Scavenger receptor B1 (SR-B1) substrates inhibit the selective uptake of high-density-lipoprotein cholesteryl esters by rat parenchymal liver cells

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High-density lipoprotein cholesteryl esters (HDL-CE) are selectively taken up by liver parenchymal cells without parallel apolipoprotein uptake, and this selective uptake route forms an important step in reverse cholesterol transport. Recent data from Acton, Rigotti, Landschulz, Xu, Hobbs and Krieger [(1996) Science **271**, 518–520] provide evidence that scavenger receptor B (SR-B1) can mediate selective uptake of HDL-CE. In order to identify if selective uptake of HDL-CE by rat liver parenchymal cells can be mediated by a protein with scavenger receptor properties we performed competition experiments *in vivo* with substrates for scavenger receptors. Addition of either low-density lipoprotein (LDL), acetylated LDL (AcLDL) or oxidized LDL (OxLDL) only marginally ($< 10\,\%$) decreased the association of HDL particles to parenchymal cells as measured by ^{125}I-labelled HDL. HDL-CE association was inhibited by AcLDL by $35\,\%$, while addition of OxLDL did inhibit HDL-CE association by $80\,\%$, thereby completely blocking the selective uptake of HDL-CE. Studies with HDL labelled with a fluorescent cholesteryl-ester analogue confirmed that OxLDL mediated complete inhibition of HDL-CE selective uptake by rat liver parenchymal cells. The inhibition of HDL-CE selective uptake by OxLDL was insensitive to the additional presence of polyinosinic acid (poly I), indicating that the inhibitory effect did not involve a poly I-sensitive site. Anionic phospholipid liposomes inhibited HDL-CE association by $40\,\%$, while neutral liposomes were ineffective. The inhibition of the selective uptake of HDL-CE in liver parenchymal cells by modified LDL, in particular OxLDL and anionic phospholipids suggests that, in liver, the SR-B1 is responsible for the efficient uptake of HDL-CE.

INTRODUCTION

High-density lipoproteins (HDL) may exert the established anti-atherogenic effects by various mechanisms [2,3]. Reverse cholesterol transport, first proposed by Glomset [4], is the best established mechanism of action. In this concept HDL accepts excess cholesterol from extrahepatic cells for transport to the liver [5]. Peripheral cholesterol is primarily accepted by small HDL particles with pre-β mobility [6]. After esterification by lecithin–cholesterol acyltransferase, the high-density lipoprotein cholesteryl esters (HDL-CE) are delivered to the liver either directly or by LDL as a result of the action of cholesteryl ester (CE) transfer protein. The direct uptake route of HDL-CE by liver parenchymal cells is characterized by the selective uptake of the CEs without simultaneous uptake of the holoparticle [7,8]. The selective uptake route is efficiently coupled to bile acid formation and secretion [5].

The mechanism of selective uptake of HDL-CE is largely unestablished. It is restricted to the adrenals, ovary and liver [7,9], while within the liver the parenchymal cells are solely responsible for the selective uptake of HDL-CE [3,5]. Rinninger et al. [10] showed that there are distinct sites on liver parenchymal cells for the binding of the protein moiety of HDL and selective CE uptake.

Several proteins have been described which can specifically bind HDL [11,12]. Recently, Acton et al. [1] provided evidence that scavenger receptor class B (SR-B1), a member of the CD 36 family [13], can not only mediate HDL binding but also selective CE uptake *in vitro* with murine SR-B1 transfected CHO cells. SR-B1 was found to bind a broad spectrum of ligands, including both modified lipoproteins, native lipoproteins and also anionic phospholipids [14]. However, SR-B1 does not bind the broad array of polyanions [e.g. fucoidin, polyinosinic acid (poly I)] which are classical ligands for scavenger class A receptors. *In vivo*, SR-B1 is expressed mainly in the adrenals, ovary and to a much lesser extent in the liver of rats and mice [15,16]. In the present work the potential role of SR-B1 in the selective uptake of HDL-CE by rat liver parenchymal cells is investigated.

Previous studies from our group provided evidence for the presence of a lipoprotein binding site on rat liver parenchymal cells capable of binding multiple types of lipoprotein [17]. It was also shown that rat liver parenchymal cells possessed a high affinity binding site for AcLDL and OxLDL that was insensitive to poly I [18].

In the present study we have investigated the potential role of SR-B1 in the selective HDL-CE uptake by rat liver parenchymal cells, By performing competition studies *in vitro* with ligands specific for SR-B1.

EXPERIMENTAL

Materials

[1α,2α(n)-^{3}H]Cholesteryl oleate ([^{3}H]Ch$_{18:1}$) and ^{125}I (carrier free) in NaOH were obtained from Amersham (Little Chalfont, Buckinghamshire, U.K.). 22-(*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino) (NBD) cholesteryl linoleate was obtained from Molecular Probes (Eugene, Oregon, U.S.A.). Egg yolk phosphatidylcholine was obtained from Fluka (Buchs, Switzerland). The phospholipids kit, the cholesterol oxidase–peroxidase aminophenazone kit and the glycerolphosphate oxidase–peroxidase

Abbreviations used: AcLDL, acetylated low-density lipoprotein; CE, cholesteryl ester; Ch$_{18:1}$, cholesteryl oleate; DMEM, Dulbecco's modified Eagle's medium; HDL, high-density lipoprotein; HDL-CE, high-density lipoprotein cholesteryl esters; LDL, low-density lipoprotein; NBD, 22-(*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino); OxLDL, oxidized low-density lipoprotein; poly I, polyinosinic acid; SR-B1, scavenger receptor class B.

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aminophenazone kit was from Boehringer Mannheim (Mannheim, Germany). Ethylmercurithiosalicylate, BSA (fraction V) and collagenase type I and type IV were from Sigma (St. Louis, MO, U.S.A.). Dulbecco's modified Eagle medium (DMEM) was from Gibco (Irvine, Scotland, U.K.). All other chemicals were of analytical grade.

Animals

Male Wistar WU rats (200–250 g), with free access to food and water, were used throughout the study. Before the experiments, the rats were anaesthetized with Nembutal given intraperitoneally.

Phospholipid liposome preparation

Unilamellar liposomes were obtained by sonication of egg yolk phosphatidylcholine/phosphatidylserine/cholesterol in a molar ratio of 1:1:1. The lipids were mixed in chloroform and dried under a stream of N_2. Sonication (MSE Soniprep 150) was for 40 min (amplitude 12 μ) at 52 °C under a constant stream of argon in 0.1 M KCl/10 mM Tris/1 mM EDTA/0.025 % (w/v) NaN_3, pH 8.0. Particles with a density of 1.03 g/ml were isolated by density-gradient ultracentrifugation and dialysed against PBS containing 1mM EDTA. The liposomes were stored at 4 °C under argon. The phospholipid content of the particles was measured by an enzymic colorimetric assay. The average diameter of the liposomes was determined by photon correlation spectroscopy (System 4700 C, Malvern Instruments, Malvern, U.K.).

Isolation and labelling of lipoproteins

Human HDL and LDL were isolated from the blood of healthy volunteers by differential ultracentrifugation as described by Redgrave et al. [19]. HDL and LDL were dialysed against PBS containing 1mM EDTA. HDL was labelled with [^{3}H]$Ch_{18:1}$ or NBD cholesteryl linoleate by exchange from donor particles as reported previously [3]. The specific activity of the [^{3}H]$Ch_{18:1}$-labelled HDL was 1000–2000 d.p.m./μg HDL protein, and that of ^{125}I-labelled HDL was 50000–100000 c.p.m./μg. The labelled HDL was dialysed against PBS containing 1mM EDTA and passed through a heparin-Sepharose affinity column to remove apoE-containing particles [20]. Routinely, the HDL fraction was checked for the absence of apoE by SDS/PAGE (10 % acrylamide gels), followed by staining with Coomassie Blue. After the labelling procedure the radiolabelled HDL was checked for hydrolysis of the CE labels by a Bligh and Dyer extraction [21] followed by TLC. Hydrolysis of the CE was always less than 5 %. The effect of the labelling procedure on the composition of HDL was analysed by measurement of phospholipid, cholesterol, CE and triglyceride content (using the phospholipid kit, cholesterol oxidase–peroxidase aminophenazone kit and glycerolphosphate oxidase–peroxidase aminophenazone kit respectively). The density, electrophoretic α-mobility and particle size (photon correlation spectroscopy, System 4700 C, Malvern Instruments) were also analysed. Labelled HDL was used only when no change was observed in the measured composition or physical characteristics compared with the original unlabelled HDL. HDL was iodinated by the ICl method of McFarlane [22], modified by Bilheimer et al. [23].

Before use, LDL was dialysed against PBS containing 10 μM EDTA. Acetylation of LDL was performed with acetic anhydride, as described previously [24]. LDL was oxidized by exposure to $CuSO_4$ as described, in detail, previously [18].

Studies *in vitro* with freshly isolated rat hepatocytes

Parenchymal liver cells were isolated by perfusion of rat liver with collagenase at 37 °C, as described previously [24]. The viability (> 95 %) of the parenchymal cells obtained was checked by Trypan Blue exclusion. The cells were resuspended in oxygenated DMEM supplemented with 2 % (w/v) BSA, pH 7.4. For competition studies, 1–2 mg of parenchymal cell protein was incubated with different amounts of protein from unlabelled human HDL, LDL, AcLDL, OxLDL, or with neutral or anionic liposomes in the presence of radiolabelled HDL in 1 ml of DMEM containing 2 % (w/v) BSA at 37 °C for varying time periods. Cell incubations were performed in a circulating shaker (Adolf Kühner AG, Basel, Switzerland) at 150 r.p.m. Every hour the incubations were briefly oxygenated. The viability of the parenchymal cells remained > 88 % during these long term incubations [24]. After incubation, the cells were centrifuged for 2 min at 50 ***g*** in an Eppendorf centrifuge and were washed × 2 in 50 mM Tris/HCl/0.15 M NaCl/0.2 % (w/v) BSA, pH 7.4 at 4 °C. Subsequently, the cell pellet was washed in a similar medium but without BSA. The cells were lysed in 0.1 M NaOH and the protein content and radioactivity were determined.

Confocal microscopy

Freshly isolated parenchymal cells were incubated on glass coverslips in 6-well plates (Costar, Cambridge, MA, U.S.A.) in DMEM supplemented with 2 % (w/v) BSA and the indicated amount of NBD-cholesteryl-linoleate-labelled HDL for 2 h at 4 °C. Cells, on the coverslips, were transferred to a Zeiss (Oberkochen, Germany) IM-35 inverted microscope with a × 63, NA 1.4 planapochromatic objective, which was equipped with an MRC600 confocal visualization system (Bio-Rad, St. Albans, Herts., U.K.). The microscope was fitted with a chamber to allow incubation of the cells at 37 °C.

Protein determination

Protein was determined by the method of Lowry et al. [25] using BSA as a standard.

RESULTS

Effect of modified lipoproteins on the selective uptake of HDL cholesterol esters.

Freshly isolated rat liver parenchymal cells were incubated for 3 h at 37 °C with HDL, either iodinated or labelled with [^{3}H]$Ch_{18:1}$. At this time point, the association of [^{3}H]$Ch_{18:1}$-labelled HDL (202 ± 14 ng HDL/mg of cell protein) apparently exceeded ^{125}I-labelled HDL (36 ± 3 ng HDL/mg of cell protein) association 5.6 times. As suggested by Pittman et al. [7], the results of CE association are expressed in terms of apparent particle uptake, i.e. the amount of apparent HDL protein taken up is calculated from the amount of CE tracer associated with the cells. Therefore the amount of radioactive CE tracer associated with the cells is expressed as apparent HDL protein association, calculated using the specific activity of the labelled HDL. The ability of (modified) lipoproteins to compete for ^{125}I-labelled HDL and HDL-CE association was tested by co-incubation with either 100 μg of protein /ml of unlabelled HDL, LDL, AcLDL or OxLDL (Table 1). Both HDL particle association, measured by ^{125}I-labelled HDL association, and [^{3}H]$Ch_{18:1}$ HDL association were inhibited by approx. 50 % when 100 μg/ml of unlabelled apo-E-free HDL protein was added. Further inhibition of both ^{125}I-labelled HDL association

Table 1 Effect of native and modified lipoproteins on the parenchymal cell association of [^{125}I]- or [^{3}H]Ch$_{18:1}$-labelled HDL

Rat liver parenchymal cells were incubated for 3 h at 37 °C with 10 μg/ml of labelled HDL in the absence or presence of 100 μg of protein/ml of unlabelled competing lipoproteins in DMEM containing 2% (w/v) BSA. The 100% value for association of [^{3}H]Ch$_{18:1}$-labelled HDL was 202 ± 14 ng HDL/mg cell protein and for [^{125}I]-labelled HDL association was 36 ± 3 ng HDL/mg of cell protein. The association is expressed as the percentage of the radioactivity measured in the absence of competitor. The results are given as means ± S.E.M. ($n = 3$ separate cell isolations). * $P < 0.005$, significant difference between the experimental and control value; ** $P < 0.001$, highly significant difference between the experimental and control value (unpaired Student's t test).

	[^{125}I]-labelled HDL	[^{3}H]Ch$_{18:1}$-HDL
Control	100	100
HDL	45.9 ± 10.5*	60.8 ± 4.8*
LDL	89.0 ± 2.3	81.6 ± 9.8
AcLDL	95.9 ± 13.4	67.9 ± 6.4*
OxLDL	89.0 ± 7.4	22.3 ± 3.9**

and [^{3}H]Ch$_{18:1}$ HDL association up to 75% was achieved by increasing the excess of unlabelled HDL to 500 μg/ml (results not shown). In contrast to HDL, addition of either 100 μg of LDL protein or modified LDL protein only marginally (< 10%) decreased the cell association of ^{125}I-labelled HDL. [^{3}H]Ch$_{18:1}$ HDL association was decreased approx. 20% by addition of LDL. However, addition of AcLDL led to a significant inhibition of 35 % ($P < 0.005$), while OxLDL decreased [^{3}H]Ch$_{18:1}$ HDL association up to 80%.

The possibility of exchange of [^{3}H]Ch$_{18:1}$ with the other lipoproteins was tested by isolating the lipoproteins after incubation for 3 h at 37 °C. Both density-gradient ultracentrifugation and agarose gel electrophoresis were performed. Less than 5% of the [^{3}H]Ch$_{18:1}$ was recovered in the LDL, AcLDL or OxLDL fraction after incubation for 3 h at 37 °C, establishing that exchange with competitors could not explain the results obtained.

Effect of OxLDL on selective HDL-CE uptake.

The inhibitory effect of OxLDL on the the selective uptake of HDL-CE was further analysed with respect to efficiency of competition. Increasing concentrations of OxLDL were added to freshly isolated parenchymal cells in the presence of [^{3}H]Ch$_{18:1}$-labelled HDL. At a concentration of 20 μg/ml OxLDL protein the association of HDL-CE was inhibited by more than 50% (Figure 1). Poly I, an established inhibitor of scavenger receptor class A [14], did not lower the cell association of ^{125}I-labelled HDL or [^{3}H]Ch$_{18:1}$ HDL, but instead increased both particle association and CE association. The simultaneous presence of increasing concentrations of OxLDL and of 100 μg/ml of poly I did not influence the inhibitory action of OxLDL, indicating that the effect of OxLDL was not due to interaction with a poly I-sensitive site.

The selective uptake of HDL-CE was also followed with confocal laser scanning microscopy using HDL labelled with a fluorescent CE analogue (NBD). The uptake of NBD cholesteryl linoleate was followed for up to 3 h of incubation with parenchymal cells. After 10 min at 37 °C the label was mainly associated with the plasma membrane (Figure 2). During the incubation period some parenchymal cell pairs, which were not separated during the isolation procedure, regained their cellular polarity and formed active bile canaliculi. These so-called parenchymal cell couplets [26] allowed us to monitor bile-directed

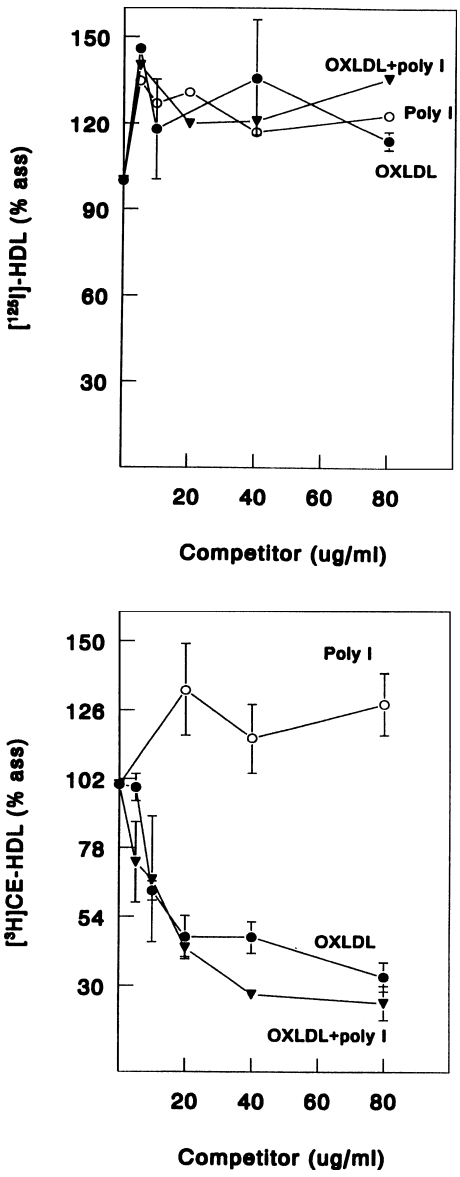

Figure 1 Effect of increasing concentrations of OxLDL and poly I on the parenchymal cell association of (upper)^{125}I- or (lower) [^{3}H]Ch$_{18:1}$-labelled HDL

Rat liver parenchymal cells were incubated for 3 h at 37 °C with 10 μg/ml of labelled HDL in the presence of the indicated amounts of OxLDL and, where shown, poly I (100 μg/ml). The association is expressed as the percentage of the radioactivity measured in the absence of competitor (% ass). The 100% value for association of [^{3}H]Ch$_{18:1}$-labelled HDL without competitor was 202 ± 14 ng HDL/mg of cell protein and for ^{125}I-labelled HDL without competitor association was 36 ± 3 ng HDL/mg of cell protein. The results are given as means ± S.E.M. ($n = 3$ separate cell isolations).

transport of NBD cholesteryl linoleate. At 3 h of incubation at 37 °C the label inside the cell was concentrated in structures at the apical side of the parenchymal cell couplets, near the bile canaliculus. Addition of 100 μg/ml of OxLDL protein abolished uptake of NBD cholesteryl linoleate almost completely.

Selective HDL-CE uptake is sensitive for anionic phospholipids

Apparently scavenger receptor class A is not involved, since the OxLDL-mediated inhibition of HDL-CE uptake by rat liver

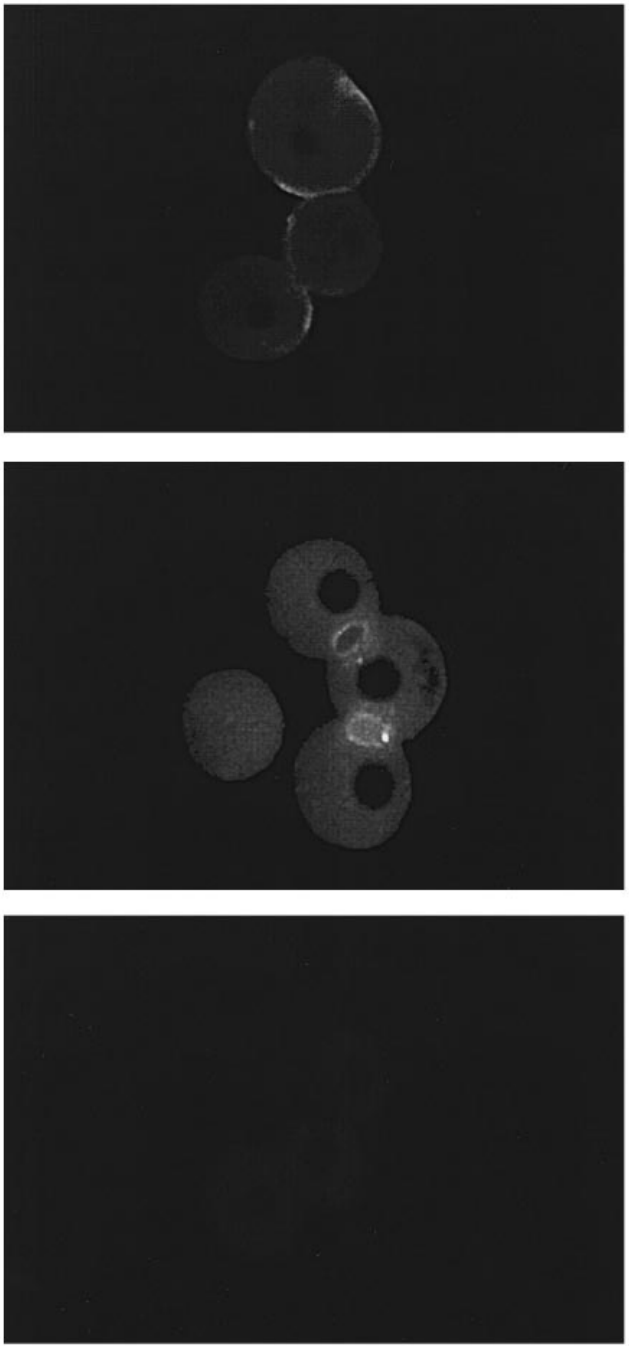

Figure 2 Visualization of the interaction of NBD-cholesteryl-linoleate-labelled HDL with liver parenchymal cell couplets and the effect of OxLDL

Freshly isolated parenchymal cells were pre-incubated on glass coverslips for 2 h at 4 °C in DMEM supplemented with 2% (w/v) BSA and 50 μg/ml of NBD-cholesteryl-linoleate-labelled HDL in the absence or presence of 100 μg/ml OxLDL. The cells were analysed with a confocal microscope fitted with a chamber to allow incubation of the cells at 37 °C. The fluorescence of NBD cholesteryl linoleate was followed, in the absence of OxLDL for 10 min (upper panel) and 120 min (middle panel), or in the presence of OxLDL for 120 min (bottom panel).

parenchymal cells was not dependent on a poly I sensitive site. We therefore specifically studied the role of SR-B1 by analysing the effect of anionic phospholipid liposomes. These liposomes effectively interact with SR-B1 [14]. Liposomes consisting of phosphatidylcholine, cholesterol and the anionic phospholipid

Table 2 Effect of phosphatidylserine liposomes and neutral liposomes on the cell association of ^{125}I- or [^{3}H]Ch$_{18:1}$-labelled HDL

Rat liver parenchymal cells were incubated for 3 h at 37 °C with 10 μg/ml of labelled HDL in the absence or presence of liposomes (100 μg of phospholipid/ml) in DMEM containing 2% (w/v) BSA. The 100% value for association of [^{3}H]Ch$_{18:1}$-labelled HDL was 202 $\pm$ 14 ng HDL/mg of cell protein and for [^{125}I]-labelled HDL, association was 36 $\pm$ 3 ng HDL/mg of cell protein. The association is expressed as the percentage of the radioactivity measured in the absence of competitor. The results are given as the means $\pm$ S.E.M. ($n = 3$ separate cell isolations). * $P < 0.005$, significant difference between the experimental and control value (unpaired Student's t test).

	[^{125}I]-labelled HDL	[^{3}H]Ch$_{18:1}$-HDL
Control	100	100
Neutral liposomes	105 $\pm$ 4	100 $\pm$ 20
Phosphatidylserine liposomes	150 $\pm$ 30	58.3 $\pm$ 8.7*

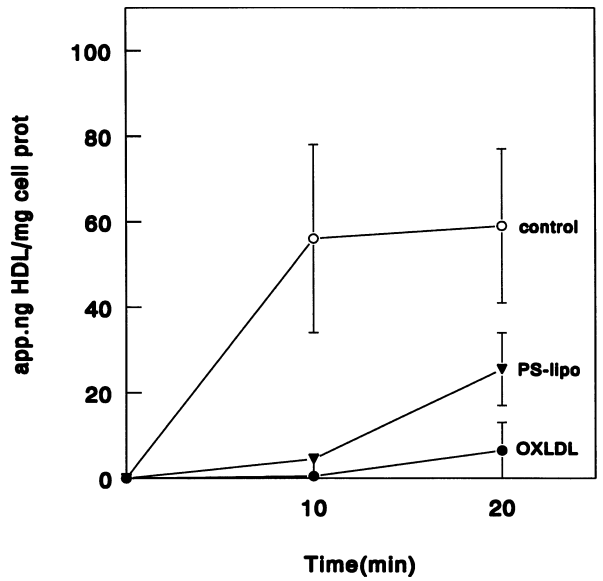

Figure 3 Time course of the cell association of [^{3}H]Ch$_{18:1}$-labelled HDL by rat liver parenchymal cells and the effect of OxLDL or phosphatidylserine liposomes

Rat liver parenchymal cells were incubated with 10 μg/ml of labelled HDL in the absence or presence of either liposomes (100 μg phospholipid/ml; PS-lipo) or OxLDL (100 μg/ml) in DMEM containing 2% (w/v) BSA at 37 °C for the times indicated. The association is expressed as apparent uptake of HDL/ng of cell protein (app.ng HDL/mg cell prot) as reported previously [7]. The results are given as the means of two experiments $\pm$ the variation of the individual experiments.

phosphatidylserine inhibited HDL-CE association by almost 50% (Table 2), while ^{125}I-labelled HDL association was increased. Neutral liposomes, consisting only of phosphatidylcholine and cholesterol, did not influence HDL-CE association. The possibility of transfer of [^{3}H]Ch$_{18:1}$ to the liposomes during the incubation was tested by re-isolating the liposomes after incubation. Both density-gradient ultracentrifugation followed by fractionation of the gradient and agarose gel electrophoresis was performed. The transfer of [^{3}H]Ch$_{18:1}$ to each type of liposome after 3 h at 37 °C was less than 1%. As a second approach, to test the potential that the inhibitory effects of both OxLDL and phosphatidylserine liposomes were caused by transfer to the inhibitors, time course studies were performed (Figure 3). Both

inhibitors inhibited the characteristic fast initial association of HDL-CE by liver parenchymal cells [3], indicating that inhibition of cellular HDL-CE association was caused by blocking the initial cellular association mechanism of HDL-CE and not by exchange with the inhibitors.

DISCUSSION

Selective delivery of CE from HDL to the liver is an important direct route for reverse cholesterol transport. The precise mechanism of this selective uptake and the cellular mediators is not known. SR-B1 might be involved in this process [1], but until now direct involvement of SR-B1 in reverse cholesterol transport has not been shown. Unlike scavenger class A receptors, SR-B1 is insensitive to polyanions such as poly I [13,27]. The presence of a scavenger receptor on rat liver parenchymal cells, which was not sensitive to poly I, has been reported by van Berkel et al. [18], and direct competition of LDL binding to parenchymal cells by HDL has also been observed [17]. However, the association of ^{125}I-labelled HDL particles with rat liver parenchymal cells was only effectively competed for by HDL itself, while the maximal competition by LDL, AcLDL and OxLDL was not significant. In contrast, the selective uptake of HDL-CE by liver parenchymal cells is completely blocked by the presence of OxLDL, while anionic phospholipids and, to a lesser extent, AcLDL are also effective inhibitors. It can be calculated that OxLDL inhibits the selective uptake of HDL-CE almost completely, as the residual association of HDL-CE equals particle association as measured with ^{125}I-labelled HDL. It thus appears that selective HDL-CE uptake by rat liver parenchymal cells is mediated by a recognition site which possesses recognition properties characteristic for SR-B1. The finding that the inhibitory effect of OxLDL on HDL-CE uptake was not influenced by the simultaneous presence of poly I is consistent with the characteristic property of SR-B1, which unlike the other scavenger receptor classes, is insensitive to poly I [27]. Both liposomes containing anionic phospholipids and poly I increase in ^{125}I-labelled HDL association. This might be caused by an increase in aspecific association mediated by the charge on poly I and anionic phospholipids acting as a bridge between cells and particles. HDL-CE association is increased similarly in the presence of poly I. However, unlike poly I, the anionic phospholipids inhibit association of HDL-CE by almost 50%. When the increase in ^{125}I-labelled HDL association mediated by anionic phospholipids is taken into account, it can be calculated that, like OxLDL, anionic phospholipids completely block selective uptake of HDL-CE.

The question arises as to why the total HDL particle association, unlike the selective CE-uptake, is only marginally affected by the inhibitors of selective HDL-CE uptake, such as OxLDL. However, it can be calculated, using our previous data on the amount of poly I-insensitive binding sites for OxLDL on liver parenchymal cells [18,28], that these sites contribute only up to 1% of all HDL binding sites. Therefore it appears that the selective uptake site of HDL-CE contributes only to a small extent to the total number of HDL binding sites, suggesting that its coupling to selective uptake is highly efficient. It also may explain that apparent HDL-CE uptake is not related to HDL particle binding as observed previously [29,30]. Since HDL-CE association exceeded ^{125}I-labelled HDL association by 5.6 times after incubation for 3 h, it can be calculated that HDL particles must exchange every 19 seconds at these sites.

In conclusion, our present results provide the first evidence that the selective uptake route of HDL-CE in rat parenchymal cells can be inhibited by competitors of SR-B1. Although SR-B1 is expressed in liver at a lower concentration than in the adrenals or ovary, the relatively high liver mass and its high efficiency suggests an important function in liver uptake of HDL-CE and thus in reverse cholesterol transport.

Financial support for this study was by the Netherlands Organisation for Scientific Research, Medical Sciences Grant No. 902-523-096.

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Received 21 January 1997/4 April 1997; accepted 15 April 1997