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Uptake of phosphatidylserine-containing liposomes by liver sinusoidal endothelial cells in the serum-free perfused rat liver

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Abstract

We studied the kinetics of hepatic uptake of liposomes during serum-free recirculating perfusion of rat livers. Liposomes consisted of phosphatidylcholine, cholesterol and phosphatidylserine in a 6:4:0 or a 3:4:3 molar ratio and were radiolabelled with [^{3}H]cholesteryl oleyl ether. The negatively charged liposomes were taken up to a 10-fold higher extent than the neutral ones. Hepatic uptake of fluorescently labelled liposomes was examined by fluorescence microscopy. The neutral liposomes displayed a typical Kupffer cell distribution pattern, in addition to weak diffuse staining of the parenchyma, while the negatively charged liposomes showed a characteristic sinusoidal lining pattern, consistent with an endothelial localization. In addition, scattered Kupffer cell staining was distinguished as well as diffuse parenchymal fluorescence. The mainly endothelial localisation of the negatively charged liposomes was confirmed by determining radioactivity in endothelial and Kupffer cells isolated following a 1-h perfusion. Perfusion in the presence of polyinosinic acid, an inhibitor of scavenger receptor activity, reduced the rate of uptake of the negatively charged liposomes twofold, indicating the involvement of this receptor in the elimination mechanism. These results are compatible with earlier in vitro studies on liposome uptake by isolated endothelial cells and Kupffer cells, which showed that in the absence of serum also endothelial cells in situ are able to take up massive amounts of negatively charged liposomes. The present results emphasize that the high in vitro endothelial cell uptake in the absence of serum from earlier observations was not an artifact induced by the cell isolation procedure.
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Keywords: Phosphatidylserine; Liposome; Liver

1. Introduction

The mechanisms involved in the elimination of liposomes from the blood compartment are still not fully understood. Upon parenteral administration, liposomes are taken up by macrophages of the spleen and the liver and, depending on liposome size and composition, hepatocytes may also significantly contribute to their uptake from the blood [1]. An important role in these uptake mechanisms has been attributed to serum proteins that, in relation to macrophage uptake, are often referred to as opsonins [2]. The involvement of serum or serum proteins in the clearance of liposomes from the blood appears to be very complex. Liposomal parameters such as lipid composition and charge are determining factors in the process of blood clearance, while also considerable species differences have been reported [3, 4]. In addition to serum-dependent liposome uptake mechanisms, serum-independent liposome uptake has been reported in a liver perfusion system in mice [5, 6]. In rats liposomes containing phosphatidylserine (PS) are efficiently eliminated from the blood by cells of the mononuclear phagocyte system, predominantly Kupffer cells in the liver. Hepatocytes have also been

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shown to contribute to the uptake of small liposomes containing PS, but we have consistently found liver endothelial cells not to participate in the elimination of liposomes without ligands [7, 8]. By contrast, in primary cultures of liver endothelial cells, we demonstrated that binding and uptake by these cells of liposomes containing PS were at least as efficient as that by Kupffer cells [9]. This in vitro association of PS-containing liposomes with liver endothelial cells was attributed to scavenger receptor activity. Depending on the PS content, the interaction of liposomes with scavenger receptors was partly or even completely inhibited by serum. The latter observation may explain at least partly the in vitro–in vivo differences in the capability of liver endothelial cells to take up liposomes containing PS. However, besides the continuous presence of serum proteins in vivo, another important difference with the in vitro situation is the obvious fact that, in vivo, liver endothelial cells co-exist in close proximity to or even in contact with Kupffer cells and hepatocytes, which may affect their functions. To gain more insight in the mechanisms underlying liposomal blood clearance by liver cells in the intact liver, we applied a controlled, isolated rat liver circulating perfusion system [10, 11].

In this study, we investigated the interaction of radioactively or fluorescently labelled negatively charged liposomes containing 30% PS and uncharged neutral liposomes in the intact perfused rat liver. In this way, hepatic liposome elimination can be studied in the absence of blood constituents but in an otherwise intact physiological environment. Liver uptake, uptake rate and intrahepatic distribution of the liposomes were determined. The possible involvement of scavenger receptors in liposome uptake in the intact perfused liver was assessed during perfusions in the presence of polyinosinic acid, an established inhibitor of scavenger receptor-mediated uptake.

2. Materials and methods

2.1. Materials

Egg phosphatidylcholine (PC) was purchased from Lipoid GmbH (Ludwigshafen, Germany). Cholesterol (Chol) was obtained from Genzyme Pharmaceuticals (Liestal, Switzerland). Soybean phosphatidylserine (PS) was supplied by the American Lecithin Company (Oxford, Connecticut, USA). [1α, 2α(*n*)-^{3}H] Cholesteryl oleyl ether (^{3}H-COE) was obtained from Amersham Pharmacia biotech (Freiburg, Germany). DiI (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate) and polyinosinic acid (Poly(I))-potassium salt were obtained from Sigma-Aldrich Co., USA. All other chemicals obtained from commercial sources were analytical grade or the best grade available.

2.2. Animals

Specified pathogen-free (SPF) male Wag/Rij rats (Harlan, Horst, The Netherlands) or Wistar rats (Boehringer Ingelheim, Germany) were kept under standard animal laboratory conditions and had free access to standard lab chow and water. The experimental protocols were approved by the local committee for care and use of laboratory animals.

2.3. Liposome preparation

The liposomes used in this study were composed of PC, Chol and PS in molar ratios of 3:4:3 (30% PS) or 6:4:0 (0% PS), respectively. The liposomes contained a tracer amount of the radioactive label [1α, 2α(*n*)-^{3}H] Cholesteryl oleyl ether. In some experiments 0.25 mol% of the fluoresecent marker 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) was incorporated in the liposomal bilayers. Liposomes were produced by a conventional rotary evaporation method [12] and sized by extrusion through polycarbonate filters with a pore size of 200 nm (Costar Nuclepore, Tübingen, Germany) using a commercially available device (EmulsiFlex-C5, Avestin Inc., Ottawa, Ontario, Canada). PC content was measured by a phospholipase D/cholinoxidase/PAP-test (Phospholipids B, WAKO Chemicals GmbH, Neuss, Germany) and the liposome concentration was adjusted to 10 mg of total lipid/ml using Krebs–Henseleit buffer (118 mM NaCl, 4.74 mM KCl, 0.59 mM KH_2PO_4, 0.59 mM Na_2HPO_4, 24.90 mM $NaHCO_3$, and 5.50 mM D-glucose) without $MgCl_2$ and $CaCl_2$. Liposome size was determined by dynamic light scattering either using a Zetasizer 4 or a Zetasizer 3000HS (Malvern GmbH, Herrenberg, Germany). The diameter of the liposome preparations used was 200±38 nm with a polydispersity coefficient of not larger than 0.2. ζ-potential of the liposomes was determined using a NICOMP model 380 ZLS zeta potential/particle sizer (NICOMP particle sizing systems, Santa Barbara, CA, USA). The ζ-potentials of 0% PS and 30% PS liposomes were −4.6 and −27.3 mV, respectively. Prior to the perfusions, the liposomes were diluted to a concentration of 0.4 mg/ml with Krebs–Henseleit buffer, the medium was warmed to 37 °C and the pH was adjusted to 7.4. Addition of 1.25 mM $CaCl_2$ and 1.19 mM $MgCl_2$ (final concentrations) was done immediately before the perfusion.

2.4. Oxygenation of the perfusion medium

For the oxygenation of the perfusion medium, Krebs–Henseleit buffer (washing buffer) or liposomal preparation was passed through a highly efficient homemade oxygenator, constructed from an HPLC inline degasser cassette (1 channel chamber, OmniLab AG, Mettmenstetten, Switzerland) using the vacuum connection as a carbogen (95% O_2, 5% CO_2) inlet. The perfusion medium was pumped

through the degasser cassette that was flushed with a gentle stream of carbogen. In control experiments this device had demonstrated its ability to saturate a buffer solution pumped through the cassette at a rate of 13 ml/min up to oxygen partial pressures of pO_2=600 mm Hg as estimated with a Radiometer ABL3 (Radiometer, Copenhagen, Denmark).

2.5. Perfusion system

Livers from rats weighing 200–250 g were perfused in situ with Krebs–Henseleit medium, pH 7.4, saturated with carbogen at a flow rate of 12.5 ml/min and a hydrostatic pressure of 8–12 cm water. For perfusion and liposome administration, the portal vein was cannulated and venous outflow was collected from a cannula, which was positioned at the upper central vein. After an initial perfusion with liposome-free buffer for a period of 15 min, allowing the liver to recover from the surgery, circulating perfusions with liposomal formulations were started as illustrated in Fig. 1. The perfusion buffer contained 0.4 mg/ml liposomes The livers were perfused in this circulation mode for a period of 1 h. Every 3 min, aliquots of the upper central venous effluent and from the perfusion reservoir were collected and radioactivity in these samples was determined. Concentrations of liposomes in the perfusion medium were calculated from the specific radioactivity of liposomes. The rate of hepatic extraction was calculated from: flux$\times(c_{\mathrm{influx}}-c_{\mathrm{outflow}})$ [mg/min].

To validate the experimental system, the functional integrity of the isolated perfused liver system was tested using standard kits for LDH (Merck, Switzerland) and transaminase GOT and GPT kit (Boehringer Mannheim, Switzerland) measurements as cell damage markers in the perfusate of the recirculating perfusion system.

2.6. Single-pass perfusions with polyinosinic acid

To examine a possible role of scavenger-receptors in the uptake of 30% PS-liposomes in this perfusion model, single-pass perfusions were performed over a period of 20 min. The perfusions were carried out with or without the addition of 25 μg/ml poly(I) in the liposomal perfusion medium. After the surgical preparation, the livers were perfused with liposome free buffer for 15 min in case of control liver and for 13 min in case livers were to be perfused with poly(I)-containing media. In the latter case the livers were perfused for an additional 2 min with Krebs–Henseleit buffer containing 25 μg/ml poly(I) before perfusion with liposome-containing medium was started.

The livers were perfused with a flow rate of 12.5 ml/min and a hydrostatic pressure of 8–12 cm water. Aliquots from the central venous outflow were taken every 15 s during the first 6 min and every 30 s for the following 14 min and assayed for radioactivity by liquid scintillation counting. The liposomal uptake rate was calculated as in the circulating perfusion experiments.

2.7. Endothelial and Kupffer cell isolation

After circulating liposomal perfusion for 1 h, liver endothelial cells and Kupffer cells were isolated by pronase perfusion and digestion of the organ and subsequent gradient centrifugation and counterflow centrifugal elutriation as described previously [7]. The numbers of cells in the

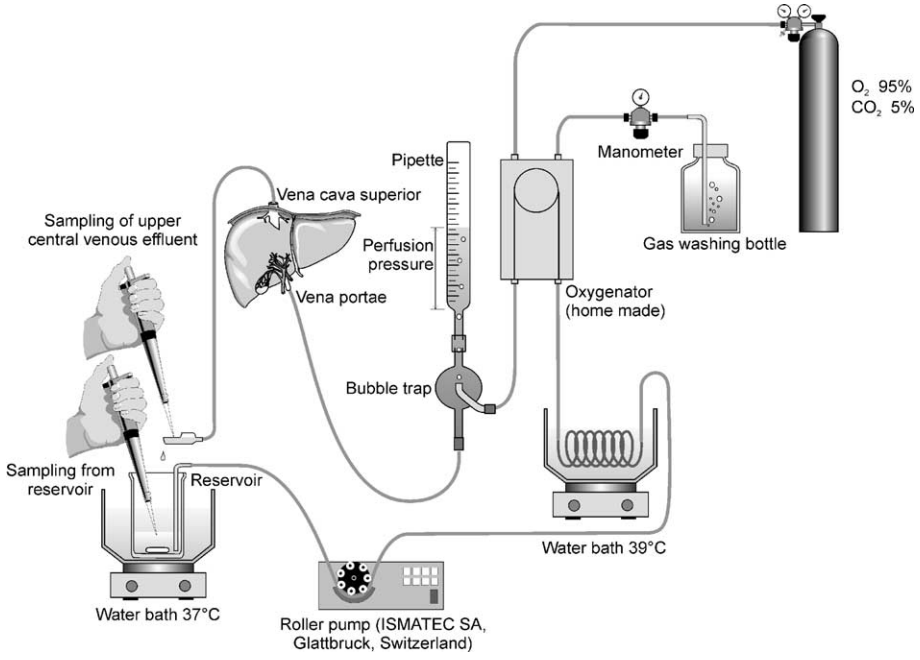

Fig. 1. Illustration of the perfusion system in the recirculation mode.

different fractions were determined by light microscopy. Liposome uptake by the different cell types was determined by measurement of the cell-associated radioactivity.

2.8. Microscopy

After perfusion of the liver with DiI-labelled liposomes and perfusion with liposome-free Krebs–Henseleit buffer for a washing period of 2 min, randomly collected pieces of liver (10–20 mm^3) were dissected, placed on small cork-discs, covered with Shandon Cryomatrix (Life Science International (Europe) Ltd, Cheshire, England), and rapidly frozen in liquid nitrogen. Frozen liver specimens were sliced to sections of 5-μm thickness with a cryo-microtome (Frigocut, Reichert Jung, Nussloch) and examined for the intrahepatic distribution of DiI fluorescence by fluorescence microscopy (Leica DM R with filters A4, L5, N3, and Y5, Leica Microsystems, Wetzlar, Germany).

3. Results

3.1. The isolated perfused liver system

Measurement of LDH, GOT and GPT was performed in a recirculating system with differently composed liposomes in the perfusate. The enzyme levels in the perfusate were monitored during 150 min. The LDH levels are shown in Fig. 2. There is no significant increase in the LDH levels for at least 60 min. Similar data were obtained for the other cell damage marker enzymes GOT and GPT (data not shown).

3.2. Uptake of liposomes by the perfused liver

Fig. 3 shows the hepatic elimination of liposomes from the recirculating perfusate for 0% PS (A) and 30% PS (B) liposomes. Total lipid uptake of the PS liposomes calculated as the area under the uptake rate curve from min 3 to min 60 was more than ninefold higher than that of liposomes without PS, i.e., 8.19±1.09 and 0.88±2.05 mg for the 30% PS and the 0% PS liposomes, respectively. Uptake rate data are presented as means±S.D. of four (A) or three (B) experiments, respectively. The *t*-test on the two groups of perfusions resulted in a *P* value of 0.00264, indicating that the two means are significantly different at the 0.01 level. Both liposome types show a rapid initial uptake phase, leveling off to a lower, nearly constant rate within 3 min. The rapid phase probably does not represent cellular uptake, since it is observed for both liposomes types. It is, in all likelihood, reflecting a dilution effect of the initial effluent perfusate with fluid present in the liver or both as has also been described by Kiwada et al. [13]. If this is correct, it is justified to compare the uptake values for the two liposome formulations between 3 and 60 min. The hepatic accumulation of neutral liposomes reaches a maximum value after about 30 min of perfusion. From then on, no further increase is seen, or maybe a slight decrease. The latter might indicate a release from initial adsorption sites.

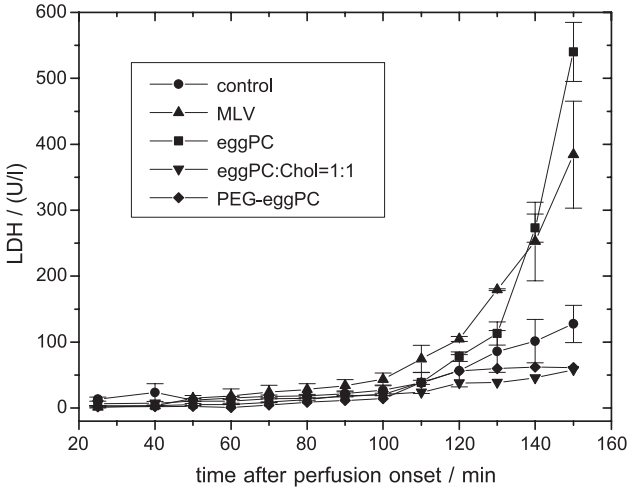

Fig. 2. LDH release in the perfusate of perfused rat liver (recirculation mode). The perfusate consisted of buffer as described in Section 2 and contained liposomes of the indicated compositions. Perfusate flux was 25 ml/min.

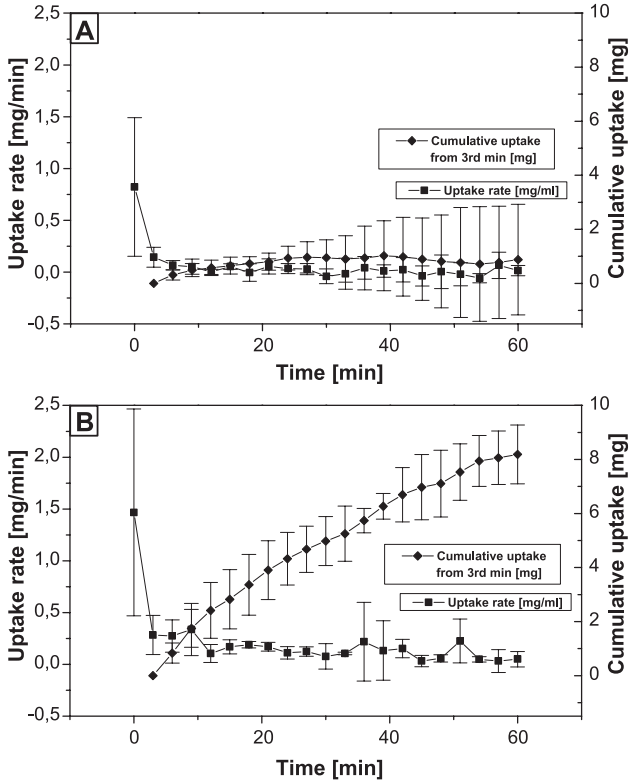

Fig. 3. Uptake of 0% PS (A) and 30% PS (B) liposomes by the perfused liver. Uptake rates (■) were calculated from the liquid scintillation data of the samples taken at 3-min intervals from the upper central venous effluent and the perfusion reservoir during a 1-h perfusion period as described in Section 3. Cumulative uptake (◆) was estimated from the third minute onward by calculating the area under the uptake rate curve. Uptake rates are presented as means±S.D. of four (A) or three (B) experiments, respectively.

3.3. Intrahepatic distribution investigated with fluorescence microscopy

In order to establish which cells in the liver are responsible for the observed liposome elimination, we labelled the liposomes with the fluorescent marker DiI and examined liver cryosections by fluorescence microscopy. Fig. 4 shows the results for neutral 0% PS liposomes (A, C) and negatively charged 30% PS liposomes (B, D). In Fig. 3A and C fluorescence appears mainly concentrated in discrete spots displaying a typical Kupffer cell distribution, although overall fluorescence intensity is low. In Fig. 3B and D fluorescence intensity in the liver is much higher and distributed in a typical sinusoidal pattern, which is compatible with a predominantly endothelial localisation. In addition, scattered areas are revealed where fluorescence appears as more concentrated spots, not unlike the distribution in Fig. 3A and C. Likewise, these spots, indicated by arrows, most probably represent Kupffer cells.

3.4. Endothelial and Kupffer cell isolation

To obtain additional information on intrahepatic distribution by an independent experimental approach, livers were perfused with liposomes, radiolabelled with ^{3}H-COE and, after 1 h, non-parenchymal liver cells were isolated and fractionated into a Kupffer cell and an endothelial cell fraction. Radioactivity assay confirmed that both cell types take up substantial amounts of liposomal label (Fig. 5). On a per cell basis, Kupffer cell uptake was approximately twice as high as endothelial cell uptake. Taking into consideration that the endothelial cells outnumber the Kupffer cells about threefold [14], the total endothelial cell population takes up about 50% more liposomal lipid than the Kupffer cell population. These results are fully compatible with the observations by fluorescence microscopy (Fig. 4).

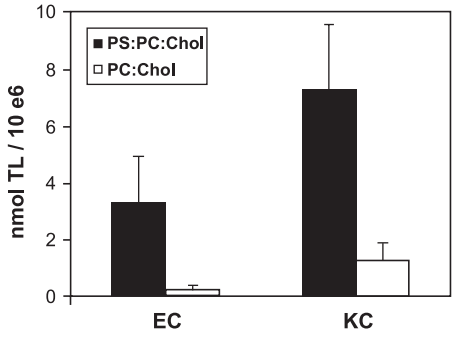

Fig. 5. Non-parenchymal liver cell uptake of 0% PS (open bars) and 30% PS (filled bars) liposomes after 1 h of circulating liver perfusion. The liposomes (initial concentration, 0.4 mg total lipid/ml) were labelled with [^{3}H]cholesteryl oleyl ether. Intrahepatic distribution was determined after isolation of liver endothelial cells (EC) and Kupffer cells (KC) as described in Section 3. Data are presented as means±S.D. (n=4).

3.5. Effect of polyinosinic acid on the uptake of 30% PS liposomes

The high uptake of 30% PS liposomes by endothelial cells is compatible with earlier observations reported by

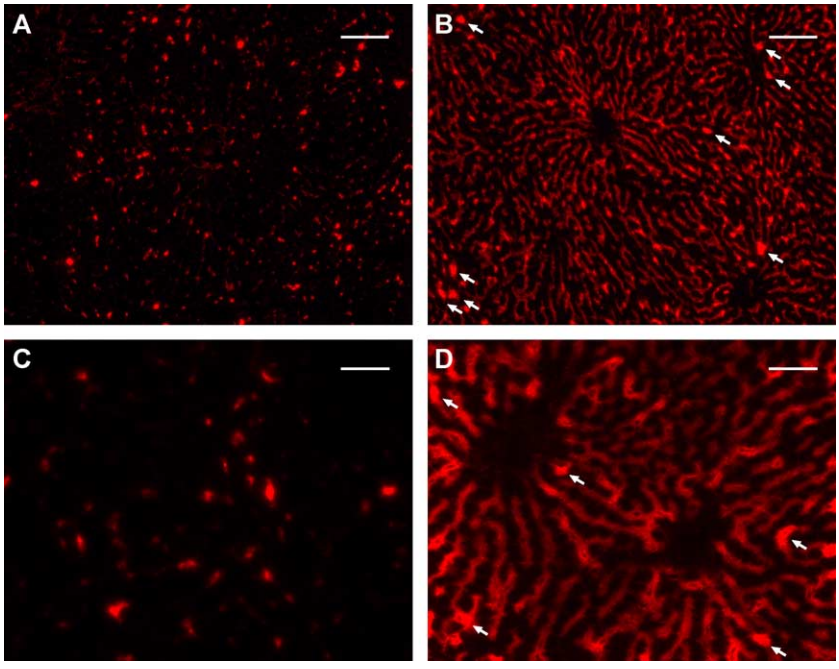

Fig. 4. Visualisation of the uptake of 0% PS (A, C) and 30% PS (B, D) liposomes after 1 h of circulating liver perfusion. Liposomes (initial concentration 0.4 mg total lipid/ml) were fluorescently labelled with 0.25 mol% 1,1′ -dioctadecyl-3,3,3′ ,3′ -tetramethylindocarbocyanine perchlorate (DiI). Post-perfusion processing of the liver tissue and preparation of specimens for fluorescence microscopy as described in Section 2. Small arrows in (B, D) are pointing at fluorescent spots representing liposome uptake by Kupffer cells as can also be observed in (A, C). Bars: 50 μm (A, B) and 25 μm (C, D), respectively.

us on in vitro experiments with isolated endothelial cells [9]. In this study, we could attribute the extensive uptake of 30% PS liposomes by endothelial cells to scavenger receptor activity. In order to determine whether also under the present isolated liver perfusion conditions this receptor is involved in liposome elimination from the perfusate, we performed the perfusion in the presence of poly(I), an established inhibitor of scavenger receptor activity. Fig. 6 shows the results of this experiment. Fig. 6A depicts the elimination of liposomes in control livers. Fig. 6B shows the elimination characteristics in the presence of poly(I).

Considering that the initial phase of the uptake curves mainly represents incomplete mixing rather than true uptake (see Sections 3.2 and 4), we calculated the cumulative uptake for the perfusions only from the third minute onward. It then becomes clear that the addition of poly(I) to the perfusion medium decreased the liposomal uptake by the livers to less than half the level of the poly(I)-free control group, i.e., 4.24±0.52 mg for the perfusions without poly(I) versus 2.03±0.98 mg for the poly(I)-containing perfusions, respectively. The P value of 0.02593 for an independent t-test on these means (n=3) confirms the significance of this difference at the 0.05 level.

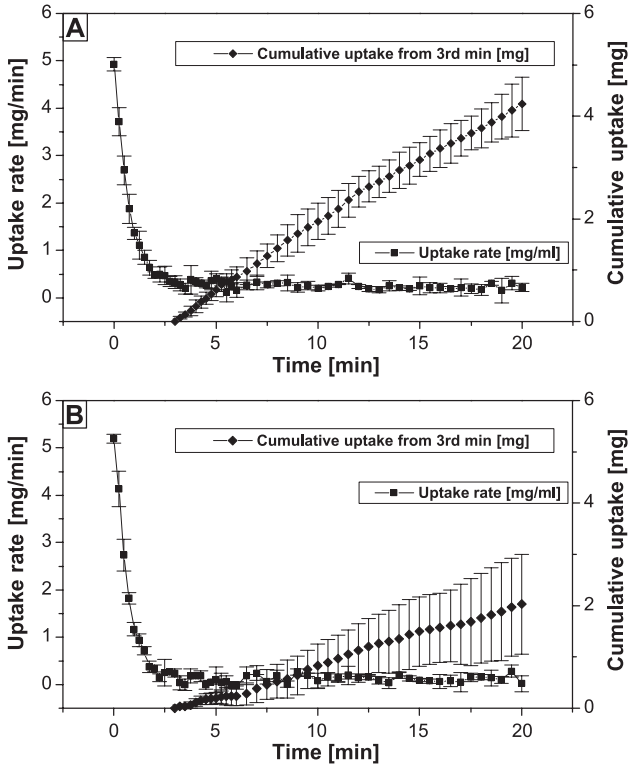

Fig. 6. Influence of polyinosinic acid on the uptake of 30% PS liposomes. [1α, 2α(n)-^{3}H] Cholesteryl oleyl ether-labelled liposomes were perfused without (A) or with (B) 25 μg/ml poly (I) in the perfusion buffer. Perfusions were performed in a single-pass mode over a period of 20 min. Perfusion conditions and estimation of uptake as described in Section 2. Data points represent means±S.D. of three rats.

4. Discussion

From our earlier work, it has become clear that under in vivo conditions liver endothelial cells do not contribute to any significant extent to total hepatic clearance of liposomes from the blood circulation, irrespective of liposome size and composition [15]. On the other hand, we did report an avid interaction of (strongly) negatively charged liposomes with liver endothelial cells in vitro, followed by endocytic internalization [9]. We demonstrated that this in vitro uptake is mediated by scavenger receptor activity and can be effectively blocked by serum. We proposed on the basis of these studies that, in vivo, plasma components (proteins) bind to the negatively charged surface of the liposomes and thus prevent proper interaction of the liposomes with the receptor on the endothelial cells. It could be argued, however, that the in vitro ability of endothelial hepatic cells to take up liposomes is induced as a result of the cell isolation procedures, which might reveal or induce surface structures which in situ are latent or absent. The results described in this paper, however, clearly indicate that also the endothelial cells in their structurally intact environment of the perfused liver can still interact with and take up negatively charged liposomes, if only there is no plasma or serum present. The negligible levels of hepatic enzymes in the perfusate during the first 90 min of perfusion indicate that our perfusion conditions are very mild, virtually excluding the possibility that significant damage was done to the liver cells and thus that liposome uptake activity was induced as an artifact of the perfusion.

The perfusion experiments closely reflect the behaviour of neutral and negatively charged liposomes in earlier reported in vitro results with Kupffer cells or endothelial cells: neutral liposomes showed very little uptake by either cell type while the negatively charged 30% PS liposomes were taken up avidly [9]. Both liposome types show a high initial uptake rate, which levels off rapidly within 1–3 min. Since this is seen for both liposome types, this phenomenon is likely to be attributable to a dilution effect of the perfusate within the liver. The perfusate may not instantly reach all available aqueous space in the liver, e.g., the space of Disse between the endothelial lining and the hepatocytes is only accessible through the limited surface area covered by the endothelial fenestrations [16]. Thus, equilibration may conceivably take the approximately 1–3 min required to reach the steady state rate of uptake in the second phase.

The cumulative uptake data show that after 1-h perfusion with the negatively charged liposomes, approximately 8 mg of liposomal lipid had been cleared from the perfusate, i.e., 10% of the initial amount in the perfusate. In terms of ("plasma") clearance this is equivalent to ~20 ml/h, which compares favorably with in vivo observations of the clearance of liposomes containing PS (unpublished results).

Total uptake of neutral liposomes reaches a maximum of approximately 1 mg after about 30 min. From then on it levels off or even tends to diminish somewhat after 40 min.

This might reflect some release of radioactivity from liposomes that did not become internalized.

Our observations on the effect of poly(I) are compatible with the notion outlined above that the initial rapid elimination phase does not represent uptake by cells. This initial phase is hardly influenced by poly(I), which argues against the involvement of the scavenger receptor in this phase. On the other hand, the slower phase of the uptake curve (the 3–20-min interval) shows a significant inhibitory effect of the poly(I), confirming the involvement of the scavenger receptor in the elimination mechanism.

Visualisation by fluorescence microscopy revealed a conspicuous difference between the neutral and negatively charged liposomes. While the neutral ones display a typical Kupffer cell distribution pattern, the charged particles show a typical sinusoidal distribution, characteristic of an endothelial cell localisation. It is clear, however, that superimposed on the sinusoidal pattern numerous more intensely fluorescent spots can be distinguished, representing uptake by Kupffer cells. Participation of both cell types was confirmed by the results on the recovery of radioactivity in isolated endothelial cells and Kupffer cells, which indicated higher uptake per cell by the Kupffer cells than by the endothelial cells. From in vivo experiments we know that both neutral and charged small liposomes can be taken up to significant extents by hepatocytes as well. In the micrographs this does not show up, probably because of the dilution of the fluorescent marker in the 50 times larger mass of the parenchymal cells as compared to that of the non-parenchymal cells, i.e., five times larger number and 10 times larger volume. In the cell isolation experiments, hepatocytes were not included because the non-parenchymal cells were isolated by means of pronase digestion, which destroys the hepatocytes.

In conclusion, this study demonstrates that in the intact rat liver, in serum-free conditions, endothelial cells are able to take up relatively large amounts of negatively charged liposomes presumably via a scavenger receptor-mediated mechanism.

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