ability of these recombinant chylomicrons to direct LPS to liver parenchymal cells. In addition, we describe the possible site(s) of interaction between LPS and recombinant chylomicrons, since the mechanism by which chylomicrons protect against the toxic effects of LPS has not been clarified. The results indicate that LPS selectively interacts with the apoE moiety of the recombinant chylomicron in vivo, thus mediating the redirection of LPS from Kupffer cells to liver parenchymal cells.

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1. Abbreviations used in this paper: CHD, cyclohexadione; LBP, LPS-binding protein.

Methods

Animals. 9–10-wk-old male Wistar rats of mass 250–310 g from Broekman Instituut BV (Someren, The Netherlands), fed ad libitum with regular chow were used for the in vivo experiments.

Chemicals. Recombinant human apoE was a generous gift from Tikva Vogel, Bio-Technology General, Ltd. (Rehovot, Israel), and was supplied as a lyophilized powder containing 76% apoE, 11.7% L-cysteine, and 12.0% NaHCO₃ (24). LPS from *Salmonella minnesota* R595 (Re) was obtained as a lyophilized powder from List Biological Laboratories, Inc. (Campbell, CA). ¹²⁵I (carrier-free) in NaOH and [$1\alpha,2\alpha$ -³H]cholesteryl oleate were purchased from Amersham International (Little Chalfont, UK). Triolein (99% pure), egg yolk phosphatidylcholine (98% pure), and methyl 4-hydroxybenzimidate hydrochloride were from Fluka (Buchs, Switzerland). L- α -Lysophosphatidylcholine (99% pure), cholesterol (> 99% pure), BSA (fraction V), collagenase (type I), and 3,3'-diaminobenzidine were obtained from Sigma Chemical Co. (St Louis, MO). Cholesteryl oleate (97% pure) and 1,2-cyclohexadione (CHD) were from Janssen (Beerse, Belgium), and metrizamide from Nycomed A/S (Oslo, Norway). Pronase, cholesterol oxidase, cholesterol esterase, peroxidase type II (200 U/mg), Precipath[®] L, and EDTA were from Boehringer Mannheim (Mannheim, Germany). Hepes was from Merck (Darmstadt, Germany), and Lipofundin-S 20% containing 20% soybean oil (triglycerides), 1.5% soybean phosphatides, and 2.5% glycerin was from Braun Medical B.V. (Uden, The Netherlands). All other chemicals were of analytical grade.

Preparation and characterization of recombinant chylomicrons. Emulsions were prepared according to the sonication and ultracentrifugation procedure from Redgrave and Maranhao (25) from 100 mg total lipid at a weight ratio triolein/egg yolk phosphatidylcholine/lyso-phosphatidylcholine/cholesteryl oleate/cholesterol of 70:22.7:2.3:3.0:2.0, using a soniprep 150 (MSE Scientific Instruments, Crawley, UK) at 18 μ m output and equipped with a water bath for temperature maintenance (23). The final weight ratio of these lipids in the obtained emulsions has previously been reported (23). The obtained emulsions were homogeneous with respect to size (low polydispersity of 0.11–0.15), and the mean particle diameter was 77.0 ± 2.9 nm (mean \pm SD; $n = 5$) as determined by photon correlation spectroscopy (4700 C system; Malvern Instruments, Malvern, UK). Measurements were performed at 25°C and a 90° angle between laser and detector. The triolein content was determined using the Boehringer Mannheim enzymatic kit for triacylglycerols. Precipath[®] L was used as an internal standard. Emulsions were stored at 20°C under argon and used for characterization and metabolic studies within 5 d after preparation, in which period no physicochemical changes occurred.

LPS assay. LPS concentrations were determined based on the 2-keto-3-deoxyoctonate (KDO) method of Karkhanis et al. (26), taking into account that KDO comprises 11.3% (wt/wt) of the LPS used in this study.

Radiolabeling of apoE and LPS. Human apoE was radioiodinated at pH 10.0 with carrier-free ¹²⁵I according to a modification (27) of the ICl method (28). LPS was radioiodinated after derivatization with *p*-OH methylbenzimidate at pH 8.0, using the chloramine T procedure as described by Ulevitch (29). Free ¹²⁵I was removed by Sephadex G25 gel filtration (in case of apoE) and extensive dialysis against 8 mM PBS containing 1 mM EDTA, pH 7.4 (PBS/EDTA), with repeated changes of buffer. More than 97% of the label in apoE was trichloroacetic acid precipitable. The specific activities of ¹²⁵I-apoE and ¹²⁵I-LPS were 353–473 and 199–409 dpm/ng, respectively.

Liver uptake, serum decay, and organ distribution. Rats were anesthetized by intraperitoneal injection of sodium pentobarbital (15 mg/kg body wt) and the abdomens were opened. ¹²⁵I-LPS (2.5–5.0 μ g), ¹²⁵I-apoE (5.0–400 μ g), or [³H]cholesteryl oleate-labeled emulsions (10 mg of triglycerides) were injected via the vena cava inferior. When indicated, ¹²⁵I-LPS (5.0 μ g) was preincubated with unlabeled emulsion (10 mg of triglycerides) and/or apoE (400 μ g) for 30 min at 37°C. ¹²⁵I-LPS was also injected in the presence of apoE without prior

incubation. In some experiments, Lipofundin was injected with ¹²⁵I-LPS after preincubation (30 min at 37°C) at 500 mg/kg body wt. At the indicated times, blood samples of 300 μ l were taken from the vena cava inferior and allowed to clot for 30 min. The samples were centrifuged for 3 min at 16,000 g, and 100 μ l serum samples were counted for ¹²⁵I radioactivity. In case [³H]cholesteryl oleate-labeled emulsions or Lipofundin were injected, triglyceride levels were determined in serum samples using the Boehringer Mannheim enzymatic kit for triacylglycerols and corrected for endogenous serum glycerol. The total amount of radioactivity or triglycerides in the serum was calculated using the equation: serum volume (ml) = [0.0219 \times body weight (g)] + 2.66 (30). To determine liver uptake, liver lobules were tied off, excised, and weighed at the indicated times. The amount of liver tissue tied off during the experiment did not exceed 15% of the total liver weight. At 30 min after injection, rats were killed and organs were excised and weighed. Uptake by the various organs was corrected for the radioactivity in the serum assumed to be present in the tissues at the time of sampling (liver, 73.4; spleen, 59.8; lungs, 107.8; heart, 66.8; bone marrow, 85.0; and adrenals, 128.0 μ l serum per gram wet wt). Correction values had been obtained earlier by our laboratory by performing organ distributions of ¹²⁵I-BSA at 10 min after injection (our unpublished data). The amounts of LPS administered in the present study did not alter the total and captured serum volumes.

Liver cell distribution. Rats were anesthetized and injected intravenously with ¹²⁵I-apoE or ¹²⁵I-LPS in the absence or presence of unlabeled emulsion and/or apoE as described above. After 10 min of circulation, the liver was perfused at < 8°C with Ca²⁺-free Hanks' solution containing 10 mM Hepes, pH 7.4, at a flow rate of 14 ml/min. 8 min after starting the perfusion, a lobule was removed for determination of total liver uptake. Subsequently, the liver was perfused for 20 min with Hanks' solution containing 10 mM Hepes and 0.05% (wt/vol) collagenase, pH 7.4. Parenchymal, endothelial, and Kupffer cells were isolated by differential centrifugation and counterflow elutriation as described in detail elsewhere (31). The contribution of the various liver cell types to total liver uptake was determined taking into account that parenchymal, endothelial, and Kupffer cells contribute 92.5, 3.3, and 2.5% to the total liver protein mass, respectively (32, 33). Kupffer and endothelial cells were > 95% pure as judged from peroxidase staining for 20 min at 37°C (0.1% [wt/vol] 3,3'-diaminobenzidine in 0.05 M Tris-HCl, 7% [wt/vol] sucrose, 0.1% [vol/vol] of 30% [vol/vol] H₂O₂, pH 7.4).

Density gradient ultracentrifugation. To investigate the interaction of ¹²⁵I-LPS with recombinant apoE or rat serum (lipo)proteins, incubation mixtures (30 min at 37°C) or serum samples, isolated at 1, 10, or 30 min after injection of ¹²⁵I-LPS (alone or complexed with emulsions and/or apoE), were subjected to density gradient ultracentrifugation. The mixtures were adjusted to a final volume of 3.8 ml and a density of 1.21 g/ml with KBr. Then they were placed at the bottom of a centrifuge tube (Kontron Instruments, Intl., Milan, Italy) and overlaid with 2.8 ml KBr solutions (all including 0.2 M NaCl and 0.3 mM EDTA, pH 7.4) with densities of 1.050, 1.019, and 1.006 g/ml, respectively. After centrifugation for 18–22 h at 40,000 rpm, 4°C, top fractions were collected by aspiration after tube slicing. The remaining volumes were fractionated at a flow rate of 1.2 ml/min using a Bromma 2132 Microperpex peristaltic pump (LKB, Uppsala, Sweden), starting at the bottom of the tubes. All fractions were assayed for density using a DMA 40 digital density meter from Mettler (Graz, Austria) and ¹²⁵I-radioactivity. Occasionally, the distribution of endogenous rat apoE over the fractions was determined using a mouse apoE-specific ELISA that cross-reacts with rat apoE (manuscript in preparation).

Agarose gel electrophoresis. To determine the in vitro interaction of LPS with the lipid and/or protein moiety of recombinant chylomicrons, ¹²⁵I-LPS was incubated (30 min at 37°C) in the absence or presence of unlabeled emulsions with or without apoE at the indicated weight ratios. When indicated, apoE was modified with CHD as detailed below. Aliquots of incubation mixtures were subjected to electrophoresis in 0.75% (wt/vol) agarose gels at pH 8.8 using 0.075 M

Tris-HCl, 0.080 M hippuric acid, and 0.65 mM EDTA buffer. Bromophenol blue served as front marker. Resulting gels were air dried and ^{125}I -spots were detected and quantified after exposure of dried gels to X-OMAT films (Eastman Kodak Co., Rochester, NY) or imaging using a Packard Instant Imager (Hewlett-Packard Co., Palo Alto, CA).

Arginine modification of apoE. Arginine residues of apoE were selectively modified with CHD according to the procedure of Patthy and Smith (34), as described in detail (35). Due to charge neutralization of these basic amino acids, a 1.3-fold increased electrophoretic mobility of apoE on agarose gel toward the anode was observed.

Statistical analysis. Statistical significance was determined by using a two-tailed Student's *t* test. Values are expressed as means, and errors represent the variation between data points (in case of $n = 2$) or standard deviation ($n \geq 3$).

Results

Previous experiments have shown that upon injection into rats, the emulsion acquires apolipoproteins (e.g., apoE and apoCs) from the blood circulation and is subsequently taken up by liver parenchymal cells via apoE-specific receptors just like endogenous chylomicrons. The rate of liver uptake is greatly increased by preloading the emulsion with apoE (23). To gain insight into the therapeutic effects of chylomicrons on endotoxin-induced rat lethality by altering endotoxin's metabolism as observed by Harris et al. (21), it was attempted to reveal the mechanism of interaction between LPS and chylomicrons using the recombinant model.

Effect of emulsion and/or apoE on the liver uptake and serum decay of ^{125}I -LPS. Upon injection of ^{125}I -LPS (2.5–5.0 μg ; derived from *Salmonella minnesota* R595) into rats, a biphasic serum decay was observed, and $\sim 80\%$ of the LPS was cleared from the serum in the α -phase ($t_{1/2\alpha} < 1$ min). At 20 min after injection, $64.3 \pm 3.1\%$ of the injected dose was taken up by the liver (Fig. 1). Preincubation of ^{125}I -LPS with the protein-deficient emulsion (10 mg of triglycerides) had no apparent effect on the serum decay and liver uptake of ^{125}I -LPS. In contrast, preincubation with the emulsion that was previously enriched with apoE (400 μg) did alter the in vivo behavior of

LPS by retarding both the liver uptake and the serum decay. Approximately 45% of the injected ^{125}I -LPS was cleared in the α -phase and $48.1 \pm 2.8\%$ was taken up by the liver at 20 min after injection. Preincubation of ^{125}I -LPS with pure apoE (400 μg) also resulted in a reduced serum decay of ^{125}I -LPS in the α -phase ($\sim 60\%$), and a significantly reduced liver uptake ($40.4 \pm 5.3\%$ at 20 min after injection, $P < 0.0001$). The same effects were observed when ^{125}I -LPS and apoE were coinjected without previous incubation at 37°C (not shown). ^{125}I -LPS was also injected after incubation with a large excess of Lipofundin (500 mg of triglycerides/kg). This commercial protein-deficient lipid emulsion did not have any effect on the in vivo behavior of ^{125}I -LPS. At the dosage used in this study, the serum half-lives of the triglycerides within our emulsion and Lipofundin were 23.9 ± 2.8 min ($n = 2$) and 26.8 ± 2.6 min ($n = 2$), respectively (not shown).

Association of ^{125}I -LPS with serum components. To investigate the distribution of LPS over serum, ^{125}I -LPS (1.0 μg) was incubated with rat serum (3.0 ml) at a similar ratio of weight over volume as applied in vivo, followed by density gradient ultracentrifugation (Fig. 2). Within rat serum, 5.1% of the apoE was associated with triglyceride-rich lipoproteins ($d \leq 1.006$ g/ml, fraction 20), 50.3% with cholesteryl ester-rich HDL ($1.05 < d < 1.10$ g/ml, fractions 7–13), and 44.6% of apoE is either in the free tetrameric state or associated with more dense HDL ($d > 1.10$ g/ml, fractions 1–6). A similar distribution of ^{125}I -LPS was observed, with 3.3% associated with triglyceride-rich lipoproteins and 36.8% with cholesteryl ester-rich HDL. The $d > 1.10$ g/ml fraction contained 59.9% of the recovered ^{125}I -LPS, representing LPS that is either bound to more dense HDL, free tetrameric apoE, or is in the free (micellar) state.

When ^{125}I -LPS was administered with or without previous incubation with apoE, the protein-deficient emulsion, or Lipofundin, the vast majority ($> 75\%$) of radioactivity in the serum, harvested at 10 or 30 min after injection, was recovered in the HDL density fractions ($1.050 < d < 1.21$ g/ml). The remaining radioactivity was recovered in the lipoprotein-defi-

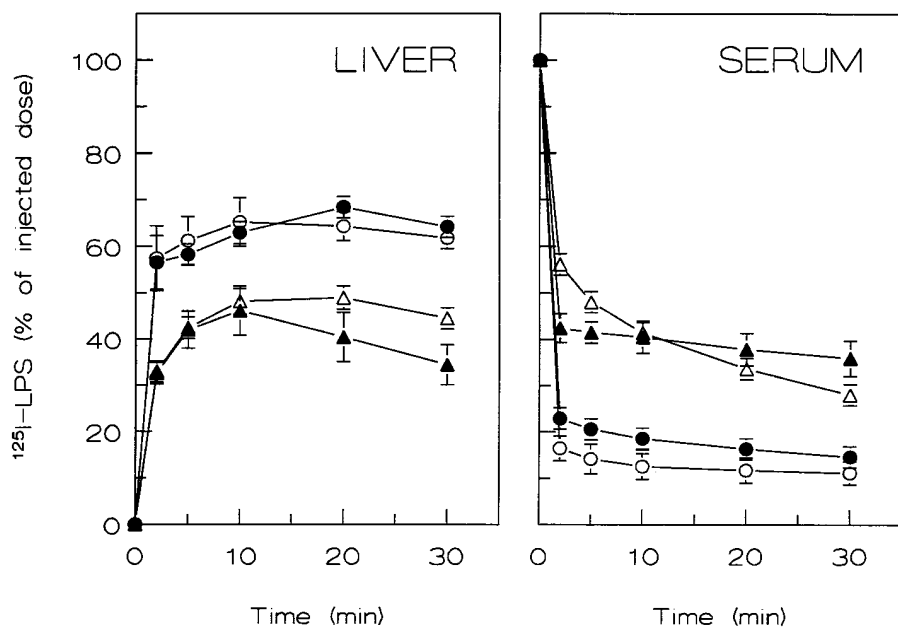


Figure 1. Effect of emulsion and/or apoE on the liver uptake and serum decay of ^{125}I -LPS. ^{125}I -LPS (2.5–5.0 μg) was injected into anesthetized rats without (\circ , $n = 5$) or with previous incubation with emulsion (10 mg triglycerides) (\bullet , $n = 2$), emulsion (10 mg triglycerides) in combination with apoE (400 μg) (Δ , $n = 2$), or apoE alone (400 μg) (\blacktriangle , $n = 5$). At the indicated times, the liver uptake (left) and serum decay (right) were determined. Liver values are corrected for serum radioactivity.

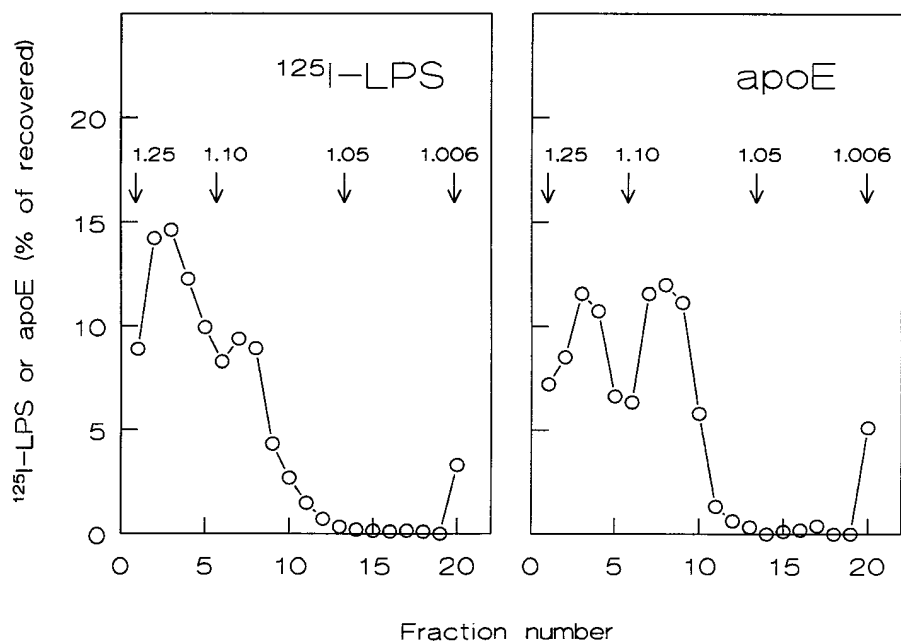


Figure 2. Distribution of $^{125}\text{I-LPS}$ over serum components. Rat serum (3.0 ml) that was incubated (30 min at 37°C) with $^{125}\text{I-LPS}$ (1.0 μg) (left) and control serum (right) were subjected to density gradient ultracentrifugation as described. After centrifugation, the tubes were subdivided from bottom (fraction 1) to top (fraction 20) according to their density, and assayed for radioactivity (left) and apoE (right). Arrows indicate fraction densities (grams per milliliter).

cient serum ($d > 1.21$ g/ml), and a low percentage ($\leq 8.6\%$) with triglyceride-rich lipoproteins ($d \leq 1.006$ g/ml). Similarly, the radioactivity recovered in the serum at 1 min after injection of the amount of $^{125}\text{I-apoE}$ that altered the in vivo behavior of LPS (400 μg) was predominantly associated with HDL ($> 94\%$). Different patterns were observed in case $^{125}\text{I-LPS}$ was injected after incubation with the apoE-enriched emulsion. At 1 min after injection, as much as 47% of the serum radioactivity was recovered in the fraction containing emulsion ($d \leq 1.006$ g/ml), and 35% was still associated with this fraction at 10 min after injection.

Effect of emulsion and/or apoE on the extrahepatic distribution of $^{125}\text{I-LPS}$. The relative contribution of several extrahepatic organs to the serum removal of $^{125}\text{I-LPS}$ was determined at 30 min after injection (Fig. 3). As LPS is known to bind to cells of the reticuloendothelial system (4, 6), uptake by the spleen and lungs was examined. Only the spleen showed a high uptake of $^{125}\text{I-LPS}$ ($7.3 \pm 1.6\%$ of the injected dose/g wet wt), exceeding that of the liver ($5.5 \pm 0.9\%$ g wet wt). The uptake of $^{125}\text{I-LPS}$ by the spleen (per gram wet weight) was markedly reduced upon preincubation with the apoE-enriched emulsion ($1.9 \pm 0.3\%$) or apoE alone ($1.5 \pm 0.4\%$, $P < 0.0001$). These values are comparable to the uptake of $^{125}\text{I-apoE}$ ($1.4 \pm 0.1\%$). Spleen uptake of $^{125}\text{I-LPS}$ was not largely reduced in the presence of the protein-deficient emulsion ($6.5 \pm 2.1\%$) or Lipofundin ($6.4 \pm 0.5\%$).

Effect of emulsion and/or apoE on the liver cell distribution of $^{125}\text{I-LPS}$. The aforementioned data already suggest that apoE is the constituent of recombinant chylomicrons responsible for altering the in vivo behavior of LPS. To further substantiate these observations, the effect of emulsion and/or apoE on the distribution of LPS over the various liver cell types was examined (Fig. 4). $^{125}\text{I-LPS}$ showed a high specific uptake (i.e., per milligram of cell protein) by Kupffer cells ($1,029 \pm 295\%$ of the injected dose $\times 10^3/\text{mg}$ cell protein) as compared with endothelial cells ($233 \pm 68\%$) or parenchymal cells ($20 \pm 7\%$). This pattern was not altered in the presence of the protein-free emulsion, with a specific uptake of $1,650 \pm 566\%$ by Kupffer cells.

In contrast, the specific uptake of $^{125}\text{I-LPS}$ by Kupffer cells was eight- to ninefold reduced after incubation with the apoE-enriched emulsion ($125 \pm 21\%$) or apoE alone ($116 \pm 57\%$, $P < 0.01$), and approached that of $^{125}\text{I-apoE}$ ($90 \pm 30\%$). A similar

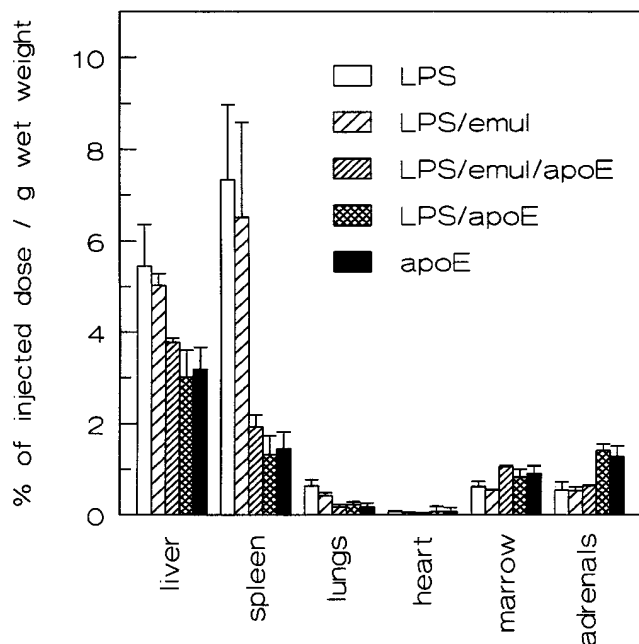


Figure 3. Effect of emulsion and/or apoE on the organ distribution of $^{125}\text{I-LPS}$. $^{125}\text{I-LPS}$ (2.5–5.0 μg) was injected into anesthetized rats, without (open bars, $n = 5$) or with (hatched bars, $n = 2$) previous incubation with emulsion (10 mg triglycerides), emulsion (10 mg triglycerides) in combination with apoE (400 μg) (densely hatched bars, $n = 2$), or apoE alone (400 μg) (cross-hatched bars, $n = 5$). $^{125}\text{I-apoE}$ (5.0 μg) (closed bars) was also injected. At 30 min after injection, the contribution of various organs to the uptake of $^{125}\text{I-LPS}$ and $^{125}\text{I-apoE}$ was determined. Values are corrected for serum radioactivity. Recoveries of ^{125}I -radioactivity in the rats exceeded 92% in all distribution studies.

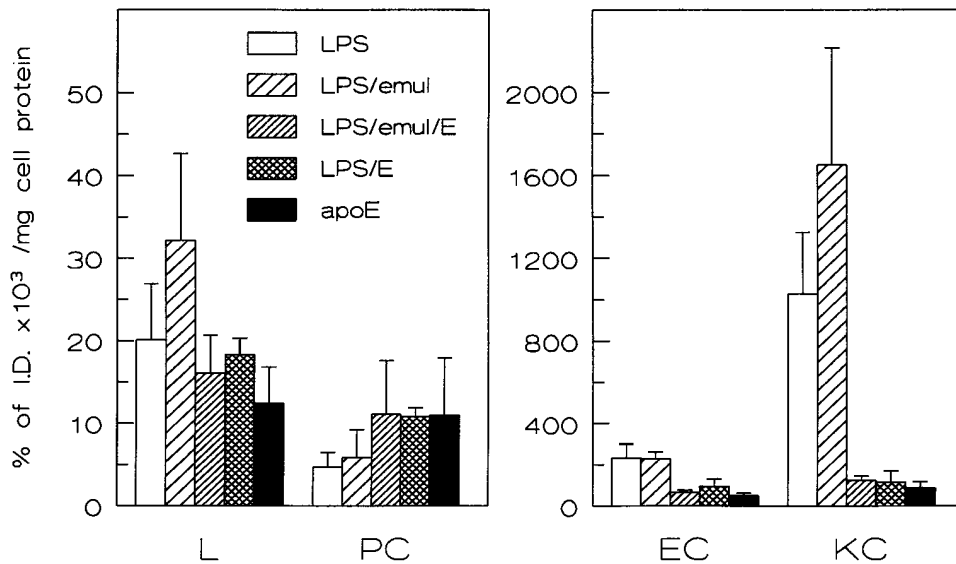


Figure 4. Effect of emulsion and/or apoE on the liver cell distribution of ^{125}I -LPS. ^{125}I -LPS (5.0 μg) was injected into anesthetized rats without (open bars, $n = 3$) or with (hatched bars, $n = 2$) previous incubation with emulsion (10 mg triglycerides), emulsion (10 mg triglycerides) in combination with apoE (400 μg) (densely hatched bars, $n = 2$), or apoE alone (400 μg) (cross-hatched bars, $n = 3$). ^{125}I -apoE (5.0 μg) was also injected (closed bars, $n = 2$). At 10 min after injection, the liver was perfused at $< 8^\circ\text{C}$ to prevent (further) degradation of internalized ligand. After 8 min of perfusion, the total liver association was determined (L) and parenchymal (PC), endothelial (EC), and Kupffer (KC) cells were subsequently isolated at $< 8^\circ\text{C}$ by differential centrifugation and counterflow elutriation as described.

but less pronounced effect (three- to fourfold reduction of ^{125}I -LPS uptake) was observed for endothelial cells.

Despite the markedly decreased uptake by Kupffer cells, a significant portion of the injected ^{125}I -LPS was still recovered in the liver upon modification of its *in vivo* behavior by the apoE-enriched emulsion or free apoE (Figs. 1 and 3). It appeared that the specific uptake of ^{125}I -LPS by parenchymal cells ($4.7 \pm 1.8\%$ of the injected dose $\times 10^3/\text{mg}$ cell protein) increased approximately twofold in the presence of emulsion-apoE ($11.1 \pm 9.2\%$) or pure apoE ($10.8 \pm 1.1\%$, $P < 0.01$). Therefore, it can be calculated that preincubation of ^{125}I -LPS with emulsion-apoE or pure apoE resulted in a 19.6-fold increase in the ratio of uptake of ^{125}I -LPS by parenchymal cells over Kupffer cells.

Characterization of the mechanism of interaction between LPS and apoE. The *in vivo* distribution of ^{125}I -LPS was modified by apoE at a 1:80 weight ratio. In fact, the distribution over the body and between the various liver cell types of ^{125}I -LPS was virtually identical to that of ^{125}I -apoE. It was therefore attempted to elucidate their interaction. ^{125}I -LPS and ^{125}I -apoE were subjected to density gradient ultracentrifugation and were recovered at mean densities of 1.202 and ≥ 1.242 g/ml, respectively. After incubation of ^{125}I -LPS with unlabeled apoE at a 1:80 weight ratio for 30 min at 37°C , the majority of radioactivity was recovered at an intermediate mean density of 1.226 g/ml, indicating interaction between LPS and apoE withstanding ultracentrifugational forces (Fig. 5).

To further characterize the mechanism of interaction between apoE and LPS, agarose gel electrophoresis was used as a tool (Figs. 6 and 7). The emulsion, ^{125}I -apoE, and ^{125}I -LPS migrated towards the anode with relative electrophoretic mobilities of 0.15, 0.45, and 0.6–1.0, respectively (Fig. 6). ^{125}I -LPS was fully recovered with apoE upon incubation at an LPS/apoE weight ratio of 1:30. *In vivo*, the ratio of LPS over apoE was 2.7-fold lower, which implies that LPS is also quantitatively bound to apoE in this situation. ^{125}I -apoE that had been incubated with the emulsion below saturating conditions

(emulsion-triglycerides/apoE = 50:0.3) (35) comigrated with the emulsion. The same pattern was observed at a ratio of emulsion over apoE of 50:2, which mimics the *in vivo* situation (not shown). ^{125}I -LPS was only partially (33%) recovered at the position of the emulsion (relative electrophoretic mobility = 0.15) upon incubation, at an LPS/emulsion ratio that is

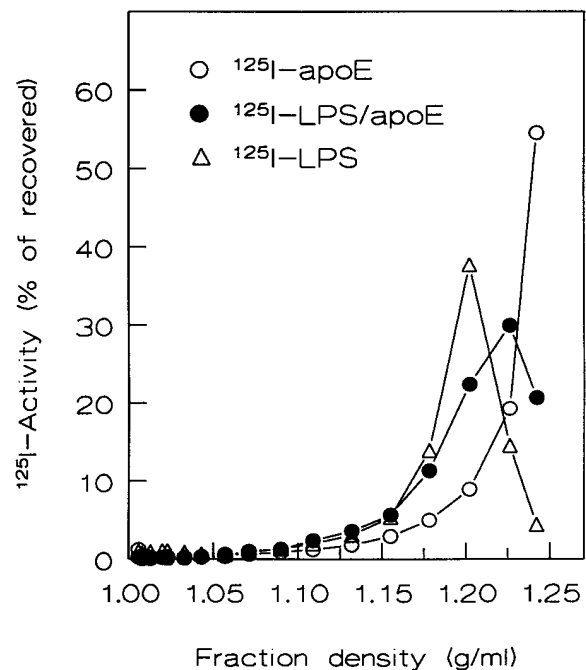


Figure 5. Effect of apoE on the density distribution of LPS. ^{125}I -LPS, ^{125}I -apoE, or the incubation mixture of ^{125}I -LPS and apoE (1:80, wt/wt) were subjected to density gradient ultracentrifugation as described. After centrifugation, the tubes were subdivided according to their density and assayed for radioactivity.

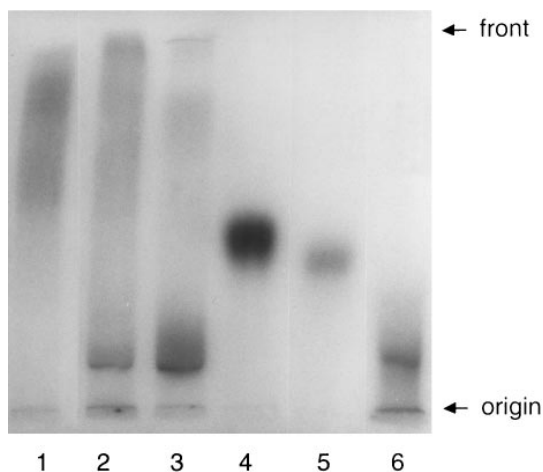


Figure 6. Effect of emulsion and/or apoE on the electrophoretic mobility of LPS. ^{125}I -LPS ($1.0\ \mu\text{g}$) (lane 1) was incubated with emulsion ($200\ \mu\text{g}$ triglycerides) (lane 2), emulsion ($200\ \mu\text{g}$ triglycerides) in combination with apoE ($4.0\ \mu\text{g}$) (lane 3), or apoE alone ($30\ \mu\text{g}$) (lane 4). Also, ^{125}I -apoE ($0.3\ \mu\text{g}$) (lane 5) was incubated with emulsion ($50\ \mu\text{g}$ triglycerides) (lane 6). Aliquots of the incubation mixtures ($\sim 10^5$ dpm) were subjected to electrophoresis in a 0.75% (wt/vol) agarose gel at pH 8.8. The resulting gel was dried and assayed for radioactivity by autoradiography.

10-fold higher than applied in vivo. Addition of apoE to the incubation mixture of emulsion and LPS (emulsion-triglycerides/apoE = 50:1) resulted in an increased (61%) comigration of ^{125}I -LPS with the emulsion, but not with free apoE (relative electrophoretic mobility = 0.44). Apparently, apoE is able to connect ^{125}I -LPS with the emulsion, and LPS does not interfere with the particle-binding characteristics of apoE.

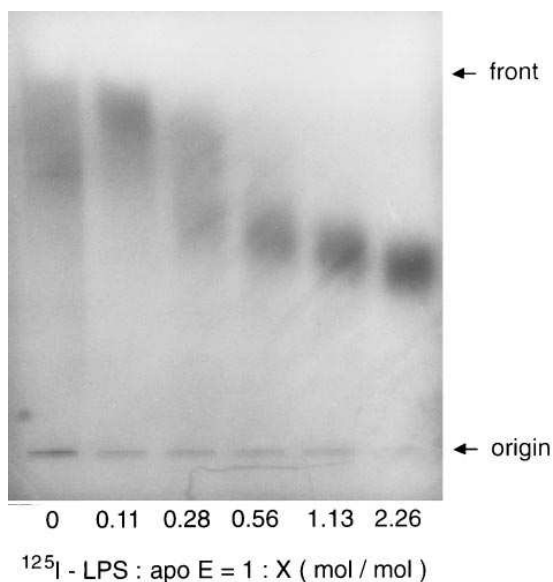


Figure 7. Concentration-dependent effect of apoE on the electrophoretic mobility of LPS. ^{125}I -LPS was incubated with apoE at the indicated molar ratios. Aliquots of the incubation mixtures ($\sim 10^5$ dpm) were subjected to electrophoresis in a 0.75% (wt/vol) agarose gel at pH 8.8. The resulting gel was dried and assayed for radioactivity by autoradiography.

The LPS-binding capacity of apoE was subsequently determined (Fig. 7). Incubation of ^{125}I -LPS with excess of apoE (1:2.26 molar ratio) resulted in a quantitative recovery of ^{125}I -LPS at the apoE position. At increasing ^{125}I -LPS/apoE molar ratios, a gradually increasing mobility of apoE and the appearance of free ^{125}I -LPS were observed. It could be calculated from the emergence of an apoE-bound and free ^{125}I -LPS population that apoE can bind approximately two molecules of LPS.

The affinity of apoE for LPS was strongly decreased upon CHD modification of apoE in borate buffer as evidenced from the emergence of unbound ^{125}I -LPS already at an LPS/CHD-apoE = 1:2.26 molar ratio. Consequently, the beneficial effects of apoE on the in vivo behavior of LPS were completely abolished. The serum kinetics of ^{125}I -LPS were not altered upon preincubation with CHD-apoE, and no effects were observed on the specific uptake by the spleen ($7.1 \pm 0.3\%$ of the injected dose/gram wet wt, mean \pm SD [$n = 3$], $P > 0.05$) or Kupffer cells ($2,495 \pm 868\%$ of the injected dose $\times 10^3$ /mg cell protein, mean \pm SD [$n = 3$], $P > 0.05$) (results not shown).

Discussion

Endotoxin derived from Gram-negative bacteria is a major risk factor for septic shock-induced lethality in the clinic (1). Within the circulation, LPS binds to LBP and the complex primarily interacts with CD14 on macrophages such as Kupffer cells (5, 6), which subsequently release mediators responsible for the metabolic and physiological changes preceding death (9, 10). Mice that were sensitized with D-galactosamine ($1,000\ \text{mg/kg}$) were protected against LPS-induced death by coinjection of LPS ($11\text{--}13\ \mu\text{g/kg}$) with VLDL and chylomicrons at a triglyceride dosage of $90\text{--}300$ and $15\text{--}90\ \text{mg/kg}$, respectively (20). Similarly, death was prevented in rats when LPS ($14\ \mu\text{g/kg}$) was coinjected with chylomicrons ($500\text{--}1,000\ \text{mg/kg}$), and a higher recovery of LPS in parenchymal liver cells and a more rapid secretion of LPS in the bile were observed (21, 22).

In search of a therapeutic treatment of endotoxemia, we investigated whether recombinant chylomicrons were able to alter the in vivo fate of LPS, and which mechanism is involved in the protection of triglyceride-rich lipoproteins against endotoxin-induced death. In our experiments, we applied doses of LPS ($9\text{--}18\ \mu\text{g/kg}$), emulsions ($40\text{--}50\ \text{mg/kg}$), and Lipofundin ($500\ \text{mg/kg}$) similar to those used by Harris and co-workers (20, 21). Upon injection into rats, LPS rapidly and specifically associated with cells of the reticuloendothelial system, such as Kupffer cells and, presumably, splenic macrophages, as observed by others (4, 6). Indeed, recombinant chylomicrons were able to alter the in vivo serum kinetics, the liver cell distribution, and the organ distribution of LPS. Both the serum decay and liver uptake were delayed and the specific uptake by the spleen was 4.1-fold reduced. Within the liver, uptake of LPS shifted from Kupffer cells to liver parenchymal cells, resulting in a 19.6-fold increased ratio of uptake by parenchymal over Kupffer cells. Surprisingly, the apoE-deficient emulsion did not influence the in vivo kinetics and distribution pattern of LPS at a dose of $40\text{--}50\ \text{mg/kg}$, which is comparable to the dose of chylomicrons that protected mice against LPS-induced death (20). Intravenous administration of emulsions at higher dosage was precluded because further concentration of the emulsions induces physical changes such as aggregation. We therefore tested the possibility that protein-free emulsions can alter the metabolism of LPS by infusing a commercial protein-

free emulsion (Lipofundin) at a high dose of 500 mg/kg, which is equivalent to the dose of chylomicrons that prevented LPS-induced death in rats (21). Again, no effects were observed on the in vivo distribution of LPS. Consistently, induction of severe hypertriglyceridemia (10-fold) by infusion of Intralipid did not inhibit the production of LPS-induced mediators (TNF, IL-1, and IL-8) in humans (36). These results suggest that lipid-lipid interaction does not play a dominant role in the beneficial effect of recombinant chylomicrons on the in vivo distribution of LPS. In fact, pure apoE appeared to be able to redirect the uptake of LPS from splenic and hepatic macrophages to liver parenchymal cells. The distribution of LPS over both the various liver cell types and the body resembled that of apoE when preincubated at an LPS/apoE = 1:80 weight ratio. Preincubation of LPS with apoE was not needed for obtaining the observed effects, which is an important finding with respect to the clinical potential of apoE in sepsis.

Studies on the interaction between LPS and the protein-deficient emulsion using agarose gel electrophoresis showed that some binding of LPS to the emulsion did occur. ApoE appeared to bind approximately two molecules of LPS and greatly enhanced the association of LPS to the emulsion. A significant portion of LPS was associated with the apoE-enriched emulsion in the blood circulation, but not with the protein-deficient or commercial emulsion. Apparently, LPS readily dissociates from emulsions but not from apoE in vivo, and apoE is able to alter the in vivo distribution of LPS in both an emulsion-bound and free state. As chylomicrons collected from mesenteric lymph contain apoE, our observations might explain why Harris et al. (21) and Read et al. (22) observed an effect of chylomicrons on the metabolic fate of LPS in rats, albeit at high triglyceride concentrations (500–1,000 mg/kg). The relevance of our findings is further substantiated by the observation that the LPS distribution over the various components of rat serum in vitro closely resembled that of endogenous apoE, while a small fraction of LPS is present in a presumably unbound state.

The mechanism by which lipoproteins interact with endotoxin has been the subject of speculation before. Upon intravenous injection into rats, the LPS that remained in the serum after the rapid clearance phase was mainly recovered at the HDL densities. It is known that endotoxin binds to plasma HDL and that it circulates as a complex until it is cleared from the circulation (37). Recent observations indicate that LBP is associated with apoA-I, the main protein of HDL, and it has been postulated that LBP may have an LPS-neutralizing function when associated with HDL in plasma (38). The fact that HDL can bind and neutralize LPS (18, 37, 39) has been adapted to show that experimentally elevated levels of circulating HDL provide protection against endotoxin-induced death in mice (40). Both Emancipator et al. (41) and Flegel et al. (18) demonstrated that purified human apoA-I is capable of binding LPS, indicating that the lipid component of HDL is not an absolute requisite for LPS inactivation. A proposed mechanism is the constitution of mixed micelles by apoA-I and LPS as a consequence of the amphipathic structures of apoA-I and the hydrophobic lipid A part of LPS (18). The present data preclude a lipid-lipid interaction mechanism between LPS and apoE. First, agarose gel electrophoresis showed that the presence of apoE enhanced the binding of LPS to emulsion particles, whereas binding of LPS to the lipid part of apoE would interfere with the ability of apoE to incorporate into the

emulsion. Second, selective elimination of the positive charge on arginine residues of apoE resulted in a largely reduced binding of LPS to apoE and abolished the effect of apoE on the in vivo behavior of LPS. Third, lactoferrin is a glycoprotein with an arginine/lysine-rich sequence at positions 25–31 resembling the receptor binding site (amino acids 142–148) of apoE (42), but lacks a hydrophobic domain as in apolipoproteins. Still, lactoferrin has previously been shown to bind LPS (43, 44) and to decrease cytokine production upon LPS challenge (45). The high affinity LPS binding site of human lactoferrin has recently been deduced to the basic 28-34 loop region in its NH₂ terminus (46). Therefore, it is suggested that the cationic arginine and/or lysine sequences of apoE may interact with the lipid A portion of LPS that contains a number of negatively charged pyrophosphate groups (47), as shown for both lactoferrin (48) and the cationic peptide polymyxin B (49). It is not clear if LBP can play a bridging role between LPS and apoE, as has been demonstrated for HDL (38) and a recently described novel lipoprotein-like particle (50). The studies using agarose gel electrophoresis clearly show that LBP is not required for the apoE-mediated binding of LPS to emulsions, but an additional LPS-neutralizing role of LBP through binding to apoE in vivo cannot be excluded from the present experiments.

In summary, apoE has been demonstrated to bind LPS instantaneously through electrostatic interactions that are tight enough to redirect LPS from Kupffer cells to liver parenchymal cells in vivo. We suspect that apoE is the component of chylomicrons that is mainly responsible for protecting mice and rats against a lethal dose of endotoxin (20, 21). Therefore, it is anticipated that human recombinant apoE may serve as a therapeutic agent to protect against LPS-induced endotoxemia.

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