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The role of apolipoprotein E in the elimination of liposomes from blood by hepatocytes in the mouse

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Abstract

We evaluated the role of apolipoprotein E (apoE) in the clearance of neutral and negatively charged liposomes by hepatocytes in apoE-deficient mice. Negatively charged liposomes were cleared at identical rates in apoE-deficient and wild-type mice; neutral liposomes were cleared at a 3.6-fold slower rate in apoE-deficient mice. ApoE deficiency did not affect hepatic uptake of negatively charged liposomes but lowered that of neutral liposomes >5-fold. Hepatocyte uptake of neutral liposomes was reduced >20-fold in apoE-deficient mice; that of negatively charged liposomes remained unchanged. We conclude that uptake of neutral liposomes by hepatocytes is nearly exclusively apoE-mediated.

Keywords: Liposome opsonization; Apolipoprotein E-deficient mice; Hepatocytes

Despite extensive research on the use of liposomes as drug or gene carriers, there is still a lack of understanding of the mechanisms responsible for liposome elimination from the blood circulation. Many studies report a major involvement of tissue macrophages, residing mainly in liver and spleen, in the clearance of intravenously administered liposomes. The participation of hepatocytes in liposome elimination is often ignored, despite studies in our own as well as other laboratories indicating that hepatocytes may play a major role in this process [1–4]. Understanding the mechanisms by which liposomes are taken up by hepatocytes may facilitate the rational design of liposomes for drug or gene delivery.

Upon exposure to serum or plasma, liposomes become readily coated with proteins [5–10]. Both type and amount of plasma proteins adsorbing to the liposomal surface may vary considerably with the physicochemical characteristics of the vesicles [5–7,10,11]. Individual proteins associated with liposomes could serve as ligands guiding the particles to specific receptors on cell surfaces. Most investigators of the role of opsonization in liposome clearance single out the role of these opsonizing proteins as ligands for the selective delivery of liposomes to cells of the mononuclear phagocyte system (macrophage-directed opsonins) [7,9,12]. We have, on the other hand, emphasized that there may also be a role for certain plasma proteins that, upon adsorption to the liposomes, direct these to receptors on the hepatocyte surface, which could therefore be considered ''hepatocyte-directed opsonins'' [13]. Hepatocytes play a key

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role in lipid and lipoprotein metabolism, and some apolipoproteins serve as ligands for lipoprotein uptake by hepatocytes. Therefore, apolipoproteins are attractive candidates to play a role as such hepatocyte-directed liposomal opsonins. It has been shown previously that apolipoprotein (apo) E and apoA-I can bind to liposomes [14,15]. Additionally, Bisgaier et al. [16] showed that uptake of small sonicated neutral liposomes by HepG2 cells was enhanced specifically by apoE but not by apoA-I and apoA-IV. However, direct evidence showing the role of apoE in liposome clearance by hepatocytes in vivo has not been reported.

In the present study, we confirm the adsorption of apoE from serum to small neutral and negatively charged liposomes, and determined the pharmacokinetics of these liposomes in apoE-deficient and wild-type mice. In addition, we singled out the role of the liver and more specifically the hepatocytes in the elimination process. We evaluated the potential involvement of low density lipoprotein (LDL) receptors in hepatic clearance of liposomes by treating rats with 17α-ethinyl estradiol (EE), an agent known to stimulate expression of hepatic LDL receptors [17,18].

Materials and methods

Animals. Male Wag/Rij rats (TNO, Rijswijk, The Netherlands) were used for the study of the inhibitory effect of surface-grafted poly(ethylene glycol) (PEG) on liposome clearance by hepatocytes and the effect of EE-treatment on hepatic clearance of liposomes. ApoE-deficient (*Apoe*−/−) mice were generated on a C57BL/6J background as described before [19]. Female C57BL/6J (wild-type) and *Apoe*−/− mice were used at 3–4 months of age. On normal lab chow, plasma cholesterol levels in wild-type and *Apoe*−/− mice were 1.94 ± 0.26 and 11.54 ± 1.27 mM, respectively (values represent means ± SD). The animals were kept under standard laboratory animal conditions and had free access to water and food. All experimental protocols were approved by the Local Committee for Care and Use of Laboratory Animals.

Materials. 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (PC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-L-serine] (PS) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Cholesterol (Chol) was obtained from Sigma (St. Louis, MO, USA). Poly(ethylene glycol)-distearoylphosphatidylethanolamine (PEG-DSPE) was a generous gift of Sequus Pharmaceuticals (Menlo Park, CA, USA). [1α,2α (*n*)-^{3}H]Cholesteryl oleyl ether ([^{3}H]COE) was from Amersham (Buckinghamshire, UK). Bio-Gel A-1.5m gel was from Bio-Rad (Hercules, CA, USA). Amplex Red Cholesterol Assay kit and 1,1′dioctadecyl-3,3,3′,3′-tetramethyl indocarbocyanine perchlorate (DiI) were purchased from Molecular Probes (Eugene, OR, USA). Pronase, collagenase, and nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate (NBT/BCIP) were from Roche Diagnostics (Mannheim, Germany). All other chemicals were of analytical grade or of the highest grade available.

Liposome preparation. Lipids from stock solutions of PC, PS, and Chol in chloroform/methanol (9:1) were mixed in the following molar ratios: PC/Chol (6:4), PC/Chol/PS (5:4:1), and PC/Chol/PS (5:4:3). A trace amount of [^{3}H]COE was added to each preparation as a non-metabolizable marker. For some experiments, DiI was added to the lipid mixtures as a fluorescent marker. When appropriate, PEG-DSPE was also added to the lipid mixture (6 mol%). The lipid mixtures were dried under reduced nitrogen pressure, dissolved in cyclohexane, and lyophilized. The lipids were then hydrated in HN buffer (10 mM Hepes, 135 mM NaCl, pH 7.4) and vortexed. The liposomes formed were sized by repeated extrusion (13 times) through polycarbonate filters (Costar, Cambridge, MA, USA), pore size 50 nm, using a high-pressure extruder (Lipex, Vancouver, Canada). Phospholipid phosphorus of each liposome preparation was determined by phosphate assay after perchloric acid destruction [20]. Liposome size and size distribution were determined by dynamic light scattering using a Nicomp Model 380 Submicron Particle analyzer (NICOMP particle sizing systems, Santa Barbara, CA, USA). The diameter of the liposome preparations, which was obtained from the volume distribution curves produced by the particle analyzer, was 80.6 ± 3.3 nm for neutral liposomes and 78.4 ± 1.8 nm ($n = 5$, mean ± SD) for liposomes containing 10% PS.

Isolation of liposomes from mouse serum using spin columns. Sixty microliters of DiI-labeled liposomes (total lipid concentration 5 mM) was incubated with 140 μl mouse serum at 37 °C for 1 h. The liposomes were then isolated from the serum by a spin column procedure slightly modified after [6]. Briefly, 1-ml tuberculin syringes plugged with glass wool were filled with Bio-Gel A-1.5 m gel equilibrated with HN buffer and centrifuged. Several fills and centrifugations were performed until the bed volume approximated 1 ml. Aliquots of the incubation mixture (50 μl) were then applied to spin columns and immediately centrifuged (800 rpm, 1 min). Column fractions were collected in glass tubes by applying 50 μl HN buffer to the columns and subsequent centrifugation (800 rpm, 1 min). The liposome content of the column fractions was determined by measuring DiI-fluorescence using a FL500 fluorescence plate reader (Bio-Tek Instruments, Winooski, VT, USA).

Analysis of apoE adsorption to liposomes. Equal amounts of liposomes isolated from serum incubations (see above) were loaded onto a 10% SDS–polyacrylamide gel, and the proteins associated with liposomes were separated by SDS–PAGE under reducing conditions. After electrophoresis, the proteins were transferred to nitrocellulose membranes. The blots were blocked in Tris-buffered saline/Tween 20 supplemented with 2% bovine serum albumin for 2 h. Subsequently they were incubated for 3 h with rabbit anti-mouse apoE antibody (Biodesign, Saco, ME, USA), diluted 1:500 in blocking buffer. This was followed by washing and incubation with alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma) and the detection of signals with NBT/BCIP developing solution. The intensity of the protein bands was quantified using software Quantity One (Bio-Rad).

Animal studies. For the study of the inhibitory effect of surface-grafted PEG on liposome clearance by hepatocytes, pentobarbital anesthetized Wag/Rij rats (220–260 g) were injected with 5 μmol liposomal lipid via the penile vein. After 48 h, hepatocytes and Kupffer cells were isolated and the cell-associated radioactivity was determined as described previously [21]. For the study of the effect of EE treatment on hepatic clearance of liposomes, rats were injected subcutaneously for 5 days with 5 mg/kg body weight of EE in propylene glycol. Control rats received an equal volume of propylene glycol alone. Then the EE-treated and control rats were anesthetized and injected with 10 μmol/kg body weight of liposomal lipid via the penile vein. After 6 h, the liver was removed and processed for measurement of radioactivity as described before [22].

For in vivo studies in mice, the animals were anesthetized and a blood sample was collected from the retro–orbital sinus for blood cholesterol determination. Subsequently, liposomes (20 μmol liposomal lipid/kg body weight) were administered by retro–orbital injection in the left eye. At the indicated time points, blood samples were taken by retro–orbital bleeding from the right eye. 120 min after injection liver, spleen, kidney, lung, and heart were removed and processed for measurement of radioactivity as described previously [22]. For determination of liposome uptake by hepatocytes, the liver was perfused with collagenase containing buffer and hepatocytes were isolated as described before [23]. The number of hepatocytes was determined microscopically and the cell-associated radioactivity was determined

by scintillation counting. The blood disappearance of neutral and negatively charged liposomes was analyzed using the program Multifit (J.H. Proost, Department of Pharmacokinetics and Drug delivery, University of Groningen, The Netherlands) [24] using a one-compartment model. Liposomal elimination rates were calculated as clearance.

Statistical analysis. Statistical significance of differences was evaluated by a two-tailed unpaired Student's *t* test.

Results

Fig. 1 demonstrates that incorporation of PEG on the surface of neutral liposomes causes a strong reduction in uptake not only by the Kupffer cells but even more so by the hepatocytes. It is generally acknowledged that the "stealth" effect of PEG is based on a reduced opsonin adsorption, which in turn causes a reduced elimination rate from the blood compartment. The strongly diminished hepatocytic uptake of PEG liposomes would imply that PEG also reduces adsorption of proteins that cause liposomes to interact with the hepatocytes.

A likely candidate to serve as such a hepatocyte-specific opsonin is apoE. Fig. 2 shows the adsorption of apoE to differently formulated liposomes, essentially confirming earlier observations [13]. ApoE adsorbed to both neutral and negatively charged liposomes. However, liposomes containing PS bound 1.5- to 1.8-fold more apoE than neutral liposomes, as determined by densitometry. In order to establish whether the apoE adsorbing to liposomes plays a role in liposome clearance, we determined blood elimination and tissue distribution of neutral and negatively charged liposomes in apoE-deficient and wild-type mice. For negatively charged liposomes, containing 10% PS, plasma elimination kinetics were nearly identical for both types of mice (Fig. 3). Clearance of these liposomes in wild-type mice

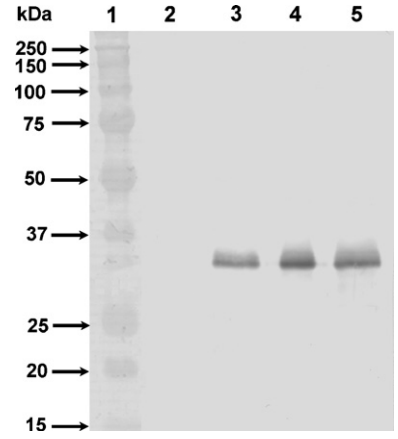

Fig. 2. ApoE adsorption to neutral and negatively charged liposomes. Following incubation with mouse serum, liposomes were isolated from the serum as described under Materials and methods. Subsequently, equal amounts of liposomes were applied to a 10% SDS–polyacrylamide gel, and the proteins associated with liposomes were separated by SDS–PAGE followed by Western blotting. 1, protein standards; 2, control (without liposomes); and 3–5 are liposomes containing 0% (neutral), 10%, and 30% PS. The experiments were repeated three times, and similar results were obtained.

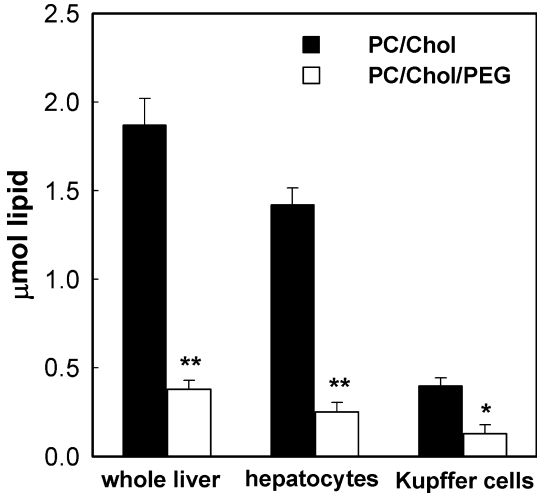

Fig. 1. Effect of PEG-DSPE on uptake of neutral liposomes by both Kupffer cells and hepatocytes. PC/Chol liposomes, with (open bars) or without (filled bars) 6 mol% PEG-DSPE and labeled with [^{3}H]COE, were injected (5 μmol of total lipid) i.v. into rats and 20 h later radioactivity was determined in the whole liver, isolated Kupffer cells, and hepatocytes. Results are given as micromoles of total lipid associated with the liver or whole cell fractions (mean ± SD, $n = 4$). *$P < 0.005$, **$P < 0.001$, versus PC/Chol liposomes.

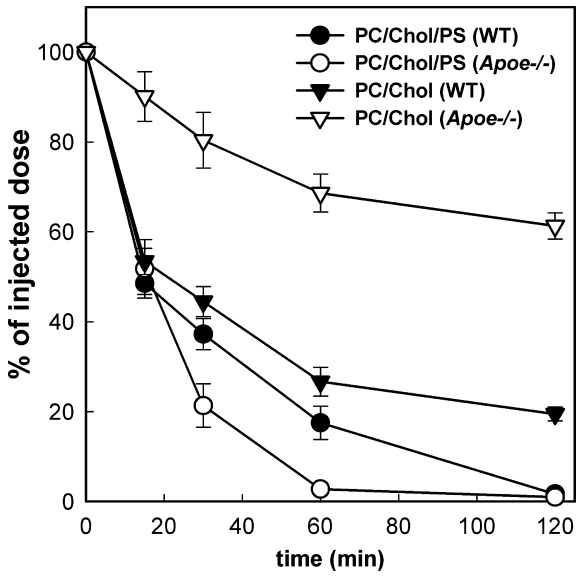

Fig. 3. Plasma disappearance of neutral and negatively charged liposomes in wild-type and *Apoe*−/− mice. After injection of [^{3}H]COE-labeled liposomes containing no PS (▼, ▽) or 10% PS (●, ○) into wild-type mice (closed symbols) or *Apoe*−/− mice (open symbols), blood samples were taken at indicated time points and radioactivity was determined as described in Materials and methods. Data are presented as percentage of injected dose (WT, wild-type; means ± SEM, $n = 5$).

and apoE-deficient mice was 1.41 and 2.11 ml/min/kg ($P > 0.05$), respectively. However, the clearance of neutral liposomes was only 0.17 ml/min/kg in apoE-deficient mice, 3.6-fold slower than in wild-type mice (0.61 ml/min/kg) ($P < 0.001$). Two hours after administration of neutral liposomes still more than 60% of the injected dose resided in the blood of apoE-deficient mice; in wild-type mice only 20%. In terms of hepatic uptake, the difference between apoE-deficient and wild-type mice was much more conspicuous (Fig. 4). Regardless of the type of mouse, the liver largely accounted for blood elimination of both liposome types, while the spleen accounted for ~12% of PS-containing and ~2% of neutral liposomes. Kidney, heart, and lung did not significantly contribute to the uptake of either neutral or PS-containing liposomes in either type of mice (<1% of the injected dose). For liposomes containing 10% PS no difference in liver uptake was found between the two types of mice, but hepatic uptake of neutral liposomes was as much as five times lower in apoE-deficient than in wild-type mice. In order to confirm the specific role of hepatocytes in the hepatic uptake of neutral liposomes, we isolated the hepatocyte fraction of mouse livers after liposome injection and determined liposome uptake (Fig. 5). Analogous to the results on blood disappearance and tissue distribution, we observed no difference in the uptake of PS-containing liposomes by hepatocytes (in both types of mice we found 87 nmol total lipid/1×10^8 cells). In sharp contrast, the uptake of neutral liposomes by hepatocytes in apoE-deficient mice had nearly vanished: hepatocyte uptake in apoE-deficient mice was >20 times lower than that in wild-type mice.

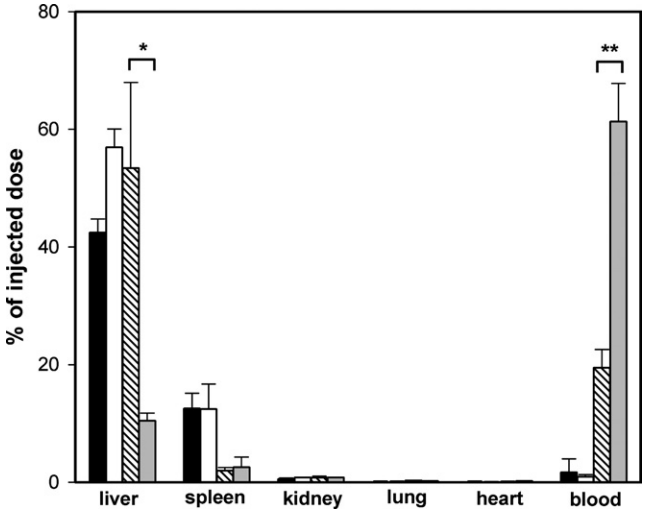

Fig. 4. Tissue distribution of neutral (PC/Chol, 6:4) and negatively charged (PC/Chol/PS, 5:4:1) liposomes in wild-type and *Apoe*−/− mice. One hundred twenty minutes after injection of [^{3}H]COE-labeled liposomes into mice, liver, spleen, kidney, lung, and heart were removed and processed for measurement of radioactivity as described under Materials and methods. Negatively charged liposomes in wild-type mice (black bars) and in *Apoe*−/− mice (open bars); neutral liposomes in wild-type mice (hatched bars) and in *Apoe*−/− mice (grey bars). Data are presented as percentage of injected dose associated with each organ (WT, wild-type; means ± SD, $n = 4$). *$P < 0.005$, **$P < 0.001$.

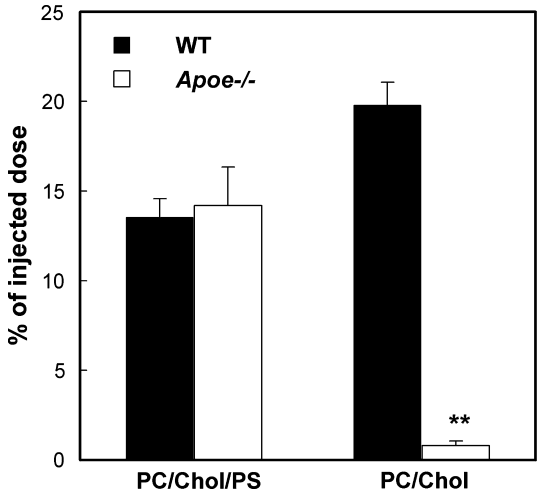

Fig. 5. Hepatocyte uptake of neutral (PC/Chol, 6:4) and negatively charged (PC/Chol/PS, 5:4:1) liposomes in wild-type mice (filled bars) and in *Apoe*−/− mice (open bars). [^{3}H]COE-labeled liposomes were injected into mice, 120 min later hepatocytes were isolated as described under Materials and methods, and cell-associated radioactivity was measured. Data are expressed as percentage of injected dose associated with the cells (WT, wild-type; means ± SD, $n = 4$). **$P < 0.001$ versus wild-type mice.

ApoE is known to bind with high affinity to the LDL receptor [25]. In order to evaluate whether this receptor is involved in hepatic uptake of neutral and negatively charged liposomes, we treated rats with EE to stimulate hepatic LDL receptor expression. As shown in Fig. 6,

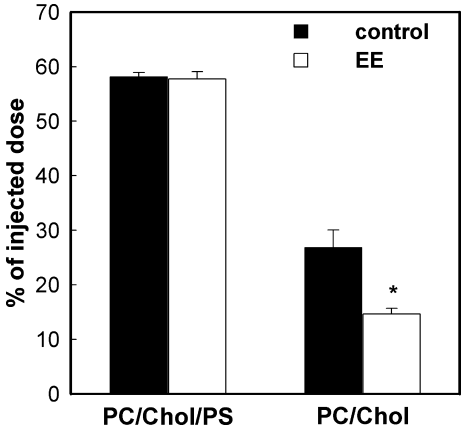

Fig. 6. Effect of EE treatment on hepatic uptake of neutral (PC/Chol, 6:4) and negatively charged (PC/Chol/PS, 5:4:1) liposomes in rats. [^{3}H]COE-labeled liposomes were injected into control rats (filled bars) or rats treated with EE (5 mg/kg) for 5 days (open bars). Six hours later radioactivity was determined in the whole liver. Results are expressed as percentage of injected dose associated with the liver (means ± SD, $n = 3$). *$P < 0.005$ versus control rats.

hepatic uptake of negatively charged liposomes was not affected by EE treatment, whereas that of the neutral vesicles was decreased by 50%.

Discussion

The finding that PEG causes a severe reduction in in vivo uptake of liposomes by hepatocytes led us to postulate the involvement of plasma proteins in liposome–hepatocyte interaction.

ApoE is a 34-kDa apolipoprotein and a constituent of chylomicrons, VLDL, IDL, and HDL [26]. In view of the indispensable role of apoE in receptor-mediated lipoprotein clearance by the liver and more specifically by hepatocytes [27], apoE may be an ideal candidate to function as a putative "hepatocyte-directed opsonin." In agreement with earlier studies [13] we confirmed that apoE does adsorb to liposomes during incubation of liposomes with mouse serum. We demonstrated that there is no effect of apoE deficiency on the elimination of 10% PS-containing liposomes. There was, however, a dramatic effect of apoE deficiency on the clearance of neutral liposomes by hepatocytes. These results strongly suggest that the uptake of small neutral liposomes by hepatocytes is almost exclusively apoE-mediated. The uptake of PS-containing liposomes by hepatocytes, on the other hand, is mediated by an apoE-independent mechanism, despite the more abundant adsorption of apoE to these liposomes (Fig. 2). This may be explained by the consideration that the receptor-binding region of apoE, which is situated in the vicinity of amino acid residues 140–160, contains a high proportion of basic residues [27]. Conceivably, the negatively charged PS interacts with the basic residues in the receptor binding domain of apoE allowing substantial adsorption of apoE to these liposomes, but at the same time completely impairing apoE receptor recognition by hepatocytes.

ApoE is a high-affinity ligand for the LDL receptor, which has been shown to be essential for high affinity hepatic clearance of remnant lipoproteins [28–30]. We studied LDL receptor effects in EE-treated rats since EE treatment in mice does not result in LDL receptor up-regulation [31]. Our data on rats treated with EE (Fig. 6) argue against a role of this receptor in liposome uptake. Hepatic uptake of negatively charged liposomes in rats treated with EE was unchanged as compared to control animals, which is compatible with our current finding in mice that the LDL receptor probably plays no role in uptake of such liposomes. Remarkably, the EE treatment resulted in a 50% decrease in the hepatic uptake of the neutral liposomes, counter-indicative of a possible involvement of LDL receptors in the uptake process. It is worth noting, however, that scavenger receptor class B type I (SR-BI), a key player in selective uptake of cholesterol esters from HDL, is down-regulated in the liver of EE-treated rats [18]. SR-BI could engage HDL particles at the cell surface in a transient interaction [32], while it has also been reported that apoE could bind to this receptor [33,34]. However, recently we found that SR-BI is involved in the uptake of liposomal constituents by hepatocytes from negatively charged liposomes but not from neutral liposomes [35]. In addition to the LDL receptor, in the liver apoE also binds to LDL receptor-related protein/α2-macroglobulin receptor (LRP) [36] and heparan sulfate proteoglycans (HSPGs) [37]. Assessment of the role of these receptors in liposome clearance awaits further studies with, for instance, LDL receptor/apoE double knockout mice, SR-BI deficient mice, and inhibitors of LRP and HSPGs.

The hepatocytes accounted for less than 50% of the hepatic clearance of the small neutral liposomes (Figs. 4 and 5). Obviously, in mice also non-parenchymal cells, in particular Kupffer cells, contributed significantly to the uptake of these liposomes. Since total liver uptake of neutral liposomes is 5-fold lower in apoE-deficient mice than in control mice, while uptake by hepatocytes is as much as 20-fold lower, liposome uptake by Kupffer cells must be operational. Earlier studies from our laboratory have shown that in the rat liver small neutral liposomes distribute in large majority to the hepatocytes [4]. For mice no such data are available. Species differences in liposome pharmacokinetics were reported before [38].

In conclusion, we have demonstrated that the hepatocyte uptake of small neutral liposomes in mice is mediated by apoE, while negatively charged PS-containing liposomes are taken up by hepatocytes via a non-apoE mediated mechanism, probably not involving LDL receptors.

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