Uptake of Lipoproteins by In Situ Perfused Rat Ovaries: Identification of Binding Sites for High Density Lipoproteins

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ABSTRACT We have examined the uptake and distribution of 125I-labeled human high density lipoprotein, apolipoprotein E-free (hHDL3), 125I-rat high density lipoprotein (HDL), and human HDL (hHDL) reconstituted with [3H]cholesteryl linoleate after their in situ vascular perfusion to ovaries of gonadotropin-primed immature rats on days 6–9 post human chorionic gonadotropin (hCG)-injection. Some rats were treated with 4-aminopyrazolopyrimidine to reduce plasma lipoproteins and ovarian cholesteryl ester stores. Perfused ovaries were analyzed biochemically and autoradiographically, and progestin content of the ovarian effluent was quantified. Infusion of ovine luteinizing hormone and hHDL increased ovarian progestin secretion severalfold, indicating that the perfused ovary was functional. After perfusion with HDL reconstituted with [3H]cholesteryl linoleate, radioactive progestin appeared in the effluent; thus, sterol carried by exogenous HDL was converted to steroid. At 37°C, uptake of 125I-hHDL3 was greatest after 15 min of perfusion with label. This was decreased by 80% when the perfusion was carried out at 4°C and by 70–95% when excess unlabeled hHDL, but not human low density lipoprotein (hLDL), was included in the perfusate with 125I-hHDL. Amino-pyrazolopyrimidine treatment enhanced 125I-hHDL uptake twofold. After perfusion for 15 min with 125I-hHDL3, radioactivity in the ovary was high for 3–30 min of HDL-free wash, then declined 75% by 30–60 min. With light and electron microscope autoradiography, 125I-hHDL3 was localized to corpora lutea, both along luteal cell surfaces and over their cytoplasm. The plasma membrane grains appeared to be associated with segments that lacked bristle coats. Perfusion with 125I-rat HDL produced a similar pattern of labeling. In ovaries perfused with 125I-BSA, silver grains were concentrated over macrophage-like cells but were sparse over luteal cells. We conclude that the in situ perfused rat ovary takes up 125I-hHDL3 by a temperature-dependent, lipoprotein-specific process, and that this lipoprotein is accumulated by luteal cells.

Highly luteinized ovaries of rats appear to use exogenous cholesterol in the form of high density lipoproteins (HDL) as a primary source of sterol for the biosynthesis of steroid hormones (2, 23, 30, 33). HDL is taken up by steroid-secreting cells of the rat through a saturable process that seems to differ from the well characterized pathway for the uptake of low density lipoproteins (LDL) (3, 7, 19). Additional data supporting this view come from work of Kita et al. (18). They have shown that antibodies to the bovine adrenal LDL receptor do not block the uptake of 125I-HDL by mouse adrenal gland in vivo, whereas they inhibit the uptake of 125I-LDL by that tissue. Moreover, the sites involved in the uptake of HDL and LDL differ in several ways, including their requirements for divalent cations and sensitivity to proteases, and in the ability of heparin to displace the bound ligand (9, 10). The sites with which HDL associates thus appear to be distinctly different from those for LDL.

However, the identity of the cells that bear the putative HDL binding sites remains in question. Schuler et al. (30)
have suggested that in rat ovaries HDL is accumulated by parenchymal cells. On the other hand, Kovanen et al. (19), in studies of adrenal cortex, have raised the possibility that binding sites for HDL need not reside on adrenocortical cells; e.g., HDL may be taken up and processed by capillary endothelial cells. It is particularly important to identify which specific structures and cells in the ovary take up HDL because this organ has several compartments which make steroids, i.e., follicles, corpora lutea, and interstitial cells. Moreover, each contains several types of cells.

Most of the information available on the uptake and metabolism of lipoproteins, including HDL, by the ovary and other tissues has emerged from studies of dispersed or cultured cells or from work on whole animals. Each of these approaches, however, has certain limitations. For example, when cells are removed from their normal environment and placed in culture, they may express phenotypes not normally expressed in situ. As a consequence, even though in vitro studies have provided much valuable information on lipoprotein metabolism, it is not yet clear whether similar processes are used in the intact organ. There are also a number of problems in studies using whole animals, where labeled lipoproteins are injected into the systemic circulation. They include (a) metabolism of exogenous lipoproteins by other organs before they reach the tissue of interest; (b) exchange of lipoprotein components in the circulation; and (c) dilution of labeled lipoproteins by endogenous lipoproteins.

Clearly, there is a strong need to examine lipoprotein metabolism as it occurs in the intact organ and to identify by morphological means those compartments and cells that take up HDL. To this end, we studied the distribution and metabolism of 125I-labeled human and rat HDL after administration of these lipoproteins to ovaries of rats via in situ vascular perfusion. This approach offers several advantages over other systems previously employed. First, it permits the responses of ovarian cells to be studied under conditions that mimic the normal physiological situation. During in situ perfusion the ovary remains relatively undisturbed and, importantly, its constituent cells maintain their structural relationships with each other and the surrounding tissues. Moreover, the exogenous lipoproteins must cross the vascular wall to gain access to ovarian cells, as would occur in the normal situation. Secondly, lipoproteins are delivered directly to the ovary, thus avoiding the complications accompanying systemic administration.

Our main probe in these studies has been 125I-labeled human HDL3 (hHDL3). Subclasses of human HDL contain various amounts of apolipoproteins A-I, A-II, Cs, and E (12). Since apolipoprotein E is recognized by binding sites for LDL (21), we used human HDL that was essentially free of apolipoprotein E to insure that we were examining the HDL and not the LDL pathway.

**MATERIALS AND METHODS**

**Animals:** Highly luteinized ovaries were produced in immature CD-1 strain rats 21 d old (Charles River Breeding Laboratories, Inc., Wilmington, MA) by priming the rats with 50 IU of pregnant mare serum gonadotropin (Organon Inc., Oss, The Netherlands), followed 60 h later with 25 IU of human chorionic gonadotropin (hCG; Sigma Chemical Co., St. Louis, MO). Rats were used on days 6-9 after hCG injection, when HDL uptake is maximal (33). Some gonadotropin-primed rats received intraperitoneal injections of 4-aminopyrazolopyrimidine (APP, Aldrich Chemical Co., Inc., Milwaukee, WI), 10 mg/kg body wt, dissolved in sodium phosphate buffer, pH 3, for three successive days prior to use. This treatment lowers plasma cholesterol from 50 mg/dl to <15 mg/dl within 24 h and reduces ovarian cholesteryl ester stores (31).

**Isolation and Labeling of Lipoproteins and Albumin:** Human HDL (density = 1.019-1.063 g/ml) and human HDL (density = 1.125-1.21 g/ml) were isolated from blood of normal human donors or rats according to Havel et al. (14). HDL was prepared in this manner is essentially free of apolipoprotein E (10).

The lipoproteins were labeled with 35S by the iodine monochloride technique of Bilheimer et al. (5) to specific activities of 150-450 cpm/ng of HDL protein. Labeled rat lipoproteins contained somewhat higher amounts of 125I in lipid (10%) than did human lipoproteins (<2%). The percentage of radioactivity not precipitable by trichloroacetic acid (TCA) was always <2% for all labeled lipoproteins. BSA (Sigma Chemical Co.) was labeled with 125I using lactoperoxidase to a specific activity of 300 cpm/ng.

**Reconstitution of HDL with [3H]Cholesteryl Linolate:** 1.2,6,7-[3H]Cholesteryl linolate was reconstituted into hHDL apolipoproteins by the procedure of Hirz and Scanu (15). HDL was delipidated by the procedure of Scanu and Edelstein (28). Delipidated apolipoproteins were sonicated with a mixture of lipids such that the apolipoproteins constituted 57% by weight; egg phosphatidylcholine, 26% by weight; free cholesterol, 2% by weight; ester cholesterol ([3H]cholesterol linolate) 11% by weight; and tripalmitin, 4% by weight. The mixture of apolipoproteins and lipid was subjected to sonic irradiation and the sonicated material was then adjusted to a density of 1.215 g/ml and centrifuged to isolate hHDL, which was dialyzed extensively against 0.15 M NaCl containing 0.01% EDTA, pH 7.4.

Reconstituted HDL had a weight ratio of protein/sterol of 2:1. The specific activity of the [3H]cholesterol linolate was 5,077-9,787 dpm/nmol. Less than 1.6% of the H was in free cholesterol. Radioactivity in these preparations migrated with native HDL on cellulose acetate electrophoresis, and the reconstruction preparation gave a line of identity with HDL upon double immunodiffusion using a rabbit anti-human HDL antiserum.

**Analytical Methods:**

- **Progesterone** (11) and 20-a-hydroxyprogren-4-en-3-one (24) were quantitated by specific radioimmunoassays. Protein was determined by the method of Lowry et al. (20) using BSA as a standard. In some experiments, ovaries were homogenized in 2 ml of 0.9% saline, and aliquots were added to an equal volume of cold 20% TCA. After standing at 4°C for 30 min, the mixture was centrifuged at 3,000 g for 10 min and the resultant supernatant was counted. Aliquots of the homogenate were extracted with 5 vol of chloroform/methanol (2:1, vol/vol) and the radioactivity in the organic phase was quantified.

- A single mean was derived for all animals in a given treatment group and the means were compared among groups with one-way analysis of variance and subsequent Newman-Keuls' test.

**Perfusion of the Ovary and Collection of the Effluent:** Labeled and/or unlabeled human or rat lipoproteins, 125I-BSA, or ovine luteinizing hormone (LH) were delivered to the ovary using a modification of the perfusion method of Paavola (25). No attempt was made to separate the uterine artery from its companion vein since manipulation of these vessels brought about their constriction. Thus, the ligation securing the in-flow cannula was placed around both uterine vessels instead of around only the artery, as is the case for other animals. The ovary was then perfused at a flow rate of 1.5-1.5 ml/min, in sequence for varying periods of time with (a) Krebs-Ringer-bicarbonate buffer (KRB; 37°C, 300 mOsmol, pH 7.4, 2 mg/ml glucose, without lipoproteins) to wash out blood; (b) KRB containing labeled and/or unlabeled lipoproteins, labeled albumin, or LH; and (c) lipoprotein-free KRB to wash out unbound label. The ovary was then either (a) excised, freed of adhering tissue, weighed, subjected to gamma spectrometry (counting efficiency: 70%), and then frozen for subsequent biochemical analyses or fixed by immersion in Bouin's fluid; or (b) perfused for an additional 45 min with cold 1% paraformaldehyde-1% glutaraldehyde-0.01% trinitroresol (16) in 0.1 M sodium cacodylate buffer at pH 7.4, excised, counted using a gamma spectrometer, and processed for autoradiography as described below.

In some studies, the effluent from the ovary was collected as follows. After cannulating the uterine artery, a second cannula (No. 23 gauge needle attached to a short length of No. 60 polyethylene tubing containing heparin) was placed in the ovarian vein and secured with a ligature. The samples of ovarian effluent were then centrifuged as necessary to remove any blood, frozen, and subsequently subjected to steroid analysis. In these studies, 2% BSA was added to the effluent to prevent clotting.

To study the conversion of [3H]cholesterol linolate carried by reconstituted HDL into steroids, animals were pretreated with APP for 3 d prior to the experiment, which was performed on day 6 post-hCG injection. Ovaries were first perfused with KRB for 30 min and then for a 20-min period with KRB containing 15 µg of reconstituted HDL/ml (9.6 x 105 dpm/ml) with or without 3 µg of native HDL/ml.

The venous effluent was extracted with 6 vol of chloroform/methanol (12:1, vol/vol)
The organic phase was dried under nitrogen and the residue subjected to thin-layer chromatography on Whatman K5 plates (Whatman Laboratory Products Inc., Clifton, NJ) using hexane/ethyl acetate (7:3, vol/vol) as a solvent system. Areas where \(^{[1]}\) progesterone and \(^{[3]}\) H]-cholesterol reconstituted HDL were identified under UV light using authentic "cold" standards, were collected into vials for liquid scintillation counting using Biofluor (New England Nuclear, Boston, MA).

**Autoradiography:** Immersion-fixed ovaries were processed by routine methods for light microscopy and embedded in paraplast or glycolmethacrylate. Ovaries previously fixed by perfusion were washed overnight at 4°C in 0.1 M glycolmethacrylate. Fixed tissue were placed on acid-cleaned, chrome alum-embedded in low viscosity epoxy resin (32), and the other was embedded in glycolmethacrylate.

For light microscope autoradiography, sections of immersion- and perfusion-fixed ovaries were processed by routine methods for light microscopy and embedded in paraplast or glycolmethacrylate. For perfusion-fixed ovaries, sections were coated with Ilford L-4 nuclear emulsion (Ilford Ltd., Ilford, Essex, England) (diluted 1:1 with distilled water) by the loop method of Caro and van Tubergen (8). After autoradiographic exposure, the sections were developed (undiluted Kodak D-19 developer, 2.5 min, 19°C), washed, and fixed by photographic means. Following washing and air-drying, they were stained with hematoxylin-eosin (paraffin) or methylblue-azure II (glycolmethacrylate, epoxy) and viewed with a bright field microscope. To ensure that no sites of uptake of \(^{125}\)-lipoprotein were overlooked, slides were processed at weekly intervals until the maximum number of silver grains was formed.

For electron microscope autoradiography, pale-gold thin sections of eposymphon embedded tissue were picked up on 0.5% celloidin-coated copper grids. The sections were coated with Ilford L-4 nuclear emulsion (Ilford Ltd., Ilford, Essex, England) (diluted 1:1 with distilled water) by the loop method of Caro and van Tubergen (8). After autoradiographic exposure, the sections were developed in freshly prepared D-19 for 2 min at 19°C, washed briefly with distilled water, fixed, and washed again. After air drying, the sections were treated with 1 N NaOH to remove gelatin from the emulsion, and with amyl acetate to remove celloidin; this greatly improved visibility of the tissue. They were then stained with uranyl acetate for 30 min and with lead citrate (26) for 5-15 min. For purposes of quantitation, cells to be photographed were selected in a predetermined manner to insure randomness, i.e., for each interval studied, the first 30-48 cells encountered that were overlain by one or more silver grains were photographed. The final prints were enlarged to 16,000 \(\times\). Grains were considered to be associated with the plasma membrane if they were within 250 nm of this structure and to be intracellular if they were >500 nm from the plasmalemma.

Controls were carried out to rule out the presence of positive or negative chemography. The specificity of the autoradiographic localizations was evaluated by perfusing a 350-fold excess of unlabeled hHDL or hLDL to the ovary simultaneously with labeled lipoprotein.

**RESULTS**

**Steroid Secretion by the Perfused Ovary**

To determine whether the ovary remained functional during in situ perfusion, we examined its steroidogenic response to lipoproteins and LH. The pattern of steroid secretion was examined for ovaries of non-APP treated (control) and APP-treated rats perfused with lipoprotein-free KRB for 165 min, the maximum length of perfusion used in this work. APP, an adenine analogue, reduces lipoprotein secretion by the liver and produces a marked hypercholesterolemia (1). As shown in Fig. 4, the perfused ovaries accumulated \(^{125}\)-HDL, with uptake reaching a maximum after 15 min of perfusion with label. The mean uptake of label by ovaries perfused for 15 min with \(^{125}\)HDL was 271,370 \(\pm\) 42,243 cpm/ovary (mean \(\pm\) SE, \(n = 7\)).

To determine whether uptake of \(^{125}\)-HDL by the perfused ovary was specific, a 350-fold excess of either unlabeled human HDL or human LDL (0.87 mg of HDL or LDL protein/ml of perfusate) was administered to the ovary simultaneously with \(^{125}\)HDL. Specificity of accumulation was then evaluated after a 15-min perfusion with label, when lipoprotein uptake was maximal in our studies. When excess unlabeled HDL was included in the perfusate, uptake of \(^{125}\)-HDL was decreased by 70-95% (cpm/mg ovary, wet wt) (Figs. 4 and 5). In contrast, addition of an excess of unlabeled LDL to the perfusate diminished uptake of \(^{125}\)-HDL by only 32% (Fig. 5).

To establish the effect of temperature on the uptake of HDL by the perfused ovary, we examined accumulation of \(^{125}\)HDL at 37° and 4°C in APP-treated animals. Uptake at 37°C was carried out as described above. Uptake in the cold was accomplished by switching to perfusate maintained...
at 4°C, and by surrounding the ovary with ice chips and flushing it with ice-cold saline. Perfusion of the ovary with 125I-hHDL at 4°C reduced accumulation of the ligand by ~80% compared with ovaries perfused at 37°C. At 4°C, the ovary took up 425 ± 35 cpm/mg ovary (mean ± SE, n = 3), whereas at 37°C it accumulated 2274 ± 584 cpm/mg ovary (mean ± SE, n = 5).

Finally, the possibility that the ovary was selectively accumulating degraded materials, such as free or lipid-associated 125I, was ruled out as follows. Ovaries from rats perfused at 37°C for 15 min with 125I-hHDL, and washed for 3 min with KRB were homogenized and aliquots subjected to TCA precipitation and chloroform/methanol extraction. The amount...
TABLE I
Metabolism of [1,2,6,7-3H]Cholesteryl Linoleate Reconstituted into Human HDL by Perfused Rat Ovaries

| Treatment                   | Perfusion No. | Ovarian wt mg | Radioactivity in venous effluent dpm x 10^-4 | Radioactivity in progestosterone and 20-a-hydroxyprogren-4-en-3-one Conversion dpm x 10^-4 | Conversion % |
|-----------------------------|---------------|---------------|---------------------------------------------|--------------------------------------------------------------------------------------|--------------|
| Reconstituted 3H-HDL        | 1             | 91.8          | 7.14                                        | 19.4                                                                                 | 2.72         |
|                             | 2             | 75.5          | 3.86                                        | 17.49                                                                                | 4.53         |
|                             | 3             | 62.2          | 0.70                                        | 3.53                                                                                 | 5.02         |
|                             | 4             | 104.1         | 0.55                                        | 2.69                                                                                 | 4.62         |
|                             | 5             | 55.6          | 9.87                                        | 10.91                                                                                | 1.11         |
|                             | 6             | 101.3         | 7.77                                        | 8.51                                                                                 | 1.09         |
| Reconstituted 3H-HDL and "cold" HDL | 6 | 101.3         | 7.77                                        | 8.51                                                                                 | 1.09         |

Animals were pretreated with 4-APP for 3 d prior to the experiment, which was performed on day 6 post-hCG treatment. The ovaries were first perfused with KRB for 30 min and then for a 20-min period with KRB containing 18 ~g/ml (9.6 x 10^5 dpm) reconstituted HDL (1, 2, 5, and 6), 21 ~g/ml (0.95 x 10^5 dpm) reconstituted HDL (3 and 4), or reconstituted HDL and 333 ~g/ml of unlabeled HDL (5 and 6). The ovarian effluent was collected for analysis of radioactive products as described in the text.

**Figure 4** Time course of accumulation of 125I-hHDL3 by perfused rat ovaries. Rats (not treated with APP) were used on day 6 post-hCG injection. Ovaries were perfused briefly with KRB buffer to flush out the blood, then for 5–35 min with 125I-hHDL3 (10^6 cpm/ml of perfusate; specific activity, 400 cpm/ng of HDL protein), after which they were perfused for 3 min with buffer to remove unbound label. Ovaries were excised and counted. Each point represents a separate animal. This experiment was repeated twice with similar results.

**Figure 5** Specificity of lipoprotein uptake by perfused rat ovaries. Rats (not treated with APP) were used on day 6 post-hCG injection. After a brief rinse with KRB buffer, ovaries were perfused for 15 min with 125I-hHDL3 (10^6 cpm/ml of perfusate) with or without a 350-fold excess (0.87 mg/ml of perfusate) of either unlabeled hHDL or unlabeled LDL. They were then perfused for 3 min with buffer alone to remove unbound label and handled as described in the legend to Fig. 4. Each point represents a separate animal. Similar results were obtained on three separate occasions for HDL and on two separate occasions for LDL.

cpm/mg tissue in order to carry out autoradiography successfully within a reasonable amount of time. We therefore examined uptake of 125I-hHDL in rats that had been treated with APP for 3 d before use.

We found that perfusion of 125I-hHDL3 to ovaries of APP-treated rats resulted in at least a 2-fold increase in uptake of label compared to rats receiving gonadotropins alone. Uptake of label in APP-treated animals was rarely ≤1,000 cpm/mg ovary and reached as high as 6,000 cpm/mg ovary.

**Pulse-Wash Studies with 125I-hHDL**

For these studies, ovaries of rats, either with or without APP treatment, were perfused first for 15 min with 125I-hHDL, then for 3–150 min with KRB lacking both label and lipoproteins. While the pattern of uptake was similar in both non-APP treated and APP-treated rats, in the latter case the curve was shifted to the left (Figs. 6 and 7). The amount of label accumulated initially was high and more or less constant on a per milligram ovary basis for up to 15 min of wash for non-
APP treated rats and up to 30 min for APP-treated animals. By 30 (non-APP treated rats) or 60 (APP-treated rats) min of postlabel perfusion, the amount of radioactivity within the ovary declined by ~75%, and remained at the same level for the duration of the infusion period.

**Autoradiographic Localization of ¹²⁵I-labeled Lipoproteins and Albumin**

The compartments accumulating lipoprotein were localized in paraffin sections of immersion-fixed tissue by light microscope autoradiography after perfusion of the ovary for 15 min with ¹²⁵I-hHDL₃ and a brief buffer wash. In this material, silver grains were numerous over corpora lutea, but sparse over follicles, stroma, and ova (Fig. 8a). When excess unlabelled hHDL was perfused to the ovary along with ¹²⁵I-hHDL₃, the number of silver grains over corpora lutea was markedly reduced (Fig. 8b). In contrast, the level of labeling in this compartment was not appreciably altered when "cold" hLDL was infused to the ovary simultaneously with ¹²⁵I-hHDL₃ (Fig. 8c).

To identify the specific types of ovarian cells accumulating ¹²⁵I-hHDL₃, uptake of this lipoprotein was also studied in plastic sections of ovaries fixed in situ by vascular perfusion. The quality of morphological preservation in these preparations was superior to that of tissue fixed by immersion. Moreover, blood was washed out of the vascular bed and there was a slight shrinkage of the cells. As a result, the boundaries of adjacent cells were discernible, allowing the cells taking up ¹²⁵I-hHDL to be recognized easily.

After perfusing for 15 min with ¹²⁵I-hHDL₃ and washing for 3 min to remove unbound label, it was clear that in such preparations most silver grains were concentrated over luteal cells (Fig. 9). Silver grains were associated with the cell surfaces and also occurred over their cytoplasm. Silver grains were especially abundant along those surfaces of luteal cells that faced capillaries (Fig. 10a), although they were also observed at interfaces between adjacent luteal cells (Fig. 10b). In ~20-25% of the luteal cells in a given field, silver grains were near the nucleus (Fig. 10c). In all cases, a variable number of grains was clustered in or near blood vessels.

Silver grains occurred in locations similar to those just described after being washed with lipoprotein-free buffer for 15 (non-APP treated rats) and 30 (APP-treated rats) min (Fig. 11a). However, in ovaries "washed" for longer periods with buffer (i.e., 30-150 min for non-APP treated rats and 60-120 min for APP-treated rats) the number of grains over luteal cells was drastically reduced (Fig. 11b). The concentration of silver grains in or near blood vessels appeared unchanged as compared with that observed at shorter wash times.
FIGURE 8  Light microscope autoradiographs of immersion-fixed, paraffin-embedded ovaries perfused with $^{125}$I-human HDL$_3$. Ovaries were perfused briefly with lipoprotein-free KRB buffer, then for 15 min with $^{125}$I-hHDL$_3$ (10$^6$ cp/m of perfusate; specific activity, 400 cp/m/ng of HDL protein) with or without a 350-fold excess (0.87 mg/ml of perfusate) of unlabeled hHDL or hLDL, followed by a brief buffer wash to remove unbound label. (a) Silver grains (small black dots) were numerous over corpora lutea, but sparse over follicle and stromal cells in ovaries perfused with $^{125}$I-hHDL$_3$. (b) In contrast, few silver grains occurred over corpora lutea (CL) of ovaries perfused simultaneously with $^{125}$I-hHDL$_3$ and excess unlabeled hHDL. (c) Inclusion of excess unlabeled hLDL in the perfusate with $^{125}$I-hHDL$_3$ did not markedly alter the number of silver grains over corpora lutea. CL, corpus luteum; ct, connective tissue at center of corpus luteum; st, stroma. All, no APP treatment; day 6 post-hCG injection; 14-d autoradiographic exposure; x 600.
As shown for immersion-fixed, paraffin-embedded tissue, the concentration of grains associated with luteal cells in plastic sections of perfusion-fixed material decreased when excess unlabeled hHDL was added to the perfusate along with \(^{125}\text{I}\)-hHDL\(_3\) (Fig. 10, d and e). There was a similar paucity of silver grains over corpora lutea when \(^{125}\text{I}\)-hHDL\(_3\) was perfused to ovaries prechilled to 4°C (Fig. 10f). These comparisons of relative grain concentrations were made among tissue sections exposed in the dark for similar amounts of time.

Some ovaries were perfused with \(^{125}\text{I}\)-rat HDL to rule out the possibility that heterologous (human) lipoprotein might have a distribution among various ovarian compartments or cell types different from that for homologous (rat) lipoproteins. As shown in Fig. 11, c and d, silver grains were numer-

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**Figure 9** Localization of hHDL\(_3\) in light microscope autoradiographs of perfusion-fixed, glycolmethacrylate-embedded ovary. After a brief perfusion with buffer, the ovary was infused with \(^{125}\text{I}\)-hHDL\(_3\) (10⁶ cpm/ml of perfusate) for 15 min and washed for 3 min with buffer, after which it was perfused-fixed in situ with FGC-cacodylate. The quality of morphological preservation in these preparations was markedly improved compared with ovaries fixed by immersion. As a result, it was clear that silver grains were primarily associated with luteal cells (arrows); few grains were observed over cells of follicles or stroma. Luteal cells can be identified by their abundant cytoplasm and large, round nuclei which often contain prominent nucleoli. The small, dark irregularly shaped nuclei belong primarily to endothelial cells. Blood cells were washed out of the vascular bed by the perfusion (bv, blood vessel). APP-treated; day 7 post-hCG injection; 6-wk autoradiographic exposure; ∗1,100.
FIGURE 10 Light microscopic autoradiographs of ovaries perfused with $^{125}$I-hHDL$_3$. (a–c) Detail of $^{125}$I-hHDL$_3$ distribution in corpora lutea. The experiment was done as described in the legend to Fig. 9. Silver grains were especially numerous along those luteal cell surfaces facing the vascular bed (a, arrows), although some were also observed at interfaces between adjacent luteal cells (b, arrows). Silver grains were frequently near the nucleus, often encircling it (c, arrow). All, $\times$ 1,800. (d–f) Competition control and effect of temperature on $^{125}$I-hHDL$_3$ uptake. At 37°C, ovaries perfused with $^{125}$I-hHDL$_3$ (10$^6$ cpm/ml of perfusate) showed numerous silver grains which occurred over the cytoplasm and along the surfaces of luteal cells (d, arrows). In contrast, in autoradiographs of ovaries perfused with either $^{125}$I-hHDL$_3$ and an excess of unlabeled hHDL at 37°C (e, arrow) or $^{125}$I-hHDL$_3$ at 4°C (f, arrow), few silver grains were over luteal cells. Blood vessels, bv. All, $\times$ 1,200. a, b, and d–f: APP-treated; day 7 post-hCG injection; perfusion-fixation; 6-wk autoradiographic exposure. c: No APP treatment; day 6 post-hCG injection; perfusion-fixation; 7-mo autoradiographic exposure.
Figure 11 (a and b) Uptake of $^{125}$I-hHDL$_3$, pulse-wash studies. Silver grain distribution in ovaries perfused for 15 min with $^{125}$I-hHDL$_3$ (10$^6$ cpm/ml of perfusate) and washed for up to 15 (non-APP treated rats) or 30 (APP-treated rats) min was the same as that in ovaries washed for 3 min (compare with Fig. 9); i.e., grains were concentrated over luteal cells (a, arrows). However, after longer washes the number of grains associated with luteal cells was markedly reduced (b, arrow; 120 min of wash). Blood vessels, bv. Both, APP-treated; day 7 post-hCG injection; 21-d autoradiographic exposure; x 1,200. (c and d) Uptake of $^{125}$I-rat HDL by perfused rat ovaries. In autoradiographs of ovaries perfused for 15 min with homologous lipoprotein ($^{125}$I-rat HDL; 10$^6$ cpm/ml of perfusate; specific activity, 400 cpm/ng of HDL protein) and washed for 3 min, silver grains were numerous along luteal cell surfaces and over their cytoplasm (c, arrows). In ovaries washed for longer periods after labeling, grains were sparse over luteal cells (d, arrow indicates a silver grain over a luteal cell). Blood vessels, bv. Both, APP-treated; day 7 post-hCG injection; 14-d autoradiographic exposure. c, x 1,200; d, x 1,800.

Figure 12 (a) Uptake of $^{125}$I-rat HDL (rat and human), since albumin may be present as a contaminant in some lipoprotein preparations. In each of three animals, the results were identical. After perfusion for 15 min with $^{125}$I-BSA followed by a 3-min buffer wash, silver grains were concentrated over blood vessels and over cells that resembled macrophages (Fig. 12) but were sparse over luteal cells. This pattern of grain distribution contrasted markedly with that of $^{125}$I-human or rat HDL.

An examination of silver grain distribution in electron micrographs confirmed that luteal cells, identified by their

ous over luteal cells at 3 min of wash, whereas at 90 min of wash few grains occurred over luteal cells. At the later wash period, most of the grains were associated with blood vessels.

Finally, we examined the uptake of $^{125}$I-BSA by the perfused ovary and compared its distribution with that of $^{125}$I-HDL (rat and human), since albumin may be present as a contaminant in some lipoprotein preparations. In each of three animals, the results were identical. After perfusion for 15 min with $^{125}$I-BSA followed by a 3-min buffer wash, silver grains were concentrated over blood vessels and over cells that resembled macrophages (Fig. 12) but were sparse over luteal cells. This pattern of grain distribution contrasted markedly with that of $^{125}$I-human or rat HDL.

An examination of silver grain distribution in electron micrographs confirmed that luteal cells, identified by their characteristic fine structure, were the predominant type of cell that accumulated $^{125}$I-hHDL$_3$. In electron microscope autoradiographs, as described above for the light microscope autoradiographic studies, silver grains were more abundant after shorter periods of wash (3-30 min) than after longer periods (60-120 min). The grain distribution over labeled luteal cells was quantified after various periods of wash as described above. After shorter periods of wash (e.g., 3-30 min), 65% of 236 grains in 48 cells were associated with the plasma membrane (i.e., on or within 250 nm of this structure), with 35% over the interior of the luteal cell. Of the plasma membrane grains, >50% were localized over processes, i.e., thin cytoplasm-filled extensions of the plasma membrane (Fig. 13a).

It appeared that most of the plasma membrane grains were associated with regions of the plasmalemma that lacked a
morphologically identifiable bristle coat (Fig. 13b). After longer wash periods there appeared to be a shift in silver grain distribution. By 90 min of wash, the percentage of grains along the plasma membrane had declined slightly, to 51%, with a corresponding rise in that over the cytoplasm, to 49% (118 grains in 31 cells). A further decrease in the percentage of grains over the plasma membrane, to 34%, was observed at 120 min of wash; at this time 66% of 167 grains in 30 cells were associated with luteal cell cytoplasm (Fig. 13c).

DISCUSSION
Our results indicated that the in situ perfused ovary of the gonadotropin-primed immature rat is a suitable model for the study of lipoprotein metabolism. The ovary remains functional during the periods of perfusion used, since it remains able to secrete steroid hormones and to respond to trophic stimuli and lipoproteins. In APP-treated rats, the addition of hHDL$_3$ to the perfusate brought about a stimulation of progestin secretion by the ovary, raising it two- to threefold above that for ovaries not receiving lipoproteins. This response occurred even though the levels of hHDL$_3$ used supplied cholesterol at concentrations only one-third to one-half of the normal circulating values for this sterol. It is unlikely that the elevated levels of progestin were merely a consequence of heightened secretion. After perfusion of the ovary with HDL reconstituted with $[^3]$Hcholesteryl linoleate, tritium-labeled progestin appeared in the effluent, indicating that sterol carried by HDL was converted to steroid. Infusion of LH also produced a vigorous secretory response in non-APP treated rats, even when this gonadotropin was delivered to the ovary 120 min after the perfusion was initiated.

Our studies further demonstrated that perfused ovaries take
up $^{125}$I-labeled human HDL$_3$ by a process that is temperature dependent and lipoprotein specific. At 37°C, accumulation of $^{125}$I-hHDL$_3$ by the ovary reached a maximum after 15 min of perfusion with label. Thus, the time course for uptake of radiolabeled hHDL by the perfused ovary was remarkably similar to that for both dispersed ovarian cells and ovaries in vivo (30, 33). When binding was carried out in the cold, however, uptake of $^{125}$I-hHDL$_3$ by the perfused ovary was reduced by 80%. The inclusion of excess unlabeled hHDL in the perfusate markedly suppressed the accumulation of $^{125}$I-hHDL$_3$, suggesting that the uptake of labeled ligand by the perfused ovary involves a finite number of binding sites. We also found that unlabeled hLDL competed less effectively for $^{125}$I-hHDL$_3$ uptake than did unlabeled hHDL, indicating a lipoprotein specificity to the uptake process. Taken together, these data suggest that the accumulation of $^{125}$I-hHDL$_3$ occurred at the physiologic binding site, presumably the HDL receptor.

Treatment of gonadotropin-primed immature rats with APP reduced plasma cholesterol concentrations from 50 mg/dl to <15 mg/dl. In these animals, uptake of $^{125}$I-hHDL$_3$ by perfused ovaries was severalfold greater than that for ovaries of rats not treated with APP. This finding is presumably due to the reduced occupancy of HDL receptors by endogenous lipoproteins and, perhaps, to an increase in HDL binding sites as a consequence of lowered plasma cholesterol levels.
We identified corpora lutea as the ovarian compartment which most actively accumulates $^{125}$I-hHDL$_3$. These observations constitute the first morphological localization of HDL accumulation in intact steroid secreting tissue. By using $^{125}$I-human HDL essentially free of apolipoprotein E as our probe, we insured that only the binding sites for HDL were visualized, and not those for LDL. Moreover, the lipoprotein-specific nature of our autoradiographic localization was verified by competition controls in which ovaries were perfused simultaneously with $^{125}$I-hHDL$_3$ and an excess of unlabeled hHDL or hLDL. In addition, both $^{125}$I-Trat HDL and $^{125}$I-human HDL labeled autoradiographically the same compartments in the perfused ovary. Thus, we can assume that the use of a heterologous lipoprotein as a probe did not give rise to an abnormal pattern of binding among ovarian structures. Finally, by perfusing some ovaries with $^{125}$I-BSA, we ruled out the possibility that albumin, a contaminant of some lipoprotein preparations, was localized instead of HDL.

Besides localizing $^{125}$I-hHDL$_3$ to corpora lutea, light and electron microscope autoradiography have pinpointed luteal cells as the main type of cell that accumulates this lipoprotein. Schuler et al. (30) suggested, in an earlier study of dispersed cells, that HDL was taken up by ovarian parenchymal cells. However, the precise identity of the cells was not established in that work. In discussing the possible mechanisms involved in the metabolism of HDL by adrenal gland in vivo, Kovansen et al. (19) pointed out that binding sites for HDL need not reside on adrenocortical parenchymal cells but could occur on other nearby cells, such as capillary endothelial cells. Our findings, however, appear to rule out this possibility as a major mechanism by which sterol is delivered to luteal cells. On the contrary, they imply that luteal cells are directly responsible for obtaining the substrate they use in the synthesis of steroids.

Our light microscopic autoradiographic observations suggested that $^{125}$I-hHDL$_3$ accumulates at the surfaces of luteal cells. Uptake was especially heavy on those surfaces that faced the vascular spaces. However, grains were also observed along the interfaces between adjacent luteal cells, indicating that binding sites for the lipoprotein were not restricted to the perivascular surfaces of luteal cells. These impressions were confirmed by electron microscope autoradiography, which clearly demonstrated that binding sites for $^{125}$I-hHDL$_3$ were associated with the plasma membranes of luteal cells. These findings support the suggestion, largely from biochemical studies, that the surfaces of steroid-secreting cells bear specific binding sites for HDL. For example, Christie et al. (10) have reported that uptake of $^{125}$I-hHDL is greatest in that fraction of rat ovaries containing the highest 5'-nucleotidase activity, an enzyme considered to be a marker for plasma membrane. Of the silver grains we found associated with luteal cell plasma membranes, 50% occurred over processes, slender cytoplasm-filled projections of the plasmalemma. Receptors for $^{125}$I-hHCG are also particularly numerous along villous processes of rat luteal cells (13, 22). The majority of the binding sites for $^{125}$I-hHDL$_3$, whether on processes or not, do not appear to be concentrated over coated pits or over linear bristle-coated segments of the plasma membrane. On the other hand, LDL receptors are associated with such specializations in both human fibroblasts (4) and rat granulosa cells in vitro (L. G. Paavola and J. F. Strauss, unpublished observations). Thus, HDL binding sites of rat luteal cells appear to differ from the classical LDL receptors in several ways, including their biochemical properties and location along the plasma membrane.

In conclusion, the data presented here provide evidence...
that \(^{125}\)I-hHDL\(_3\) is taken up by in situ perfused ovaries of rats, and that this accumulation involves a specific interaction between the lipoprotein and a binding site located on the plasma membrane of luteal cells. We have alluded to the possibility that HDL or some portion of it may be internalized by luteal cells. Studies in progress are aimed at providing further information on how the ovary processes HDL at the cellular level.

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REFERENCES

1. Andersen, J. M., and J. M. Dietschy. 1977. Regulation of sterol synthesis in 15 tissues of rat. II. Role of rat and human high and low density lipoprotein lipoproteins and of rat chylomicron remnants. J. Biol. Chem. 252:3653-3659.

2. Andersen, J. M., and J. M. Dietschy. 1978. Relative importance of high and low density lipoprotein cholesterol in the regulation of cholesterol synthesis in the adrenal gland, ovary and testis of the rat. J. Biol. Chem. 253:9024-9032.

3. Andersen, J. M., and J. M. Dietschy. 1981. Kinetic parameters of the lipoprotein transport systems in the regulation adrenal gland of the rat determined in vivo. J. Biol. Chem. 256:7362-7370.

4. Anderson, R. G. W., M. S. Brown, and J. L. Goldstein. 1979. Receptor-mediated endocytosis: insights from the lipoprotein receptor system. Proc. Natl. Acad. Sci. USA. 76:3330-3337.

5. Brown, M. S., and J. L. Goldstein. 1979. Receptor-mediated uptake of lipoprotein-cholesterol and its utilization for sterol synthesis in the adrenal cortex. Rec. Prog. Horm. Res. 35:213-257.

6. Caro, L., and R. P. van Tubergen. 1962. High resolution autoradiography. I. Methods. J. Cell Biol. 15:171-188.

7. Chen, Y.-J., F. R. Kraemer, and G. M. Reaven. 1980. Identification of specific high density lipoprotein-binding sites in rat tissues and regulation of binding by human chorionic gonadotropin. J. Biol. Chem 255:9162-9167.

8. Christie, M. H., J. T. Gwynne, and J. F. Strauss. III. 1981. Binding of high density lipoproteins to membranes of luteinized rat ovaries. J. Steroid Biochem. 14:671-678.

9. DeVilla, G. O., K. Roberts, W. G. Weist, G. Mikhail, and G. Flickinger. 1972. A specific radioreceptor assay of plasma progesterone. Int. J. Endocrinol. Metab. 35:458-460.

10. Gwynne, J. T., and J. F. Strauss. III. 1982. The role of lipoproteins in steroidogenesis and cholesterol metabolism in steroidogenic glands. Endocr. Rev. 3:299-329.

11. Hain, S. S. H. J. Rajanmuni, M. F. Cho, A. N. Hirschfield, and A. R. Midgley, Jr. 1974. Gonadotropin receptors in rat ovarian tissue. II. Subcellular localization of LH binding sites by electron microscope autoradiography. Endocrinology. 95:589-598.

12. Havel, R. J., H. A. Eder, and J. H. Bragdon. 1955. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. J. Clin. Invest. 34:1345-1353.

13. Ito, S., and M. J. Karnovsky. 1968. Formaldehyde-glutaraldehyde fixatives containing trinitro compounds. J. Cell Biol. 39(2, Pt. 2):1856a (Abstr.).

14. Jansen, H., C. Kalkman, J. C. Birkenhager, and W. C. Hulsman. 1980. Demonstration of a heparin-releasable liver-lipase-like activity in rat adrenals. FEBS (Fed. Eur. Biochem. Soc.) Lett. 112:30-34.

15. Kita, T., U. Beisegel, J. L. Goldstein, W. J. Schneider, and M. S. Brown. 1981. Antibody against low density lipoprotein receptor blocks uptake of low density lipoprotein (but not high density lipoprotein) by the adrenal gland of the mouse in vivo. J. Biol. Chem. 256:4701-4705.

16. Kovanen, P. T., W. J. Schneider, G. M. Hillman, J. L. Goldstein, and M. S. Brown. 1978. Separate mechanisms for the uptake of high and low density lipoprotein by mouse adrenal gland in vivo. J. Biol. Chem. 253:5498-5505.

17. Lowry, O. H., N. J. Rosebrugh, A. L. Fan, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagents. J. Biol. Chem. 193:265-275.

18. Mahley, R. W., K. H. Weggraber, and T. Innerarity. 1979. Interaction of plasma lipoproteins containing apolipoproteins B and E with heparin and cell surface receptors. Biochem. Biophys. Acta. 575:81-91.

19. Markkanen, S. O., and H. J. Rajaniemi. 1980. Role of internalization and degradation of the low density lipoprotein receptor, an acidic glycoprotein of 164,000 molecular weight. FEBS (Fed. Eur. Biochem. Soc.) Lett. 112:30-34.

20. McNamara, B. C., R. Booth, and D. A. Stansfield. 1981. Evidence for an essential role for a high density lipoprotein in progesterone synthesis by rat corpus luteum. FEBS (Fed. Eur. Biochem. Soc.) Lett. 14:79-82.

21. Orczyk, G. P., M. Michenga, G. Arth, and H. R. Behrman. 1979. Progestrone and 20-

22. Markkanen, S. O., and H. J. Rajaniemi. 1980. Role of internalization and degradation of the low density lipoprotein receptor, an acidic glycoprotein of 164,000 molecular weight. FEBS (Fed. Eur. Biochem. Soc.) Lett. 112:30-34.

23. McNamara, B. C., R. Booth, and D. A. Stansfield. 1981. Evidence for an essential role for a high density lipoprotein in progesterone synthesis by rat corpus luteum. FEBS (Fed. Eur. Biochem. Soc.) Lett. 14:79-82.

24. Orczyk, G. P., M. Michenga, G. Arth, and H. R. Behrman. 1979. Progestrone and 20-

25. Paavola, L. G. 1977. The corpus luteum of the guinea pig. Fine structure at the time of maximum progesterone secretion and during regression. Am. J. Anat. 150:565-584.

26. Reynolds, E. S. 1963. The use of lead citrate at high pH as an electron opaque stain in electron microscopy. J. Cell Biol. 17:290.

27. Rosenblum, M. F., C. R. HuUler, and J. F. Strauss, Ill. 1981. Control of sterol metabolism in cultured rat granulosa cells. Endocrinology. 107:1153-1161.

28. Schneider, W. J., U. Beisegel, J. L. Goldstein, and M. S. Brown. 1982. Purification of the low density lipoprotein receptor, an acidic glycoprotein of 164,000 molecular weight. J. Biol. Chem. 257:2664-2673.

29. Schuler, L. A., K. K. Langenberg, J. T. Gwynne, and J. F. Strauss. III. 1981. High density lipoprotein utilization by dispersed rat luteal cells. Biochim. Biophys. Acta. 644:55-601.

30. Schuler, L. A., K. K. Langenberg, J. T. Gwynne, and J. F. Strauss. III. 1981. High density lipoprotein utilization by dispersed rat luteal cells. Biochim. Biophys. Acta. 644:55-601.

31. Schuler, L. A., L. Scavo, T. M. Kirch, G. L. Flickinger, and J. F. Strauss. III. 1979. Regulation of de novo biosynthesis of cholesterol and progesterone, and formation of cholesterol ester in rat corpus luteum by exogenous sterol. J. Biol. Chem. 254:8662-8668.

32. Spur, A. R. 1969. A low-velocity epoxy resin embedding medium for electron microscopy. J. Ultrastr. Res. 26:31-43.

33. Strauss, J. F., III, L. C. MacGregor, and J. T. Gwynne. 1982. Uptake of high density lipoprotein by rat ovaries in vivo and dispersed ovarian cells in vitro. Direct correlation of high density lipoprotein uptake with steroidogenic activity. J. Steroid Biochem. 15:525-531.

606 THE JOURNAL OF CELL BIOLOGY - VOLUME 97, 1983