Published in final edited form as:
Biochemistry. 2011 March 22; 50(11): 1818–1830. doi:10.1021/bi101657j.

Negatively Cooperative Binding of High Density Lipoprotein to the HDL Receptor SR-BI[†]

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Abstract

Scavenger receptor class B, type I (SR-BI) is a high-density lipoprotein (HDL) receptor, which also binds low density lipoprotein (LDL), and mediates the cellular selective uptake of cholesteryl esters from lipoproteins. SR-BI also is a co-receptor for hepatitis C virus and a signaling receptor that regulates cell metabolism. Many investigators have reported that lipoproteins bind to SR-BI via a single class of independent (not interacting), high affinity binding sites (one site model). We have re-investigated the ligand concentration dependence of ^{125}I-HDL binding to SR-BI and SR-BI-mediated specific uptake of [^{3}H]CE from [^{3}H]CE-HDL using an expanded range of ligand concentrations (<1 μg protein/ml, lower than previously reported). Scatchard and non-linear least squares model fitting analyses of the binding and uptake data were both inconsistent with a single class of independent binding sites binding univalent lipoprotein ligands. The data are best fit by models in which SR-BI has either two independent classes of binding sites, or one class of sites exhibiting negative cooperativity due to either classic allostery or ensemble effects (‘ lattice model’). Similar results were observed for LDL. Application of the ‘infinite dilution’ dissociation rate method established that the binding of ^{125}I-HDL to SR-BI at 4 °C exhibits negative cooperativity. The unexpected complexity of the interactions of lipoproteins with SR-BI should be taken into account when interpreting the results of experiments that explore the mechanism(s) by which SR-BI mediates ligand binding, lipid transport and cell signaling.

High density lipoprotein (HDL) cholesterol levels in plasma are inversely and low density lipoprotein (LDL) cholesterol levels are directly associated with the risk of atherosclerosis (1–3). The apparently protective effect of HDL may be due, at least in part, to reverse cholesterol transport (RCT) by which cholesterol moves via lipoproteins from peripheral organs to the liver where it or its metabolic products (e.g., bile acids) are excreted into the bile (4–7). Among the cell surface receptors playing major roles in regulating plasma lipoprotein cholesterol levels and metabolism is the HDL receptor SR-BI (scavenger receptor, class B, type I). SR-BI is highly expressed in the liver, steroidogenic organs and intestines (8), and it can also be found in other types of cells (e.g., macrophages, endothelial cells) (7).

[†]This work was supported by the National Institutes of Health grants to MK

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SR-BI's participation in reverse cholesterol transport is probably responsible for at least some of its potent atheroprotective effects in mice. Indeed, increasing the expression of hepatic SR-BI increases reverse cholesterol transport and reduces atherosclerosis in mice, despite accompanying decreased plasma HDL cholesterol concentration (5–7,9–11). Homozygous inactivation of the SR-BI gene in mice causes an ~2.2-fold increase in plasma cholesterol due to accumulation of abnormally large and unesterified cholesterol enriched HDL particles (12,13). Loss of SR-BI results in a number of abnormal phenotypes, including a decrease in biliary cholesterol secretion (14), female infertility (15,16), stress-induced adrenal insufficiency (17,18) and red blood cell (19,20) and platelet abnormalities (21,22). SR-BI and apolipoprotein E double knockout mice fed a normal chow diet exhibit hypercholesterolemia, severe occlusive coronary arterial atherosclerosis, and at very young ages (5–8 weeks of age) myocardial infarction, cardiac dysfunction and premature death (13,23). In addition to its role in transporting lipids, SR-BI appears to be a signaling receptor; HDL binding to SR-BI in endothelial cells activates a signal transduction pathway that leads to the activation of multiple kinase cascades and endothelial nitrous oxide synthase (eNOS) (24–26). It can also serve as an hepatic co-receptor of hepatitis C virus (27–29).

SR-BI is a 509 amino acid, fatty acylated, integral membrane glycoprotein (7,30), comprising a heavily N-glycosylated extracellular loop, two transmembrane domains and short intracellular amino and carboxy termini. Initial immunoblotting observations in tissues raised the possibility that SR-BI might be able to dimerize with either itself or other proteins (31,32) and subsequent co-immunoprecipitation, biochemical and biophysical studies have provided evidence that SR-BI can form homodimers or higher order oligomers, with putative dimers most readily detected in cells and tissues exhibiting the highest levels of SR-BI expression (33–36).

SR-BI binds to HDL with high affinity (discussed in detail below). We previously reported that the density of the subfraction of HDL influences its interaction with SR-BI, with larger, less dense HDL particles exhibiting apparently tighter binding compared to smaller, denser HDL particles (37). Similar results were also reported by de Beer et al. (38). HDL binding results in the SR-BI-mediated transfer of HDL's core cholesteryl esters (CE) into the cell via a process called selective lipid uptake, whose mechanism is not well understood (8,39,40). Selective lipid uptake appears to involve a two-step process, first high affinity lipoprotein binding and then receptor-mediated transfer of cholesteryl esters from the core of the lipoprotein particle into the cell (41,42). Subsequently, the lipid-depleted lipoprotein particle dissociates from the receptor, freeing the receptor to bind another lipoprotein (7,37,43). Unlike LDL receptor-mediated endocytosis of LDL (3), SR-BI mediated selective uptake does not require internalization or subsequent lysosomal degradation of the whole lipoprotein particle to release the lipids into the cell (39,40,44) (reviewed in (5)). In addition, SR-BI mediates the bidirectional transfer of unesterified cholesterol between cells and lipoproteins (45–47).

SR-BI can also bind LDL (48) with higher affinity than HDL (49) and mediate selective uptake of its core CEs, although not as efficiently as uptake from HDL (50,51). LDL's interaction with SR-BI differs from that of HDL. Simultaneous mutation of two residues in the extracellular loop of SR-BI (Q418R together with either G401R or Q402R) abrogates most detectable, direct HDL binding but does not appear to influence LDL binding, clearly showing that the interactions of SR-BI with LDL and HDL are not identical (49). Curiously, the interactions of LDL and HDL with SR-BI exhibit nonreciprocal cross competition (52), a process in which an excess of one ligand (HDL) efficiently inhibits most of the binding of a relatively low concentration of a second ligand (LDL), whereas the reverse is not the case despite both ligands exhibiting similar high affinity binding (7,8,49). Several other groups

also have reported evidence of nonreciprocal cross completion exhibited by SR-BI (32,53). These nonreciprocal cross competition and mutational analyses have raised the possibility that SR-BI might exhibit multiple, nonidential binding sites. However this explanation does not appear to be consistent with previous direct measurements of the concentration dependence of ligand binding, which suggest that SR-BI exhibits only one class of binding site for each of the lipoprotein ligands examined. Indeed, we and many others have previously described the concentration dependence of both the cell surface binding of ^{125}I-labeled lipoproteins to SR-BI expressed in cultured mammalian cells at 37° and 4°C and SR-BI-mediated cellular uptake of lipids, such as [^{3}H]cholesteryl esters ([^{3}H]CE) from these lipoproteins at 37°C. When we first reported the high affinity binding of ^{125}I-LDL to hamster SR-BI expressed as a cDNA transgene in COS cells (48), we examined binding over an ^{125}I-LDL concentration range of 2–50 μg protein/ml. We concluded that there was a single class of independent (not interacting), high affinity binding sites with a Kd of ~5 μg protein/ml (~10 nM, assuming a single apolipoprotien B/particle with a protein molecular weight of 512,000 (54,55)). We and others subsequently reported Kd values for LDL binding to a single site on SR-BI from several species that vary between 3–15 μg protein/ml (~6–29 nM) in varying mammalian cell culture systems (48,49,56,57) (also see (53) for studies in insect (Sf9) cells). Studies of ^{125}I-HDL binding and [^{3}H]CE-HDL uptake performed over relatively high HDL concentration ranges reached similar conclusions – a single class of HDL binding site - with Kd's for binding (Km's for lipid uptake) in the range of 9–44 μg protein/ml (~62–306 nM) (37,38,48,56,58–61) (also see (53) for studies in Sf9 cells).

In the current study we have re-evaluated the concentration dependence of the binding of ^{125}I-LDL and ^{125}I-HDL to SR-BI and the SR-BI mediated uptake of [^{3}H]CE from these lipoproteins using lower lipoprotein concentrations than reported previously. The extended concentration range uncovered previously unrecognized complex binding, such that when represented by Scatchard plots (62,63) the data exhibit nonlinear, concave up curves. These results strongly suggest that HDL and LDL binding to SR-BI and subsequent selective uptake are best described by either a two independent binding site model or a model in which there is one class of binding sites exhibiting negative cooperativity due either to allostery or lattice/ensemble effects, which are described in detail below. Direct determination of cooperativity using the 'infinite dilution' dissociation rate method of De Meyts et al (64–66) established that the binding of HDL to SR-BI at 4 °C involves negative cooperativity.

EXPERIMENTAL PROCEDURES

Lipoproteins

Each preparation of human LDL and HDL was isolated from freshly harvested, EDTA-treated plasma (from two units of pooled blood from two donors) using self-forming KBr gradients (67,68) and a zonal rotor with a reorienting core as previously described (37). The gradient was fractionated and the location of the positions of the lipoproteins in the gradient were determined by measuring absorption at 494 nm. There was baseline, or near baseline separation between the HDL and LDL containing fractions. All HDL or LDL fractions that were subsequently processed as described below were first supplemented with the antioxidant butylated hydroxytoluene (BHT, final concentration 20 μM). For LDL, the peak two fractions (density ~1.026 g/ml, determined by gravimetry) and most of the two adjacent fractions (~1.019 and 1.032 g/ml) were pooled (combined calculated density of ~1.026 g/ml), concentrated by centrifugation (4° C, 20–24 hrs, 49,000 rpm, Beckman Type 70 Ti rotor) after adjustment of the density to 1.063 g/ml by addition of KBr, and then dialyzed against 8 changes of 6L of LDL buffer (0.9% NaCl, 0.3 mM Na_2EDTA, pH adjusted to 7.4 and degassed immediately prior to use). For the 8 fractions containing HDL, the density of

each fraction was determined by gravimetry and individual fractions or pools of two or more fractions were concentrated by centrifugation after adjustment of the density to 1.215 g/ml and subsequently dialyzed as for LDL above. After concentration, all preparations were stored under argon gas and protected from light to minimize oxidation. The density (mean ± SD density, (g/ml)) of each fraction determined from 13 independent preparations was: fraction #1, 1.155±0.018; #2, 1.149±0.017; #3, 1.143±0.017; #4, 1.137±0.013; #5, 1.129±0.013; #6, 1.123±0.013; #7, 1.113±0.012; #8, 1.102±0.022. For most of the studies reported here, HDL fraction 7 was used for radiolabeling and the binding and uptake studies. An equal volume mixture of fractions 5 and 6 (mean density 1.126 g/ml) or fraction 1 (1.155g/ml) were used for some of the studies as indicated. Protein concentrations were determined by the method of Lowry (69) using bovine serum albumin as a standard. Approximate molar concentrations of lipoproteins were calculated as follows: for HDL, 1 μg protein/ml ~6.944 nM; for LDL, 1 μg protein/ml~1.95 nM (70).

HDL and LDL protein components were labeled with ^{125}I (^{125}I-HDL and ^{125}I-LDL) and with [^{3}H]cholesteryl oleate (Perkin Elmer #NET746L) ([^{3}H]CE, [^{3}H]CE-HDL or [^{3}H]CE-LDL) as previously described (71,72). The ranges of specific activities (cpm/ng protein) of these labeled preparations were: ^{125}I-HDL, 361–2044; ^{125}I-LDL, 162–249; [^{3}H]CE-HDL, 8–26 ; and [^{3}H]CE-LDL, 38. For many of the assays described below, master stock solutions of radioactive lipoproteins were prepared immediately before the assay at the following concentrations (μg protein/ml assay medium): 0.2, 2, 20, 150 and 300. These stock solutions were then further diluted into aliquots at the final lipoprotein concentrations (between 0.1 and 150 μg/ml) without or with a 40-fold excess of the corresponding unlabeled lipoprotein. The unlabeled HDL used to determine nonspecific binding (see below) was a mixture of fractions, ranging from all fractions combined (1–8) to fraction 8 alone. In half of the experiments, the unlabeled HDL contained only the lowest density (highest affinity) fractions (one or more of fractions 6–8).

Cells

LDL receptor-deficient Chinese hamster ovary (CHO) cells (ldlA-7) (73) that are stably transfected with a cDNA encoding wild-type murine SR-BI (ldlA[mSR-BI]) (8) were maintained in CO_2 incubators at 37°C in medium A (Ham's F12 containing, 2 mM L-glutamine, 50 units/ml penicillin/50 μg/ml streptomycin and 0.25 mg/ml G418) supplemented with 5% fetal bovine serum (FBS) (medium B). Untransfected control ldlA-7 were maintained in the same medium without G418.

Cell binding and lipid uptake assays

For most experiments, cells were seeded in 24-well plates at a density of 50,000 cells per well in medium B and binding and uptake assays were performed two days later as previously described (8,74–76). For binding and uptake assays at 37°C, on the day of the assay, the cells were washed twice with prewarmed (37° C) medium C (Ham's F12 containing, 2 mM L-glutamine, 50 units/ml penicillin/50 μg/ml streptomycin). For binding assays performed at 4°C, the assay plates were removed from the incubator and precooled for 30 min on ice in a cold room (4°C). The cells were then washed twice with cold (4°C) medium C supplemented with 10 mM HEPES, pH 7.4 (medium D). For the assays at either temperature, the medium overlaying the cells was removed by aspiration and then fresh assay media (at 37 °C, medium E (medium C plus 0.5% (w/v) bovine serum albumin (BSA)); at 4 °C, medium F (medium D plus 0.5% BSA), 200–250 μl per well) containing the indicated concentrations of labeled and unlabeled lipoproteins were added, and the cells were incubated for 2 hrs either as stationary plates in a CO_2 incubator (37°C) or on a slowly rotating platform in a cold room (4°C). The radioactive assay media (37° and 4° C assays) were then removed and the cells washed rapidly in the cold room two times with ice cold

wash buffer 1 (0.9% NaCl, 50 mM Tris HCl, pH 7.4) containing 2 mg/ml of BSA and once with wash buffer 1 without BSA. For binding studies, the wash buffer was removed and cells were lysed in 0.75 ml of 0.1 N NaOH and samples were taken for counting (500 µl) and protein determination (200 µl) by the method of Lowry (69). For [^{3}H]CE uptake experiments, the wash buffer was removed and lipids were extracted from cells into 1 ml isopropanol for 30 min at room temperature, and the entire volume of isopropanol extract subjected to liquid scintillation counting. The remaining lipid-depleted cell extracts were then lysed in 0.1N NaOH and protein content determined as described above. The amounts of cell-associated [^{3}H]cholesteryl oleate (uptake) are expressed as the equivalent amount of [^{3}H]CE-HDL protein (ng of the protein component of the lipoprotein /mg cell protein) to permit direct comparison of the relative amounts of ^{125}I-HDL binding and [^{3}H]CE uptake (77). The values presented represent SR-BI specific binding or specific uptake and were calculated as the differences between the average of replicates of total cell associated binding or uptake minus the average value for control wells representing nonspecific binding or uptake. For most experiments, total cell associated binding or uptake values are based on quadruplicate determinations and nonspecific values from duplicate determinations made in the presence of either 400 µg protein/ml (for labeled lipoprotein concentrations less than 10 µg protein/ml) or a 40-fold excess of the corresponding unlabeled lipoprotein and presented in the figures as mean values ± standard error of the mean (SEM). However, for experiments in which cellular uptake of [^{3}H]CE from [^{3}H]CE-LDL was measured, total uptake and nonspecific uptake were based on duplicate determinations each and nonspecific uptake was obtained using untransfected ldlA-7 cells in place of the excess of unlabeled lipoproteins. The total and nonspecific, as well as the specific, values are illustrated for several representative experiments in Supplemental Figure S1.

^{125}I-HDL cell dissociation assays

For cell dissociation assays at 4°C, cells were plated in the wells of 24 well dishes and subsequently incubated on a slowly rotating platform for two hours at 4°C in medium F with 10 µg protein/ml of ^{125}I-HDL (891 cpm/ng)in the presence (single incubations) or absence (duplicate or triplicate incubations) of 400 µg protein/ml of unlabeled HDL as described above. The cells then were washed twice with 1 ml of medium F. Some of the cells were then harvested (“0 min” time point) by washing once with 1 ml of ice cold wash buffer 1 then lysing in 0.1N NaOH, and the amounts of bound ^{125}I-HDL and protein in the lysates were determined. The remaining cells were refed with 2 ml of medium F without or with 400 µg protein/ml unlabeled HDL and then incubated on a slowly rotating platform at 4 °C for the indicated times (5–240 minutes). After the additional incubation period to permit dissociation of the bound ^{125}I-HDL, the cells were washed twice with 2 ml of cold wash buffer 1 with BSA and once with 2 ml of wash buffer 1 without BSA before lysing with NaOH and counting as described above.

Data Analysis

All data were analyzed using Prism 5 (GraphPad Software, Inc., San Diego, CA). Non-linear regression analysis was performed on the specific binding and uptake data using the standard equations for a one site binding model, a two site binding model and a one site binding model plus a Hill Slope (Hill Coefficient). For each experiment, all three models were fit and standard statistical pairwise comparisons of the models were made using the Graphpad Prism software using an extra sum-of-squares F test. The P values for all comparisons are given in Supplemental Table S2. The P values for pairwise comparisons of one site vs either two sites or one site plus Hill Slope are shown in Supplemental Table S2. In all cases, either the two site or one site plus Hill Slope models were preferred over the simple one site model.

Average values for parameters and their standard deviations were calculated using Excel. Weighted averages of parameters presented in Table 1 were calculated as follows:

$$\overline{X} = \sum_{i=1}^{n} (X_i/\sigma'^2_i) / \sum_{i=1}^{n} (1/\sigma'^2_i)$$

$$\sigma^2_X = \frac{1}{\sum_{i=1}^{n} (1/\sigma'^2_i)},$$

where x_i is the parameter value determined by Graphpad Prism 5 and σ'_i (%SD) = ((standard deviation of x_i)/x_i))*100. This weighting scheme reduces the inappropriate influence of large values of x_i that have large associated standard deviation when unweighted averages are calculated (see Table S1). Our experience suggests that this weighting scheme provides a less inappropriately biased set of average parameters that either the unweighted averages or averages weighted using σ'_i = standard deviation. However, the calculated errors of these %SD weighted averages, $(\sigma^2_X)^{0.5}$ appear at face value to systematically underestimate the uncertainty in the weighted averages. We therefore present the unweighted average values (and the range of x_i values) in Table S1.

The determination of significant outliers was based on the Grubbs test (78), $P<0.05$ as implemented by Graphpad (http://www.graphpad.com/quickcalcs/grubbs2.cfm).

The data for the time dependence of ^{125}I-HDL dissociation were fit using linear, single exponential or double exponential curves and the best fits determined by Graphpad Prism 5 as described above. In all cases, a single exponential decay curve was preferred over a straight line. The uncertainty in the half-lives was calculated using the percent standard deviation in the corresponding exponential rate constant K (half-life = 0.69/K).

RESULTS

During the course of investigation of the lipoprotein binding properties of murine SR-BI, we re-evaluated the concentration dependence of the cell association of ^{125}I-LDL with SR-BI expressed as a stable transgene in a LDL receptor-negative Chinese hamster ovary cell line, ldlA[mSR-BI] (8). Figure 1A shows results from a typical experiment for the specific binding of ^{125}I-LDL at 37°C determined over a range of concentrations (0.1–125 μg protein/ml) that included significantly lower values than we previously reported. Figure 1B shows the same data in the form of a Scatchard plot. Strikingly, the Scatchard plot is non-linear (concave up). This suggested that there were either more than one class of binding site or that there were more than one members of a single class of binding site that exhibit negative cooperativity (one site plus Hill coefficient). [Other potential explanations are described under Discussion.] This was confirmed by direct non-linear least squares analyses of the binding data (Table 1) using Graphpad Prism software that significantly preferred models of either two classes of independent binding sites (‘two site’ model) or one site with a Hill coefficient of <1.0 (negative cooperativity, ‘one site + Hc’ model) to a simple single class (“one site”) binding model (see Supplemental Table S2 for P values). The weighted average binding parameters from six independent experiments for the two preferred models are presented in Table 1 (LDL, 37°C, Binding). For the two site model they include the apparent dissociation constants (Kd1, Kd2) and apparent maximum binding for each site (Bm1, Bm2), while for the one class of site with negative cooperativity they include the apparent dissociation constant (K^hd), apparent maximum binding (B^hm) and Hill coefficient (Hc). The weighted averages were calculated by weighting each parameter by its associated %

standard deviation (see Methods) to reduce distortion in the means by values with differing % uncertainty. Unweighted average parameter values and the ranges of the parameters for all experiments are presented in Table S1.

For the two site model of ^{125}I-LDL binding at 37°C, the weighted average apparent Kd1 (1.9±0.2 μg protein/ml, ~3.7 nM) was ~20-fold lower than that of the apparent Kd2 (39±4 μg protein/ml, ~75 nM). Another receptor that exhibits ligand binding with nonlinear Scatchard plots and has a similar ratio of Kd2:Kd1 (15-fold difference) is the EGF receptor (79). The unweighted average of the ratios of apparent Kd2 to apparent Kd1 for all experiments was 47±34. The apparent Bm1 and Bm2 values are expected to vary from experiment to experiment because they reflect the average number of receptors expressed per cell in each experiment. For cultured, transfected cells (ldlA[mSR-BI]) this varies depending on growth conditions, passage number, variations in growth medium (e.g., serum lot), etc.. Thus absolute values of Bm1 and Bm2 (Table 1) do not directly reflect the intrinsic properties of SR-BI, but rather provide only an approximate estimate of the receptor activity in the cell line examined. In as much as the ratios of the apparent Bm2:Bm1 values in any one experiment reflect the true ratio of maximal binding at equilibrium, they provide a rough estimate of the relative number of low and high affinity sites for a two-site model. The unweighted average of the ratios of Bm2:Bm1 for the six independent experiments was 3.4±2.3, suggesting that there may have been as many as 3 times more lower affinity than higher affinity sites, if there were two classes of independent binding site. For the one site plus Hc model, the weighted average apparent K^hd was 28±3 μg protein/ml (~55 nM) and the Hill coefficient was 0.65±0.02. We conclude that several previous studies of the binding of LDL by SR-BI did not sample the binding at low enough concentrations of the LDL ligand to robustly detect the complex nature of the interaction between LDL and SR-BI.

We also determined the concentration dependence of the specific uptake of [^{3}H]CE from [^{3}H]CE-LDL in ldlA[mSR-BI] cells. Figure 1C shows the [^{3}H]CE-LDL concentration dependence of cell associated [^{3}H]CE for one experiment; Figure 1D shows Scatchard analysis of these data; and the weighted averaged parameters from two experiments are shown in Table 1 (unweighted averages in Table S1). As was the case for binding, the Scatchard plot is non-linear (concave up) and either a two site model or one site plus Hc (negative cooperativity) was significantly preferred over a simple one site model by Graphpad Prism analysis (see Supplemental Table S2 for P values). The calculated parameters describing the lipid uptake represent the Michaelis constants (Km1, Km2 or K^hm, rather than dissociation constants) and maximum uptake rates (Vm1, Vm2 or V^hm, rather than binding maxima). The weighted average K^hms and Hill coefficient parameters and the averaged Vm2:Vm1 ratio were similar, though not identical (see Discussion), to the comparable parameters for 37° binding, with the most similar of the uptake parameters to those for binding being the Km1 of 3.5±0.6 μg protein/ml (~7 nM), the Hill coefficient of 0.65+0.02 and the average Vm2:Vm1 ratio of 4.5±0.8. As expected, because SR-BI mediates selective lipid uptake at 37° C, Vmax values for uptake were greater than the apparent Bmax values for binding at 37°C (e.g., for one site with Hc, V^hm for uptake was 4.5-fold greater than the apparent Bhm for binding). We conclude that at 37°C the concentration dependence of lipid uptake from LDL by SR-BI mirrors that of the binding and is best described by either a two site model or a one site plus Hc (negative cooperativity) model. The results suggest that all LDL binding sites can mediate lipid uptake in addition to binding.

We also reexamined ^{125}I-HDL binding to SR-BI and uptake of [^{3}H]CE from [^{3}H]CE-HDL by SR-BI at 37°C using cultured ldlA[mSR-BI] cells using HDL concentrations that ranged between 0.05 and 150 μg protein/ml. Many of these experiments involved sampling at 20 different ligand concentrations to enable discovery of potential high affinity binding sites

not heretofore detected. In preliminary experiments using HDL's isolated by zonal centrifugation containing particles with a relatively broad range of densities (fractions 1–8, 1.102–1.155 g/ml, see Experimental Procedures), we found it difficult to interpret the results because heterogeneity of the HDL ligand (multiplicity of apparent Kd values (37,38) apparently interfered with qualitative and quantitative analysis of binding. Therefore, we prepared ^{125}I-HDL and [^{3}H]CE-HDL from HDL with a relatively narrow range of buoyant densities (> 1.102 and <1.123 g/ml, mean density of 1.113 g/ml), containing larger, lower density particles in the HDL2 density range (70).

Figures 2A and C show the specific binding of ^{125}I-HDL and the uptake of [^{3}H]CE from [^{3}H]CE-HDL at 37°C at concentrations of 0.05–150 μg protein/ml. Figures 2B and 2D show the same data in the form of Scatchard plots. Just as in the case with LDL, addition of data from a lower lipoprotein concentration range resulted in non-linear (concave up) Scatchard plots for specific binding of ^{125}I-HDL and specific uptake of [^{3}H]CE from [^{3}H]CE-HDL. [We have observed concave up Scatchard plots for experiments using HDL with different mean densities (1.126 g/ml and 1.155 g/ml, see Figure S2), establishing that this phenomenon is not limited to HDL with a mean density of 1.113 g/ml.] Quantitative analysis of the binding data using GraphPad Prism software established that a one site model does not fit the data well, but that either a two site model or one site plus Hc (negative cooperativity, Hill coefficient <1) are significantly preferred for both HDL binding and lipid uptake from HDL (see Supplemental Table S2 for P values). The weighted averaged parameters from three independent experiments each for HDL binding and uptake at 37° are shown in Table 1 (unweighted averages and ranges in Table S1). Again as for LDL, the apparent Kd, Km and Hill coefficient values were similar, though not identical, for binding and uptake. The weighted averages of apparent Kd and Km values for binding and uptake (μg protein/ml, respectively) were: apparent Kd1 of 7.2±3.1 (~50 nM) for binding and Km1 of 11.9±1.1 for uptake; apparent Kd2 of 42±12 (~291 nM) and Km2 of 33+4; apparent $K^{h}d$ of 26±2 (~181 nM) and $K^{h}m$ of 31±2. The Hill coefficients were 0.85±0.02 (binding) and 0.82±0.02 (uptake). For HDL, the ratios of Kd2:Kd1 (Km2:Km1) appeared to be lower than those for LDL and correspondingly, the Hill coefficients for HDL were closer to one than for LDL (~0.8 vs ~0.6, respectively), indicating less negative cooperativity for HDL than LDL. For ^{125}I-HDL binding at 37°C, the unweighted average of the ratios of apparent Bm2:Bm1 from three independent experiments was 2.9±0.4, similar to that for LDL binding at 37°C (3.4±2.3). Also, the absolute amount of [^{3}H]CE uptake (Vm) relative to surface binding (Bm) was higher for HDL (~13-fold difference) than for LDL (~4-fold difference) and the affinity of SR-BI for LDL was higher than for HDL (lower molar Kd). These data suggest, as previously reported (49–51), that SR-BI-mediated selective uptake is more efficient from HDL than from LDL even though binding to LDL is tighter than for HDL.

All the experiments described above were performed at 37°C and permit calculation of 'apparent' binding affinities and binding maxima of HDL and LDL for SR-BI. These do not represent thermodynamically rigorous binding dissociation constants (Kd) because the experiments were not performed under equilibrium conditions (e.g., selective uptake from the lipoproteins occurred during the incubations with the cells). To evaluate binding at equilibrium, we examined ^{125}I-HDL binding at 4°C. [The cellular selective uptake of [^{3}H]CE from [^{3}H]CE-HDL was not examined because it is dramatically reduced at low temperatures (e.g., 4°C) (60,80–82)]. Figure 3 shows results from a typical experiment for specific binding (panel A) and the same data in the form of a Scatchard plot (panel B). The weighted average results from the quantitative analysis (Graphpad Prism) of six independent experiments are summarized in Table 1 (unweighted averages and ranges in Table S1). The binding data at 4°C confirmed our findings at 37°C of non-linearity of the Scatchard plot and a significant preference for either a two site model or a one site plus Hc (negative cooperativity) model relative to a one site model (see Supplemental Table S2 for P values).

The weighted average binding constants (μg protein/ml) were: Kd1, 4.0±0.07 (~28 nM); Kd2, 61±6 (~423 nM); and K^hd, 54±5 (~372 nM). The Hill coefficient was 0.70+0.02. For ^{125}I-HDL binding at 4°C, the unweighted average of the ratios of Bm2:Bm1 from five independent experiments was 3.3±1.8[1], similar to those for ^{125}I-HDL and ^{125}I-LDL binding at 37°C (2.9+0.4 and 3.4±2.3, respectively). All three types of binding experiment suggest that the ratio of lower to higher affinity sites is ~3 in the two binding site model. Because of the qualitatively similar results for both LDL and HDL binding and uptake observed using multiple independently isolated lipoprotein preparations from multiple donors, we conclude that the complexity of LDL and HDL binding to SR-BI (non-linear concave up Scatchard plots) is unlikely to be an artifact and most likely reflects the intrinsic binding properties of SR-BI and the lipoproteins.

To determine directly if the binding of HDL to SR-BI involves negative cooperativity, we used a simple, yet powerful, two-step kinetic method initially described by De Meyts and colleagues (64–66). In the first step of the experiment, cells are incubated for two hours at 4°C with a sub-saturating level of labeled ligand (10 μg protein/ml ^{125}I-HDL). Medium containing unbound ^{125}I-HDL is removed and the cells rapidly washed. In the second step the cells with their bound ^{125}I-HDL are incubated for varying times (0–240 min) in medium (1 ml/well in 24 well dishes) at 4 °C in the absence or presence of a large excess of unlabeled ligand (400 μg protein/ml HDL). The unlabeled ligand is expected to rapidly bind to most ligand binding sites that remained unoccupied after the first step. If a radioactive ligand is bound to non-interacting receptor sites (no cooperativity), the rate of its dissociation should be independent of the occupancy of other, independent receptors on the same cell. That is, addition of the excess unlabeled ligand would not influence the dissociation rate. In contrast, if the population of receptors exhibits negative cooperativity, the rate of dissociation of any given bound radioactive ligand will depend on the occupancy of other, interacting receptors. As a consequence, the dissociation rate of the labeled ligand will be higher when other interacting receptors have ligands bound compared when those interacting receptors are unoccupied. The relatively large volume of medium present during the second incubation step results in the essential 'infinite dilution' of any ^{125}I-HDL that dissociates from the cells during that step. Thus, rebinding of ^{125}I-HDL during the second incubation will not significantly confound the results (64–66).

Figure 4 shows the averaged results from two independent dissociation experiments (the results from each experiment were similar to the combined values). Dissociation in the absence of excess unlabeled HDL (open circles) was best fit by a one-phase exponential decay curve with $t_{1/2}$= 51 ± 9 min. Dissociation was substantially faster in the presence of excess unlabeled HDL (filled circles) and those data were best fit with a two-phase exponential decay curve. The corresponding two $t_{1/2}$ values were 3 ± 0.7 min, and $t_{1/2}$= 51 ± 11 min. These results are comparable to those reported for many other receptors that exhibit negative cooperativity (65,79,83,84),76). Thus, negative cooperativity contributes to, and may be the primary source of, the concave up curvilinear shapes of the Scatchard plots of HDL binding to SR-BI. It is striking that about half of the pre-bound ^{125}I-HDL was shifted from slowly dissociating ($t_{1/2}$= 51 ± 11 min) to rapidly dissociating (3 ± 0.7 min) by the addition of the excess unlabeled HDL. Future studies will be required to elucidate the molecular mechanism(s) underlying the negative cooperativity (see Discussion below).

DISCUSSION

In the current study we have extended to less than 1 μg protein/ml the range of lipoprotein concentrations used to characterize the interactions of LDL and HDL with the HDL receptor

[1]One ratio of 13.4 was a significant outlier based on the Grubbs test, P<0.05, and was therefore omitted from this calculation.

SR-BI highly expressed on the surface of transfected cells (ldlA[mSR-BI]) in culture. We measured as a function of LDL (0.1–125 μg protein/ml) or HDL (0.05–150 μg protein/ml) concentration the specific binding of ^{125}I-LDL and ^{125}I-HDL to SR-BI and the SR-BI-mediated specific uptake of [^{3}H]CE from [^{3}H]CE-LDL and [^{3}H]CE-HDL. Previous studies that employed lipoprotein concentrations above the lower levels used here suggested that binding, and at 37° consequent lipid uptake, were adequately described by a single independent binding site (one site) model that is characterized by a linear Scatchard plot (see Introduction). The extended concentration range resulted in binding and uptake concentrations curves that form non-linear, concave up, Scatchard plots. Non-linear least squares analysis of all data showed that they were poorly described by a one site model, in that the data were statistically significantly better fit (most - 14 or 15 out of 20 experiments - with $P<0.0001$, see Table S2) by either of two alternative models: 1) two independent classes of binding site (two site model), or 2) one class of sites that exhibit negative cooperativity (one site + Hill coefficient (Hc)).

Non-linear Scatchard plots for ligand binding to a receptor can arise either because the binding is more complex than a single class of binding sites on both the receptor and ligand or because of artifacts due to heterogeneity in the ligands even though the receptor truly may have a single uniform class of non-interacting binding site. We have concluded that ligand heterogeneity is unlikely to be the principal source of the non-linear Scatchard plots. We will next consider two potential sources of ligand heterogeneity – intrinsic biological heterogeneity of lipoproteins, especially for HDL, and heterogeneity introduced during the isolation and storage of or as a consequence of radiolabeling the lipoproteins - and how we attempted to minimize these sources of artifacts. We then describe models that we conclude are most likely to account for the genuine, complex binding properties of SR-BI.

The first potential source of lipoprotein heterogeneity -– intrinsic biological heterogeneity – arises because of their complex biosynthesis, intravascular remodeling and metabolism that can influence particle composition (apolipoproteins, lipids) and physical properties (charge, diameter (70)). HDL particles are often very heterogenous (85). As noted in the Results, previous studies have shown that the apparent affinity of SR-BI for HDL varies as a function of density, with lower density particles binding more tightly (37,38). Thus, to minimize this source of heterogeneity we selected from the total HDL pool isolated by density gradient zonal centrifugation (37,67,68) a small subset of lower density particles (in the HDL2 density range (70)) with a narrow range of buoyant densities (> 1.102 and <1.123 g/ml, mean density of 1.113 g/ml). This subfraction appeared to strike a reasonable balance between the practical requirements for obtaining sufficient HDL to perform experiments and obtaining as narrow a range of densities (homogeneity) as possible to avoid binding artifacts. Using this relatively homogenous HDL (as well as similarly narrow density range fractions with mean densities of 1.126 or 1.155 g/ml), we observed binding data that generated classic nonlinear Scatchard plots and were readily fit successfully by either two site or one site plus Hill coefficient models. In contrast, when we used as the ligand HDLs with a much broader size distributions (>1.102 and <1.1155 g/ml), the data produced irregular Schatchard plots that could not be fit using standard models, probably because of heterogeneous binding affinities of differing HDL particles in such preparations. [However, Mendel et al. (86) have suggested that lipoprotein heterogeneity might not be responsible for non-linear, concave up, Scatchard plots of binding to receptors in at least some circumstances.] There is relatively low intrinsic structural heterogeneity of LDL, with all particles carrying a single copy of apolipoprotein B and limited heterogeneity due primarily to differences in the amount of lipid carried per particle. This limited heterogeneity is reflected in the buoyant densities and diameters of the particles (small dense vs large buoyant particles over the 1.019–1.063 g/ml density range, 18.0–23.3 nm in diameter) (87,88). We are not aware of data indicating heterogeneity in the LDL used in this study

impacts binding to SR-BI. Because our results were qualitatively similar for LDL and several distinct subfractions of HDL, we conclude that it is unlikely that the nonlinear, concave up, Scatchard plots were consequences of the inherent size/density heterogeneity of the lipoproteins.

The second potential source of ligand heterogeneity involves alterations introduced during the isolation and storage (e.g., oxidation) or as a consequence of radiolabeling the lipoproteins (chemical modification, time dependent radiolytic damage/oxidation/protein crosslinking), especially in the case of iodination. To avoid these pitfalls, lipoproteins were isolated with antioxidants and stored using degassed buffers under argon gas with minimal exposure to light, and preparations were generally used within two weeks after labeling. Furthermore, the quality of labeled HDL preparations was monitored periodically by gel electrophoresis as previously described (37) to examine the influence of aging on storage. The covalent modification of tyrosines in the apolipoproteins of HDL by iodination does not appear to affect binding, because we have shown that binding of freshly prepared (and undegraded) ^{125}I-HDL is virtually identical to the binding of unlabeled native HDL measured using an immunoreceptor assay (37). Nonlinear, concave up Scatchard plots were obtained using both ^{125}I- and [^{3}H]CE-labeled lipoproteins. Thus, it is extremely unlikely that radioisotope-specific modifications of the lipoproteins (e.g., ^{125}I-induced radiolytic/oxidative damage) led to heterogeneity that was responsible for the nonlinear Scatchard plots.

Ligand heterogeneity, however, may have contributed to the inter-experiment, quantitative variations we observed in the calculated and Bmax/Vmax and Kd/Km values (Tables 1 and S1). Sources of this ligand heterogeneity include: a) intra- and inter-individual variations in lipoprotein metabolism in plasma donors that can lead to variation in the composition and physical/chemical state of the lipoproteins, b) varying extents of radiolabeling (specific activity) resulting in varying rates of radiolytic degradation, and c) varying intervals between lipoprotein isolation, labeling and assays, which in some cases appears to influence the extent of radiolabeling and quantitative results. Another potential sources of inter-experimental variability in the calculated binding parameters is variable surface receptor expression in the transfected cells, which would directly influence Bmax/Vmax values and might also influence Kd/Km (79). Differences in Bmax and Kd parameters observed at 4° and 37°C may be explained by effects of temperature on the structure of the lipoprotein ligands. For instance LDL, but not HDL, undergoes a distinct lipid core phase transition between 4°C and 37°C (89–94). There may be temperature dependence of the state of the receptor on the cell surface, which may include multimerization, SR-BI surface expression level, interaction with other cellular components, and lipid raft phase transition (30,95).

There are several potential, not necessarily mutually exclusive, interpretations of the nonlinear Scatchard plots of HDL and LDL binding. These involve A) multiple independent receptor binding sites with distinct ligand affinities (two site models), B) a single class of receptor binding sites exhibiting negative cooperativity (single site with negative cooperativity models), or C) homogeneous, but multivalent ligands with distinct sites that can bind to receptors with different affinities (multivalent ligand models). Two site models include: 1) multiple classes of independent binding sites on either SR-BI monomers or on SR-BI homooligomers. Multiple independent sites on a single SR-BI monomer might required rather tight packing of ligands at saturation, because the parameters for maximal binding for the two site model suggest that there may be as many as four binding sites (ratios of Bm2:Bm1 of ~3:1) for HDL and LDL. So many sites for multiple, very large, lipoprotein particles on a relatively small monomeric protein (8,48) seems less likely than multiple independent sites on an oligomer, although it cannot be excluded. There could also be, 2) presence of both monomeric and homooligomeric SR-BI proteins, each exhibiting a

distinctive, but noncooperative, single class of binding site, as has recently been proposed for EGF receptors (96), and 3) presence of SR-BI molecules either associated or not associated with other surface molecules (e.g., other proteins or a subset of lipids such as those found in lipid rafts), each exhibiting a distinctive, but noncooperative, single class of binding site.

Single site with cooperativity models involve SR-BI monomers, each possessing a single class of binding site that, when clustered as monomers or when associated into homodimers or homoologomers exhibit negative cooperativity (Hill coefficient <1.0). Such negative cooperativity could be due to either 4) classic allostery (97–100) caused by changes in receptor structure accompanying initial ligand binding (such as those reported for the bacterial aspartate receptor (101) and EGF receptors (102)) or 5) ensemble effects (or the ‘ lattice model’). In a lattice model, binding of a large ligand to one site in a relatively closely clustered group of identical binding sites, either on a single receptor molecule or on adjacent molecules, can sterically interfere with the approach of a second ligand toward one of the neighboring binding sites (103–106). The lattice effect can occur without any changes in the structure or binding properties of each of the individual receptor molecules, yet it effectively increases the Kd after the first ligand has bound for subsequent ligand binding (negative cooperativity). Lattice effects may be responsible for the negative cooperativity observed for several receptors (107,108), including binding of acetylated LDL to the scavenger receptor, class A, types I and II (52). The propensity of SR-BI to cluster on the surface of ldlA[mSR-BI] and other cells (30,95) and to apparently form dimers or oligomers (31–36) is expected to bring its lipoprotein binding sites into the close apposition required for lattice-based negative cooperativity. Furthermore, its lipoprotein ligands are large, LDL and HDL particles in typical preparations (densities of 1.019–1.063 and 1.063–1.210 g/ml, respectively) have diameters of ~18.0–23.3 nm and ~7.3–15.4 nm, respectively (109,110). Indeed, in the current study the larger LDL exhibited greater negative cooperativity (smaller Hill coefficient) than the smaller HDL (~0.65 vs ~0.8) for both binding and uptake at 37°C (Table 1), as one would predict from the lattice model.

In multivalent ligand models, the complexity of the binding might be due to multiple, distinct sites on the ligand that can differentially interact with one (or more) class of site on the receptor. Both HDL and LDL are large particles. The very large apolipoprotein B on LDL (~512 kDa) distributed over the surface of the particle, a flattened ellipsoid with planar opposing faces (~250 Å × ~240 Å × ~166 Å, (111)) could easily provide multiple distinct sites for receptor binding. The HDLs used in this study are expected to have multiple apolipoproteins on each particle (70), and thus also potentially expose multiple distinct binding sites. Indeed, the ability of SR-BI to bind to multiple lipoproteins and apolipoproteins incorporated in vitro into phospholipids particles, and analysis of binding to truncated and mutant apolipoprotiens, suggested that SR-BI is likely to recognize common structures, e.g., amphipathic helices, present in one or more copies per apolipoprotein rather than specific amino acid sequences in its apolipoprotein ligands (32,37,46,74,112,113). Thus, it is possible that a heterogeneous population of binding sites on a homogenous preparation of lipoproteins might contribute to binding data that result in nonlinear Scatchard plots.

We used a two step dissociation kinetics assay pioneered by De Meyts and colleagues (64–66) to determine directly whether or not negative cooperativity contributes to the nonlinear shape of the Scatchard plots. In the first step a sub-saturating level of ^{125}I-HDL was bound to SR-BI and in the second step the rate of dissociation of the prebound ^{125}I-HDL was measured in the absence or presence of a large excess (saturating level) of unlabeled HDL. The dramatic increase in dissociation rate in the presence of the excess unlabeled HDL

strongly indicates that SR-BI expressed in the cultured cells used in these studies employs a binding mechanism involving negative cooperativity (64–66).

In summary, the data presented here raise the possibility of three different models of HDL and LDL binding to SR-BI that may account for the nonlinear, concave up Scatchard plots of lipoprotein binding to SR-BI: (1) the two site model, (2) binding involving negative cooperativity model (one site plus Hill coefficient (<1.0)) due either to allostery or a lattice effect, or (3) the multivalent ligand model. These models are not mutually exclusive. For example, there could be multiple distinct binding sites that also exhibit negative cooperativity or multivalent ligand binding to sites exhibiting negative cooperativity. Dissociation kinetic assays have established that negative cooperativity plays a significant role in the binding of HDL to SR-BI. Thus, we choose the follow the principle of parsimony (Occam's razor) and conclude that the concave up curvilinear shapes of the Scatchard plots described here are most likely due to the one site plus Hill coefficient (<1.0) model. The physiologic relevance, if any, for negative-cooperativity of binding by SR-BI is not clear. Koshand and Hamadani have reviewed several evolutionary rationales for ligand binding cooperativity (114). Positive cooperativity confers enhanced sensitivity to ligands relative to noncooperative, single site binding, and conversely negative cooperativity results in relatively decreased sensitivity. Reduced HDL or other ligand sensitivity of SR-BI may be physiologically relevant in the context of SR-BI-mediated cell signaling, as is the case of SR-BI-mediated regulation of eNOS by HDL in endothelial cells (24–26). An alternative rationale for negative cooperativity is that it extends the concentration range for substrates over which a response is generated. In other words, saturation in the context of negative cooperativity requires higher ligand concentrations than would otherwise be the case for a noncooperative process using the same binding site. Thus, it is possible that negative cooperativity exhibited by SR-BI might permit it to operate efficiently over a wide range of HDL and other ligand concentrations. Whatever the underlying reason, negative cooperativity binding should be considered when evaluating the as yet poorly defined process of nonreciprocal cross competition and the effects of drugs (44,115–117), experimentally induced (35,36,49,59,61) or naturally occurring (118,119) mutations, or other manipulations on the activities SR-BI, especially in the context of studies designed to elucidate the mechanism(s) by which this receptor mediates ligand binding, lipid transfer and cell signaling. Future experiments will be required to determine the molecular mechanism(s) responsible for the complex lipoprotein binding exhibited by SR-BI.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

[^{3}H]CE	[^{3}H]cholesteryl oleyl ester
HDL	high density lipoprotein
SR-BI	scavenger receptor, class B, type I
LDL	low density lipoprotein
CE	cholesteryl ester
apoA	apolipoprotein A
RCT	reverse cholesterol transport
HCV	Hepatitis C Virus

Hc Hill Coefficient

Acknowledgments

We thank Leonid Gaidukov Andrew Nager, Ayce Yesilaltay, and Harvey Motulsky for helpful discussions and suggestions.

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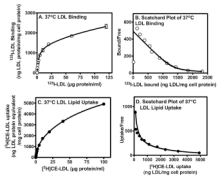

Figure 1. Concentration dependence (A,C) and corresponding Scatchard analysis (B,D) of specific SR-BI-mediated ^{125}I-LDL binding (A, B) and uptake of [^{3}H]Cholesteryl Ester from [^{3}H]CE-LDL (C,D) at 37°C

Transfected LDL-receptor negative ldlA-7 cells expressing murine SR-BI (ldlA[mSR-BI] cells) and mSR-BI-negative control untransfected ldlA-7 cells were plated on day 0 at a density of 50,000 cells per well in 24-well dishes. On day 2, the cells were washed and the indicated amounts of ^{125}I-LDL or [^{3}H]CE-LDL in the absence or presence (duplicate determinations) of a 40-fold excess of unlabeled LDL were added in fresh medium E. After a 2-h incubation at 37° C, ^{125}I-LDL cell surface binding (panel A) and cell associated [^{3}H]CE (uptake, panel C) were measured as described in Experimental Procedures. Nonspecific background (single determinations) was subtracted from the average of duplicate determinations of the total values measured in the absence of unlabeled LDL to calculate the specific values. The specific data from individual representative experiments are shown (averaged data for all experiments summarized in Tables 1 and S1). For ^{125}I-LDL binding experiments (n=6), nonspecific background was measured in the presence of a 40-fold excess of unlabeled LDL. For [^{3}H]CE uptake experiments (n=2), nonspecific background was measured in the same experiment as the total binding to untransfected ldlA-7 cells..

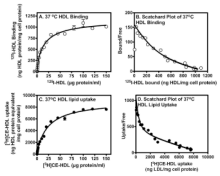

Figure 2. Concentration dependence (A,C) and corresponding Scatchard analysis (B,D) of specific SR-BI-mediated ^{125}I-HDL binding (A, B) and uptake of [^{3}H]Cholesteryl Ester from [^{3}H]CE-HDL (C,D) at 37°C

ldlA[mSR-BI] cells were plated on day 0 at a density of 50,000 cells per well in 24-well dishes.. On day 2, the cells were washed and the indicated amounts of ^{125}I-HDL or [^{3}H]CE-HDL in the absence (quadruplicate determinations) or presence (duplicate determinations) of a 40-fold excess of unlabeled HDL were was added in fresh medium E. After a 2-h incubation at 37° C, ^{125}I-HDL cell surface binding (panel A) and cell associated [^{3}H]CE (uptake, panel C) were measured as described in Experimental Procedures. Nonspecific background measured in the presence of a 40-fold excess of unlabeled HDL was subtracted from the average of quadruplicate determinations of the total values measured in the absence of unlabeled HDL to calculate the specific values. The specific data from individual representative experiments are shown (averaged data for all experiments (n=3 each for binding and for uptake) summarized in Tables 1 and S1).

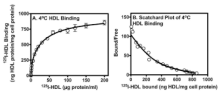

Figure 3. Concentration dependence (A) and corresponding Scatchard analysis (B) of specific SR-BI-mediated ^{125}I-HDL binding at 4°C

ldlA[mSR-BI] cells were plated on day 0 at a density of 50,000 cells per well in 24-well dishes. On day 2, the cells were prechilled at 4° C for 30 min, washed once with medium D and the indicated amounts of ^{125}I-HDL without (quadruplicate determinations) or with a 40-fold excess of unlabeled HDL (duplicate determinations) were added in fresh medium F. After a 2-h incubation at 4° C, ^{125}I-HDL cell surface binding was measured as described in Experimental Procedures. Nonspecific background measured in the presence of a 40-fold excess of unlabeled HDL was subtracted from the average of quadruplicate determinations of the total values measured in the absence of unlabeled HDL to calculate the specific values. The specific data from individual representative experiments are shown (averaged data for all experiments (n=6) summarized in Tables 1 and S1).

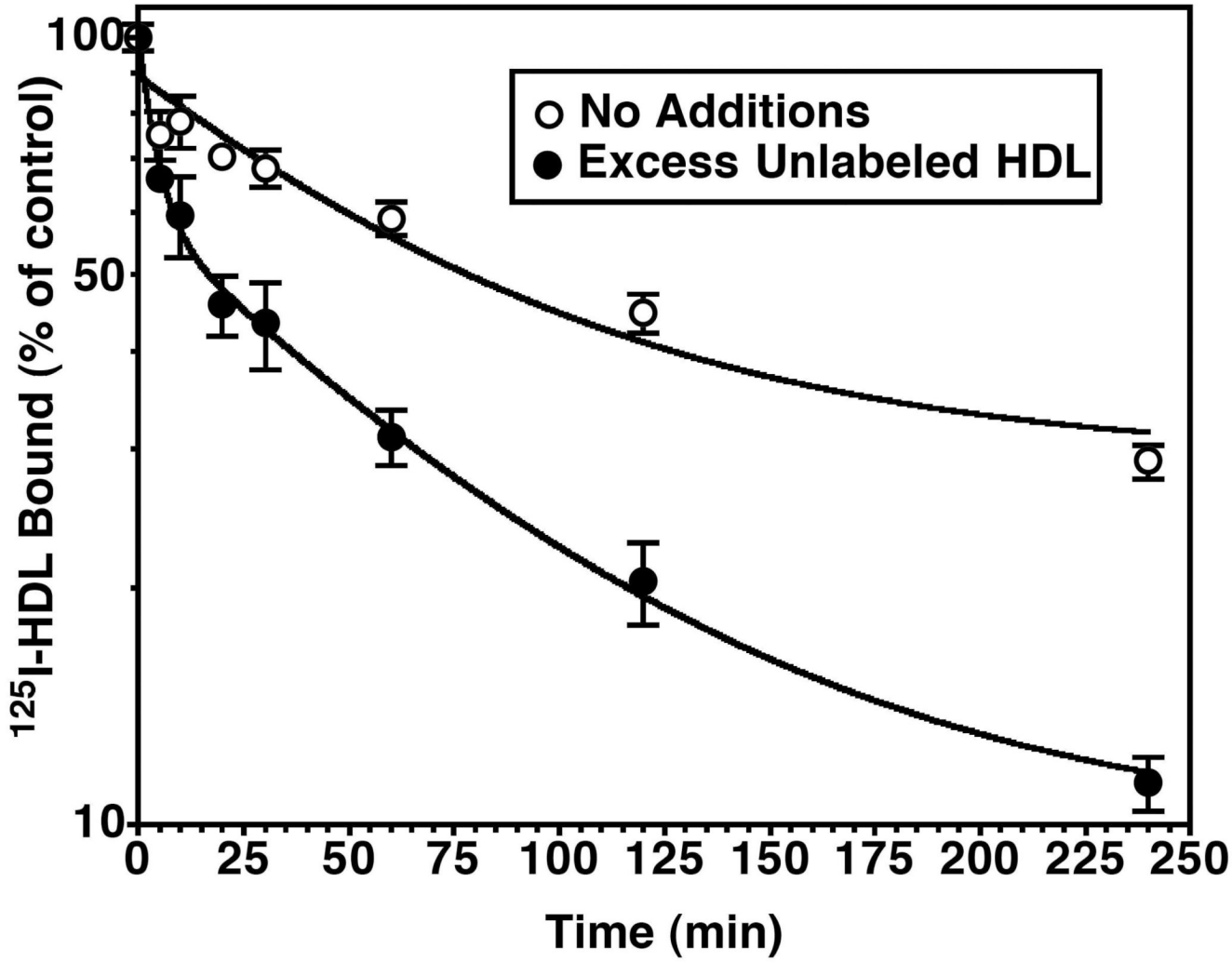

Figure 4. Rates of dissociation of SR-BI-bound ^{125}I-HDL in the absence or presence of excess unlabeled HDL at 4°C

In two independent experiments ldlA[mSR-BI] cells were plated on day 0 at a density of 50,000 cells per well in 24-well dishes. On day 2, the cells were prechilled at 4° C for 30 min, washed once with medium D and 10 μg protein/ml of ^{125}I-HDL without or with a 40-fold excess of unlabeled HDL were added in fresh medium F. After a 2-h incubation at 4° C (step 1), ^{125}I-HDL cell surface binding was measured ("0 min") as described in Experimental Procedures, or the cells were washed, refed fresh medium F supplemented with or without 400 μg protein/ml unlabeled HDL. After additional incubation at 4° C (step 2) for the indicated times (5–240 min), the cells were washed and the amounts of cell bound ^{125}I-HDL remaining were determined. For both conditions (absence and presence of unlabeled HDL during step 2), nonspecific background measured in the presence of a 40-fold excess of unlabeled HDL (single determinations) during the initital ^{125}I-HDL incubation of step 1 was subtracted from the total values measured in the absence of unlabeled HDL in step 1 (duplicate or triplicate determinations) to calculate the specific values. All values in each experiment were normalized to the 100% control values at time 0 (251 or 293 ng protein of HDL bound per mg of cell protein). In the combined data set for the two experiments shown in the figure, n=5 for each datum except for t=5 min (n=3 from a

single experiment). The error bars represent standard deviations determined by Graphpad Prism 5.

Table 1

Percent standard deviation-weighted average values for binding and uptake parameters of two site and one site with Hill Coefficient models

		Two Site Model[a]				One Site plus Hill Coefficient Model[a]		
Assay	**Ligand, Temp**	**Kd1[b,c] (μg/ml)**	**Kd2 (μg/ml)**	**Bm1 (ng/mg cell protein)**	**Bm2 (ng/mg cell protein)**	**K^hd (μg/ml)**	**Hill Coef.**	**B^hm (ng/mg cell protein)**
Binding	LDL, 37°C n=6	1.9±0.2	39±4	993±65	2150±51	28±3	0.65±0.02	2755±84
	HDL, 37°C n=3	7.2±3.1	42±12	282±134	762±98	26±2	0.85±0.02	1003±23
	HDL, 4°C n=6	4.0±0.7	61±6	138±16	469±10	54±5	0.70±0.02	748±21
Assay	Ligand, Temp	Km1[b,c] (μg/ml)	Km2 (μg/ml)	Vm1 (ng/mg cell protein)	Vm2 (ng/mg cell protein)	K^hm (μg/ml)	Hill Coef.	V^hm (ng/mg cell protein)
[^{3}H]CE Uptake	LDL, 37°C n=2	3.5±0.6	100±17	1554±198	7905±344	142±28	0.65±0.02	12280±867
	HDL, 37°C n=3	11.9±1.1	33±4	8367±669	8716±222	31±2	0.82±0.02	12801±256

[a] The errors were calculated using the % standard deviation-weighting approach (see Experimental Procedures).

[b] Abbreviations: Kd1, Kd2, apparent (37°C) or true equilibrium (4°C) dissociation constants; Bm1, Bm2, apparent (37°C) or true equilibrium (4°C) maximum binding values; K^hd, apparent (37°C) or true equilibrium (4°C) dissociation constant for the one class of site with negative cooperativity model; B^hm, apparent (37°C) or true equilibrium (4°C) maximum binding value; Km1, Km2, Michaelis constants; Vm1 Vm2, maximum lipid uptake rates; K^hm, Michaelis constant for the one class of site with negative cooperativity model; V^hm, maximum lipid uptake rate for the one site with negative cooperativity model; and Hill Coef, Hill coefficient.

[c] For experiments performed at 37°C the binding values represent apparent dissociation constants and maximal binding, as the measurements were not made under equilibrium conditions (see text).