Identification of Specific High Density Lipoprotein-binding Sites in Rat Testis and Regulation of Binding by Human Chorionic Gonadotropin*

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Yii-Der I. Chen‡, Fredric B. Kraemer, and Gerald M. Reaven

From the Department of Medicine, Stanford University School of Medicine and Geriatric Research, Education and Clinical Center, Veterans Administration Medical Center, Palo Alto, California

Freshly prepared rat testicular membranes bind iodinated rat high density lipoprotein (HDL) with high affinity ($K_d = 32 \mu g$ of HDL protein/ml). This high density lipoprotein binding differs from low density lipoprotein binding by cultured human fibroblast cells in two ways: it is not affected by Ca2+ or ethylenediaminetetraacetic acid, and it is not sensitive to pronase and trypsin. Testicular binding activity is primarily found in interstitial tissue containing Leydig cells and can be modulated by human chorionic gonadotropin administration in vivo (2-fold increase of binding capacity, with no affinity change) with 250 units/kg of human chorionic gonadotropin injection daily for 4 days. The interstitial high density lipoprotein binding site also recognizes rat very low density lipoprotein, as shown by displacement experiments. When membrane preparations of various other tissues were assessed for their high density lipoprotein binding, we found that the adrenal gland binds rat high density lipoprotein with similar affinity and capacity, while spleen, kidney, and heart showed no high affinity binding. In addition, when iodinated rat low density lipoprotein was tested for its ability to bind to testicular membranes, no high affinity saturable binding was observed. We conclude that there are specific high density lipoprotein-binding sites present in steroidogenic tissues, but these binding sites are not found in the nonsteroidogenic tissues tested. Furthermore, no high affinity low density lipoprotein-binding sites can be demonstrated in the testis; thus it appears that high density lipoprotein, rather than low density lipoprotein, is the major cholesterol-carrying lipoprotein recognized by the rat testis.

Several recent papers have suggested that high density lipoprotein, rather than low density lipoprotein, serves as the major source of cholesterol for rat steroidogenic tissues. For instance, Gwynne et al. (1) have indicated that the transfer of labeled cholesterol from HDL¹ into incubated adrenal glands was three times greater than from LDL. While the importance of HDL as a source of cholesterol for steroidogenic tissue has been reinforced by studies using the drug, 4-amino-pyrazolopyrimidine, an adenine analog which inhibits hepatic lipoprotein secretion and results in markedly reduced plasma cholesterol levels (2, 3). Sterol synthesis by steroidogenic and other extrahepatic tissues was increased in rats treated with APP (4–9), and infusion of HDL was more effective than infusion of LDL in suppressing sterol synthesis in steroidogenic tissues (6, 7, 8). In contrast, the increase in sterol synthesis in nonsteroidogenic tissue (intestine, kidney, etc.) from APP-treated rats was suppressed more effectively by LDL infusion (7, 8). These data suggest that both HDL and LDL can serve as a source of intracellular cholesterol and that differences in the location of specific cell surface lipoprotein receptors might play a role in regulation of cholesterol uptake by various tissues. In this regard, Kovanen et al. (9) have demonstrated that in one steroidogenic tissue, the mouse adrenal gland, HDL and LDL are taken up by two distinct mechanisms, and that the LDL cholesterol uptake is via receptor-mediated absorptive endocytosis (10, 11). However, essentially no information is available concerning the mechanism of HDL cholesterol uptake. If adrenal gland, testis, and ovary take up and utilize HDL cholesterol in preference to LDL cholesterol, it seems reasonable to assume the existence of specific HDL receptors on these cells. Such receptors have yet to be described in rat steroidogenic tissues. Therefore, the present experiments were undertaken in an effort to see if specific HDL-binding sites could be demonstrated in the testis and the adrenal gland.

EXPERIMENTAL PROCEDURES

Materials
Male Sprague-Dawley rats (200 to 250 g or retired breeders) were obtained from a local breeder (Simonsen Laboratories, Gilroy, CA). Hepes, Tris-HCl, collagensesodium EDTA, sodium azide, pronase, and human chorionic gonadotropin were purchased from Sigma Chemical Co. (St. Louis, MO). Bovine serum albumin was obtained from Armour Pharmaceutical Co. (Chicago, IL). Aquadide was purchased from Calbiochem-Behring Co. (San Diego, CA). Trypsin (1:250) was obtained from Difco Laboratories, Inc. (Detroit, MI). Newborn calf serum was ordered from Grand Island Biological Co. (Santa Clara, CA). Sodium (14)iodide (carrier free) in NaOH was obtained from New England Nuclear (Boston, MA). Sephadex G-25 (coarse) was purchased from Pharmacia Fine Chemicals, Inc. (Piscataway, NJ). All other chemicals were reagent grade from J.T. Baker Chemical Co. (Phillipsburg, NJ) or Fisher Scientific Co. (Pittsburgh, PA).

Methods

Serum Lipoprotein Isolation

Serum was pooled from 20 male retired breeders fed a fat-free diet overnight. Rat VLDL (d < 1.000), LDL (d = 1.019 to 1.040), and HDL (d = 1.090 to 1.215) were isolated from this serum by sequential...
ultracentrifugation (12) using solid KBr for density adjustment (13) in a 60 Ti rotor at 55,000 rpm for 20 h. Human VLDL (d < 1.005), LDL (d = 1.019 to 1.063), and HDL (d = 1.090 to 1.215) were isolated similarly from nonpyrogenic pooled human plasma. (The bulk of the chylomicrons was removed from the top of the spun sample after preliminary centrifugation at 10,000 × g for 1 h.)

The collected lipoproteins were dialyzed exhaustively against 0.15 M NaCl, 10 mM Tris-HCl (pH 7.4), and 0.001% sodium azide; they were subsequently concentrated by loosely packing the outside of the dialysis bag with Aquacide and sterilized by passing through a 0.45-μm Millipore filter. After addition of 10 μg/ml of gentamicin sulfate, 0.02% sodium azide, and 0.01% EDTA, the lipoproteins were stored at 4°C for no more than 40 days. The purity of the lipoproteins was verified by agarose electrophoresis (14) using 0.5% agarose for human lipoproteins and 1% agarose for rat lipoproteins (15).

**Iodination**

The lipoproteins were iodinated using the iodine monochloride method as described by MacFarlane (16) and modified by Bielheimer (17). Free 125I was separated from iodinated lipoproteins by passing through two G-25 columns packed in 20-cc syringes, followed by exhaustive dialysis against isotonic saline containing 10 mM Tris-HCl (pH 7.4) and 0.001% sodium azide. Iodinated lipoproteins were sterilized and stored as described above. Specific activities of iodinated rat VLDL, LDL, and HDL varied between 20 and 150 cpm per ng of lipoprotein. Iodinated lipoproteins retained their electrophoretic characteristics on agarose gels, migrating slightly faster than noniodinated lipoproteins.

**Tissue Preparations**

Tissue from decapitated rats was rinsed in chilled saline, decapsulated, and stored in parafilm pockets in liquid nitrogen. On the day of the experiment, testis samples were weighed, thawed, and homogenized in 4 volumes (w/v) of Tris-HCl buffered saline (0.15 M) in a Duell glass homogenizer. The homogenate was centrifuged at 500 × g for 5 min. In some experiments, the supernatant (500 × g) was centrifuged again at 8000 × g for 15 min. Since whole homogenate, 500 × g supernatant, or 8000 × g supernatant gave similar binding results, 500 × g supernatant samples were chosen for most studies and will be referred to as “crude membrane preparations” in this report. Similar 500 × g supernatants were prepared from homogenates of other tissues studied.

**Binding Assay**

A. Lipoprotein Binding — Incubation of lipoproteins with testicular crude membrane preparations was carried out in disposable tubes (12 × 75 mm) precoated with 5% BSA. The final volume was 0.25 ml containing 20 mM Tris-HCl (pH 7.4), 0.5% BSA, isotonic saline, and varying concentrations of 125I-labeled rat VLDL, LDL, or HDL for each experiment. Non-specific binding was estimated by addition of at least 100-fold excess of unlabeled human lipoproteins to duplicate tubes. Aliquots of the sample (200 μl) were layered onto 1 ml of chilled newborn calf serum, and bound lipoproteins were separated from free lipoproteins by centrifugation (30,000 × g for 30 min). The pellet was washed once with 1 ml of chilled calf serum, and then the tip of the tube (containing the pellet material) was cut off and counted in a γ scintillation counter (Searle Analytic Inc., model 1185).

B. hCG Binding — hCG binding was performed according to Chen and Payne (18), with the following modifications. Supernatants (500 × g) of the tissue homogenates were incubated with varying amounts of 125I-hCG (0.7 ng to 7.0 ng; specific activity 30,000 cpm per ng of CR 121 hCG) in 0.2 ml final volume of the same reaction mixture as described in paragraph A. Non-specific binding was estimated by addition of at least 100-fold excess of unlabeled hCG (Sigma, 250-unit ml) to duplicate tubes. The binding reaction was terminated with addition of 1 ml of chilled isotonic saline containing 50 mM Tris-HCl and 2% BSA and centrifuged at 30,000 × g for 30 min. The resulting pellets were washed with 2 ml of the same buffered saline, and bound radioactivity was determined by a γ scintillation spectrometer.

**Separation of Interstitial Tissue from Seminiferous Tubules**

Interstitial tissue containing Leydig cells was separated from seminiferous tubules according to Shaw et al. (19). Briefly, the testis was treated with 0.5 mg of collagenase in 2 ml of Hepes-Ringer buffer containing 0.5% glucose and 1% BSA for 5 min at 37°C. The tissue was placed in a chilled Petri dish containing buffer, and sharp tipped forceps were used to tease the seminiferous tubules away from the interstitial tissue. The separated interstitial tissue and tubular fractions were cleaned further and rinsed 3 to 5 times in chilled buffer.

**Crude membrane preparations (500 × g supernatant) were obtained from these fractions as described above.**

**hCG Administration**

hCG obtained from Sigma (5,000 IU) was prepared in isotonic saline (250 units/ml). Rats, weighing 200 to 250 g, were injected subcutaneously with 250 units/kg of body weight of hCG daily for 4 days. Control rats received an equal volume of saline solution. No significant difference in plasma cholesterol levels (mg/dl; mean ± S.E., n = 4) was observed between control (57 ± 4) and hCG-treated rats (45 ± 5).

**Protein Assays**

Protein in human and rat VLDL and LDL preparations was estimated by the Lowry method (20) with sequential extraction of lipid with chloroform and ether after color development (21). Protein in HDL and tissue preparations was estimated by the Coomassie colorimetric method (22), since it is more rapid. The results of the Coomassie assay were verified using the Lowry method as described above. No significant discrepancies were observed, except with triglyceride-rich lipoproteins. Bovine serum albumin was used as a standard for both assays.

**RESULTS**

The data in Fig. 1 show the time course of 125I-HDL binding to crude testicular membrane preparations (2 mg of membrane protein/ml; 24 μg of 125I-HDL/ml) at 37°C. The binding reaction reached equilibrium by 40 min and remained constant for at least 2 h. 125I-HDL binding was reduced by 80 to 95% in the presence of a 100-fold excess of unlabeled HDL.

Fig. 2 shows 125I-HDL binding at 37°C with varying concentrations of crude testicular membrane protein. A linear increase in binding activity was found as the testicular membrane protein concentration was increased from 0.8 to 3.2 mg/ml.

Fig. 3A shows the amount of 125I-HDL binding to the testicular membrane preparations at 37°C as a function of the concentration of 125I-HDL in the assay mixture. Specific binding (total binding minus the binding in the presence of excess unlabeled HDL) displayed saturation kinetics, with maximal binding achieved at approximately 90 μg of HDL protein/ml. When these data are transformed into a Scatchard plot (Fig. 3B), a single binding site is seen with a Kd of approximately 2.0 × 10–9 M. The data in Fig. 1 show the time course of 125I-HDL binding to crude testicular membrane preparations (2 mg of membrane protein/ml; 24 μg of 125I-HDL/ml) at 37°C. The binding reaction reached equilibrium by 40 min and remained constant for at least 2 h. 125I-HDL binding was reduced by 80 to 95% in the presence of a 100-fold excess of unlabeled HDL.

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are binding to the same site through the recognition of an apoprotein (apo E?) common to both. In order to further evaluate this possibility, we assessed the ability of VLDL to displace the binding of \(^{125}\text{I}-\text{HDL}\). These data are displayed in Fig. 5. The fact that VLDL displaced \(^{125}\text{I}-\text{HDL}\) indicates that VLDL can bind to the HDL-binding site. On the other hand, LDL did not displace \(^{125}\text{I}-\text{HDL}\) significantly. This is consistent with the observation that iodinated LDL did not show any saturable high affinity binding to testicular membranes.

Since less than 1% of the testes consist of steroiogenetic tissue (the Leydig cells), it is important to clarify whether the HDL binding sites are present uniformly throughout the whole testis or localized in the Leydig cell fraction. Testicular tissue was separated into interstitial tissue (Leydig cell-rich fractions) and seminiferous tubules, and crude membranes were prepared as previously described. Since hCG receptors are located solely on Leydig cells (23), purity and contamination of the fractions were determined by \(^{125}\text{I}-\text{hCG}\) binding. As displayed in Fig. 6A there is approximately a 10-fold enrichment of \(^{125}\text{I}-\text{hCG}\) binding in the interstitial fraction, however, there is clearly a small amount of contamination of the tubular fraction by Leydig cells. When \(^{125}\text{I}-\text{HDL}\) and \(^{125}\text{I}-\text{VLDL}\) binding was performed on these fractions as previously described,

32 \(\mu\text{g}\) of HDL protein/ml and a binding capacity (Bo) of 1100 ng of HDL protein/mg of testicular membrane protein. Also shown in Fig. 3B is the Scatchard plot for \(^{125}\text{I}-\text{HDL}\) specific binding activity at 4°C, which has a higher \(K_d\) (approximate 70 \(\mu\text{g}\) of HDL protein/ml) and a lower Bo (approximately 660 ng of HDL protein/mg of membrane protein).

The ability of LDL and VLDL to bind to testis membranes is illustrated in Fig. 4. When \(^{125}\text{I}-\text{LDL}\) was used as the ligand, no saturable specific binding was observed (Fig. 4A). However, when \(^{125}\text{I}-\text{VLDL}\) was used as the ligand, a high affinity saturable binding site was found (Fig. 4B), with a \(K_d\) of approximately 4 \(\mu\text{g}\) of VLDL protein/ml and a Bo of 720 ng of VLDL protein/mg of membrane protein when analyzed with Scatchard plot. The VLDL binding displayed similar physical characteristics to the HDL binding such as temperature effect, time course, cation effect, and insensitivity to trypsin digestion (see Fig. 9 and Table I), and it is likely that the two ligands

![Fig. 3. Saturation curve (A) and Scatchard plot (B) of \(^{125}\text{I}-\text{rat HDL}\) binding to testicular membranes.](image)

![Fig. 4. Binding of \(^{125}\text{I}-\text{rat LDL}\) (A) and \(^{125}\text{I}-\text{rat VLDL}\) (B) to testicular membranes.](image)

![Fig. 5. Displacement of \(^{125}\text{I}-\text{rat HDL}\) binding by unlabeled rat HDL (C), rat VLDL (D), and rat LDL (A). Binding of \(^{125}\text{I}-\text{rat HDL}\) at concentrations of 60 \(\mu\text{g}\) of HDL protein/ml was used as 100% bound. Testicular membrane proteins, 2.4 mg/ml.](image)
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Figure 6. Scatchard plots of hCG binding (A), HDL binding (B), and VLDL binding (C) to crude membranes of separated testicular components. ○, interstitial fraction; ●, seminiferous tubular fractions. Binding condition is the same as described for Fig. 3 with the Scatchard plots (Fig. 6, B and C) of the binding data clearly show that HDL and VLDL binding to the Leydig cell fraction have greater affinity and capacity than the tubular fraction. In fact, most of the 125I-VLDL and a portion of the 125I-HDL binding to the tubular fraction can be attributed to contamination with Leydig cells. Thus, HDL-binding sites appear to be concentrated in the steriodogenically active portion of the testis.

In order to further establish that the HDL-binding sites are localized in the Leydig cells and to see if this binding can be modulated physiologically, we examined the effect of hCG administration on HDL binding to membrane preparations of separated testicular components. hCG is known to stimulate steroid (testosterone) production following its binding to specific receptors located solely on Leydig cells (23). Fig. 7 shows that hCG (50 units/day for 4 days) caused a 220% increase in HDL binding to membranes of the interstitial tissue fraction with no effect on binding affinity. The increased binding is not a function of Leydig cell hyperplasia or hypertrophy since the data are expressed per mg of interstitial tissue membrane protein. Thus, the results demonstrate an increase in the number of HDL-binding sites per unit of protein. In addition, no effect of hCG administration was demonstrated on HDL binding to membranes of the tubule fraction. This result further strengthens the conclusion that the Leydig cells are the testicular component which possess specific binding sites for HDL.

To further verify the concept that HDL binding occurs predominantly in steroidogenic tissue, we examined several

Figure 7. Effect of hCG on 125I-rat HDL binding to membranes of separated testicular components depicted as Scatchard plots. ○, interstitial tissue fractions; ●, seminiferous tubular fractions.

Figure 8. Binding of 125I-rat HDL to various tissue preparations. Membrane protein concentrations added to binding assays were 2.1 mg/ml, 0.8 mg/ml, 4.4 mg/ml, 3.2 mg/ml, and 5.2 mg/ml for testis ( ○), adrenal ( ●), spleen ( ▲), heart ( ■), and kidney ( ●), respectively.

interstitial tissue protein concentration equaled 200 μg/ml while seminiferous tubular proteins equaled 2.4 mg/ml.
Other tissues in the rat. As seen in Fig. 8, adrenal tissue possessed HDL-binding sites with affinity and capacity similar to testicular binding sites. Kidney, heart, and spleen failed to display any specific saturable HDL binding.

The effects of cations on the specific binding activity are shown in Fig. 9. Mn²⁺, Ca²⁺, EDTA, and EGTA caused no significant changes in HDL binding. These effects are similar to those seen in the binding of chylomicron remnants and HDL to liver (24, 25) but are different from the Ca²⁺-dependent binding of LDL to cultured human fibroblasts (26) and bovine adrenal membranes (27). Similar results were observed with ¹²⁵I-VLDL binding.

Crude testicular membranes were treated with pronase in order to test whether these binding sites were sensitive to proteolytic enzymes. The results are shown in Table I. HDL binding was not reduced with either low or high concentrations of enzyme. A cleaner membrane preparation was obtained from isolated interstitial fractions and was treated with high doses of trypsin. Again, no significant change of HDL binding was demonstrated (Table II). Thus, HDL-binding sites appear to be inert to at least two preparations of proteolytic enzymes.

**DISCUSSION**

In this paper we have demonstrated the existence of high affinity saturable binding sites for HDL in testis and adrenal glands, thus extending previous observations (1, 5–9) which suggested that there is selective utilization of HDL cholesterol in steroidogenic tissues in the rat. (At 37°C the Kᵦ for HDL binding was 32 µg/ml and the Bₒ was 1100 µg/mg of membrane protein. LDL did not effectively compete for the binding site, while both HDL and VLDL effectively displaced labeled HDL. The HDL binding was affected by temperature but displayed no cation dependence or sensitivity to proteolytic enzymes.) We could not demonstrate HDL binding sites in the three nonsteroidogenic tissues we tested, suggesting that the HDL-binding sites are organ specific. Furthermore, hCG was shown to increase the number of binding sites in the testis.

The HDL binding observed in our studies resembles the human HDL binding to rat liver membranes described by Kovanen et al. (25) in several aspects. First, half-maximal binding to liver membranes at 4°C was 75 µg/ml, which is similar to the Kᵦ at 4°C of 70 µg/ml that we found. Second, no effects of cations (Ca²⁺ or EDTA) or of pronase were noted in either system. Third, LDL did not compete with HDL for binding. The characteristics of this HDL binding are in contrast to the LDL system where the binding site requires divalent cations for activity, is destroyed by treatment with pronase, and displays a preference for binding LDL as compared with HDL (28).

In an effort to more clearly define the relationship between HDL binding and steroidogenesis, we separated testicular tissue fractions rich in either Leydig cells or seminiferous tubules. HDL binding to testicular tissue from hCG-treated rats was, for all practical purposes, confined to the Leydig cell fraction, and the HDL binding in the tubular fraction could be almost entirely attributed to contamination. Thus, there appeared to be a striking relationship between steroidogenesis and HDL binding. However, under basal conditions there did appear to be HDL binding to the tubular fraction, which could not be entirely attributed to contamination with Leydig cells. On the other hand, when ¹²⁵I-VLDL binding was performed to further assess the distribution of binding sites in the testicular fractions, the results were similar to the ¹²⁵I-hCG binding, suggesting that binding sites are not present in the tubes. In any event, the HDL-binding data raise the possibility that LDL receptors might also exist on seminiferous tubules. If so, the biological significance of these sites is unclear at present.

Since binding was specific for HDL with cross-reactivity to VLDL, the recognition site for binding should be related to apoprotein constituents that are common to both HDL and VLDL. In the rat, HDL and VLDL contain significant percentages of apo E and apo C with only a small amount of apo A being found in VLDL. We are currently attempting to see which of these are involved in the binding reaction.

Although these data suggest that rat steroidogenic tissues obtain cholesterol as the result of the interaction between HDL and specific HDL receptors, they do not define the mechanism of the cholesterol transfer. At the present time, two different models of cholesterol transfer from lipoprotein into cells have been described. In the case of LDL, the entire molecule is internalized via "receptor-mediated endocytosis" and after a sequence of steps, free cholesterol is produced (28). However, in the case of chylomicron binding to endothelial cells in culture, it appears that cholesterol enters the cell without internalization of the apoprotein (29). The mechanism responsible for cholesterol transfer from rat HDL to steroidogenic tissue is currently being studied in our laboratory.

Finally, at least two classes of membrane-specific lipoprotein receptors have been identified in the rat to facilitate cholesterol transfer. One class of receptors, located in the liver, is specific for remnant binding (24) and is responsible for chylomicron remnants (25) and VLDL (26). The mechanism responsible for cholesterol transfer from LDL to steroidogenic tissues is currently being studied in our laboratory.
for hepatic removal of dietary cholesterol. The second class of lipoprotein receptors we have now described appears to provide cholesterol for steroidogenesis from HDL which carries 60% of the total plasma cholesterol. Whether other tissues in the rat utilize LDL or HDL or both particles as a source of cholesterol remains to be elucidated.

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