SR-BI-directed HDL-cholesteryl ester hydrolysis

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Abstract We have examined the metabolic fate of HDL cholesteryl ester (CE) delivered to cells expressing scavenger receptor class B type I (SR-BI). Comparison of SR-BI with a related class B scavenger receptor, CD36, showed a greater uptake and a more rapid and extensive hydrolysis of HDL-CE when delivered by SR-BI. In addition, hydrolysis of HDL-CE delivered by both receptors was via a neutral CE hydrolase. These data indicate that SR-BI, but not CD36, can efficiently direct HDL-CE to a neutral CE hydrolytic pathway. In contrast, LDL-CE was delivered and hydrolyzed equally well by SR-BI and CD36. Hydrolysis of LDL-CE delivered by SR-BI was via a neutral CE hydrolase but that delivered by CD36 occurred via an acidic CE hydrolase, indicating that SR-BI and CD36 deliver LDL-CE to different metabolic pathways. Comparison of inhibitor sensitivities in Y1-BS1 adrenal, Fu5AH hepatoma, and transfected cells suggests that hydrolysis of HDL-CE delivered by SR-BI occurs via cell type-specific neutral CE hydrolases. Furthermore, HDL-CE hydrolytic activity was recovered in a membrane fraction of Y1-BS1 cells. These findings suggest that SR-BI efficiently delivers HDL-CE to a metabolically active membrane compartment where CE is hydrolyzed by a neutral CE hydrolase.—Connelly, M. A., G. Kellner-Weibel, G. H. Rothblat, and D. L. Williams. **SR-BI-directed HDL-cholesteryl ester hydrolysis.** ***J. Lipid Res.*** **2003.** 44: **331–341.**

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Scavenger receptor class B type I (SR-BI) is the first physiologically relevant HDL receptor to be identified and is one of the major participants in reverse cholesterol transport (RCT). RCT is the process whereby cholesterol is transported from peripheral tissues via plasma HDL to the liver for bile acid synthesis and secretion or to endocrine tissues for steroid production (1). A variety of studies indicate that SR-BI mediates the selective uptake (SU) of HDL cholesteryl ester (CE), the process whereby the HDL core CE is taken into the cell without degradation of the whole particle and its apolipoproteins. The SU pathway is the major route for delivery of HDL-CE to the liver and steroidogenic tissues of rodents in vivo and in vitro (2–7). In addition, SR-BI stimulates the bidirectional flux of free cholesterol (FC) between cultured cells and lipoproteins (8–12), an activity that may be responsible for the rapid hepatic clearance of FC from plasma HDL and its resultant secretion into bile (13, 14). SR-BI's role in the RCT pathway may be one of the contributing factors to how HDL protects against the development of atherosclerosis.

Although numerous studies have been conducted on the mechanism of SR-BI-mediated HDL-CE SU, little is known about the fate of the HDL-CE once incorporated into the cell. It is well known that LDL-CE internalized via the LDL receptor pathway is hydrolyzed by a lysosomal acid cholesteryl ester hydrolase (ACEH) (15–17). In contrast, a limited number of studies indicate that HDL-CE internalized via SU is hydrolyzed extra-lysosomally (18) by a neutral cholesteryl ester hydrolase (NCEH) (19, 20). To complicate matters, total NCEH activity varies greatly among different tissues, and there appear to be multiple NCEH activities in some tissues, as suggested by studies with inhibitors and a limited amount of biochemical characterization. In general, tissues with an active cholesterol metabolism, such as liver, adrenal gland, ovary, testis, macrophage, and the gastrointestinal tract have the highest CEH activity. Interestingly, these are the tissues that express SR-BI at the highest levels, particularly adrenal and liver cells (21, 22), raising the possibility that a specific NCEH in these cells may be responsible for hydrolysis of HDL-CE delivered by SR-BI. Identifying the enzyme(s) responsible for the hydrolysis of HDL-CE in cells, which nat-

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Abbreviations: ACEH, acid cholesteryl ester hydrolase; ACTH, adrenocorticotropic hormone; CE, cholesteryl ester; ECD, extracellular domain; FC, free cholesterol; [^{3}H]CE, [^{3}H]cholesteryl oleate; NCEH, neutral cholesteryl ester hydrolase; PMA, phorbol 12-myristate 13-acetate; RCT, reverse cholesterol transport; SR-BI, scavenger receptor class B type I; SU, selective uptake; UBP, diethylumbelliferyl phosphate.

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urally express SR-BI, is essential for understanding the complex metabolism of HDL-CE and the role played by SR-BI in RCT.

The present study investigates the fates of HDL and LDL-CE when presented to the cell by either SR-BI or CD36 receptors. Our results indicate that HDL-CE taken up by SR-BI is rapidly directed to a membrane-associated NCEH, whereas HDL-CE delivered by CD36 is slowly and inefficiently hydrolyzed. Differential inhibitor sensitivities in various cell types suggest that efficient SR-BI-directed HDL-CE hydrolysis occurs via cell type-specific NCEH(s).

MATERIALS AND METHODS

Maintenance of tissue culture cells and transient transfection of COS-7 cells

WI38-VA13 human lung fibroblasts were maintained in Dulbecco's minimal essential medium (DMEM) (Invitrogen Corp./GIBCO/BRL/Life Technologies, Inc., Carlsbad, CA), 10% fetal bovine serum (FBS) (Atlanta Biologicals, Norcross, GA), 50 U/ml penicillin, 50 μg/ml streptomycin, and 2 mM L-glutamine. WI38-VA13 cells, stably expressing SR-BI, were maintained in growth medium supplemented with 800 μg/ml geneticin (G418) (Sigma, St. Louis, MO). Fu5AH rat hepatoma cells were maintained in DMEM, 5% FBS, 50 U/ml penicillin, 50 μg/ml streptomycin, and 2 mM L-glutamine. Y1-BS1 murine adrenocortical cells were maintained in Ham's F-10 medium (Sigma) with 12.5% horse serum (Sigma), 2.5% FBS, 2 mM L-glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin. Y1-BS1 cells were treated for 24 h with 100 nM Cortrosyn (Organon, West Orange, NJ), a synthetic adrenocorticotropic hormone (ACTH) analog, in growth medium unless otherwise indicated. THP-1 human macrophage cell line was maintained in RPMI with 25 mM Hepes, 10% FBS, 2 mM L-glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin. THP-1 human monocytic cells were differentiated for 3 days with 50 ng/ml phorbol 12-myristate 13-acetate (PMA) (Sigma) to increase SR-BI expression. All cells were cultured in a humidified 95% air-5% CO_2 incubator at 37°C.

COS-7 cells were maintained in DMEM, 10% calf serum (Gemini Bio-products, Calabasas, CA), 2 mM L-glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, and 1 mM sodium pyruvate, and transfected as previously described (23). The following day, two 10 cm dishes of transfected cells were trypsinized, resuspended in a total volume of 12 ml with fresh medium, and 0.5 or 1 ml was dispensed to each 22 (12 well plate) mm or 35 (6 well plate) mm well, respectively. The cells were assayed 48 h post-transfection unless otherwise indicated.

Immunoblot analysis

Transiently transfected cells expressing SR-BI (in 35 mm wells) were washed twice with PBS (pH 7.4) and lysed with 300 μl NP-40 cell lysis buffer (24, 25) containing 1 μg/ml pepstatin, 0.2 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, and 10 μg/ml aprotinin. Protein concentrations were determined by the Lowry method (26). Immunoblots with antibodies directed to SR-BI and CD36 confirmed their expression (data not shown).

Preparation of [^{3}H] and [^{125}I,^{3}H]hHDL$_3$

Human (h) HDL$_3$ (1.125 g/ml $< \rho <$ 1.210 g/ml) and LDL (1.006 g/ml $< \rho <$ 1.066 g/ml) were isolated by sequential ultracentrifugation (27). The hHDL$_3$ and hLDL were labeled with [^{3}H]cholesteryl oleate ([^{3}H]CE) (Amersham Pharmacia Biotech, Piscataway, NJ) using recombinant cholesteryl ester transfer protein as described (28) with the following modifications. HDL or LDL and cholesteryl ester transfer protein were incubated with [^{3}H]CE (dried down on the glass tube) for 5 h at 37°C. Labeled particles were reisolated by gel exclusion chomatography on a 25 ml Superose 6 (Amersham Pharmacia Biotech) column. The average specific activity of the [^{3}H]CE-hHDL$_3$ particles that were used was ~19 dpm/ng protein and ~94 dpm/ng CE, and that of [^{3}H]CE-hLDL particles was ~22 dpm/ng protein and ~19 dpm/ng CE. To obtain double-labeled particles, [^{3}H]CE-hHDL$_3$ was labeled with ^{125}I-dilactitol tyramine as previously described (23). Particles were dialyzed versus four changes of 150 mM NaCl, 10 mM potassium phosphate buffer, pH 7.4, 1 mM EDTA, and stored at 4°C under Argon. The average specific activity of the ^{125}I-dilactitol tyramine-[^{3}H]CE-hHDL$_3$ particles that were used was ~530 dpm/ng protein for ^{125}I and ~8 dpm/ng protein and ~49 dpm/ng CE for ^{3}H.

The [^{3}H]FC content of the [^{3}H]CE-hHDL$_3$ or [^{3}H]CE-hLDL was determined by lipid extraction (29), running the extracts on polysilica acid gel impregnated instant thin layer chomatography (ITLC-SA) plates (Gelman Sciences Inc., Ann Arbor, MI), and calculating the amount of [^{3}H]FC versus [^{3}H]CE (see below). [^{3}H]CE-hHDL$_3$ and [^{3}H]CE-hLDL were not used unless the [^{3}H]FC content was less than 2%.

CE hydrolysis assays

Transiently transfected COS-7 cells (in 22 or 35 mm wells) were preincubated for 4 h (unless otherwise indicated) at 37°C in the presence of an acyl CoA:cholesterol acyltransferase (ACAT) inhibitor, CP113,818 (gift from Pfizer, Groton, CT), at a final concentration of 2 μg/ml in serum free medium. The cells were washed twice and [^{3}H]CE-hHDL$_3$ particles were added at a concentration of 10 μg protein/ml in serum-free medium containing 2 μg/ml ACAT inhibitor. After incubation at 37°C for 4 h (unless otherwise indicated), the medium was removed and the monolayers were washed three times with PBS and allowed to dry. The lipids were extracted from the cell monolayers with isopropanol. One tenth of the extracted lipids was counted for total cell associated radioactivity. The remainder of the lipids was dried down under N_2 and resuspended in 100 μl chloroform-methanol (1:1, v/v) with FC and [^{3}H]CE as unlabeled standards. The samples were run on activated silica gel ITLC plates in a TLC tank with 95% petroleum ether-5% diethyl ether-1% acetic acid as the solvent system. The plates were air dried, and spots corresponding to CE and FC were visualized with iodine, cut from the strip, and analyzed by liquid scintillation counting to determine the fraction of [^{3}H]FC versus [^{3}H]CE. Statistical comparisons were made by Student's two-tailed *t*-test.

[^{3}H]diisopropylfluorophosphate labeling of cellular proteins

Y1-BS1 or Fu5AH cells were plated in 35 mm wells. The following day the Y1-BS1 cells were incubated in the presence or absence of ACTH (100 nM) overnight in growth medium. On day 3, the confluent cells were incubated in the presence or absence of 400 μM diethylumbelliferyl phosphate (UBP) or 10 μM RHC-80267 in serum-free medium for 30 min prior to the addition of 6 mM [^{3}H]diisopropylfluorophosphate (DFP) (Amersham Pharmacia Biotech) and then incubated for 1 h at 37°C. Protein lysates were made, as described above, and the proteins were separated on preformed (Biorad Laboratories, Inc., Hercules, CA) 4–15% gradient gels. The gels were impregnated with fluor using EN^{3}HANCE (NEN Life Science Products, Inc., Boston, MA), dried, and exposed to film for 7 days at −80°C.

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Membrane purification

Four of eight flasks of Y1-BS1 cells were treated with ACTH (100 nM) overnight in growth medium. The cell monolayers were washed with serum-free medium, placed on ice, and scraped into 10 ml 10 mM Tris, pH 7.5/2.0 mM $MgCl_2$. Cells were broken by N_2 cavitation after holding at 500 psi for 20 min, and unbroken cells removed by centrifugation for 10 min at 1,000 *g* at 4°C. The supernatant was centrifuged for 30 min at 100,000 *g* in a Ti75 rotor at 4°C to pellet the microsomal membrane fraction. Pellets were washed by suspension in 3 ml 10 mM Tris, pH 7.5/2.0 mM $MgCl_2$ and centrifuged again for 30 min at 100,000 *g* in a Ti75 rotor at 4°C. Microsomal membrane pellets were suspended in 10 mM Tris, pH 7.5/2.0 mM $MgCl_2$ and divided into three equal aliquots. One aliquot was heated for 20 min at 65°C, one was preincubated for 15 min with 1,000 μM UBP, and the last aliquot was used as the untreated control. The samples were incubated for 1.5 h at 37°C with [^{3}H]CE-hHDL$_3$ at a final concentration of 10 μg/ml. Samples were then washed twice at 100,000 *g* as described above to remove excess labeled HDL particles. Pellets were resuspended in 10 mM Tris pH 7.5-2.0 mM $MgCl_2$ and aliquots were removed for protein determination by Lowry assay and for lipid extraction. One tenth of the extracted lipids was counted for total radioactivity and the remainder was analyzed by ITLC to determine the fraction of [^{3}H]FC versus [^{3}H]CE.

RESULTS

SR-BI efficiently directs HDL-CE for hydrolysis but CD36 does not

Previous studies from several laboratories have indicated that SR-BI and CD36, a closely related class B scavenger receptor, bind HDL with high affinity (21, 23, 30–32). However, SR-BI mediates the SU of nonhydrolyzable HDL cholesteryl oleyl ether (HDL-COE) (33) or 1,1'-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyaine perchlorate (21) with a much higher efficiency than CD36, although most of the HDL-COE delivered by CD36 is also via the SU pathway as opposed to an endocytic pathway (disposition of HDL-COE with CD36: 91% SU vs. 0.1% endocytic uptake, with the remaining 8.9% COE associated with intact HDL particles within the cell or on the cell surface, unpublished data). This observation raised the question of whether there might be a difference in the hydrolysis of HDL-CE delivered to the cell via SR-BI versus CD36. In order to address this question, COS-7 cells were transiently transfected to express SR-BI or CD36 and then compared for their ability to hydrolyze HDL [^{3}H]CE. As previously demonstrated for HDL-COE, SR-BI delivered much more HDL-CE to COS-7 cells than CD36 (**Fig. 1A**). Notably, however, 75% of the HDL-CE delivered by SR-BI was hydrolyzed, whereas less than 20% of the HDL-CE delivered by CD36 was hydrolyzed (Fig. 1B). As seen in previous studies in transiently transfected COS-7 cells (23, 33), when double-labeled ^{125}I-dilactitol tyramine-[^{3}H]CE-hHDL$_3$ particles were used in these hydrolysis experiments, similar levels of SR-BI and CD36 were expressed on the cell surface as determined by the amount of HDL binding (data not shown). Despite similar amounts of cell surface HDL binding with these two receptors, HDL-CE is not efficiently transferred from the HDL particle to the cell membrane by the CD36 receptor, nor is it efficiently hydrolyzed. Therefore, we believe that HDL-CE delivered to COS-7 cells via SR-BI is efficiently directed toward hydrolysis but HDL-CE delivered by CD36 is not.

Previous experiments revealed that high-efficiency SU of HDL-COE requires the extracellular domain (ECD) of SR-BI but not the transmembrane domains or the cytoplasmic C-terminal tail (23, 32). This was also the case for a spectrum of other SR-BI-stimulated activities, including bidirectional FC flux, membrane cholesterol accumulation, and cholesterol oxidase sensitivity of plasma membrane FC (33). The transmembrane and cytoplasmic domains of CD36 may be substituted for those in SR-BI with no loss of these activities, whereas the converse substitutions did not confer these activities on the ECD of CD36. In order to address whether SR-BI's ECD also directs the metabolism of the HDL-CE, hydrolysis experiments were performed using SR-BI-CD36 chimeric receptors (Fig. 1C) (33). As with the other SR-BI-mediated activities, the data showed that the ECD, but not its transmembrane domains or the cytoplasmic tails, is responsible for high-efficiency HDL-CE hydrolysis (Fig. 1D). With chimeric receptors containing the ECD of SR-BI (SR-BI, SR-CDT, CD-SR-CD, and SR-BI-D1), 80% of the delivered HDL-CE was hydrolyzed, whereas with chimeric receptors containing CD36s ECD (CD36, CD-SRT, SR-CD-SR) only 30% of the delivered HDL-CE was hydrolyzed. Substitution of the SR-BI C-terminal tail for that of CD36 (SR-CDT) gave a small increase in HDL-CE hydrolysis by CD36, but deletion of the C-terminal tail from SR-BI (SR-BI-D1) had no effect (Fig. 1D). Additionally, as compared with SR-BI, no reduction of hydrolysis activity was observed with chimeric receptors containing the ECD of SR-BI when cell incubations were carried out at 22°C, at which only 30% of the SR-BI-delivered HDL-CE was hydrolyzed (data not shown). Thus, as previously established with other SR-BI-dependent activities, efficient HDL-CE hydrolysis requires the ECD, but not the other domains of SR-BI.

SR-BI directs HDL-CE more efficiently for hydrolysis than LDL-CE

Previous work indicated that SR-BI was able to mediate selective COE uptake from LDL (34) and stimulate the esterification of cellular cholesterol in response to LDL (12). Also, the fractional transfer of lipoprotein COE by SR-BI was found to be ∼7-fold greater from the core of an HDL than from an LDL particle (34). Similar to HDL-COE, most of the LDL-COE delivered to the cell by SR-BI or CD36 was delivered in the SU pathway (SR-BI: 81% SU vs. 1.0% endocytic uptake vs. 18% associated with intact LDL; CD36: 83% SU vs. 2.0% endocytic uptake vs. 15% associated with intact LDL; data not shown). These data raised the question: Is LDL-CE hydrolyzed as efficiently as HDL-CE? To address this question, we measured the hydrolysis of LDL [^{3}H]CE delivered to COS-7 cells by SR-BI versus CD36. Note that the contribution of the LDL receptor in these experiments was minimized by growing the cells in serum-containing medium to down-regulate

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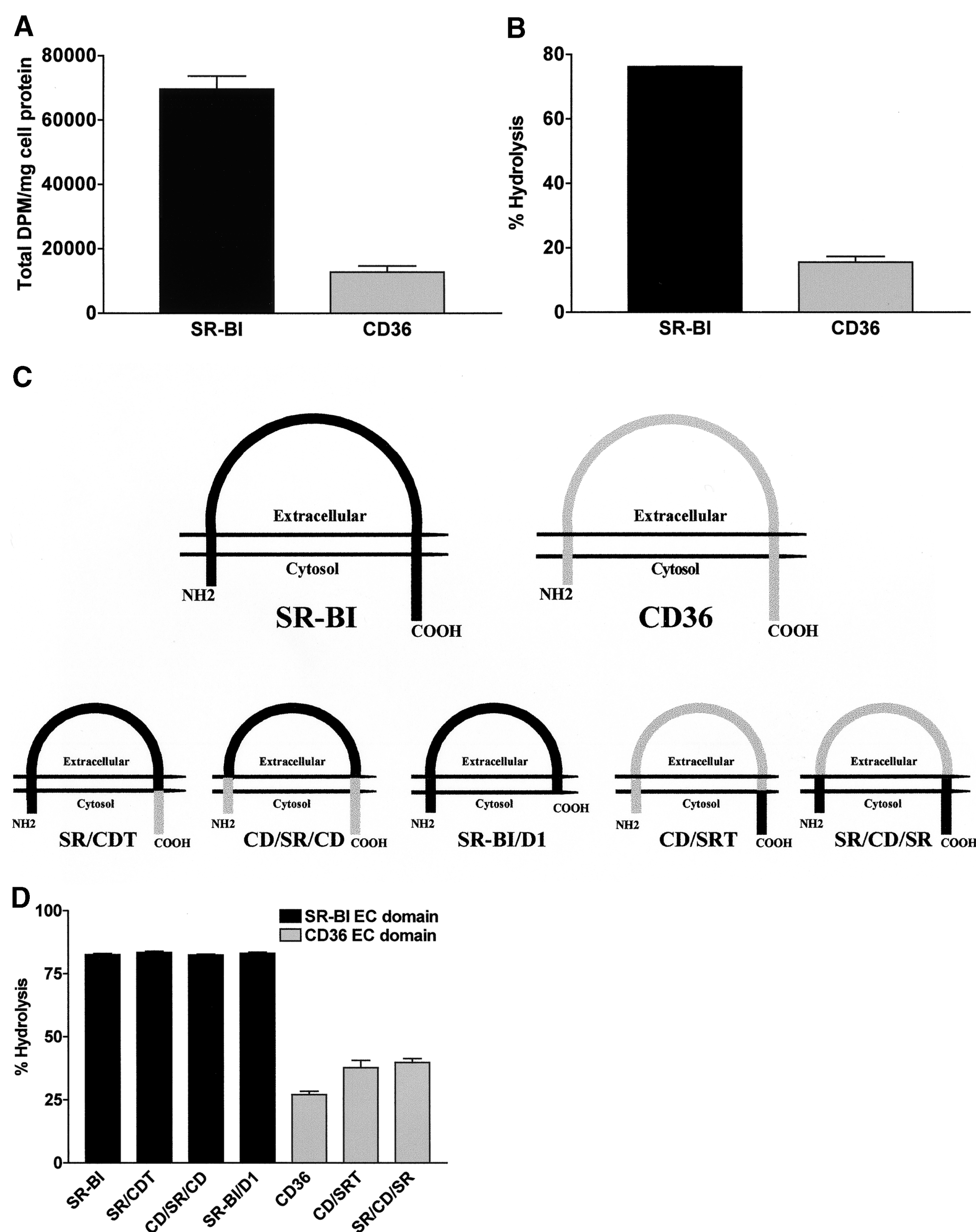

Fig. 1. Uptake and hydrolysis of HDL-cholesteryl ester (CE) by scavenger receptor class B type I (SR-BI) and CD36. COS-7 cells transfected with vector, pSG5, or expressing CD36 or SR-BI were incubated at 37°C for 4 h with 10 μg/ml [^{3}H]cholesteryl oleate ([^{3}H]CE)-hHDL$_3$ in the presence of an acyl CoA: cholesterol acyltransferase (ACAT) inhibitor at a final concentration of 2 μg/ml. After incubation, the cells were processed to determine the total cell associated DPM/mg cell protein (A) and the percentage of the total number of counts that were [^{3}H]free cholesterol (FC) (B). COS-7 cells transfected with vector, pSG5, or transiently expressing SR-BI, SR-CDT, CD-SR-CD, SR-BI-D1, CD36, CD-SRT, or SR-CD-SR (C) (23, 31) were incubated at 37°C for 4 h with 10 μg/ml [^{3}H]CE-hHDL$_3$, in the presence of an ACAT inhibitor at a final concentration of 2 μg/ml. After incubation, the cells were processed to determine the percentage of the total number of counts that were [^{3}H]FC (D). The amino acid delineations of the chimeras are as follows: SR-CDT = mSR-BI a.a. 1–467 and rCD36 a.a. 459-472; CD-SR-CD = rCD36 a.a. 1–40 and 441–472 and mSR-BI a.a. 43–440; SR-BI-D1 = mSR-BI a.a. 1–467; CD-SRT = rCD36 a.a. 1–458 and mSR-BI a.a. 464–509; and SR-CD-SR = rCD36 a.a. 44–435 and mSR-BI a.a. 1–41 and 436–509. Values represent the mean ± SD of triplicate determinations after subtraction of values obtained with vector transfected cells. This graph is representative of five experiments. Note: The amount of HDL-CE uptake per experiment varied up to 2-fold among multiple experiments due to differences in receptor expression levels; however, the percentage of hydrolysis varied usually less than 5% from one experiment to another.

the LDL receptor and by subtracting the background uptake and hydrolysis with the vector transfected cells. The results revealed that, in contrast to HDL-CE, LDL-CE was delivered to the cell (**Fig. 2A**) and hydrolyzed (Fig. 2B) approximately as well by both SR-BI and CD36 receptors. Furthermore, the extent of LDL-CE hydrolysis was about half that of HDL-CE delivered by SR-BI. Similar amounts of CE by mass were delivered by SR-BI to the cells from HDL versus LDL (note: in Figs. 1, 2 the specific activity of HDL-CE was ~5 times that of LDL-CE, see Materials and

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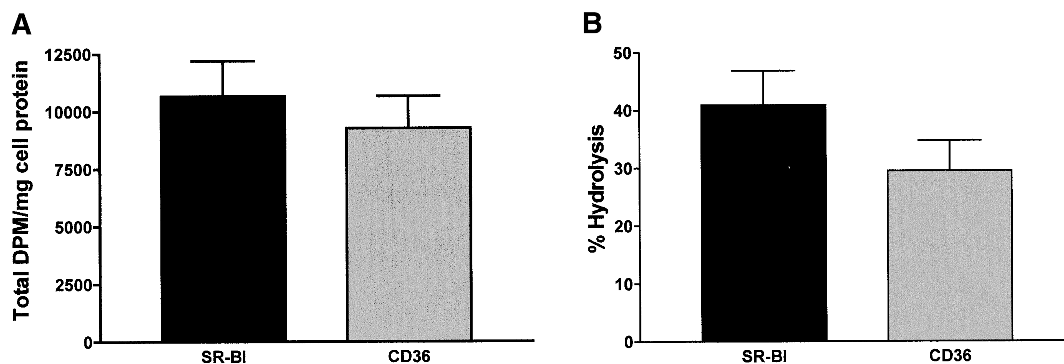

Fig. 2. Uptake and hydrolysis of LDL-CE by SR-BI and CD36 COS-7 cells transfected with vector, pSG5, or expressing CD36 or SR-BI were incubated at 37°C for 4 h with 10 μg/ml [^{3}H]CE-hLDL in the presence of an ACAT inhibitor at a final concentration of 2 μg/ml. After incubation, the cells were processed to determine the total cell associated DPM/mg cell protein (A) and the percentage of the total number of counts that were [^{3}H]FC (B). Values represent the mean ± SD of triplicate determinations after subtraction of values obtained with vector alone cells. These graphs are representative of three experiments. Note: The amount of HDL-CE uptake per experiment varied up to 2-fold among multiple experiments due to differences in receptor expression levels; however, the percentage hydrolysis varied usually less than 5% from one experiment to another.

Methods). Therefore, the difference in the degree of hydrolysis was not due to saturation of the hydrolase by an excess of CE delivery from the LDL core. In other words, even when SR-BI delivers approximately the same amount of CE from HDL as LDL, the % CE hydrolysis is higher for HDL-CE than for LDL-CE. Hence, SR-BI directs HDL-CE to hydrolysis more efficiently than LDL-CE.

HDL-CE and LDL-CE delivered by SR-BI are hydrolyzed by a NCEH

LDL-CE internalized via the LDL receptor pathway is hydrolyzed by a lysosomal ACEH (15–17). In contrast, several studies have suggested that HDL-CE internalized via SU is hydrolyzed extralysosomally (18) by an NCEH (19, 20). In order to address the question of whether SR-BI directs lipoprotein CE to an ACEH or an NCEH for hydrolysis, we performed HDL-CE and LDL-CE hydrolysis assays in the presence or absence of either chloroquine or UBP. Chloroquine inhibits ACEH localized to lysosomes or other low pH compartments, whereas UBP is an inhibitor of NCEH activity in hepatocytes, MA-10 Leydig cells, and macrophages (19, 35–37). As previously observed, with CD36 only 15% of the delivered HDL-CE was hydrolyzed in a 4 h period compared with 75% with SR-BI in the COS-7 cells (**Fig. 3A**). Furthermore, the HDL-CE that was hydrolyzed after delivery by CD36 was poorly inhibited by UBP or chloroquine. With SR-BI-delivered HDL-CE, where hydrolysis was high, there was no inhibition by chloroquine and only 13% inhibition of hydrolysis by UBP (Fig. 3A). In multiple experiments, the inhibition by UBP of hydrolysis of HDL-CE delivered by SR-BI was 21% (**Table 1**). These data indicate that most of the HDL-CE loaded into the plasma membrane by SR-BI is not acted on by an ACEH. The partial sensitivity to UBP for SR-BI delivered HDL-CE hydrolysis suggests that a fraction of the CE hydrolysis in COS-7 cells is due to a NCEH activity, although this activity in COS-7 cells appears significantly less sensitive to inhibition compared with hepatocytes (19).

In order to address the question of whether SR-BI delivered LDL-CE is hydrolyzed in the lysosomal pathway, we performed LDL-CE hydrolysis assays in the presence or absence of either chloroquine or UBP. In contrast to HDL-CE, we observed significant inhibition of CE hydrolysis by chloroquine but no inhibition by UBP when LDL-CE was delivered to COS-7 cells by CD36 (Fig. 3B). These data suggest that most of the CD36-delivered LDL-CE is hydrolyzed by an ACEH. Thus, the chloroquine sensitivity of LDL-CE hydrolysis with CD36 suggests different pathways for LDL-CE (chloroquine sensitive) and HDL-CE (chloroquine insensitive) hydrolysis with this receptor. In contrast, when LDL-CE was delivered by SR-BI, we observed partial inhibition of LDL-CE hydrolysis by UBP (~30%) and no inhibition by chloroquine (Fig. 3B). These data suggest that neither LDL-CE nor HDL-CE delivered via SR-BI is hydrolyzed by an ACEH. As noted with HDL-CE, the partial sensitivity to UBP suggests that a fraction of the LDL-CE hydrolysis occurs via an NCEH, although this inhibitor does not efficiently block CE hydrolysis in COS-7 cells.

SR-BI-delivered HDL-CE is hydrolyzed by cell type-specific NCEHs

The fact that the NCEH activity we observed in the COS-7 cells was not inhibited efficiently by UBP suggested to us that different NCEHs may be responsible for HDL-CE hydrolysis in different cell types. Since SR-BI is not normally expressed in COS-7 cells, we asked whether CE hydrolysis is similar in cell types where SR-BI is expressed endogenously. To this end, several cell types expressing SR-BI, either naturally (Fu5AH and Y1-BS1) or after transient (COS-7) or stable transfection (WI38), were incubated in the presence or absence of inhibitors. As shown in Table 1, with ACTH-treated Y1-BS1 adrenal cells, in

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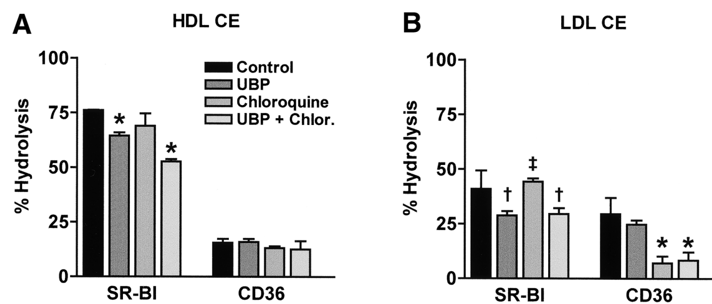

Fig. 3. Hydrolysis of HDL-CE and LDL-CE and by SR-BI and CD36 in the presence of inhibitors of CE hydrolysis COS-7 cells transfected with vector, pSG5, or expressing CD36 or SR-BI were preincubated in the presence or absence of 400 μM diethylumbelliferyl phosphate (UBP) or 50 μM chloroquine and an ACAT inhibitor at a final concentration of 2 μg/ml for 4 h at 37°C then incubated for 4 h at 37°C with 10 μg/ml [^{3}H]CE-hHDL$_3$ (A) or 10 μg/ml [^{3}H]CE-hLDL (B) in the presence or absence of UBP or chloroquine and the ACAT inhibitor. After incubation, cells were processed to determine the percentage of the total number of cell-associated counts that were [^{3}H]FC. Values represent the mean ± SD of triplicate determinations after subtraction of values obtained with vector alone cells. These graphs are representative of five experiments. Note: The amount of HDL-CE and LDL-CE uptake per experiment varied up to 2-fold among multiple experiments due to differences in receptor expression levels; however, the percentage of hydrolysis varied usually less than 5% from one experiment to another. Values designated asterisk differ from respective control value, $P < 0.001$. Values designated by a dagger differ from that designated by a double dagger, $P < 0.005$.

which HDL-CE uptake is almost entirely due to SR-BI (25), UBP inhibited HDL-CE hydrolysis by 51% but chloroquine had no effect. The lack of inhibition by chloroquine is in agreement with early studies by the laboratories of Pittman and Gwynne (18, 38). The high percent inhibition by UBP confirms the role of a NCEH in the hydrolysis of HDL-CE in Y1-BS1 cells. Fu5AH cells, which also naturally express SR-BI, have an intermediate level of UBP sensitivity and a low level of chloroquine sensitivity, whereas SR-BI-expressing COS-7 and WI-38 cells exhibit low UBP and chloroquine sensitivities. The differential inhibitor sensitivities suggest that SR-BI-directed HDL-CE hydrolysis occurs via different NCEHs in various cell types. Note also that HDL-CE hydrolysis in differentiated THP-1 macrophages was substantially inhibited by both UBP and chloroquine. This is of interest because previous studies showed that macrophages process HDL by both SR-BI-mediated SU and by endocytic uptake (39, 40), and possibly other pathways.

To further address the question of cell type-specific NCEHs, ACTH-stimulated Y1-BS1 and Fu5AH cells were incubated with a panel of NCEH inhibitors and assayed for HDL-CE hydrolysis as described above. The following were used: *1*) RHC-80267, which selectively inhibits hormone-sensitive lipase hydrolysis of diacylglycerides and triglycerides in adipocytes (41); *2*) masoprocol, a lipoxygenase inhibitor that inhibits the phosphorylation of hormone sensitive lipase, thereby inhibiting its activation (42); *3*) papaverine HCl (Pap. HCl), a phosphodiesterase inhibitor that blocks the cellular redistribution of activated hormone sensitive lipase (43); *4*) DFP and phenylmethylsulfonyl flouride (PMSF), which are active site-specific inhibitors of serine hydrolases; and *5*) deoxyglucose (DG) and NaN_3, which inhibit the translocation of hormone sensitive lipase to lipid droplets in 3T3-L1 adipocytes by abrogating cellular energy (44). The two cell types exhibited different sensitivities to each of the inhibitors implicating cell type-specific NCEHs in HDL-CE hydrolysis (**Table 2**).

In order to address the concern that the differences in inhibitor sensitivities between the Y1-BS1 and Fu5AH cells could be due to differences in cell permeability, which could cause differential inhibitor accessibility to cellular proteins, we incubated ACTH-unstimulated and -stimulated Y1-BS1 cells and Fu5AH cells with [^{3}H]DFP to label the serine hydrolases. [^{3}H]DFP labeled several proteins in

TABLE 1. Uptake and hydrolysis of HDL-CE in several cell types in the presence of inhibitors of CE hydrolysis

Cell	NG HDL-CE Influx/2H/ MG Protein	% CE Hydrolyzed	% Inhibition of Hydrolysis UBP (400 μM)	Chloroquine (50 μM)
COS	27 ± 4 (18)	37 ± 4	11 ± 4	5 ± 2
COS + SR-BI	647 ± 67 (18)	73 ± 1	21 ± 3[a]	6 ± 2
COS + CD36	124 ± 18 (18)	13 ± 1	14 ± 3	19 ± 4
WI38 – SR-BI	13 ± 1 (3)	63 ± 1	6 ± 1	19 ± 1
WI38 + SR-BI	333 ± 10 (3)	83 ± 1	16 ± 1[a]	5 ± 1
Fu5AH	993 ± 51 (12)	61 ± 1	37 ± 2[a]	5 ± 2
Y1-BS1	101 ± 13 (12)	43 ± 1	35 ± 6[a,b]	5 ± 3
Y1-BS1 + ACTH	143 ± 15 (12)	53 ± 1	51 ± 5[a,b]	0 ± 0
THP-1 diff w/ PMA	55 ± 7 (9)	47 ± 2	30 ± 7	38 ± 9

ACTH, adrenocorticotropic hormone; CE, cholesteryl ester; PMA, phorbol 12-myristate 13-acetate; SR-BI, scavenger receptor class B type I; UBP, diethylumbelliferyl phosphate. Cells were preincubated in the presence or absence of 400 μM UBP or 50 μM chloroquine and an ACAT inhibitor at a final concentration of 2 μg/ml for 2 h at 37°C then incubated for 2 h at 37°C with 10 μg/ml [^{3}H-CE]hHDL$_3$ in the presence or absence of UBP or chloroquine and the ACAT inhibitor. After incubation, the cells were processed to determine the percentage of the total number of counts that were [^{3}H]FC. Values are the mean ± SD of the number of replicates indicated in the parenthesis.

[a] Differ from no inhibitor, $P < 0.0001$.
[b] Differ from each other, $P < 0.0001$.

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TABLE 2. Percent inhibition of HDL-CE hydrolysis in Y1-BS1 and Fu5AH cells

Cell	UBP 400 μM	Chloroquine 50 μM	RHC-80267 10 μM	Masoprocol 50 μM	Pap HCl 100 μM	DFP 10 μM	PMSF 100 μM	DG/NaN_3 40 mM/0.02%
Y1-BS1	51 ± 5	0 ± 0	42 ± 7	50 ± 14	65 ± 10	47 ± 9	36 ± 14	30 ± 7
Fu5AH	37 ± 2	5 ± 2	12 ± 5	82 ± 2	28 ± 3	4 ± 6	9 ± 6	0 ± 0

Cells were preincubated in the presence or absence of diethylumbelliferyl phosphate (UBP), chloroquine, RHC-80267, Masoprocol, Papaverine HCl (Pap.HCl), diisopropyl fluorophosphate (DFP), phenylmethylsulfonylfluoride (PMSF), or deoxyglucose-sodium azide (DG-NaN_3), and an ACAT inhibitor (2 μg/ml) for 2 h at 37°C then incubated for 2 h at 37°C with 10 μg/ml [^{3}H-CE]hHDL$_3$ in the presence or absence of inhibitors. After incubation, the cells were processed to determine the percentage of the total number of counts that were [^{3}H]FC. Values are the mean ± SD of six to nine replicates each.

each cell line and, as expected, a different pattern of labeled proteins between the two cell types was observed (**Fig. 4**). Labeling of most (UBP) or some (RHC-80267) of the proteins in both cell types was inhibited by these agents (Fig. 4). PMSF also inhibited protein labeling by [^{3}H]DFP (data not shown). These data support the idea that DFP, UBP, and RHC-80267 are fully accessible to the cellular hydrolases in the intact Y1-BS1 and Fu5AH cells. The high lipid solubility of these agents is also supportive of this point. Nevertheless, we cannot completely rule out the possibility that cell type differences in inhibitor access to NCEHs could contribute to the quantitative differences among cell types. The same approach could not be used with papaverine and masoprocol since these inhibitors act indirectly to inhibit lipase activity.

Hydrolysis of HDL-CE occurs in a crude microsomal membrane fraction

Preliminary data suggested that SR-BI-mediated HDL-CE SU and subsequent CE hydrolysis are coupled and consequently may be occurring in a membrane versus a cytoplasmic compartment. Time courses at 37°C in COS-7 cells expressing SR-BI revealed that HDL-CE SU and hydrolysis occurred concomitantly (data not shown). In an attempt to dissociate HDL-CE uptake and hydrolysis and to address the compartmentalization of HDL-CE hy-

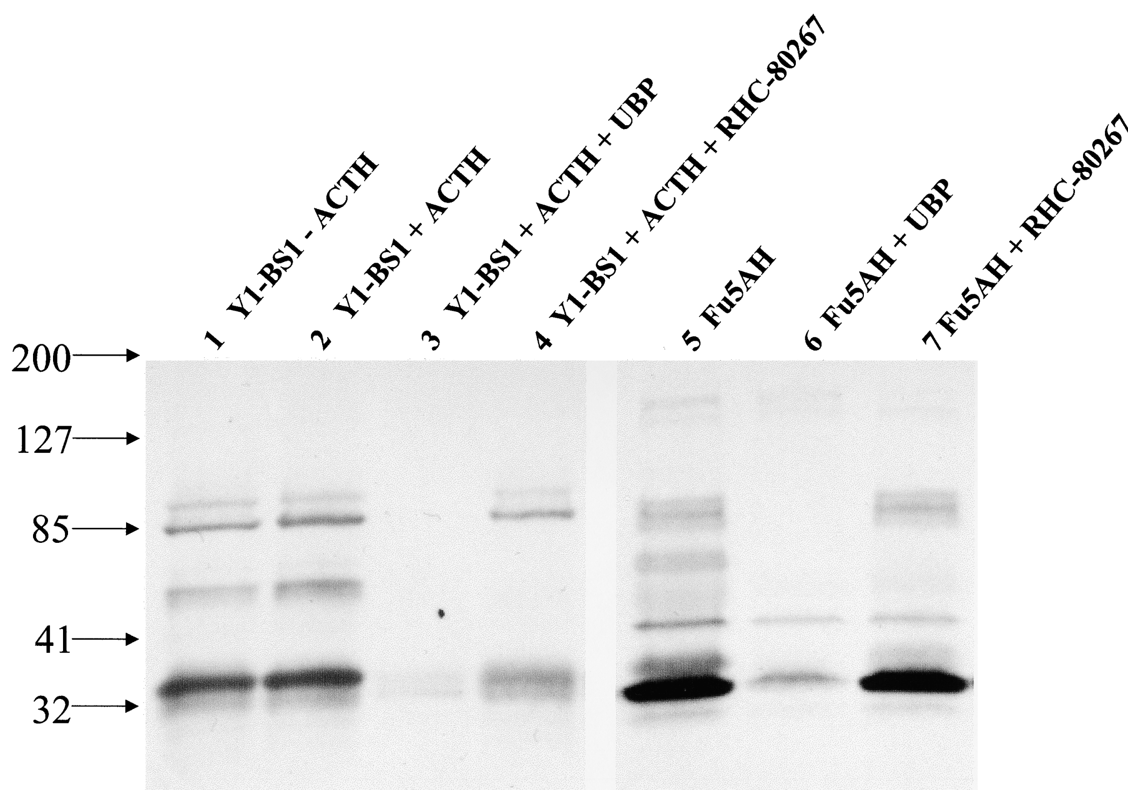

Fig. 4. [^{3}H]diisopropylfluorophosphate (DFP) labeling of serine hydrolases. Lane 1: Y1-BS1-adrenocorticotropic hormone (ACTH); Lane 2: Y1-BS1+ACTH; Lane 3: Y1-BS1+ACTH+UBP; Lane 4: Y1-BS1+ACTH+RHC-80267; Lane 5: Fu5AH; Lane 6: Fu5AH+UBP; Lane 7: Fu5AH+RHC-80267. Y1-BS1 ± ACTH or Fu5AH were incubated in the presence or absence of 400 μM UBP or 10 μM RHC-80267 in serum-free medium for 30 min prior to the addition of 6 mM [^{3}H]DFP and then incubated for 1 h at 37°C. Protein lysates were made as described above, and the proteins were separated on 4–15% gradient gels. The gels were impregnated with fluor using EN^{3}HANCE, dried and exposed to film for 7 days at −80°C. The experiment was repeated three times with the same results.

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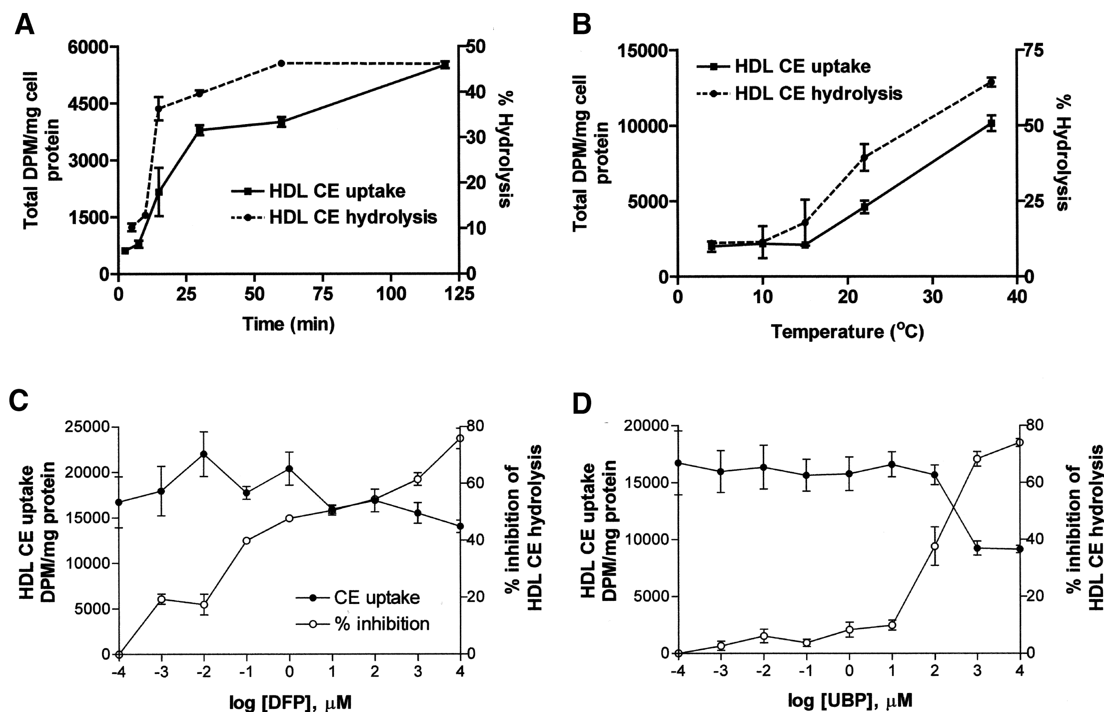

Fig. 5. Coincidence of HDL-CE uptake and hydrolysis and inhibition of HDL-CE uptake and hydrolysis in ACTH treated Y1-BS1 cells ACTH treated Y1-BS1 cells were incubated for 1.5 h at 22°C for various times (A) or at various temperatures (B) with 10 μg/ml [^{3}H]CE-hHDL$_3$ in the presence of an ACAT inhibitor at a final concentration of 2 μg/ml. After incubation, the cells were processed to determine the total cell associated DPM/mg cell protein (solid line) and the percentage of the total number of counts that were [^{3}H]FC (broken line). ACTH treated Y1-BS1 cells were incubated for 1.5 h at 37°C with 10 μg/ml [^{3}H]CE-hHDL$_3$ in the presence or absence of various concentrations of DFP (C) or UBP (D) in the presence of an ACAT inhibitor at a final concentration of 2 μg/ml. After incubation, the cells were processed to determine the total DPM/mg cell protein (closed circle) and the percent inhibition of HDL-CE hydrolysis (open circle). Values represent the mean ± SD of triplicate determinations.

drolysis in a cell type that endogenously expresses SR-BI, hydrolysis assays were carried out with Y1-BS1 cells at various temperatures using a 1.5 h incubation period, and for various times at 22°C. The results show that HDL-CE uptake and CE hydrolysis are temporally coupled (**Fig. 5A**) and coincident over a wide temperature range (Fig. 5B). HDL-CE hydrolysis appears to occur rapidly following CE uptake. Note, however, that inhibitors of HDL-CE hydrolysis appear relatively ineffective in blocking HDL-CE SU. For example, the data show substantial inhibition of HDL-CE hydrolysis by DFP (Fig. 5C) over a broad concentration range with little inhibition of HDL-CE uptake. With UBP (Fig. 5D), substantial inhibition of CE hydrolysis occurs with no effect on HDL-CE uptake up to 100 μM and partial inhibition of uptake above that. These data indicate that while HDL-CE uptake and hydrolysis are closely coupled, CE hydrolysis can be substantially inhibited with no effect on HDL-CE uptake. This led us to conclude that HDL-CE SU is not initially dependent on its subsequent hydrolysis. In addition, HDL-CE hydrolysis might be occurring in a membrane compartment since its hydrolysis is so closely coupled to its uptake.

To address whether SR-BI-directed HDL-CE hydrolytic activity was membrane associated, a crude microsomal membrane fraction prepared from ACTH-treated or -untreated Y1-BS1 cells was incubated with [^{3}H]CE HDL for 1.5 h at 37°C followed by washing of the membrane fraction. As shown in **Fig. 6**, the microsomal membrane fraction contained an HDL-CE hydrolytic activity that was sensitive both to heat and inhibition by UBP. The HDL-CE hydrolytic activity in the ACTH-treated Y1-BS1 microsomal membranes showed a modestly but consistently higher UBP inhibition than membranes from Y1-BS1 cells that were not treated with ACTH (62.5 ± 2.8% vs. 50.8 ± 0.4%, $P < 0.006$). A similar modest difference was seen with intact Y1-BS1 cells treated or not with ACTH (51 ± 5% vs. 35 ± 6%, $P < 0.05$) (Table 1). We were unable to reliably estimate [^{3}H]CE HDL hydrolytic activity in the supernatant fractions of these preparations due to the background radioactivity from the labeled HDL.

Interestingly, when [^{3}H]CE was introduced directly into the Y1-BS1 membrane sample via a small volume of dimethylsulfoxide, significant CE hydrolysis also occurred (35%), but the hydrolysis was not sensitive to inhibition by UBP (data not shown). This result suggests the presence of multiple NCEH enzymes in the microsomal membrane preparation. As judged by UBP inhibition, NCEHs, responsible for HDL-CE hydrolysis in Y1-BS1 membranes, were not active in the hydrolysis of [^{3}H]CE delivered without the aid of the HDL particle. Therefore, SR-BI-directed HDL-CE hydrolysis occurs in a microsomal membrane

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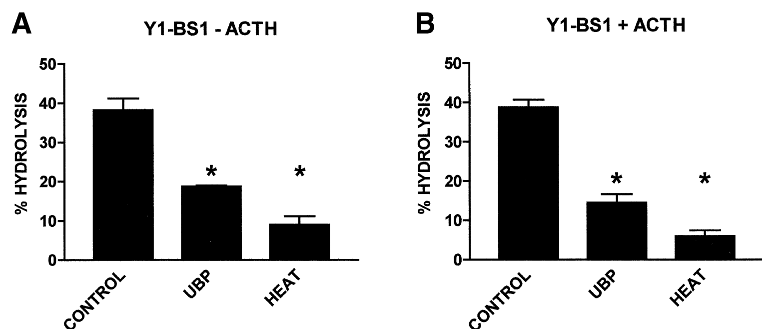

Fig. 6. Hydrolysis of HDL-CE in a membrane fraction of Y1-BS1 cells. Crude microsomal membrane fractions prepared from Y1-BS1 cells with or without ACTH treatment for 24 h were isolated by centrifugation at 100,000 *g*. The membranes were incubated with 10 μg/ml [^{3}H]CE-hHDL$_3$ for 1.5 h at 37°C in the presence or absence of UBP or heating to 65°C and reisolated at 100,000 *g*. A Bligh Dyer lipid extraction was performed on the membranes, after which the lipids were processed to determine the percentage of the total number of counts that were [^{3}H]FC. Values represent the mean ± SD of four experiments. * Differs from respective control, $P < 0.001$.

fraction by an NCEH that is sensitive to the means of substrate presentation.

DISCUSSION

Although numerous studies have been conducted on the mechanism of SR-BI-mediated lipoprotein COE SU, little is known about the fate of lipoprotein CE once incorporated into the cell. The aim of this study was to investigate the fate of lipoprotein CE when presented to the cell by either SR-BI or CD36 scavenger receptors. As previously demonstrated for HDL-COE, SR-BI delivered more HDL-CE to COS-7 cells compared with CD36. In addition, the HDL-CE delivered to the cells by SR-BI was more efficiently hydrolyzed than the HDL-CE delivered by CD36. These data suggest that SR-BI not only delivers HDL-CE to the plasma membrane, but that it somehow directs the CE in such a way that it is efficiently hydrolyzed, a property not shared by CD36. These results complement previous data showing that delivery of HDL FC by SR-BI results in a 3–5-fold greater fractional esterification of newly influxed FC compared with CD36 (33,45). Furthermore, it has been shown that although CD36 can bind HDL with high affinity, it does not play a direct role in hepatic HDL metabolism, as judged by short-term expression studies using adenovirus vectors (46). Together these data support the idea that, unlike CD36, SR-BI delivers HDL-FC and CE into a metabolically active membrane pool. Whether this metabolically active pool reflects the localization of SR-BI in a physically distinct membrane domain compared with CD36 is unclear.

The data presented here indicate that 70–80% of the HDL-CE that is delivered to the cell by SR-BI is rapidly hydrolyzed. Interestingly, we have not seen a greater extent of hydrolysis of HDL-CE even with incubations up to 4 h. This may indicate that a fraction of the HDL-CE delivered to the cell by SU may be transferred to other cellular sites without hydrolysis. This possibility is supported by a recent study of SR-BI-expressing CHO cells that showed trafficking of CE to internal membranes in a complex with annexin II, cyclophilin 40, caveolin, and cyclophilin A (47). These findings suggest that there are two pathways for trafficking of SR-BI-delivered HDL-CE: one involving rapid hydrolysis and another involving transport to internal membranes prior to further metabolism.

Our results with the chimeric receptors revealed that, like its other lipid transport functions (23, 32, 33), SR-BI's ECD, but not its transmembrane domains or cytoplasmic tails, is responsible for efficient HDL-CE hydrolysis. It is unlikely that SR-BI itself has hydrolytic activity since UBP inhibits CE hydrolysis in a cell-specific manner. In addition, analysis of the SR-BI amino acid sequence failed to identify the core sequence motif that is common to all lipid hydrolases. More likely, SR-BI delivers the CE into the membrane or alters membrane organization in such a way as to facilitate CE presentation to the hydrolase. Alternatively, SR-BI may recruit an NCEH to the membrane domain into which CE is delivered. Although the mechanism of the efficient HDL-CE hydrolysis with SR-BI is unclear, it is likely that the rapid CE hydrolysis facilitates FC trafficking and utilization within the cell.

In contrast to HDL-CE, LDL-CE was delivered to the cell and hydrolyzed approximately as well by both SR-BI and CD36 receptors. Furthermore, the extent of LDL-CE hydrolysis was about half that of HDL-CE delivered by SR-BI. In this case, similar amounts of CE mass were delivered to the cells by HDL versus LDL. Therefore, the difference in the degree of hydrolysis was not due to saturation of hydrolytic activity. In other words, even when SR-BI delivers approximately the same amount of CE from HDL as LDL, the percent of CE hydrolysis is higher for HDL-CE than LDL-CE. Hence, SR-BI directs HDL-CE more efficiently than LDL-CE for hydrolysis. It has been hypothesized that there are different binding sites on SR-BI for HDL versus LDL (48). LDL, therefore, may bind to an SR-BI site that delivers the lipoprotein CE less efficiently and does not direct the CE efficiently for further metabolism. However, HDL may bind to a site on SR-BI that delivers the CE much more efficiently and allows it to be directed for metabolism.

Hydrolysis of HDL-CE delivered by SR-BI was not inhibited by chloroquine in COS-7, WI38, Fu5AH, or Y1-BS1 cells, indicating that hydrolysis is not via an ACEH. The lack of inhibition by chloroquine was previously noted in rat adrenal cells (18, 38) and appears to be a general feature of the SR-BI-mediated delivery process in other cell types as well. In contrast, UBP inhibited hydrolysis of SR-BI-delivered HDL-CE in all cell types tested, although with variable efficiencies (Table 1). Up to 75% inhibition by UBP was seen in Y1-BS1 cells (Fig. 5D). Recent studies with the hormone-sensitive lipase (HSL)-deficient mouse

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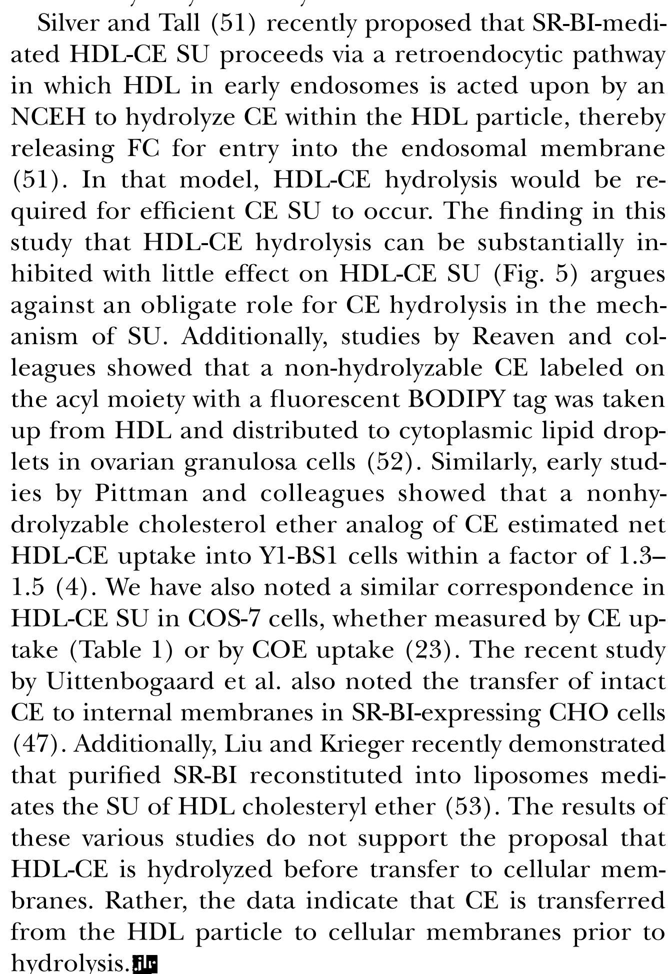
suggest that HSL is the major CE hydrolase in adrenal cells (49, 50). Whether HSL is also responsible for hydrolysis of HDL-CE in Y1-BS1 cells remains to be tested, but the profile of inhibitor sensitivities in Table 2 is consistent with this possibility.

In contrast to the situation with SR-BI, with CD36-expressing COS-7 cells most hydrolysis of LDL-CE was inhibited by chloroquine, whereas most HDL-CE hydrolysis was not. These data suggest different pathways for LDL-CE (chloroquine-sensitive) and HDL-CE (chloroquine-insensitive) hydrolysis with CD36. This difference in sensitivity to chloroquine may reflect a difference in the cellular itinerary of CD36 when liganded by HDL versus LDL, or could reflect a difference in the trafficking of HDL-CE and LDL-CE at postreceptor steps.

The present results suggest that HDL-CE hydrolysis in SR-BI-expressing cells may occur concurrently with SU. HDL-CE uptake and CE hydrolysis are temporally coupled and coincident over a wide temperature range. Additionally, HDL-CE hydrolytic activity was isolated in a microsomal membrane fraction of Y1-BS1 cells. However, these data do not distinguish between the possibilities that HDL-CE hydrolysis may occur at or near the plasma membrane in an internal membrane fraction and/or during microvesicular trafficking of CE to sites of FC-CE metabolism. Additional studies will be required to determine which cellular membranes possess the majority of the HDL-CE hydrolytic activity.



Silver and Tall (51) recently proposed that SR-BI-mediated HDL-CE SU proceeds via a retroendocytic pathway in which HDL in early endosomes is acted upon by an NCEH to hydrolyze CE within the HDL particle, thereby releasing FC for entry into the endosomal membrane (51). In that model, HDL-CE hydrolysis would be required for efficient CE SU to occur. The finding in this study that HDL-CE hydrolysis can be substantially inhibited with little effect on HDL-CE SU (Fig. 5) argues against an obligate role for CE hydrolysis in the mechanism of SU. Additionally, studies by Reaven and colleagues showed that a non-hydrolyzable CE labeled on the acyl moiety with a fluorescent BODIPY tag was taken up from HDL and distributed to cytoplasmic lipid droplets in ovarian granulosa cells (52). Similarly, early studies by Pittman and colleagues showed that a nonhydrolyzable cholesterol ether analog of CE estimated net HDL-CE uptake into Y1-BS1 cells within a factor of 1.3–1.5 (4). We have also noted a similar correspondence in HDL-CE SU in COS-7 cells, whether measured by CE uptake (Table 1) or by COE uptake (23). The recent study by Uittenbogaard et al. also noted the transfer of intact CE to internal membranes in SR-BI-expressing CHO cells (47). Additionally, Liu and Krieger recently demonstrated that purified SR-BI reconstituted into liposomes mediates the SU of HDL cholesteryl ether (53). The results of these various studies do not support the proposal that HDL-CE is hydrolyzed before transfer to cellular membranes. Rather, the data indicate that CE is transferred from the HDL particle to cellular membranes prior to hydrolysis.

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