BIOPHYSICS AND BIOCHEMISTRY

Characterization of Specific Binding Sites for High-Density Lipoproteins on Rat Hepatocytes: Effects of Estradiol and Testosterone

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Two types of binding sites for high-density lipoproteins (HDL): P_1 and P_2, K_{d1}=20 and K_{d2}=2.5 μg/ml, N_1=130 and N_2=35 ng/mg cell protein) are identified on the surface of rat hepatocytes. Conditions for predominant determination of P_2 are created by employing radiolabeled lipoproteins (^{125}I-HDL) with a high specific activity (1000 dpm) and the differences in the kinetics of the P_1- and P_2-^{125}I-HDL complex formation. P_2 predominate on hepatocytes from females. The addition of estradiol to a culture of hepatocytes from males increases the content of P_2, while the addition of testosterone to hepatocytes from females decreases the content of P_2 to the levels determined in males.

Key Words: *hepatocytes; cell culture; high-density lipoproteins; specific binding; androgens; estrogens*

The modern concept of reverse cholesterol transport maintains that excessive cholesterol is transported from peripheral tissues to the liver for oxidation to bile acids and excretion in the bile as free cholesterol—bile acid micelles. A negative correlation between the cholesterol content of high-density lipoproteins (HDL) and the risk of coronary atherosclerosis can be explained in the terms of this concept.

Receptor-mediated binding of HDL with subsequent internalization by hepatocytes has been regarded as a tentative mechanism responsible for the removal of excessive cholesterol from the body [14]. However, this mechanism has not been generally recognized, since none of the known surface receptors participates in the removal and uptake of cholesterol [10]. Meanwhile, binding sites with a high affinity for HDL (K_d=1-2.5 μg/ml) have been identified on different cell types [2]. The ability of anti-apoA-I monoclonal antibodies to block HDL binding [6] may be associated with participation of these binding sites in the removal of cholesterol via HDL.

It is unclear whether these binding sites are involved in reverse cholesterol transport, since their physico-chemical properties and interaction with HDL are poorly investigated. In the present study we attempted to determine the parameters of binding, define the conditions for identification of high-affinity receptors, and examine the effects of some sex steroids on the expression of HDL-binding sites.

MATERIALS AND METHODS

Adult mature Wistar rats (250-300 g) of both sexes were used.

HDL_3 (d=1.125-1.216 g/cm^3), low-density lipoproteins (LDL, d=1.0219-1.063 g/cm^3), and very

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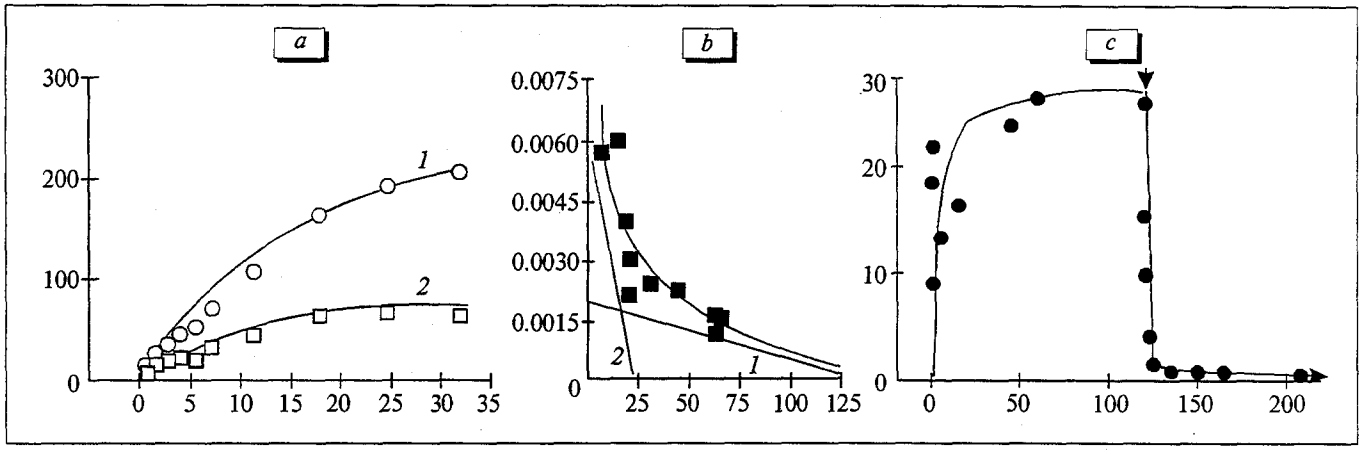

Fig 1. Kinetics of ^{125}I-HDL$_3$ binding to cultured hepatocytes. *a*) isotherms for ^{125}I-HDL$_3$ binding. Abscissa: concentration of ^{125}I-HDL$_3$, μg/ml; ordinate: bound ligand, ng/mg cell protein. Total (*1*) and specific (*2*) binding; *b*) Scatchard plots of specific binding. Abscissa: specifically bound ^{125}I-HDL$_3$, ng/mg cell protein, ordinate: the ratio between specifically bound and free ligands. High-affinity (*1*) and low-affinity (*2*) components of specific binding constructed with the use of LIGAND software. The data are the means of 3 parallel determinations in one of 5 typical experiments; *c*) rates of ^{125}I-HDL$_3$ association and dissociation from rat hepatocytes. The concentration of ^{125}I-HDL$_3$ was 5 μg/ml. The curves represent one of four typical experiments. Abscissa: time, min; ordinate: bound ^{125}I-HDL$_3$, ng/mg cell protein.

low-density lipoproteins (VLDL, d=1.006-1.019 g/cm^3) were isolated from healthy donors' plasma by preparative ultracentrifugation in a NaBr solution [8]. The purity of lipoprotein preparations was checked by the method [3]. Labeling of HDL with ^{125}I was performed as described elsewhere [3,13]. More than 98% of radioactivity was associated with protein. The specific radioactivity ranged from 300 to 1000 dpm/ng.

The hepatocyte suspension was prepared by *in situ* perfusion of the liver with a collagenase-containing solution [1]. Hepatocytes were cultured on collagen-coated plastic in William's E medium (Flow Lab.) supplemented with 5 μg/ml insulin (Sigma), 100 μg/ml kanamycin (Sigma), 20 mM HEPES (Flow Lab.), and 10^{-7} M dexamethasone (Sigma). Estradiol-17 and testosterone were added to a final concentration of 10^{-5} M (Sigma). Culture medium was replaced every day. The viability and functional activity of cultured cells were the same as in previous experiments [1,3].

The HDL$_3$—hepatocyte binding was determined by the method [3]. Hepatocytes were incubated with 0.1-40 μg/ml labeled HDL in the absence and presence of a 20-fold excess of unlabeled HDL (for determination of nonspecific binding) for 2 h at 4°C. The cells were then washed with 10 volumes of ice-cold Hanks' solution (Flow Lab.) for 1-2 sec, and incubated for 10-15 min on ice in Hanks' solution containing 100 μg/ml dispase and 100 μg/ml protease (Sigma). Specific binding was calculated as the difference between the radioactivities of the lysates obtained after incubation of hepatocytes with and without the excess of HDL$_3$. The dissociation constant (K_d) for the HDL-receptor binding and the receptor concentration (N) were determined by the method of Scatchard [11]. The protein content was measured as described [4].

For determination of the association constant (k_{+1}) cultured cells were incubated for varied time periods with 5 μg/ml ^{125}I-HDL$_3$, the reaction was terminated by adding a 20-fold excess of native HDL$_3$, and the amount of bound ligand was determined. Washing and radioactivity measurements were performed as described above. In order to de-

TABLE 1. Kinetic Parameters of ^{125}I-HDL$_3$ Binding to Rat Hepatocytes

Binding site	k_{+1}, min^{-1}(μg/ml)$^{-1}$	k_{-1}, min^{-1}	association $t_{1/2}$, min	dissociation $t_{1/2}$, min	K_d, μg/ml
High-affinity	0.041	0.123	10	3	3.075* 2.28±0.44** (12)
Low-affinity	0.00018	n.d.	50	n.d.	20±2* n.d.**

Note. *Calculated as $K_d=k_{-1}/k_{+1}$; **From the saturation analysis data; the number of determinations is given in parentheses; n.d. = not determined.

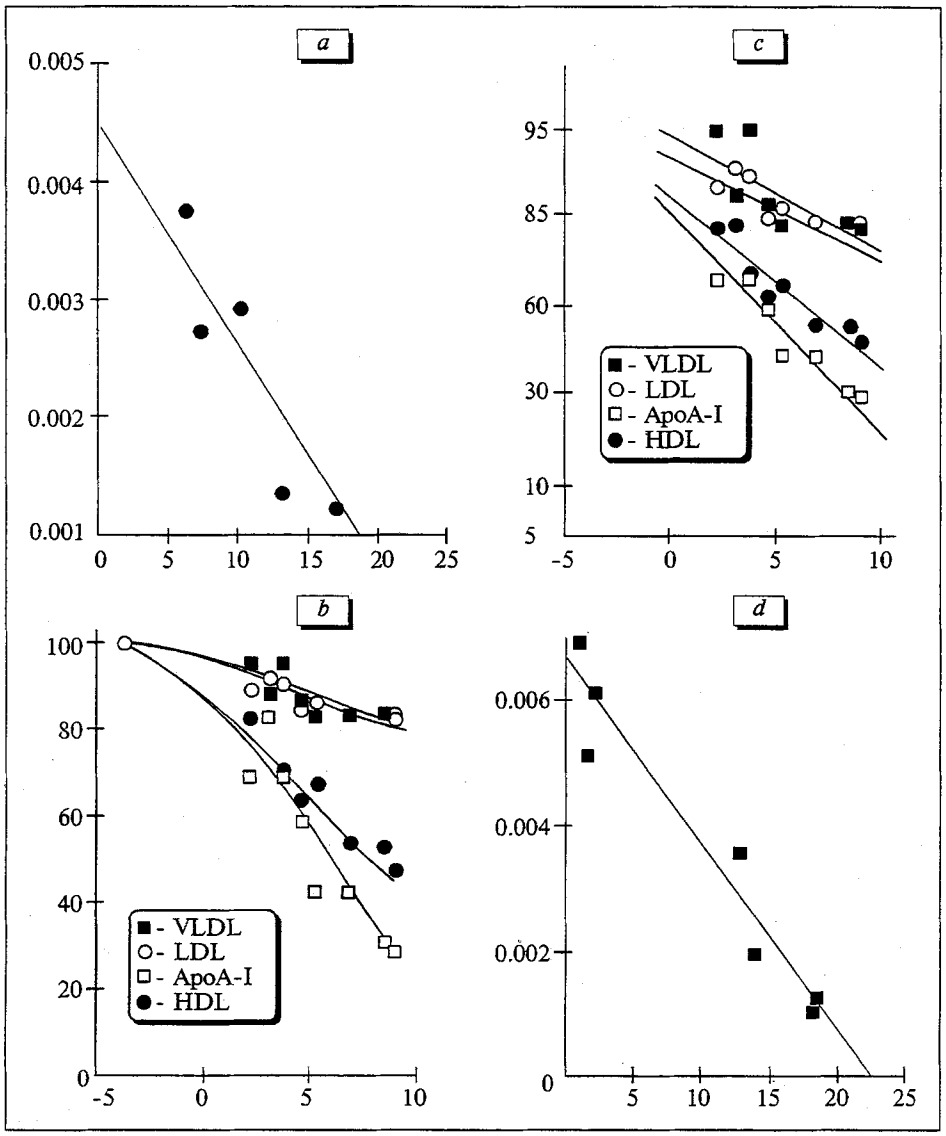

Fig. 2. Competitive inhibition of ^{125}I-HDL$_3$ binding during short-term incubation with hepatocytes. *a*) binding of ^{125}I-HDL$_3$ to hepatocytes. Abscissa: specifically bound ^{125}I-HDL$_3$, ng/mg cell protein. Ordinate: the ratio between bound and free ligands; *b*) abscissa: concentration of added compounds (lg); ordinate: bound HDL$_3$, % of the control; *c*) abscissa: concentrations of added compounds (lg); ordinate: bound HDL$_3$ in logit coordinates. Results of one typical experiment. Each point is the mean arithmetic ($M<m<10$) of three parallel determinations; *d*) Scatchard plots of the binding between delipidated apoA-I and HDL-binding sites. Abscissa: bound ^{125}I-HDL$_3$, ng/mg cell protein; ordinate: the ratio between bound and free ligands.

termine k_{+1} during the entire process of the receptor—ligand complex formation, the interaction between HDL$_3$ and the "receptors" was regarded as a second order reversible reaction; K_d and N were determined in the same experiments. Assuming that B_e and B are the equilibrium and current concentrations of the bound ligand, respectively, and F is the initial concentration of free ligand, the following equations can be written: $\ln(B_e^*-B)/(B_e-B)=k_{+1}\times t\times(B_e^*-B_e)+\ln(B_e^*/B)$ and $B_e^*=N+F+K_d-B_e$.

The association constant was calculated using the $\ln(B_e^*-B)/(B_e-B)$ *vs.* time plots. For determination of the dissociation constant (k_{-1}) hepatocytes were incubated with ^{125}I-HDL$_3$ for 2 h on ice to reach equilibrium, then a 20-fold excess of native HDL was added, the cells were washed with ice-cold Hanks' solution, and fresh growth medium was added. Under these conditions, k_{-1} can be calculated using a first-order equation and a plot of $\ln(B/B_o)$ *vs.* time, where B_o is the initial concentration of the "receptor"-bound HDL.

The specificity of the HDL$_3$-receptor binding was determined by competitive analysis [5]. Hepatocytes were incubated for 1 h at 4°C in the presence of 1.5 μg/ml ^{125}I-HDL$_3$ and increased concentrations of unlabeled competitors (LDL, apoA-I, and VLDL). Washing and radioactivity measurements were performed as described above. The logarithm of the concentration of unlabeled competitors was plotted against the amount of "receptor"-bound HDL$_3$, and the concentration at which the binding of labeled ligand is inhibited by 50% was determined. In this case the relative competitive activity of a given lipoprotein particle or apoprotein can be calculated as (concentration of HDL$_3$/concentration of the competitor)×100%, suggesting that the inhibiting concentration of a competitor is inversely proportional

to its relative affinity for the HDL-binding protein. The relative competitive activity of HDL_3 was taken as 100%. Sigmoid curves were transformed into linear curves using logit transformation for the ordinate [9]: $\text{logit}(P)=\ln[P/(100-P)]$, assuming that P is the percent of bound HDL_3.

RESULTS

In order to prevent internalization and degradation of the HDL-binding protein complexes all procedures were carried out at 4°C. Previously, we found that there is no difference in the determination of the "classic" HDL-"receptor" with the use ofHDL_3 labeled by the method [3] (specific activity 300-500 dpm) or by the method [13] (specific activity 1000 dpm).

Fig. 1, *a*, *b* illustrates dose-dependence of ^{125}I-HDL_3. Analysis of the results obtained in Scatchard plots using the LIGAND software [7] suggests the presence of two binding sites with the following parameters: K_{d1}=20 μg/ml and N_1=130 ng/mg protein (P_1, low affinity) and K_{d2}=2.4 μg/ml, N_2=25 ng/ml protein (P_2, high affinity) on the surface of rat hepatocytes. However, from the shape of the curves plotted in Scatchard coordinates the negative cooperativity occurring upon the interaction of a ligand with two mutually dependent binding centers cannot be distinguished from complexation of the ligand with two independent binding centers. This can be done with the help of kinetic curves. A concentration of 5 μg/ml ^{125}I-HDL_3 was used in kinetic experiments. Dissociation and association constants are listed in Table 1, and the corresponding curves are shown in Fig. 1, *c*. The constants were calculated using a KINETIC software [7]. The maximum ligand-"receptor" binding was observed after 2 h of incubation; however, about 80% of ^{125}I-HDL_3 was bound after 50-60 min of incubation. Analysis of the curve showed that two types of HDL-binding site complexes ($p<0.001$) were formed. These complexes have different association rates: k_{+1}=0.04[$\text{min}^{-1}(\mu\text{g/ml})^{-1}$], $t_{1/2}$=10 min and k_{+1}*=0.00018 [$\text{min}^{-1}(\mu\text{g/ml})^{-1}$], $t_{1/2}$=50 min. It should be remembered that dissociation was studied by two methods: incubation with an excess of HDL_3 followed by its removal and manifold dissolution of the system after the equilibrium had been reached. Figure 1, *c* shows the dissociation curve obtained by one of these methods. The curve is biphasic. The rapidly dissociating component has $t_{1/2}$=2 min and k_{-1}=0.123. We failed to determine these parameters for the slowly dissociating component. The equilibrium K_d calculated using the association and dissociation constants is practically the same as that obtained for the rapidly dissociating component by saturation analysis (Table 1).

TABLE 2. Effects of Estradiol-17 and Testosterone on the Expression of P_2 on Cultured Hepatocytes From Male and Female Rats (*M±m*, Three Determinations)

Experimental conditions	K_d, μg/ml	N, ng/mg cell protein
Males (control)	2.5±0.3	22±2.7
Males+estradiol-17 (10^{-5} M)	2.5±0.2	54±6.4
Females (control)	1.58±0.2	65±5.4
Females+testosterone (10^{-5} M)	1.52±0.2	38±3.4

Thus, the use of ^{125}I-HDL_3 with high specific activity and reduction in the time of washing allowed us to detect two types of binding sites on the surface of cultured hepatocytes. Since similar values for the dissociation constant were obtained by two methods, negative cooperativity can be ruled out, and the presence of two independent binding sites ("receptors") for HDL_3 on rat hepatocytes can be postulated. These binding sites differ by the affinity for HDL_3 (their equilibrium constants differ by an order of magnitude) and concentration. Our finding shows that more labile complexes are formed between HDL_3 and the high-affinity binding site is consistent with the published data [2].

Taking into account the substantial difference between the association and dissociation times for the complexes formed by HDL_3 with two types of "receptors", we shortened the incubation period and lowered the concentration of ^{125}I-HDL_3 to 0-20 μg/ml. Under these conditions, the Scatchard plot of the ligand-receptor binding looks like a unicomponent curve with K_d=2.5 μg/ml and N=22 ng/mg cell protein (data not shown), which agrees with the parameters of the high-affinity binding site (Fig. 2, *a*, Table 1).

Using this approach, we attempted to assess the specificity of the HDL_3-P_2 binding in a hepatocyte culture. As Fig. 2, *c*, *d* shows, HDL_3, and apoA-I but not LDL and VLDL complete for the binding sites. ApoA-I has the highest affinity for the high-affinity HDL binding sites. The relative competitive activity of apoA-I is 250%, which is higher than that of HDL_3. Scatchard plot of the apoA-I binding (Fig. 2, *d*) is represented by a unicomponent curve with K_d=1.92 μg/ml and N=23 ng/mg cell protein, which are similar to those for HDL_3 (Table 1). The reported competition between LDL and VLDL of the HDL-"receptor" indicates that P_1 and P_2 have different binding properties. It is likely that P_2 bind only apoA-I. The high-affinity binding sites for HDL_3 with similar characteristics were identified by others [6].

Since sex steroids modulate the expression of HDL binding sites on a cell surface [12], we decided to examine the effects of estradiol and testosterone on the expression on the high-affinity binding sites for HDL_3. Hepatocytes were incubated for 24 h with

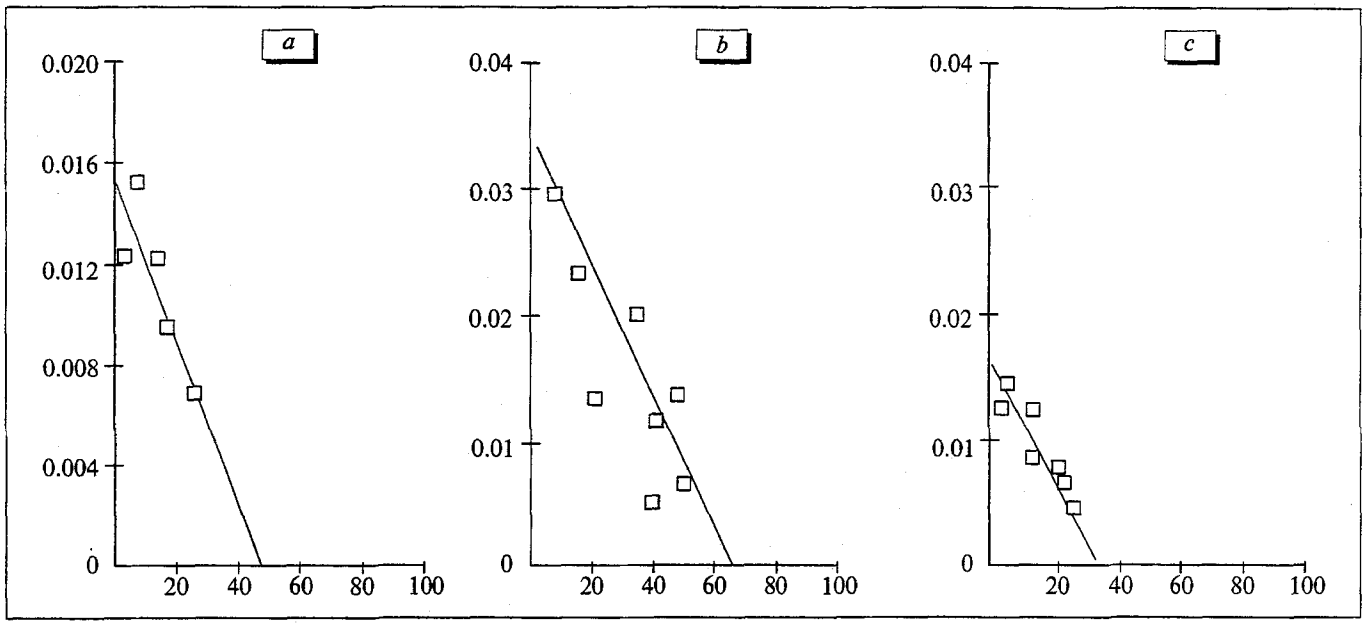

Fig. 3. Effect of estradiol and testosterone on the expression of HDL-binding sites on hepatocytes of male and female rats. Abscissa: specifically bound ^{125}I-HDL$_3$, ng/mg cell protein; ordinate: the ratio between bound and free ligand. *a*) effect of estradiol on the expression of HDL-binding sites on hepatocytes from male rats; *b*) expression of HDL-binding sites on hepatocytes from male rats; *c*) effect of testosterone on the expression of HDL-binding sites on hepatocytes from female rats.

10^{-5} M estradiol. The Scatchard plot (Fig. 3, *a*) shows that K_d remains unchanged (2.5 μg/ml), while the number of binding sites increases 2-fold.

Thus, if estrogens modify the expression of HDL binding sites via the corresponding receptors but not as sterol compounds, it is reasonable to suggest that the number of these binding sites is different in males and in females. Figure 3, *b* shows the dose-dependence for ^{125}I-HDL$_3$ binding to cultured hepatocytes from female rats. Analysis with the use of LIGAND software showed that a unicomponent curve is the best fit ($p<0.05$) for the binding between HDL$_3$ and its "receptors". The K_d calculated from this curve (1.58 μg/ml) is comparable to that for the high-affinity "receptor" on hepatocytes from males, while the number of binding sites in females is 2-fold higher. The low-affinity binding site (P_1) was not identified on cultured hepatocytes from female rats.

We then tried to find out whether testosterone regulates the expression of the high-affinity binding sites on cultured hepatocytes from female rats. After incubation with 10^{-5} M testosterone the number of P_2 decreased. It should be noted that K_d remains unchanged (Fig. 3, *c*). We did not determine the number of binding sites; however, under the standard conditions N increases 2- to 3-fold (data not shown).

Thus, two types of HDL-binding sites were identified on the surface of rat hepatocytes. These binding sites have different kinetic parameters, and different lipoproteins have different abilities to compete for them. The high-affinity binding sites predominate over the low-affinity binding sites after short-term incubation due to a higher rate of formation of the ligand-receptor complexes. Short-term incubation revealed stimulating effect of estrogen and inhibitory effect of androgen on the expression of the high-affinity HDL-binding sites (Table 2).

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