THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 274, No. 13, Issue of March 26, pp. 8893–8899, 1999
Printed in U.S.A.

Scavenger Receptor BI Mediates the Selective Uptake of Oxidized Cholesterol Esters by Rat Liver*

(Received for publication, January 5, 1999)

Kees Fluiter‡, Wolgang Sattler§, Maria C. De Beer¶, Patrice M. Connell¶, Deneys R. van der Westhuyzen¶, and Theo J. C. van Berkel‡‖

From the ‡Division of Biopharmaceutics, Leiden/Amsterdam Center for Drug Research, University of Leiden, Sylvius Laboratories, P. O. Box 9503, 2300 RA Leiden, The Netherlands, the §Institüt für Medizinische Biochemie, Universität Graz, Harrachgasse 21, 8010 Graz, Austria, and the ¶Department of Internal Medicine, University of Kentucky Medical Center, Lexington, Kentucky 40536

High density lipoprotein (HDL) can protect low density lipoprotein (LDL) against oxidation. Oxidized cholesterol esters from LDL can be transferred to HDL and efficiently and selectively removed from the blood circulation by the liver and adrenal *in vivo*. In the present study, we investigated whether scavenger receptor BI (SR-BI) is responsible for this process. At 30 min after injection, the selective uptake of oxidized cholesterol esters from HDL for liver and adrenal was 2.3- and 2.6-fold higher, respectively, than for native cholesterol esters, whereas other tissues showed no significant difference. The selective uptake of oxidized cholesterol esters from HDL by isolated liver parenchymal cells could be blocked for 75% by oxidized LDL and for 50% by phosphatidylserine liposomes, both of which are known substrates of SR-BI.

***In vivo* uptake of oxidized cholesterol esters from HDL by parenchymal cells decreased by 64 and 81% when rats were treated with estradiol and a high cholesterol diet, respectively, whereas Kupffer cells showed 660 and 475% increases, respectively. These contrasting changes in oxidized cholesterol ester uptake were accompanied by similar contrasting changes in SR-BI expression of parenchymal and Kupffer cells. The rates of SR-BI-mediated selective uptake of oxidized and native cholesterol esters were analyzed in SR-BI-transfected Chinese hamster ovary cells. SR-BI-mediated selective uptake was 3.4-fold higher for oxidized than for native cholesterol esters (30 min of incubation). It is concluded that in addition to the selective uptake of native cholesterol esters, SR-BI is responsible for the highly efficient selective uptake of oxidized cholesterol esters from HDL and thus forms an essential mediator in the HDL-associated protection system for atherogenic oxidized cholesterol esters.**

High density lipoprotein (HDL)[1] may exert its anti-atherogenic effects by various mechanisms (1, 2). First, reverse cholesterol transport, as originally proposed by Glomset (3), is a widely accepted mechanism of anti-atherogenic action. In this concept, HDL accepts excessive cholesterol from extrahepatic cells for transport to the liver parenchymal cells (3, 4). The direct uptake of native HDL cholesteryl ester (HDL-CE) by liver parenchymal cells is fundamentally different from that of the classical LDL receptor pathway in that HDL-CEs are taken up selectively without simultaneous uptake of the holoparticle (5, 6). This so-called selective uptake of HDL-CE in the liver parenchymal cells is efficiently coupled to bile acid formation and secretion (4). A second mechanism for the anti-atherogenic action of HDL is related to its antioxidative properties (7). Several mechanisms have been proposed by which HDL can prevent oxidative damage of LDL. One of the possible mechanisms that have been suggested involves the transfer of reactive oxidized lipids from LDL to HDL and the subsequent efficient transport to the liver for biliary secretion (2, 8, 9). The rapid removal of the oxidized lipids may prevent the propagation of an oxidation cascade in LDL. Bowry *et al*. (10) showed that HDL is the predominant carrier of cholesteryl ester hydroperoxides (CEOOH) in humans. The first step in LDL oxidation involves hydroperoxide formation, and it is suggested that HDL may accept CEOOH from LDL and that this process is possibly mediated by cholesteryl ester transfer protein (11). Moreover, a HDL-associated hydroperoxide-reducing activity was found that converted the reactive CEOOH to the less reactive cholesteryl ester hydroxides (CEOH) (9). Previous studies (2) have shown that oxidized cholesterol esters are taken up extremely efficiently as compared with native cholesterol esters by the liver parenchymal cells and this uptake is coupled to an increased rate of biliary secretion, indicating that the efficient liver uptake indeed might lead to the irreversible removal from serum of the reactive oxidized cholesterol esters *in vivo*. The hypothesis was put forward that this antioxidant mechanism may use the same selective uptake pathway as that used during normal reverse cholesterol transport but with a much higher efficiency (2).

Acton *et al*. (12) recently provided the first evidence that scavenger receptor class BI (SR-BI), a member of the CD 36 family (13), binds HDL and can also mediate the selective uptake of HDL-CE. SR-BI was found to bind a broad spectrum of ligands, including both modified and native lipoproteins and anionic phospholipids (14). The binding of HDL to SR-BI was shown to be mediated by the major apolipoproteins of HDL, *e.g.* apoA-I, apoA-II, and apoC-III (15). We showed recently that the selective uptake of HDL-CE by isolated rat liver parenchymal cells can be inhibited completely by ligands specific for SR-BI (16), indicating that the expression of SR-BI can be solely responsible for the selective HDL-CE uptake in this cell type.

* This study was financially supported by the Netherlands Organization for Scientific Research, Council for Medical Reseach, Medical Sciences Grant 902-523-096, The Netherlands Heart Foundation, and National Institutes of Health Grant HL59376. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‖ To whom correspondence should be addressed. Tel.: 31-71-5276216; Fax: 31-71-5276032; E-mail: T.Berkel@lacdr.LeidenUniv.nl.

[1] The abbreviations used are: HDL, high density lipoprotein; BSA, bovine serum albumin; CE, cholesteryl ester; DMEM, Dulbecco's modified Eagle's medium; EE, 17α-ethinyl estradiol; LDL, low density lipoprotein; NBD, 22-(*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino); Ox-LDL, oxidized LDL; poly I, polyinosinic acid; apo, apolipoprotein; SR-BI, scavenger receptor type B class I.

In vivo, SR-BI is expressed in the steroidogenic organs and liver of rodents (12, 17, 18), which all display selective uptake of HDL-CE. In the steroidogenic tissues, SR-BI expression is coordinately regulated with the steroidogenesis by adrenocorticotropic hormone, human chorionic gonadotropin, and estrogen (17, 19). Furthermore, SR-BI expression in adrenals is up-regulated in apoA-I knockout mice, hepatic lipase knockout mice, and lecithin:cholesterol acyltransferase knockout mice (18, 20). Unlike the steroidogenic tissues, SR-BI expression in the liver is down-regulated by estrogen treatment of rats (17). We showed recently that the down-regulation of SR-BI expression in the liver is limited to the parenchymal cells and is correlated with a decrease in the selective HDL-CE uptake (21). Surprisingly, SR-BI expression and the selective HDL-CE uptake is up-regulated in Kupffer cells after 17-α-ethinyl estradiol (EE) treatment or a high cholesterol diet, pointing to a different regulatory response in tissue macrophages (Kupffer cells) as compared with parenchymal cells (21).

Our present goal was to analyze whether SR-BI may mediate the increased hepatic uptake of oxidized cholesterol esters *in vitro* and *in vivo*. We investigated the potential role of scavenger receptor BI in the efficient removal of HDL-CEOH by rat liver cells by performing *in vitro* competition studies with ligands specific for scavenger receptor BI. *In vivo*, the effect of the regulation of SR-BI in liver cells by EE treatment or a high cholesterol diet on the selective uptake *in vivo* of oxidized cholesterol esters was studied. Finally, the uptake of both native and oxidized HDL cholesterol esters was determined in SR-BI-transfected CHO cells. These experiments indicate that SR-BI can mediate the highly efficient selective uptake of oxidized cholesterol esters from HDL.

EXPERIMENTAL PROCEDURES

Materials—[Cholesteryl-1,2,6,7-^{3}H(*N*)]-linoleate was purchased from NEN Life Science Products. [1α,2α(n)-^{3}H]Cholesteryl linoleate ([^{3}H]CH18:2) and ^{125}I (carrier free) in NaOH were obtained from Amersham Pharmacia Biotech. 2,2′-Azobis-(2,4-dimethyl-valeronitrile) was purchased from Polyscience (Warrington, Florida). 22-(*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)(NBD)-23,24-bisnor-5-cholen-3β-yl linoleate and carboxyfluorescein diacetate was obtained from Molecular Probes (Eugene, OR). Egg yolk phosphatidylcholine was obtained from Fluka (Buchs, Switzerland) the phospholipid, cholesterol oxidase-peroxidase aminophenazone, and glycerolphosphate oxidase-peroxidase aminophenazone kits from Boehringer Mannheim. Ethylmercurithiosalicylate (thimerosal), bovine serum albumin (BSA) (fraction V), and collagenase type I and type IV were from Sigma. Dulbecco's modified Eagle's medium (DMEM) was from Life Technologies, Inc. All other chemicals were of analytical grade.

Label Preparation—[^{3}H]Cholesteryl-linoleate-hydroperoxide ([^{3}H]Ch18:2-OOH) was prepared by peroxidation of (cholesteryl-1,2,6,7-^{3}H(*N*))-linoleate with a lipid-soluble peroxyl radical generator 2,2′-azobis-(2,4-dimethyl-valeronitrile). 200 μCi of [^{3}H]Ch18:2 in toluene (final volume, 1 ml) was peroxidized with 2,2′-azobis-(2,4-dimethyl-valeronitrile) (0.4 M at 37 °C for 5 h). The incubation mixture was dried under nitrogen and purified by reversed phase HPLC on an LC-18 column as described in Ref. 22. The fraction eluting between 8 and 9 min (corresponding to the retention time of an unlabeled Ch18:2-OOH standard) was collected and dried in a SpeedVac (Savant, Bierbeek, Belgium). The resulting [^{3}H]Ch18:2-OOH was then chemically reduced to the corresponding [^{3}H]Ch18:2-OH by $NaBH_4$ (10 mg) reduction in methanol (1 ml) on ice (1 h) as described by Van Kuijk *et al.* (23). [^{3}H]Ch18:2-OH was extracted into hexane and purified in a second HPLC step (LC-18 column, mobile phase acetonitrile/isopropanol/H_2O, 22:27:1, v/v) (24). Fluorescent NBD-cholesteryl linoleate was oxidized with 70% *tert*-butyl OOH and 100 μM $Fe(II)SO_4$ over night at 45 °C. This method yielded a 100% oxidation of the fluorescent cholesterol ester as checked by TLC.

Isolation and Labeling of HDL—Human HDL was isolated from the blood of healthy volunteers by differential ultracentrifugation as described by Redgrave *et al.* (25). HDL ($1.063 < d < 1.21$) was dialyzed against phosphate-buffered saline/1 mM EDTA and labeled with either [^{3}H]Ch18:2 or [^{3}H]Ch18:2-OH by exchange from donor particles as reported previously (1).

HDL was labeled by incubating HDL with donor particles (mass ratio of HDL protein:particle phospholipid, 8:1) in the presence of human lipoprotein-deficient serum as cholesteryl ester transfer protein source (1:1 v/v) for 8 h at 37 °C in a shaking water bath under argon as described (2, 16). Subsequently, the labeled HDL was dialyzed against phosphate-buffered saline/1 mM EDTA and passed through a heparin-Sepharose affinity column to remove apoE-containing particles (26). After the labeling procedure, the radiolabeled HDL was checked for hydrolysis of the cholesteryl ester labels by a Bligh and Dyer extraction (27) followed by thin layer chromatography. The effect of the labeling procedure on HDL was analyzed by measurement of phospholipid, cholesterol, cholesteryl ester, and triglyceride content (with the phospholipid, cholesterol oxidase-peroxidase aminophenazone, and glycerolphosphate oxidase-peroxidase aminophenazone kits, respectively). The density, electrophoretic α-mobility, and particle size (photon correlation spectroscopy, System 4700 C, Malvern Instruments) were also analyzed. Labeled HDL was only used when there was no change observed in the measured composition or physical characteristics as compared with the original unlabeled HDL batch. Additionally, to exclude the possibility that endogenous HDL lipids or tracers were oxidized during the labeling procedure, HPLC analyses of lipid extracts obtained from [^{3}H]Ch18:2-labeled HDL were performed. HDL lipids were extracted into methanol/hexane (1:5), and the hexane phase, containing the neutral lipids, was dried and analyzed by HPLC as described (2). HDL was iodinated by the ICl method of McFarlane (28) as modified by Bilheimer *et al.* (29).

Before use, LDL was dialyzed against phosphate-buffered saline with 10 μM EDTA. Acetylation of LDL was performed by acetic anhydride as described (30). LDL was oxidized by exposure to $CuSO_4$ as described in detail earlier (31).

Phospholipid Liposome Preparation—Unilamelar liposomes were obtained by sonication of egg yolk phosphatidylcholine/phosphatidylserine/cholesterol in a molar ratio of 1:1:1 as described previously (16).

Liver Association and Tissue Distribution—Male Wistar WU rats (200–250 g) were anesthetized by intraperitoneal injection of 20 mg of Nembutal. Body temperature was maintained with a heating lamp. After opening of the abdomen, radiolabeled HDL was injected into the inferior vena cava. At the indicated time point, blood sampling and adrenal and liver excision were performed as described previously (2).

Hepatic Cellular Distribution—The hepatic cellular distribution of HDL was studied by using a low temperature cell isolation technique as described (32). By means of centrifugal elutriation, the Kupffer cells were purified from the non-parenchymal cell preparation (33). The purity of each cell fraction was checked by light microscopy after staining for peroxidase activity. Cellular cholesterol concentrations were measured with a commercial kit as mentioned above after a Bligh and Dyer extraction (27) of the cellular lipids.

In Vitro Studies with Freshly Isolated Rat Liver Cells—Liver cells were isolated by perfusion of the livers of male Wistar WU rats (200–250 g) with collagenase at 37 °C as described (33). By means of centrifugal elutriation, the Kupffer cells were purified from the non-parenchymal cell preparation (33). The viability (>95%) of the obtained parenchymal cells was checked by trypan blue exclusion. The cells from the last centrifugation step were resuspended in oxygenated DMEM supplemented with 2% BSA, pH 7.4. For *in vitro* competition studies, the liver cells were incubated with the indicated amount of radiolabeled HDL and competitors for 180 min in 1 ml of DMEM containing 2% BSA at 37 °C. The temperature dependence studies were performed at the indicated temperatures for 15 min. All cell incubations were performed in a circulating laboratory shaker (Adolf Kühner AG, Basel, Switzerland) at 150 rpm. Every hour, the incubations were briefly oxygenated. The viability of the cells remained higher than 88% during these long term incubations (33). After incubation, the cells were centrifuged for 2 min at 50 $\times$ *g* for parenchymal cells and 500 $\times$ *g* for Kupffer cells in an Eppendorf centrifuge and washed two times in 50 mM Tris-HCl, 0.15 M NaCl, 0.2% BSA, pH 7.4 at 4 °C. Subsequently, the cell pellet was washed in a similar medium without BSA. The cells were lysed in 0.1 N NaOH, and the protein content and radioactivity were determined.

SR-BI Assays in Transfected CHO Cells—ldlA cells (clone 7), an LDL receptor-deficient CHO line (provided by M. Krieger) were cultured in Ham's F-12 medium containing 5% (v/v) fetal bovine serum, 2 mM glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin. Stable transfectants expressing mouse SR-BI were prepared as described previously using the expression vector pCMV5 (37). Expressing lines were isolated and maintained in medium containing 0.5 mg/ml G-418. Binding and uptake assays with transfected CHO cells were carried out following the procedure of Acton *et al.* (12) as described previously (37).

Confocal Microscopy—Freshly isolated parenchymal cells were incu-

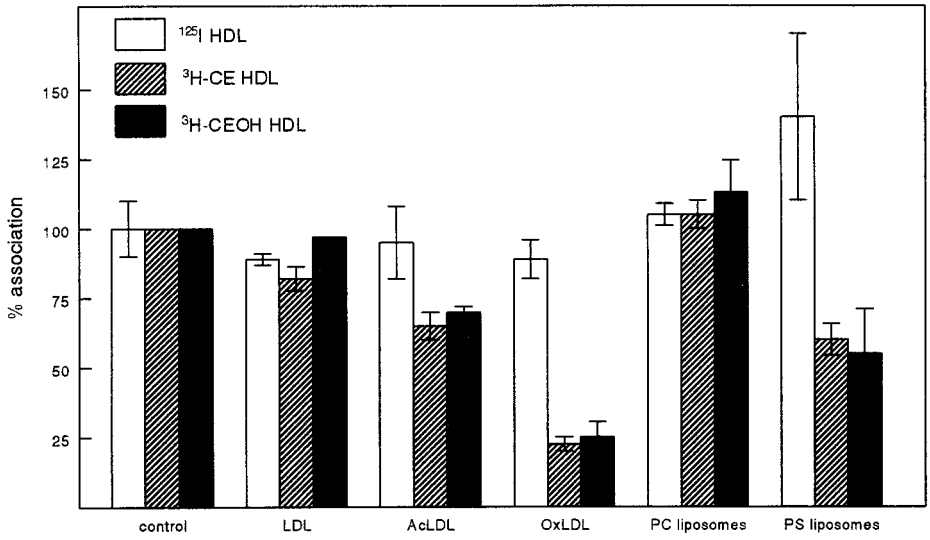

FIG. 1. **Effect of native and modified lipoproteins and neutral and phosphatidylserine liposomes on the parenchymal cell association of ^{125}I-HDL, [^{3}H]CE-, or [^{3}H]CEOH-labeled HDL.** Rat liver parenchymal cells were incubated for 3 h at 37 °C with 10 μg/ml labeled HDL in the absence or presence of 100 μg/ml unlabeled competitors in DMEM with 2% BSA. The 100% value for association of [^{3}H]CE- or [^{3}H]CEOH-labeled HDL was 202 ± 14 and 966 ± 150 ng of HDL/mg of cell protein, respectively, and for ^{125}I-HDL association, it was 36 ± 3 ng of HDL/mg of cell protein. The association is expressed as the percentage of the radioactivity obtained in the absence of competitor. The results are given as means ± S.E. (n = 3).

bated on glass coverslips in 6-well plates (Costar, Cambridge MA) for 2 h at 4 °C in DMEM supplemented with 2% BSA and the indicated amount of oxidized NBD cholesteryl linoleate-labeled HDL or carboxyfluorescein diacetate. Cells on the coverslips were transferred to a Zeiss IM-35 inverted microscope (Oberkochen, Germany) with a × 63, NA 1.4 planapochromatic objective that was equipped with a Bio-Rad MRC600 confocal visualization system. The microscope was fitted with a incubation chamber to allow incubation of the cells at 37 °C.

Quantification of SR-BI Expression—The expression levels of SR-BI in membranes of isolated parenchymal and Kupffer cells were measured on Western blot as described (21).

Protein Determination—Protein was determined according to Lowry *et al.* (34) with BSA as standard.

RESULTS

Inhibition of Selective Uptake of HDL-CEOH by Rat Liver Parenchymal Cells by Substrates of Scavenger Receptor BI—We showed earlier that the selective uptake of HDL-CE by parenchymal cells could almost be completely inhibited by OxLDL and liposomes containing phosphatidylserine, *e.g.* known substrates for SR-BI (16). As for native cholesterol esters, the increased selective uptake of HDL-CEOH by the liver is mediated by the parenchymal cells *in vivo* (2). To answer the question of whether SR-BI is mediating this increased selective uptake of HDL-CEOH, the inhibitory effects of known SR-BI substrates on HDL-CEOH uptake by isolated parenchymal cells were compared with the inhibitory effects on the selective uptake of HDL-CE and HDL particle association as measured by iodinated HDL. Freshly isolated rat liver parenchymal cells were incubated for 3 h at 37 °C with HDL, either iodinated or labeled with [^{3}H]CE or [^{3}H]CEOH. At this time point, the apparent association as calculated according to Pitmann (5) of [^{3}H]CE-labeled HDL (202 ± 14 ng of HDL/mg of cell protein) exceeded ^{125}I-HDL (36 ± 3 ng of HDL/mg of cell protein) association by 5.6 times, whereas the association of [^{3}H]CEOH-HDL (966 ± 150 ng of HDL/mg of cell protein) apparently exceeded ^{125}I-HDL association by 27 times. The ability of (modified) lipoproteins to compete for [^{125}I]HDL and selective HDL-CE and HDL-CEOH uptake was tested by co-incubation with either 100 μg/ml LDL, acetylated LDL, or OxLDL and either neutral liposomes or liposomes containing phosphatidylserine (Fig. 1). Addition of either 100 μg/ml LDL or modified LDL only marginally (<10%) decreased the cell association of [^{125}I]HDL. [^{3}H]CE-HDL and [^{3}H]CEOH-HDL association was also not significantly affected by addition of LDL. However, addition of acetylated LDL led to a 35 and 30% inhibition of [^{3}H]CE-HDL and [^{3}H]CEOH-HDL association, respectively, whereas OxLDL decreased their uptake by 75 ± 2.4 and 75 ± 5.4%, respectively. Liposomes consisting of phosphatidylcholine, cholesterol, and the anionic phospholipid phosphatidylserine inhibited both HDL-CE and HDL-CEOH uptake by almost 50% (Fig. 1), whereas [^{125}I]HDL association was increased. Neutral liposomes, consisting only of phosphatidylcholine and cholesterol, did not influence HDL-CE and HDL-CEOH uptake. The possibility of exchange of [^{3}H]CE and [^{3}H]CEOH to the other lipoproteins and liposomes was tested by isolating the lipoproteins after incubation for 3 h at 37 °C. Both gradient density ultracentrifugation and agarose gel electrophoresis were performed. Less than 5% of both [^{3}H]CE and [^{3}H]CEOH was recovered in the LDL, acetylated LDL, OxLDL, or liposomal fraction after incubation for 3 h at 37 °C, establishing that exchange to competitors could not explain the achieved results. Therefore, both OxLDL and the phosphatidylserine liposomes were effective inhibitors of HDL-CEOH uptake by the liver parenchymal cells, and the inhibition of HDL-CEOH uptake was comparable to the inhibition of HDL-CE uptake. The effective inhibition of the selective uptake of oxidized cholesterol esters, like that of native cholesterol esters from HDL, did not correlate with a similar inhibition of the HDL particle association (16). Apparently, the total amount of HDL binding sites in liver exceeds the amount of SR-BI-mediated binding sites, albeit these latter are efficiently coupled to selective uptake. This is in agreement with the limited amount of poly I-insensitive binding sites for OxLDL on parenchymal cells (31) as compared the total amount of HDL binding sites (less than 10%).

The inhibitory effect of OxLDL on the the selective uptake of HDL-CEOH was further analyzed with respect to efficiency of competition. Increasing concentrations of OxLDL were added to freshly isolated parenchymal cells in the presence of [^{3}H]CEOH-labeled HDL. Already at 20 μg/ml OxLDL, the uptake of HDL-CEOH was inhibited by more than 70% (Fig. 2). Poly I, an established inhibitor of scavenger receptor class A, did not lower the cell association of [^{3}H]CEOH-HDL. The simultaneous presence of increasing concentrations of OxLDL and 100 μg/ml 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-CEOH was also analyzed by confocal laser scanning microscopy using HDL labeled with a fluorescent analog of CEOH. The uptake of fluorescent NBD-CEOH was followed by up to 3 h of incubation with parenchymal cells. 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 (35) allow to

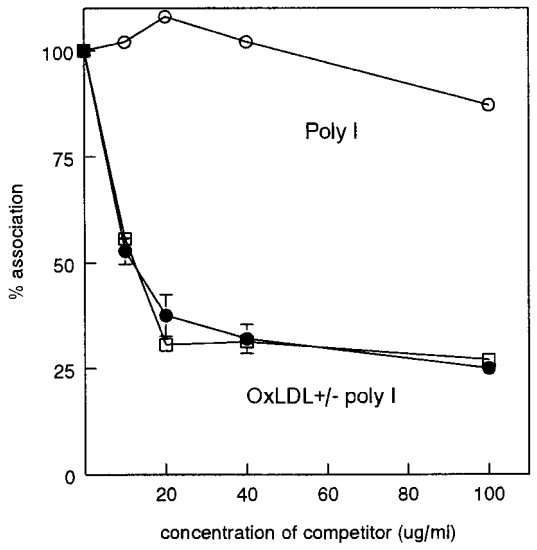

FIG. 2. **Effect of increasing concentrations of OxLDL and poly I on the parenchymal cell association of [^{3}H]CEOH-labeled HDL.** Rat liver parenchymal cells were incubated for 3 h at 37 °C with 10 μg/ml labeled HDL in the presence of the indicated amounts of OxLDL (●—●) or poly I (○—○). When OxLDL was added together with poly I (□—□), a fixed 100 μg/ml of poly I was applied. The association is expressed as the percentage of the radioactivity obtained in the absence of competitor. The results are given as means ± S.E. (n = 3).

follow bile-directed transport of fluorescent cholesterol esters. At 3 h of incubation at 37 °C, most of the label inside the cell was concentrated in the bile canalicular vacuole of the parenchymal cell couplets, indicating a very efficient biliary secretion of oxidized cholesterol esters (Fig. 3). A very punctuate labeling in the cells was also observed. Addition of 100 μg/ml OxLDL abolished uptake of fluorescent CEOH almost completely.

As a control, carboxyfluorescein diacetate was used, which is a substrate for an ATP-dependent organic anion transporter of the bile canalicular membrane and which is absent in TR$^-$ rats (42, 43). A concentration of fluorescence in the bile canalicular vacuole similar to that of the oxidized cholesterol esters was noticed.

Uptake of HDL-CEOH by the Liver and Adrenals in Vivo—The main uptake site of [^{3}H]CEOH-labeled HDL *in vivo* is the liver (2). The liver uptake of [^{3}H]CEOH-HDL 30 min after injection was 27.0 ± 1.9 injected dose% (Fig. 4) and was 2.3-fold higher than the liver uptake of native HDL-CE (n = 4). When the adrenal uptake of [^{3}H]CEOH-HDL was compared with the uptake of [^{3}H]CE-HDL, a similar 2.6-fold difference in uptake was seen. Adrenal uptake at 30 min after injection was 2.0 ± 0.7 injected dose% and 5.1 ± 0.6% for [^{3}H]CE-HDL and [^{3}H]CEOH-HDL, respectively (n = 4). No other organs have a preferential uptake of HDL-CEOH as compared with native HDL-CE (2).

Intrahepatic Cellular Uptake in Vivo of HDL-CEOH: Effect of 17α-Ethinyl Estradiol Treatment or a High Cholesterol Diet on SR-BI Expression and HDL-CEOH Uptake—Treatment of rats with EE for 5 consecutive days or a high cholesterol diet for 2 weeks lowered expression of SR-BI in the liver parenchymal cells, whereas the expression of SR-BI in the Kupffer cells was increased as previously reported (Table I) (21). Furthermore, it was found that the induced changes in hepatic SR-BI expression were well correlated to changes in the selective uptake of HDL-CE (21). In order to test whether the changes in SR-BI expression induced by either EE treatment or a high cholesterol diet also affected the increased selective uptake of HDL CEOH, the liver uptake of [^{3}H]CEOH-labeled HDL was determined, as well as the association of iodinated HDL in order to

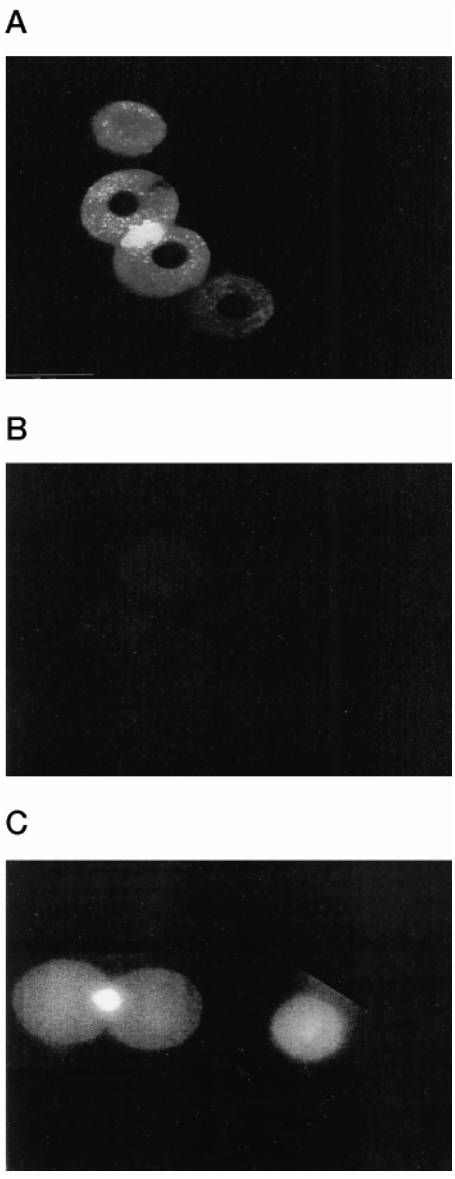

FIG. 3. **Visualization of the interaction of oxidized NBD cholesteryl linoleate-labeled HDL with liver parenchymal cell couplets and the effect of OxLDL.** Freshly isolated parenchymal cells were preincubated on glass coverslips for 2 h at 4 °C in DMEM supplemented with 2% BSA and 50 μg/ml HDL labeled with oxidized NBD cholesteryl linoleate in the presence (*B*) or absence (*A*) of 100 μg/ml OxLDL. Cells were analyzed with a confocal microscope fitted with a incubation chamber to allow incubation of the cells at 37 °C. The fluorescence of oxidized NBD cholesteryl linoleate is the result of a 3-h incubation of the cells at 37 °C. As a control, 5 μg/ml carboxyfluorescein diacetate, a substrate for an ATP-dependent organic anion transporter of the bile canalicular membrane, was used and incubated for 3 h at 37 °C with the cells (*C*).

analyze total particle association. To identify the changes in the cellular uptake sites for [^{3}H]CEOH-HDL, parenchymal cells and the liver tissue macrophages (Kupffer cells) were isolated (Fig. 5). Treatment of rats with EE for 5 days resulted in a significant 50% decrease in [^{3}H]CEOH-HDL uptake by the liver, whereas uptake of ^{125}I-labeled HDL was not significantly changed (data not shown). Thus, the selective uptake of HDL-CEOH was greatly inhibited by treatment of rats with EE, in accordance with the supposed role of SR-BI as the mediator of selective HDL-CEOH uptake. This decrease in selective uptake of HDL-CEOH by the liver can be explained by a 64% decrease in [^{3}H]CEOH-HDL uptake by the parenchymal cells after EE treatment. In contrast, the Kupffer cells showed a significant

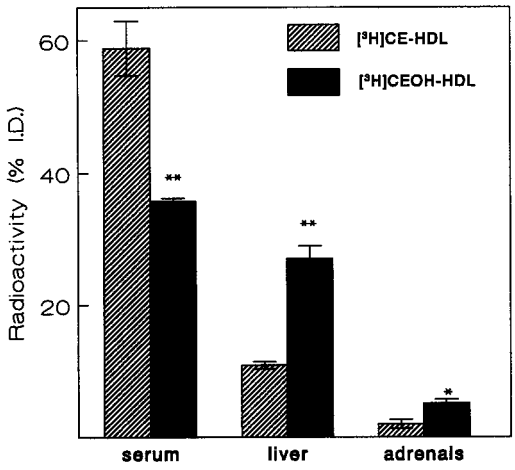

FIG. 4. **Tissue distribution of [³H]CEOH-HDL and [³H]CE-HDL at 30 min after injection in the rat.** 300–400 μg (500000 dpm) of [³H]CEOH-HDL or [³H]CE-HDL was injected into the inferior vena cava of anaesthetized rats. 30 min after injection, the amount of radioactivity was determined after combustion in a Hewlett-Packard sample oxidizer 306 and counting for radioactivity. The total recovery of label was 94 and 86% of the injected dose for [³H]CEOH and [³H]CE-HDL, respectively. Values are means ± S.E. of four experiments. ** indicates extremely significant difference (p <0.005). * indicates significant difference ($p < 0.05$) (unpaired Student's t test).

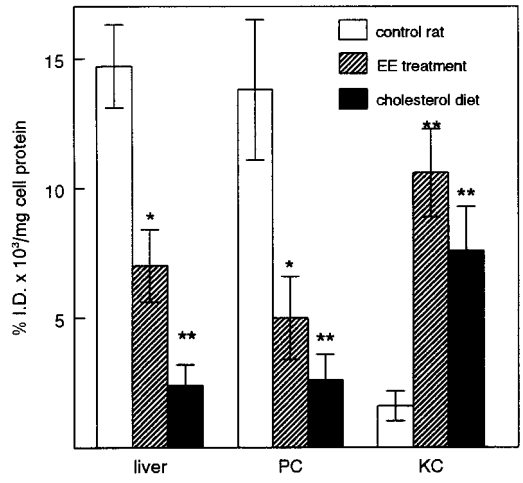

FIG. 5. ***In vivo* distribution of [³H]CEOH-HDL between parenchymal and Kupffer cells at 10 min after injection in EE-treated rats, rats fed with a high cholesterol diet, or control rats.** Shown are the results for control rats (*open bars*) and rats treated with EE (5 mg/kg) for 5 days (*hatched bars*) or put on a high cholesterol diet for 2 weeks (*black bars*). At 10 min after injection of [³H]CEOH-HDL, the liver was perfused, and parenchymal cells (*PC*) and Kupffer cells (*KC*) were isolated at 4 °C. Values, expressed as the percentage of the injected dose × 10³/mg of cell protein, are means ± S.E. of five experiments. ** indicates very significant difference ($p < 0.01$). * indicates significant difference ($p < 0.05$) (unpaired Student's t test).

TABLE I

SR-BI expression levels on parenchymal and Kupffer cell membranes

Cell membranes from parenchymal (PC) and Kupffer (KC) cells were isolated from control rats, rats treated with EE (5 mg/kg) for 5 days, or rats that had been fed a high-cholesterol diet for 2 weeks. Solubilized membrane proteins were subjected to SDS-polyacrylamide gel electrophoresis and blotted on to nitrocellulose membranes. SR-BI was visualized by immunolabeling followed by enhanced chemiluminescence detection and quantitated (21). Values are means ± S.E. of three experiments.

	Control	EE	Cholesterol diet
		% of control	
PC	100 ± 4.5	15.5 ± 4.1[a]	25.0 ± 6.0[a]
KC	100 ± 5.0	388 ± 55.0[a]	310 ± 75.0[b]

[a] Indicates very significant difference, $p < 0.005$.
[b] Significant difference, $p = 0.05$ (unpaired Student's t test).

6.6-fold increase ($p < 0.05$) in uptake of [³H]CEOH-HDL.

Rats were also fed a high cholesterol containing diet for 2 weeks. This diet increased the plasma cholesterol levels 20-fold as compared with the control animals, as described previously (21), whereas total cholesterol concentration in the liver increased more than 10-fold. The total cellular cholesterol concentration in parenchymal cells increased from the control value of 11 ± 0.9 ng/mg of cell protein up to 136 ± 18 ng/mg of cell protein ($n = 3$) (mean ± S.E.), whereas the cholesterol content in the Kupffer cells increased from 6.8 ± 0.2 ng/mg of cell protein in the control animals up to 155 ± 66 ng/mg of cell protein after the 2-week diet ($n = 3$). This diet resulted in a very significant ($p < 0.01$) 80% decrease in selective uptake of [³H]CEOH-HDL by the liver (Fig. 5). The 2-week high cholesterol diet inhibited only the parenchymal cell uptake of [³H]CEOH-HDL (81%), whereas Kupffer cell uptake of [³H]CEOH-HDL was almost 5-fold increased. The decrease in HDL-CEOH uptake by the parenchymal cells and increase in HDL-CEOH uptake by the Kupffer cells after either EE treatment or high cholesterol diet is similar, as was found with native HDL-CE, and correlates with the induced changes in hepatic SR-BI expression (21).

The effects of EE treatment or a high cholesterol diet on the selective uptake of HDL-CEOH was also studied *in vitro*. Hepatic parenchymal cells were isolated from both EE-treated rats and rats that had been fed a high cholesterol diet for 2 weeks. The concentration dependence of the cell association of [³H]CEOH-HDL was studied (Fig. 6). Data are expressed in terms of apparent particle uptake as originally devised by Pittman *et al.* (5). Parenchymal cells isolated from both EE-treated rats and from rats fed on a high cholesterol diet showed a significant decrease in [³H]CEOH-HDL association ($p < 0.05$, two-way analysis of variance) *in vitro* (Fig. 6). These *in vitro* data thus illustrate that the changes in *in vivo* uptake are also reflected *in vitro* with the isolated parenchymal cells.

SR-BI Transfection Studies—To assess the ability of SR-BI to mediate the uptake of oxidized cholesterol esters from HDL, stable CHO cell transfectants expressing mouse SR-BI were used for cell association studies. SR-BI-specific values were determined as the difference between the values for the SR-BI-transfected cells and the control ldlA cells. CHO cells expressing SR-BI showed a clear selective uptake of both native and oxidized cholesterol esters (Fig. 7) wherein the apparent association of [³H]CE-HDL and [³H]CEOH-HDL exceeded the ^{125}I-HDL association 7.8 and 20 times, respectively (30-min incubation). The rate of uptake of [³H]CEOH-HDL by transfected cells was markedly greater (3.4 ± 0.6 ($n = 3$) at 30 min of incubation) than that of native HDL cholesterol esters. The amount of cell-associated [^{125}I] HDL was also increased in SR-BI-transfected cells as compared with mock-transfected cells, but it did not differ significantly between CEOH-HDL and CE-HDL (Fig. 7, *C* and *D*).

DISCUSSION

The generally accepted anti-atherogenic effect of HDL can be based upon several mechanisms. The protective role of HDL is classically associated with reverse cholesterol transport, as first proposed by Glomset (3). In addition to this generally accepted concept, HDL may also protect against oxidation of LDL (7), thereby preventing the formation of an atherogenic form of LDL. The mechanism by which HDL exerts its antioxidant action is still speculative. It was suggested that the degree of protection during LDL oxidation was directly related to the paraoxonase activity of HDL (36). In addition, Kunitake

et al. (38) showed that HDL-associated cearuloplasmin or transferrin could scavenge metal ions and thus prevent catalysis of lipid peroxidation in LDL. However, in the same studies, ultracentrifugally prepared HDL, which looses most of its associated enzymes, still retained antioxidant activity. In addition, reconstituted HDL, not containing any undefined proteins, decreased the capacity of OxLDL to form foam cells (39). Parthasarathy *et al.* (40) suggested that HDL may act as a reservoir for lipid peroxides. HDL is suggested to break the chain of lipid peroxide propagation by taking over LDL lipid peroxides. Consistent with this theory, it was found that HDL is the major carrier of lipid hydroperoxides in human blood. More than 85% of all detectable oxidized lipids in human plasma are associated with HDL, whereas LDL is relatively peroxide-free (10). HDL-associated lipid hydroperoxides are readily reduced by an intrinsic peroxidase activity leading to less reactive hydroxides (9), an activity that may be exerted by apoA-I and apoA-II (41).

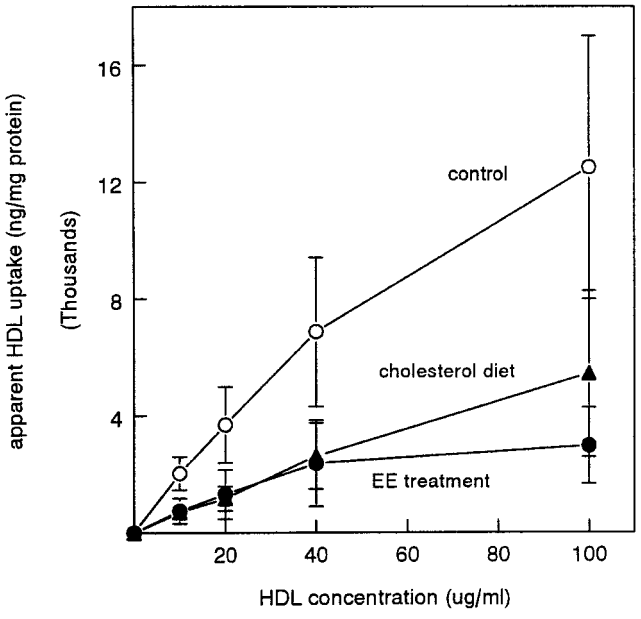

FIG. 6. **Concentration dependence of [^{3}H]CEOH-HDL association to rat liver parenchymal cells isolated from control rats, EE-treated rats, or cholesterol-fed rats.** Rat liver parenchymal cells were isolated from control rats, rats treated with EE (5 mg/kg) for 5 days, or rats on a high cholesterol diet for 2 weeks. Rat liver parenchymal cells were incubated for 3 h at 37 °C with the indicated amount of labeled HDL in DMEM with 2% BSA (w/v). Data are expressed in terms of apparent particle uptake. The values are corrected for nonspecific cell association in the presence of a 20-fold excess of HDL (n = 2 separate cell isolations).

The biological fate of HDL-associated oxidized cholesterol esters was until recently unexplored. Sattler and Stocker (8) showed that oxidized cholesterol esters in HDL are taken up to a greater extent by HepG2 cells *in vitro* than native cholesterol esters. Recently, we showed that oxidized cholesterol esters show an increased serum decay as compared with native cholesterol esters, whereas the liver uptake exceeded particle uptake, thus indicating selective delivery (2). The selective uptake of HDL-CEOH by the liver is 8.2-fold higher than native cholesterol ester uptake at 2 min after injection, whereas after 30 min, the uptake is still 2.3-fold higher. This process is, within the liver, selectively exerted by the parenchymal cells, and it is coupled to efficient biliary excretion. However, the mechanism of the highly efficient uptake of HDL-CEOH was not clear.

The liver and steroid-forming tissues are mainly responsible for the clearance of native HDL cholesteryl esters from the blood circulation (1, 5, 6), and SR-BI is now held responsible for mediating the selective uptake of native HDL-CE by these organs (12, 17, 18). The presence of hydroxyl groups in oxidized cholesterol esters yields a better solubility in water, enabling the cholesteryl hydroxides to transfer more efficiently through an aqueous phase between HDL and cellular membranes. This might explain the increased cellular uptake of HDL-CEOH. However, based upon our previous *in vivo* experiments (2), it was concluded that this increased solubility did not lead to an increased uptake by all cell types but, within the liver, apparently only by the parenchymal cells. In addition, the adrenals, which are known to have a high expression of SR-BI, show a similar increased uptake of HDL-CEOH as compared with native HDL-CE. Apparently, a cell-specific process is mediating uptake of oxidized cholesterol esters *in vivo*, similar to the uptake of native cholesterol esters. We now suggest that indeed

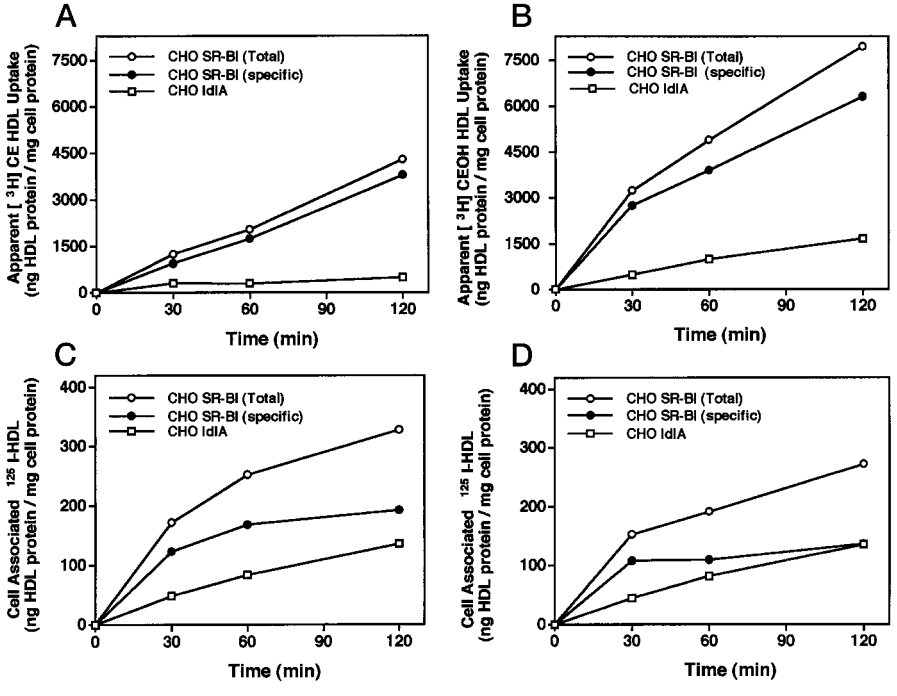

FIG. 7. **Time dependence of the selective uptake of [^{3}H]CE and [^{3}H]CEOH-HDL by SR-BI-transfected CHO cells.** Cells were incubated at 37 °C with labeled HDL (10 μg of protein/ml) for the indicated times, and cell-associated label was quantified as described under "Experimental Procedures." *A* and *B,* cells were incubated with [^{3}H]CE-HDL (*A*) or [^{3}H]CEOH-HDL (*B*). *C* and *D,* cells were incubated with ^{125}I-labeled CE HDL (*C*) or ^{125}I-labeled CEOH-HDL (*D*). Values represent the mean of duplicate determinations. Similar results were obtained in two additional experiments.

the increased selective uptake of oxidized cholesterol esters as compared with native cholesterol esters is mediated by SR-BI. We obtained five points of evidence for this involvement. First, the increased selective uptake of HDL-CEOH by isolated parenchymal cells can be blocked by oxidized LDL, acetylated LDL, and phosphatidylserine liposomes to an extent similar to native cholesterol ester uptake. Second, the effect of oxidized LDL is not influenced by the simultaneous presence of poly I, indicating a poly I-insensitive site for OxLDL interaction. Third, *in vivo* the increased uptake of oxidized *versus* native cholesterol esters in untreated rats is only exerted by the parenchymal cells of the liver and by the adrenals, the cellular sites where SR-BI is expressed. Fourth, within the liver, the uptake of oxidized cholesterol esters and native cholesterol esters are regulated to a similar extent by estradiol treatment and by a high cholesterol diet, whereby the *in vivo* uptake by parenchymal cells is decreased and the uptake by Kupffer cells is increased. These latter changes parallel the expression of SR-BI as analyzed on Western blots. Fifth, the SR-BI-transfected CHO cells show a very efficient selective uptake of oxidized cholesterol esters, exceeding particle association by 20 times at 30 min of incubation, while oxidized cholesterol ester uptake at this time point is 3.4 times higher than for native cholesterol esters.

Recently, Thuren *et al.* (44) observed that an antibody against SR-BI does block the selective uptake of native cholesterol esters by isolated parenchymal cells by up to 75%, confirming our initial findings that substrates for SR-BI inhibit the selective uptake of HDL cholesterol esters (16) in these cell types. Furthermore, the similarity in uptake behavior observed in this study between native and oxidized cholesterol esters by rat parenchymal cells, together with the antibody data, are in accordance with our suggestion for SR-BI involvement for both native and oxidized cholesterol esters from HDL.

Oxidized lipids might be recognized by types of scavenger receptors other than SR-BI, and we have shown earlier that oxidized LDL is rapidly removed from the blood circulation by Kupffer cells (31). In untreated rats, we did not observe any selective uptake of CE-OH by the Kupffer cells; thus, the Ox-LDL-specific recognition site of Kupffer cells (probably macrosialin (45)) does not interact with the oxidized cholesterol esters from HDL. Down-regulation of SR-BI by estradiol or a high cholesterol diet leads to a 64 or 81% inhibition, respectively, of the oxidized cholesterol ester uptake by parenchymal cells. It thus appears that SR-BI is a major determinant for the selective uptake of oxidized cholesterol esters, leaving only a low percentage of contribution for other receptors.

The mechanism for the more effective cellular uptake of oxidized cholesterol esters as compared with native cholesterol esters from HDL by SR-BI is presently unclear. The higher solubility of oxidized cholesterol esters as compared with native cholesterol esters might either facilitate the interaction of CEOH from HDL with the active site of SR-BI or facilitate its further transport into the cell. Whatever its mechanism, it might be concluded that the association of enzymatic activities with HDL together with the efficient clearance route of HDL-associated oxidized cholesterol esters as exerted by SR-BI on liver parenchymal cells may work synergistically to detoxify lipid hydroperoxides and thereby protect LDL from oxidation *in vivo.* This scavenging function of SR-BI for oxidized lipids is in line with the expected function of a member of the scavenger receptor family.

Acknowledgment—We thank Edward Lee Gattertdam for excellent technical assistance.

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Scavenger Receptor BI Mediates the Selective Uptake of Oxidized Cholesterol Esters by Rat Liver

Kees Fluiter, Wolgang Sattler, Maria C. De Beer, Patrice M. Connell, Deneys R. van der Westhuyzen and Theo J. C. van Berkel

J. Biol. Chem. 1999, 274:8893-8899.
doi: 10.1074/jbc.274.13.8893
