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Research Article

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Uptake of Low Density Lipoproteins by Rat Tissues

Special Emphasis on the Luteinized Ovary

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Abstract

The aim of this study was to determine how luteal cells of the hormone-primed (luteinized) ovary process low density lipoproteins (LDL). Ovary uptake of perfused ^{125}I-LDL was assessed by tissue levels of radioactivity; the distribution of LDL protein in cells was assessed on autoradiograms of the fixed tissue; and the level of stimulation of steroidogenesis, as well as degradation of LDL protein, was assessed on effluent perfusion samples. Human LDL ligand used in these studies was rigorously defined biochemically and physiologically. Homologous (rat) LDL was used as a special ligand control. Other tissue controls included the use of perfused or in vivo-infused luteinized ovaries from animals pretreated to reduce circulating lipoprotein levels, perfused ovaries from a second hormone-primed model, perfused liver from estrogen-treated rats, and isolated and cultured cells from the same ovarian tissues used in the perfusion experiments. The results show that perfused LDL promptly stimulates steroidogenesis. However, the labeled protein moiety of the LDL is not interiorized by the luteal cells, nor is there evidence of LDL protein degradation in the effluent samples. In contrast, internalization of the ligand occurs when luteal cells are incubated with the ligand in vitro. We have observed also that uptake of the ^{125}I-LDL by the ovary can be displaced equally well by excess unlabeled LDL or HDL_3. Overall, these experiments suggest that in the intact luteinized ovary, LDL binds to the same sites on the cell surface where HDL "binds," and that LDL cholesterol must be obtained by these steroid hormone-producing cells by a mechanism that does not require internalization of the intact lipoprotein particle.

Introduction

Most steroid hormone-producing tissues (e.g., ovary and adrenal) obtain the major supply of cholesterol needed for steroidogenesis from circulating cholesterol-rich lipoproteins (1, 2). In the rat, it is believed that the predominant exogenous lipoprotein source of cholesterol is the high density lipoprotein (HDL) particle (3), although it is clear that both HDL and the less available (2, 3) low density lipoproteins (LDL) can, in fact, be bound by steroid-producing cells and stimulate steroidogenesis under similar in vivo and in vitro circumstances (2). To what extent the rat's steroid hormone-producing tissues discriminate between HDL and LDL during binding or in the cholesterol-processing phase is not yet clear. Indeed, it is not yet certain whether or not the two lipoproteins are recognized by the same receptor sites on the cells.

In a recent study we began reexamining the issue of how steroid hormone-producing cells of the rat utilize HDL: we used ^{125}I-labeled human HDL (hHDL) as the ligand, and followed its time-related uptake during perfusion of hormone-primed luteinized ovaries of immature rats (4). Our results showed that the luteinized ovary responded to HDL perfusion with a dramatic increase in progesterone production, but, at the same time, the intact HDL particle was not internalized by the hormone-producing cells. Instead, we found in electron microscopic autoradiographs prepared of ovaries perfused for up to 2 h with labeled HDL, that ~90% of the exposed grains (representing sites of HDL protein) remained associated with the plasma membrane of the luteal cells. Moreover, the few exposed grains that were present within the confines of the luteal cell cytoplasm were not specifically associated with cell organelles (vesicles, vacuoles, or lysosomes) generally considered to be part of the endocytic-degradative pathway (5). It was of interest that the same results obtained whether human HDL_3 (lacking apolipoprotein [apo][1] E) or rat HDL (known to contain ~12% apo E [3]) had been used as the perfused ligand. This finding with apo E-containing HDL brought up the possibility that rat luteal cells "in vivo" may not have an active B/E receptor pathway for processing lipoproteins, and, as such, further complicated the issue of how such cells discriminated between HDL and the apo B-containing LDL.

In the current study, we examined this issue still further by directly using LDL as the lipoprotein of choice. Experiments similar to those previously described for HDL (4) were carried out except the perfused (or intravenously infused) ligand was changed to ^{125}I-hLDL. Steroidogenesis, kinetics of LDL uptake and degradation by the intact ovary, and the distribution of the labeled LDL on luteal cells, as determined by electron microscope autoradiograms, were evaluated as before.

Various other rat tissues were used as special controls. For one, perfusion experiments with ^{125}I-LDL were carried out on luteinized ovaries from rats whose circulating lipoprotein levels had been reduced to <10% of normal values by pretreatment of the animals with 4-aminopyrazolo [3,4-d] pyrimidine (6). In addition, LDL uptake by the pregnant mare serum gonadotropin (PMSG)-treated (granulosa cell-differentiated) ovary (7) was examined as a second steroidogenic tissue. LDL uptake by the perfused livers of estrogen-primed rats (8, 9) was used as a positive

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1. *Abbreviations used in this paper:* apo, apolipoprotein; 4-APP, 4-aminopyrazolo 3,4-d pryimidine; ARG, autoradiograph; EM, electron microscope; hCG, human chorionic gonadotropin; LM, light microscope; LPDS, lipoprotein-deficient fetal bovine serum; PMSG, pregnant mare serum gonadotropin.

control for tissue uptake of LDL by the classical endocytic-degradative pathway (5). The uptake of homologous (rat) LDL was used as a control for the heterologous (human) ligand used in the majority of the present experiments. And finally, isolated cultured luteal cells and granulosa cells (from the same ovarian tissues used in the perfusion experiments) and fibroblasts in culture were incubated with the labeled LDL in vitro as a means of estimating ligand quality in established systems (10).

Methods

Perfusion experiments

TISSUE MODELS. *Luteinized ovary.* Immature female Sprague–Dawley rats (Simonsen Laboratories, Fremont, CA) were hormone-primed with PMSG and human chorionic gonadotropins (hCG) as previously described (11, 12). The luteinized ovaries from these animals provided the major in situ organ perfusion model for the present study: ovarian tissue was used for biochemical and structural studies, and collagenase-isolated luteal cells from these ovaries were maintained in culture for incubation with ligand as described below.

Luteinized ovary from animals treated with 4-aminopyrazolo [3,4-d] pyrimidine (4-APP). Rats prepared as above were injected with 4-APP (15 mg/kg body weight; s.c. [6]) at 9:00 a.m. on each of the final 2–3 d before use. When used for experiments, plasma cholesterol levels of these animals were <10% of that of hormone-primed rats not given 4-APP.

Granulosa cell-differentiated ovary. Immature female rats treated only with PMSG (20 IU) for 48 h develop enlarged ovarian follicles rich in granulosa cells (7). These cells differ morphologically from luteal cells, but insofar as they produce progesterone, they provide a second steroid cell model for study. Because preliminary studies indicated that not all resident granulosa cells are labeled when perfused with radioactive lipoproteins (undoubtedly owing to poor vascularization of the follicles [2]), such ovaries were not used for biochemical studies and only cells that were adequately labeled were examined morphologically. It should be noted that isolated granulosa cells from these ovaries can be obtained simply by piercing the enlarged follicles and expressing their contents. As such, the granulosa cells provide a noncollagenase-treated cell for cultivation which is particularly useful for comparison with the identical cells examined in situ.

Estrogen-stimulated liver. Male Sprague–Dawley rats (180–200 g, Simonsen Laboratories) were injected subcutaneously with 17 α-ethynylestradiol (E_2, 5 mg/kg body weight) for 5 d prior to use to reduce circulating cholesterol levels and increase hepatic LDL receptor sites (8, 9). Livers from these animals were used for morphologic observation only and were intended to provide a nonsteroidal tissue control for the perfused ovarian tissues described above.

PERFUSION PROTOCOL. The methodology used in this study was similar to that described previously for HDL (4), except that in this study flow to all abdominal organs except the genital tract was excluded, and that perfusion effluent represented collection solely from the ovary, uterus, and some musculature surrounding these tissues. The perfusion sequence for all experiments (except those for kinetic data) was as follows: 2-min washout with oxygenated Medium 199 or McCoy's 5a media (pH 7.4, 33–35°C), followed by 50 min (occasionally 120 min) of nonrecirculating (flow-through) ligand perfusion at 1–2 ml/min and a 2-min wash with media (2 ml/min). At this point the ovaries were either excised and counted for radioactivity, or the tissues were perfusion-fixed with glutaraldehyde before processing for preparation of autoradiographs (ARGs). No differences were seen in cell morphology or in the distribution of ^{125}I-LDL after the use of Medium 199 or McCoy's 5a media.

Hepatic perfusion was carried out for morphologic purposes only. Two types of procedures were used. In some cases rat livers were perfused by precisely the same LDL ligand–media preparation and the same conditions used to perfuse the luteinized ovaries. In other cases, rat livers were perfused with ligand in Krebs–Ringer buffer supplemented with freshly prepared washed human red blood cells (25% hematocrit) and 4% albumin, as done for standard liver perfusion studies (13). No differences were detected in the distribution of ligand by the two perfusion methods.

The basic experimental protocol for this study was as follows: (*a*) determination of the kinetics of LDL uptake in luteinized ovaries using as an endpoint the accumulation of total ovary radioactivity after perfusion with ^{125}I-hLDL (3–5 μCi/ml) for varying periods of time (15–180 min) and with different concentrations of LDL (25–1,000 μg of protein/ml); (*b*) assessment of competition of 10-fold excess unlabeled hLDL or unlabeled $hHDL_3$ for the tissue-associated labeled ligands; (*c*) determination of the progesterone response of luteinized ovaries to the perfusion of varying concentrations (10, 50, 100, 500, 1,000 μg of protein/ml) of unlabeled hLDL for 1 h; (*d*) determination of the LDL degradation in perfusate effluent; and (*e*) the localization of either ^{125}I-h or rat (r)LDL (45 μCi/ml, 50–500 μg of protein/ml) on cells of each of the tissue models discussed above, using ARG at both the light (LM) and electron microscopic (EM) level.

In vivo experiments

To test the efficacy of the perfusion models of the luteinized ovary in identifying the localization of exogenously supplied ^{125}I-LDL, two experiments were carried out in which the ligand was slowly infused into 4-APP-treated, luteinized rats and permitted to circulate with the blood for 1 h before perfusion fixation of the ovaries. At the start of these experiments a cannula was inserted into the jugular vein of lightly anesthetized animals (Surital, sodium thiamylal) and ^{125}I-LDL (1.5 mCi/ml; 3.1 mg protein/ml) was infused at a rate of 0.05 ml for 15 min, then at 0.02 ml for the remaining 45 min of the experiment. The animals remained lightly anesthetized throughout the procedure. At the end of 1 h the abdomen of the animals was opened and the ovaries were prepared for in situ perfusion of fixative as before. Medium 199 was perfused for 2 min (2.5 ml/min) to flush the ovaries of blood and immediately thereafter the ovaries were perfused with glutaraldehyde fixative (4). Subsequently, the radioactivity of the intact fixed ovaries was assessed (using a LKB gamma counter, LKB Instruments Inc., Gaithersburg, MD), and the tissue was processed for LM and EM ARGs. Only a minimum number of these experiments were carried out because it was necessary to use high levels of radioactivity (2.5 mCi) for the purpose of EM ARGs, and it was difficult to protect the perfusionist from exposure during surgical preparation of the radioactive animals.

Isolated cell studies

Isolated rat luteal cells (12) and rat granulosa cells (14) were plated for 2–48 h in petri dishes with McCoy's 5a (15) medium (and in some cases with Dulbecco's modified Eagle's medium:Ham's F12 [1:1]) containing 10% lipoprotein-deficient fetal bovine serum (LPDS) before labeled (±10-fold excess unlabeled) ligand was added for 1–2-h incubation periods. After incubation with the radioactive ligand, the cells were rapidly washed four times with cold (4°C) fresh media and fixed in situ with 1% glutaraldehyde in 0.1 M cacodylate buffer. Subsequently, the cells were pelleted and prepared for EM ARGs as described previously (4).

On occasion, gold-labeled LDL was prepared (16, 17) and used for incubation of cells as described above. Although no differences in ligand uptake by cells were noted for the differently labeled LDL in vitro, it should be mentioned that gold-labeled LDL cannot be used for in situ perfusion of ovaries insofar as the gold ligand does not pass through the basement membrane that surrounds each luteal cell, unless that region is damaged by excessively high perfusion flow rates (E. Reaven, personal observation).

Miscellaneous techniques

PREPARATION OF LIPOPROTEINS. Human (h) lipoproteins were isolated from fresh plasma of healthy male donors (containing 8 U/ml of kallikrein inactivator and 1 mM diisopropyl fluorophosphate [18]) by preparative ultracentrifugation (19) in a 60 Ti rotor (Beckman Instruments, Inc., Palo Alto, CA) at 58,000 rpm. hLDL and $hHDL_3$ fractions were isolated between densities of 1.020 and 1.050 g/ml (20) and 1.125

and 1.210 g/ml (21), respectively. Each fraction was recentrifuged for 18 h at the appropriate density to minimize plasma protein contamination. rLDL was isolated between densities of 1.020 and 1.050 and used without any further purification (22). Isolated lipoprotein fractions were dialyzed against 0.15 M NaCl, 3 mM EDTA, pH 7.4, at 4°C for 24 h.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as described previously (4). As shown in Fig. 1, hLDL contained apo B, and hHDL contained apo AI as the major apoproteins.

Cholesterol in the serum, LDL, or in HDL_3 fractions was determined enzymatically by the reagents (kit) supplied by Reagents Applications, Inc., San Diego, CA. The protein content of lipoproteins was determined by a modification of the procedure of Lowry et al. (23) as described by Markwell et al. (24).

IODINATION OF LIPOPROTEINS. hLDL was iodinated using the iodine monochloride method of McFarlane (25) as modified by Bilheimer et al. (26). The specific activity of various preparations ranged from 6 to 12×10^5 cpm/μg protein. Over 99% of the radioactivity was precipitated by 15% trichloroacetic acid and <2.5% was extractable with chloroform/methanol (2:1 vol/vol).

CHARACTERIZATION OF ^{125}I-hLDL UPTAKE AND DEGRADATION BY HUMAN FIBROBLASTS, RAT GRANULOSA CELLS AND THE IN SITU PERFUSED OVARY. (*a*) Every preparation of ^{125}I-hLDL was tested for its uptake and degradation by human fibroblasts. In this system, the uptake and degradation of LDL occurs by way of the high-affinity saturable process involving the specific receptor first described by Goldstein and Brown (5, 10). Human fibroblasts were maintained for 48–72 h in 60-mm petri dishes (1 or 2×10^5 cells per dish) containing Dulbecco's modified Eagle's medium, 10% LPDS, 100 U/ml penicillin G, and 100 μg/ml streptomycin. Subsequently, the cells were incubated for 3 h at 37°C with indicated concentrations of ^{125}I-hLDL in the presence and absence of unlabeled LDL. Uptake and degradation assays were performed as described by Goldstein and Brown (10). All degradation data refer to moniodide, trichloroacetic acid-soluable radioactivity.

LDL uptake by fibroblasts was a saturable process: the equilibrium dissociation constant (K_d) was 8.6 μg of LDL protein/ml; degradation of LDL was reduced in the presence of 10-fold excess unlabeled lipoprotein. These data showed that the iodo-labeled LDL used in the present experiments was actively metabolized by fibroblasts, and suggested that in vivo and in vitro differences noted in the present studies were not due to an alteration in the lipoprotein particle itself.

(*b*) Rat granulosa cells were maintained as described above for 48 h in 35-mm dishes ($\sim 2 \times 10^6$ cells per dish) with 10% lipoprotein-deficient fetal calf serum. Subsequently the cells were incubated for 3 h at 37°C with 5, 10, 20, 40, 60, 80, and 100 μg protein/ml concentrations of ^{125}I-LDL (500 cpm/ng protein). Uptake and degradation assays were performed as described by Goldstein and Brown (10).

(*c*) For studies involving the in vivo uptake and degradation of ^{125}I-LDL, two separate sets of superovulated rats were injected either with saline or hCG (25 IU) s.c. 1 h after saline or hCG treatment, ovaries of these animals were perfused with ^{125}I-LDL (1 μCi; 100 μg protein/ml) for 1 h at a flow rate of 1.1 ml/min. 12 fractions (5 ml) each were collected and assayed for LDL degradation as described by Goldstein and Brown (10).

CHARACTERIZATION OF ^{125}I-hHDL BOUND TO OVARY TISSUE MEMBRANES. SDS-PAGE was used to determine whether radioactivity was still associated with apo B after perfusion and binding of LDL to luteinized ovaries. A luteinized ovary plasma membrane-enriched fraction was isolated as described previously (4). 50 μg of the membrane fraction from perfused ovaries or 10 μg of purified ^{125}I-LDL was loaded on SDS-PAGE and subjected to electrophoresis. Protein bands were identified with molecular weight standards and purified apo B and radioactive proteins in the samples were identified by radioautography.

MEASURMENT OF PROGESTINS. Progesterone (27) and its metabolite, 20 α-hydroxypregn-4ene-3-one (28), was quantitated by radioimmunoassay using specific antiserum.

MORPHOLOGIC TECHNIQUES. All morphologic procedures used in this study have been described in a previous report from this laboratory (4). For data analysis, 10 nucleated parenchymal cells from each of three blocks per tissue sample were photographed at a low magnification (× 4,000) so as to include an image of the entire cell. For heavily labeled cells from the luteinized ovary, selection was totally random (i.e., the first 10 nucleated cells to appear on the screen were chosen): for cells from other tissues or from cultures, some preselection was necessary to insure the analysis of cells with sufficient numbers of exposed grains. The number of exposed grains counted per cell also varied with the sample and with the length of exposure of the autoradiograph: in general, profiles of luteal cells contained from 15 to 40 grains. Thus, because ovaries from five luteinized animals perfused with hLDL were used in this study, ~3,500 exposed grains were counted for this aspect of the study alone.

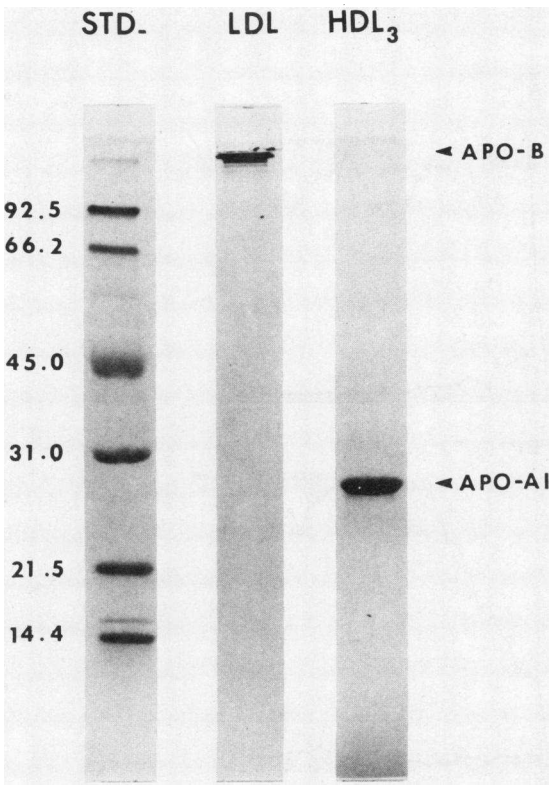

Figure 1. SDS-PAGE of hLDL and $hHDL_3$. Gels are stained with Coomassie blue. The position of apo B and apo AI are indicated. Protein standards are phosphorylase B (mol wt 92,500), bovine serum albumin (mol wt 66,200), ovalbumin (mol wt 45,000), carbonic anhydrase (mol wt 31,000), soy bean trypsin inhibitor (mol wt 21,500), and lysozyme (mol wt 14,400). 20–50 μg of delipidated lipoproteins were applied to each 12% polyacrylamide gel.

Materials

Carrier-free ^{125}I-iodine (~17 Ci/mg), [1,2-^{3}H(*N*)] progesterone (40–60 Ci/mmol) and 20 α-[1,2-^{3}H]-hydroxypregn-4-ene-3-one (40–60 Ci/mmol) were obtained from New England Nuclear, Boston, MA. Progesterone and 20 α-hydroxypregn-4-ene-3-one were purchased from Steraloids Inc., Wilton, NH. Eagle's minimum essential medium, Medium 199, McCoy's 5a medium, fetal calf serum, and antibiotics were from KC Biologicals Inc., Lenexa, KS. The antibody against 20 α-hydroxypregn-4-ene-3-one was kindly supplied by Dr. Harold R. Behrman of Yale University. The antibody against progesterone was obtained from Radioassay Systems Laboratories, Carson, CA. All other chemicals were of analytical grade.

Results

LDL uptake and response by the perfused luteinized ovary. Luteinized ovaries were washed for 2 min with Medium 199, then perfused for 30 min with increasing concentrations of ^{125}I-hLDL (3 μCi/ml). After a final 2-min wash with medium, the ovaries were excised and counted in a gamma counter (Searle Analytic Inc., Arlington Heights, IL, model 1185). Fig. 2 *A* describes the results obtained from two separate dose-response studies, each using a separate ligand preparation: the LDL uptake curve indicates that tissue saturation with ^{125}I-hLDL occurs only at high concentrations of the perfused ligand (600–1,000 μg of protein/ml). In addition, when ovaries are perfused with 1,000 μg of protein/ml ^{125}I-hLDL (3 μCi/ml) concentration for varying periods of time, uptake is linear for 2–2½ h (Fig. 2 *B*), after which the curve flattens. Whether true saturation occurs between the second and third hour of perfusion, or whether tissue function begins to decline after this prolonged procedure, is not clear.

Competition for the uptake of labeled LDL occurs when luteinized ovaries are perfused with 10-fold unlabeled hLDL or $hHDL_3$. In Table I, ovaries were perfused for 30 min with ^{125}I-hLDL (100 μg of protein/ml, 5 μCi/ml) with (or without) unlabeled (1,000 μg of protein/ml) hLDL or $hHDL_3$. As indicated, the addition of either excess LDL or HDL_3 reduces tissue uptake of LDL by ~90%.

ARGs of SDS-PAGE of plasma membrane-enriched fractions obtained from luteinized ovaries perfused for 1 h with ^{125}I-LDL (500 μg of LDL protein/ml, 5 μCi/ml) indicated that the radioactivity was exclusively associated with apo B: no other protein bands were detected (data not shown).

That perfused LDL are capable of inducing and maintaining steroid production is seen by data such as displayed in Fig. 3. Here, the luteinized ovaries of animals were perfused on the same day with saturating levels of hLDL (1,000 μg of protein/ml) for 1 h; 1-ml effluent samples obtained from the vena cava were assayed for progesterone and 20 α-hydroxypregn-4ene-3-one levels by radioimmunoassay. Fig. 3 shows that perfusion with LDL results in a dramatic increase in secretion of progesterone and its metabolite, 20 α-hydroxypregn-4ene-3-one (progestins), as compared with ovaries perfused for a similar period without LDL (control). On the other hand, less than saturating levels of LDL protein (i.e., 100–500 μg of protein/ml) are associated with a submaximal hormone response, and small levels of LDL protein (10–50 μg of protein/ml) do not induce a progestin response (Table II).

Table I. Effect of Unlabeled Lipoproteins on Uptake of ^{125}I-hLDL by Perfused Ovaries

Lipoproteins	^{125}I-hLDL uptake	% uptake
	cpm/mg ovary	
^{125}I-hLDL	5575	100
^{125}I-hLDL + hLDL	408	7
^{125}I-hLDL + $hHDL_3$	596	10

Luteinized ovaries were perfused for 30 min with Medium 199 containing 100 μg/ml, 5 μCi/ml ^{125}I-hLDL alone, or in the presence of additional unlabeled hLDL (1,000 μg/ml) or $hHDL_3$ (1,000 μg/ml). Subsequently, the ovaries were perfused with media alone for 2 min, excised and counted in a gamma counter. The results represent a mean of two experiments with each ligand combination.

Localization of labeled LDL in luteinized ovary during perfusion. LM ARGs of luteinized ovary perfused for 1 h with ^{125}I-

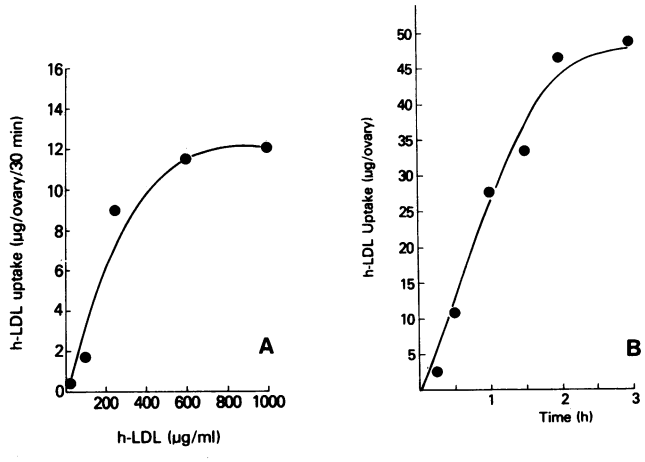

Figure 2. Concentration and time course of hLDL uptake by perfused luteinized ovaries. (*A*) Perfusion was carried out for 30 min followed by a 2 min wash with media after which the ovaries were excised and counted in a gamma counter. Saturation with LDL occurred at protein concentrations > 600 μg of protein/ml. (*B*) luteinized ovaries were perfused with ^{125}I-hLDL for varying periods of time using 1,000 μg of protein/ml. Uptake was linear until 2 h. Experiments represent two separate studies each using a separate ligand preparation.

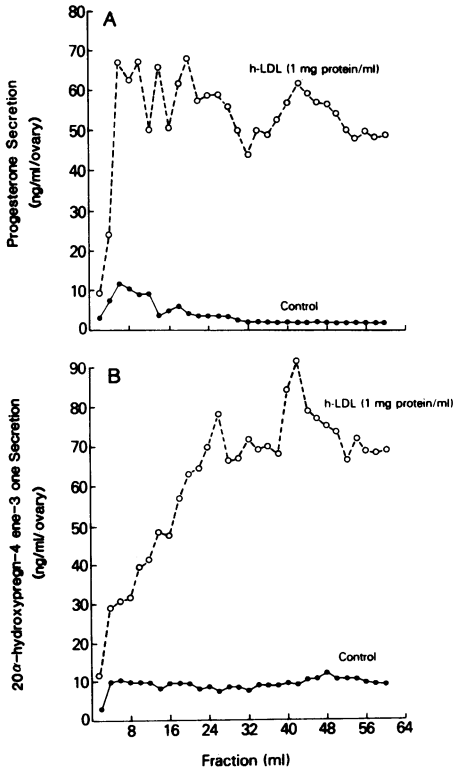

Figure 3. Typical pattern of effect of hLDL perfusion on progestin secretion by the luteinized ovary. 1-ml effluent samples were collected from ovaries perfused with (or without) hLDL (1,000 μg of protein/ml). Ovaries perfused for 1 h (total of 60 fractions) with hLDL showed a rapid and sustained increase in progesterone (*A*) and 20 α-hydroxypregn-4ene-3-one production (*B*).

Table II. Effect of Different Doses of hLDL on Progesterone and 20 α-Hydroxypregn-4ene-3-one Secretion by Perfused Luteinized Ovaries

hLDL (protein)	No. of experiments	Progesterone	20 α-Hydroxy-pregn-4ene-3-one	Total progestins
		ng per ovary per hour		
None	4	240	519	759
10 μg/ml	2	250	496	746
50 μg/ml	2	316	478	794
100 μg/ml	2	640	713	1353
500 μg/ml	2	1686	2040	3726
1,000 μg/ml	4	3184	3703	6887

Results are mean of separate experiments. Except for two experiments without LDL and two experiments with 1,000 μg of LDL/ml (representing the data displayed in Fig. 3), all other experiments were conducted with the same ligand preparation.

h- or rLDL (100–500 μg of protein/ml; 45 μCi/ml) indicate that the label has special affinity for the luteal cells of the tissue (Fig. 4). However, owing to the irregularity of the luteal cell surface and the compact nature of the tissue, it is not possible at this magnification to determine the cellular distribution of the exposed grains.

EM ARGs of the same tissues perfused with human (Fig. 5) or rat LDL show that the large majority (~90%) of the exposed grains associated with the luteal cells are present on the luteal cell plasma membrane. This occurs whether perfusion is carried out for 15 min, 1 h, or 2 h, whether LDL protein concentration is low (100 μg) or high (500 μg), or whether homologous or heterologous LDL is used. Of the exposed grains associated with luteal cells very few (<0.2%) are associated with endocytic vesicles, vacuoles, or lysosomal-like structures. A higher-magnification view of the luteal cell surface after perfusion (Fig. 6) indicates that the exposed grains are associated with both upright and inverted microvilli and in every respect resemble the distribution of labeled HDL_3 in similarly perfused luteinized ovaries (4).

Localization of labeled LDL in perfused 4-APP-treated luteinized ovary, in vivo infused 4-APP luteinized ovary, PMSG-treated (granulosa cell-differentiated) ovary, and liver. EM ARGs of ^{125}I-LDL perfused ovaries from 4-APP-treated rats show identical distribution of label to that seen in non-4-APP-treated luteinized ovaries. Moreover, EM ARGs of ^{125}I-LDL in vivo infused ovaries from 4-APP-treated rats show identical distribution of label to that seen in in situ perfused non-4-APP-treated luteinized ovaries: i.e., ~90% of the label remains with the plasma membrane of luteal cells (Fig. 7). The only notable difference in the in vivo labeled ovaries is the rather large amount of labeling of nonluteal cells (thecal, connective tissue, and endothelial cells) of the tissue (data not shown).

Similarly, EM ARGs of ^{125}I-LDL perfused ovaries of the granulosa cell model show that only the surface of the granulosa cells are labeled (29). Only rarely are exposed grains found within the cytoplasm of the cells.

In contrast, EM ARGs of ^{125}I-LDL perfused estrogen-treated liver shows that most of the exposed grains are present within cells with a large majority of the internalized grains closely associated with lysosomal-like vacuoles (Fig. 8) and/or VLDL-filled vacuoles. Similar results have been obtained from several other laboratories (17, 30, 31).

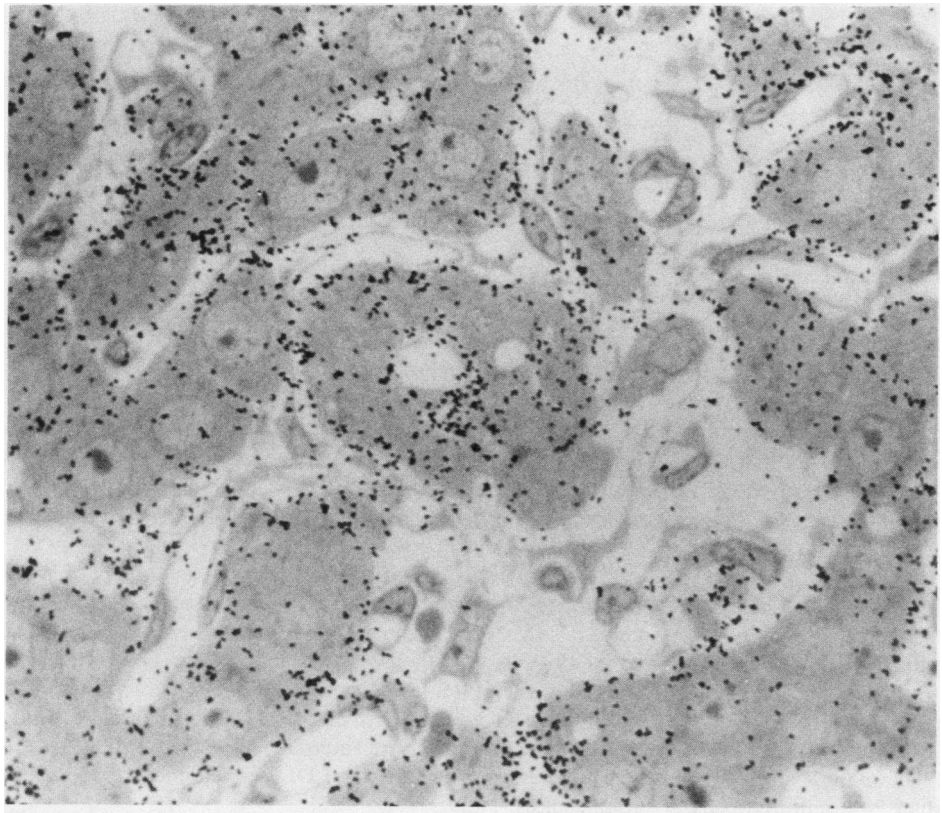

Figure 4. LM ARG of luteinized ovary perfused for 1 h with ^{125}I-hLDL (100 μg of protein/ml, 45 μCi/ml). Most of the exposed grains are associated with luteal cells (large cells with large round nuclei). With LM ARGs many grains appear to be inside cells, but inspection of the same tissue at the EM level shows the exposed grains are associated primarily with luteal cell plasma membranes.

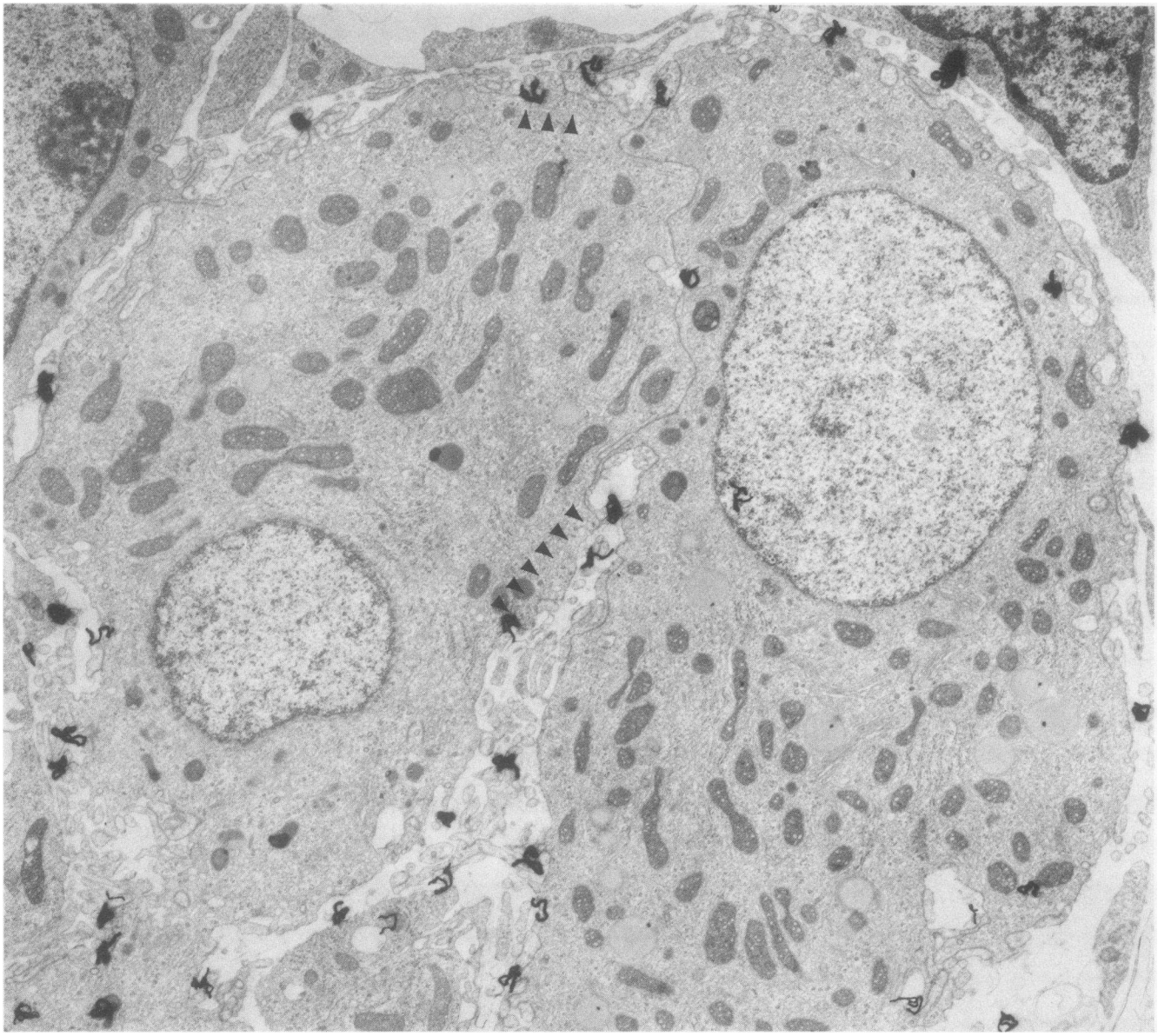

Figure 5. Low-magnification EM ARG showing exposed grains over two luteal cells after 1-h perfusion of luteinized ovary with ^{125}I-hLDL (500 μg of protein/ml; 45 μCi/ml). Quantitation of randomly photographed cells indicated that <10% of the exposed grains were associated with organelles within the cells. Most grains were associated with the microvillus surface (*arrowheads*) of luteal cells. × 8,200.

Localization of labeled LDL in fibroblasts and in freshly isolated or cultured luteal and granulosa cells. Incubation of ^{125}I-labeled LDL for 2, 6, and 24 h with cultures of fibroblasts grown in LPDS showed a time-related accumulation of label within cells: the label was associated with endocytic or lysosomal-like vacuoles, as previously described (32). These results verified fibroblast binding and degradation data obtained using the same ligand (see Methods).

Likewise, cultured luteal cells and granulosa cells incubated for 2 h with either ^{125}I- or gold-labeled LDL showed labeling within cytoplasmic vacuoles of various sorts, suggesting internalization of ligand (Figs. 9 and 10). Although both isotope and gold-labeled LDL was effectively internalized by the cells, the amount of label internalized was dependent on whether the cells were freshly isolated (and dispersed in LPDS for 2 h) or cultured for 24 h (in contact with LPDS for 24 h) before incubation with LDL: i.e., fewer freshly isolated cells than 24-h cultured cells showed internalization of any labeled LDL and less label per cell was present in the freshly isolated preparations compared with the 24-h cultured cells.

Granulosa cells, which have few microvilli in situ, had a healthy appearance in vitro at 2 and 24 h. Although the cells showed some label on their surfaces, a large proportion of either the radiolabel or gold (Fig. 9 *A* and *B*) was found inside the cells associated with structures of endocytic-lysosomal pathway.

It is important to note that the microvillar surface of luteal cells often did not survive the cell isolation process, and many cultured luteal cells, even after 24 h, had broken vesicles attached to the outer face of their plasma membranes. These disrupted structures were often associated with label, much of which was not displaceable with 100-fold excess unlabeled ligand. Despite this, luteal cells in culture internalized labeled LDL (Fig. 10) and were efficient in utilizing LDL-derived cholesterol for steroidogenesis.

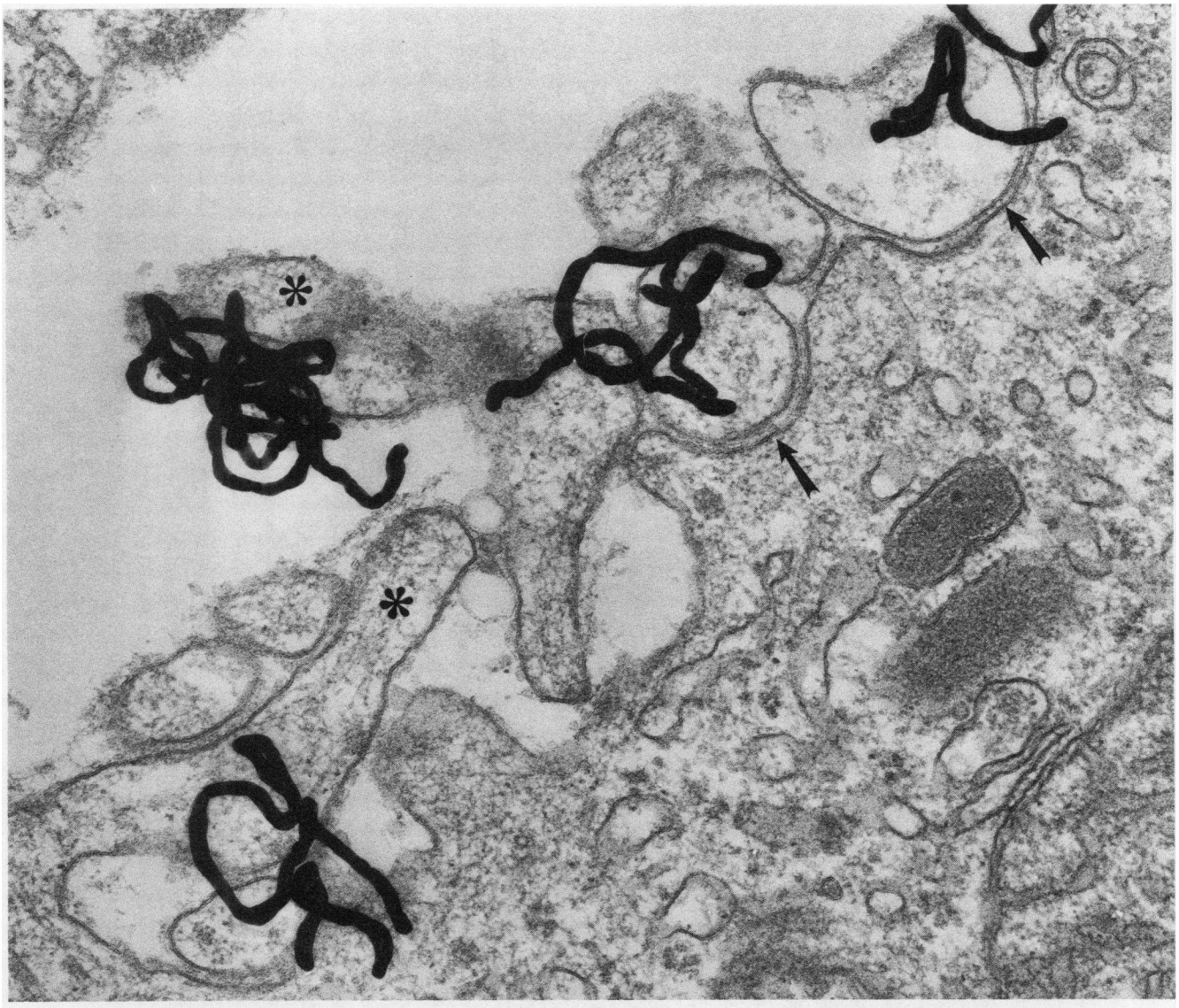

Figure 6. Higher-magnification view of association of exposed grains with luteal cell microvilli (^{125}-hLDL, 500 μg/ml, 45 μCi/ml). Grains are seen on both upright (*asterisks*) and inverted microvilli (*arrows*). This distribution of LDL resembles that seen in ovaries perfused under identical conditions with hHDL (see Reaven et al. [4]). × 80,000.

Degradation of ^{125}I-LDL by the perfused luteinized ovary and by granulosa cells in culture. In view of the unexpected morphologic differences noted in LDL uptake by steroid-producing cells in situ and in vitro, it seemed prudent to examine the issue further—this time using the degradation of LDL as an indicator of lipoprotein uptake, internalization and utilization. Perfusion effluent was collected from luteinized ovaries of "control" rats (injected with saline) and hormonally "stimulated" rats (injected with hCG 1 h prior to perfusion) and assayed for levels of LDL degradation. No evidence of degradation of the protein moiety of LDL was observed when the ovaries were perfused with ^{125}I-LDL for up to 1 h, regardless of whether the rats had been stimulated with hCG or not: i.e., trichloroacetic acid-soluble, noniodide ^{125}I accounted for 0.017% and 0.008% of the total radioactivity of the samples from saline and hCG-treated rats, respectively. On the other hand, the hCG injection resulted in a rapid and significant increase in plasma progestin levels: mean (±SE) levels of progesterone and 20 α-hydroxy preg-4-ene-3-one for control rats were 440 (±77) and 132 (±9) ng/ml, respectively, where the same plasma hormone levels in stimulated rats were 2,592 (±293) and 985 (±50) ng/ml, respectively. Thus, total plasma progestin levels for control rats equaled 571 (±84) ng/ml, whereas total plasma protestin levels for hCG-stimulated rats equaled 3,577 (±288) ng/ml—a sixfold increase.

In contrast to the lack of LDL degradation observed in the perfused ovaries, results in Fig. 11 show the uptake of ^{125}I-LDL to be a saturable and specific process in granulosa cells in vitro. The equilibrium constant (K_d) calculated for this process was 22 μg of LDL protein/ml. Fig. 11 *B* shows that the cells degraded ^{125}I-LDL with a half-maximal concentration of ~18 μg of LDL protein/ml.

Discussion

The current study resulted in several unexpected observations which suggest, on the whole, that rat ovary luteal cells (and gran-

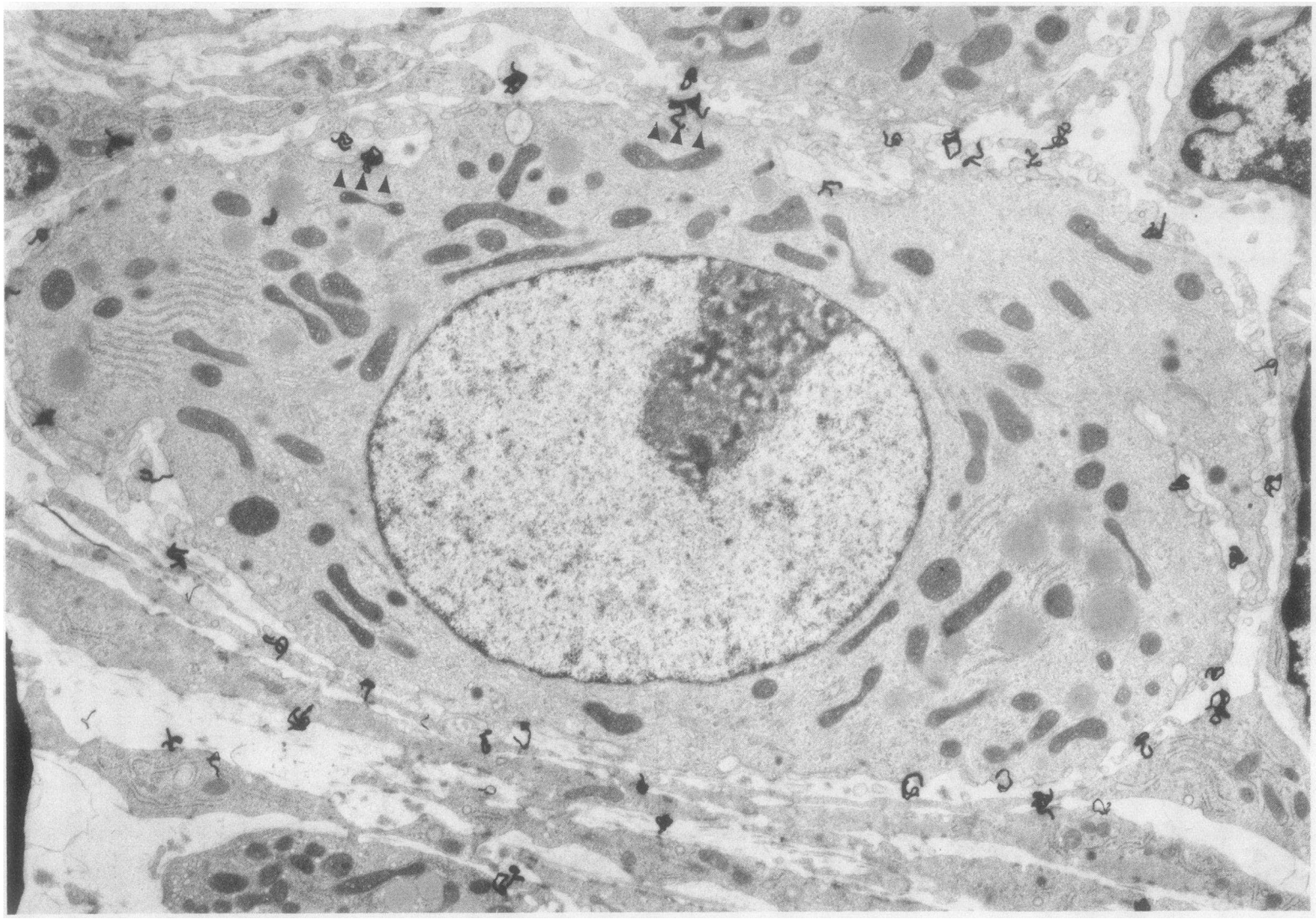

Figure 7. EM ARG of luteal cell from 4-APP-treated rat in which ^{125}I-hLDL (1.5 mCi/ml; 3.1 mg of protein/ml) was administered intravenously and permitted to circulate with blood for 1 h before perfusion fixation of the ovaries. Exposed grains are primarily associated with the cell surface (*arrowheads*). × 8,200.

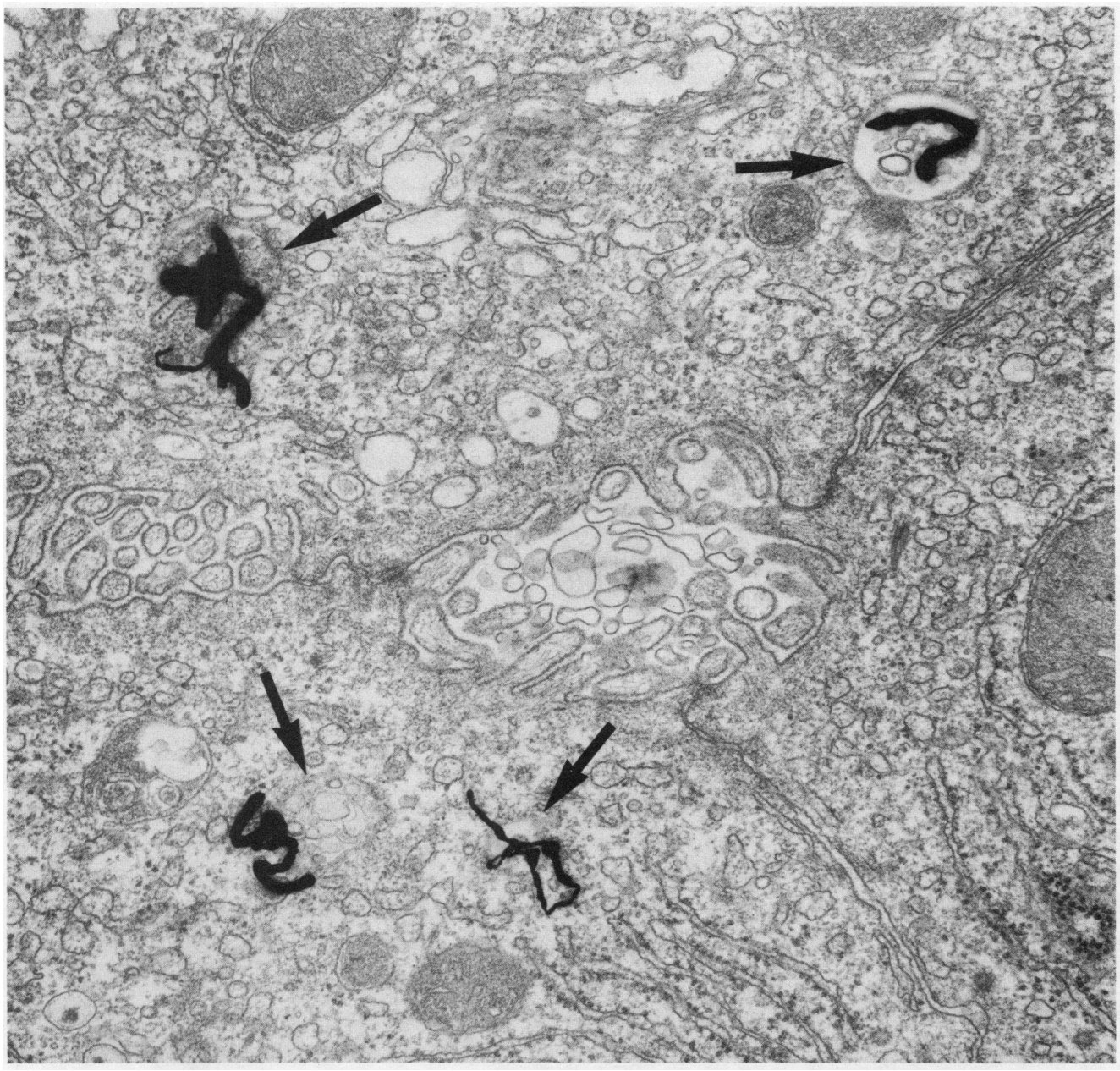

Figure 8. Autoradiograph of bile canalicular surface of hepatocytes from estrogen-treated liver perfused for 1 h with hLDL (100 μg of protein/ml; 45 μCi/ml). Exposure 6 wk. In contrast to the situation in the ovaries (Figs. 5–7), most of the exposed grains are found within the cytoplasm of hepatocytes associated with lysosomal-like vacuoles (*arrows*). × 50,000.

ulosa cells) in situ do not utilize LDL cholesterol by the classical endocytic-lysosomal pathway (5). Instead, the large majority of the labeled protein moiety of LDL (whether from homologous or heterologous sources) remains constantly associated with the plasma membrane surface of the cells, even though steroidogenesis is dramatically increased. The general distribution of the labeled LDL protein on the luteal cell surface and particular localization of the LDL protein to specialized sites of inverted microvilli strongly reminds one of the recently described association of HDL protein with the same cells (4). It is of special interest, therefore, that the uptake of ^{125}I-LDL-protein by the luteinized ovary can be displaced equally well by excess unlabeled LDL or HDL_3. Insofar as this event occurs in perfusion experiments (where defined media is used), there is little likelihood of exchange between lipoprotein particles. It is well to keep in mind, however that LDL and HDL have somewhat different "binding" characteristics in this rat tissue: as shown here, the luteinized ovary has low affinity but high capacity for hLDL, whereas the situation with hHDL in the same tissues, under identical experimental conditions, is the converse (4). In all, these experiments show that under the condition of this study, LDL "binds" to the same sites on the cell surface where HDL "binds" and suggests that LDL cholesterol (like HDL cholesterol) must be obtained by cells by a mechanism that does not require internalization of the intact lipoprotein particle (2, 4). To what extent such a process could correspond to the LDL receptor-independent pathway previously described in rats by Koelz et al. (33) remains to be seen. It should be noted that although

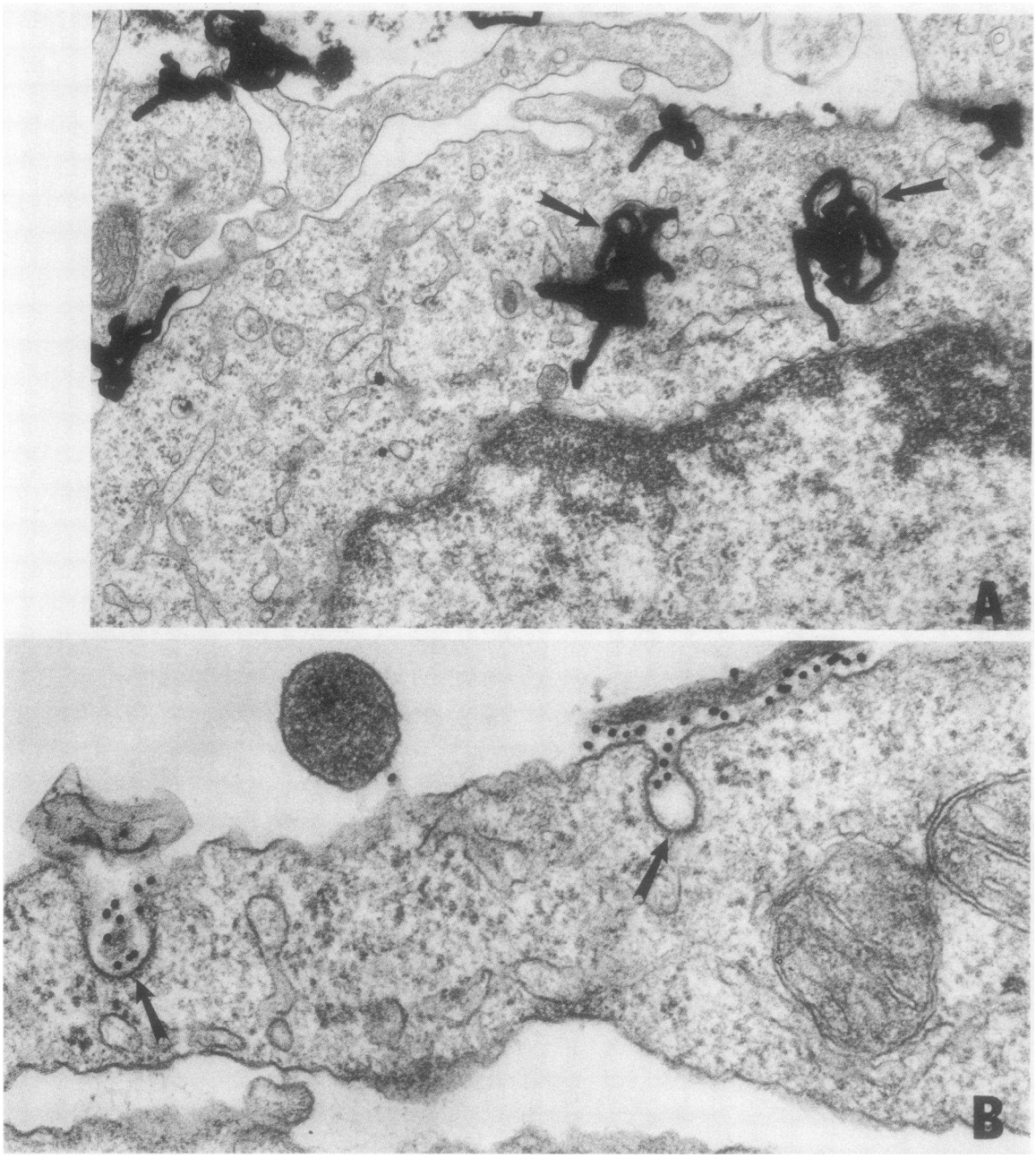

Figure 9. 24-h cultured granulosa cells incubated for 2 h with (*A*) ^{125}I-labeled hLDL (50 μCi/ml; 100 μg of protein) or (*B*) gold-labeled hLDL. These cells internalize varying amounts of label. In *A*, lysosomes are labeled (*arrows*). × 30,000. In *B*, funneling of the LDL into coated pits (*arrows*) on the outstretched extremity of one cell is clearly shown using gold-labeled ligand. × 70,000.

evidence for a common HDL and LDL receptor site on cells has been obtained in cell systems such as adipocytes (34), that previous reports using steroid tissues (35, 36) provide evidence for separate receptor sites for the uptake of LDL and HDL. Obviously, further work involving specific chemically modified lipoprotein particles will be necessary before the issue raised in the present study regarding a common HDL and LDL binding site in the rat ovary can be resolved.

To a large extent, the results of this study depend on the legitimacy of both the tissue model and the labeled ligand used in the study, and we have attempted to take every precaution in this regard. First, the ovary was examined as both a granulosa cell and luteal cell-differentiated model: furthermore, luteinized ovaries from control and/or lipoprotein-deprived (4-APP) animals were examined after the administration of ligand by two different routes—by perfusion and/or by intravenous injection. In all situations, the results were the same and showed that in situ the protein portion of the exogenously provided LDL binds to, and remains associated with, the complex microvillar surface of the steroidogenic cells during the course of the study.

In an effort to control ligand quality, each preparation of hLDL was rigorously characterized. The binding and degradation of LDL by fibroblasts was identical to that previously described (10) and the morphologic internalization and association with coated pits, endosomes, and lysosomes (32) of fibroblasts was verified. Electrophoresis of each batch of hLDL used revealed

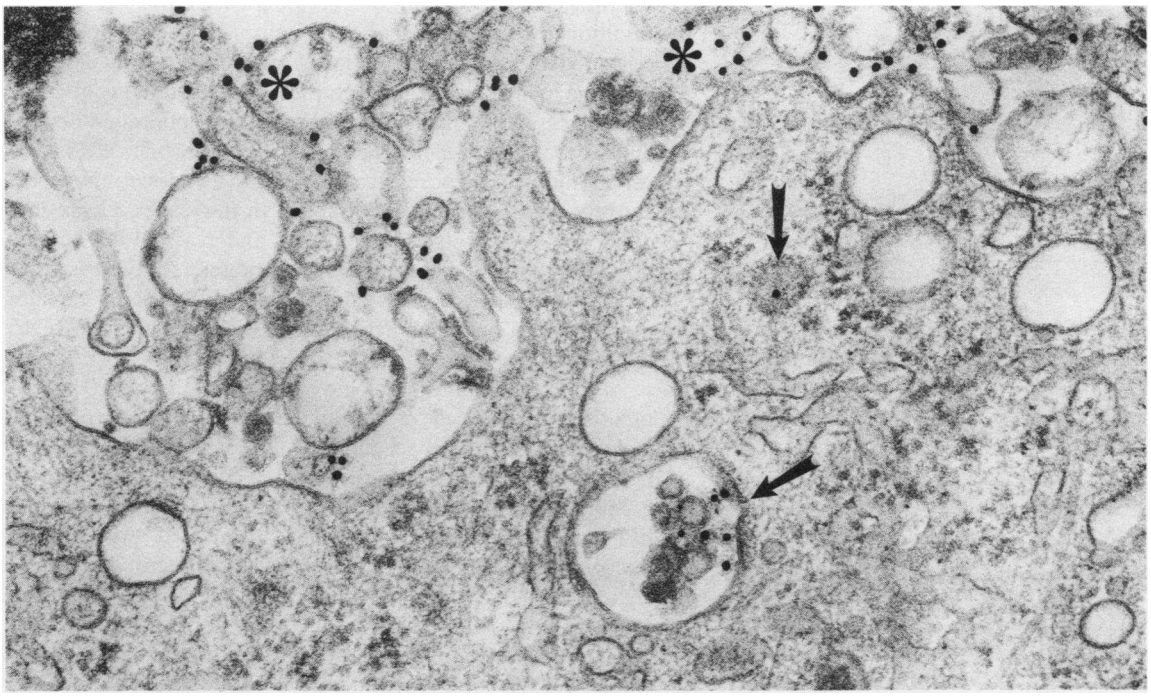

Figure 10. 24-h cultured luteal cell incubated with gold-labeled hLDL for 2 h (50 μCi/ml; 100 μg protein/ml). Like the cultured granulosa cells of Fig. 9, luteal cells internalize the radioactive or gold-labeled LDL. The label is often seen in endocytic vacuoles (*arrows*) with coated surfaces as demonstrated here. It is important to note that, although the isolated luteal cell sheds most of its complex microvillar surface after collagenase treatment, a large number of these vesiculated structures remain associated with the luteal cell surface during incubation and continue to "bind" the labeled ligand (*asterisks*). × 70,000.

only a single protein band consistent with the position for apoprotein B, and in this regard was entirely different from the lipoprotein (HDL) used where only apo A-I was present. In addition, the morphologic uptake of the labeled hLDL by estrogen-treated liver (with induced LDL receptor sites [8, 9]) or by granulosa cells in culture (37) was found to be identical to that described in other laboratories (1, 2, 5). An important control involved the use of homologous LDL. Insofar as hLDL had previously been shown to have low affinity for rat tissues (34, 38), it was necessary to compare the effect of the human-derived lipoprotein with that obtained directly from rats. Owing to the scarcity of rat LDL and the nonrecirculating nature of our perfusion studies, we could not conduct the entire study with the homologous ligand, but when radiolabeled rLDL was, in fact, used, its morphologic distribution was identical to that seen with hLDL. Of course, the final and most important control was the fact that perfusion with hLDL in luteinized ovaries or incubation with hLDL in luteal cells and granulosa cells in culture was directly associated with a strong steroidogenic response. Thus, we are confident that the ligand used in these studies was fully characteristic of LDL, and, at the same time, was a suitable biologic source of cholesterol for the steroidogenic tissues. As such, we are now proposing that rat luteal and granulosa cells in situ utilize LDL cholesterol for steroidogenesis, but appear to do so without internalizing the intact LDL particle. In this respect, the ovarian steroidogenic cells seem to process LDL in situ just as they do HDL (4).

In formulating this view we have been mindful of the fact that a small percentage (~10%) of the exposed grains labeling the luteal cells in situ appear to lie within the cytoplasm of the cells. If these grains do, in fact, represent internalized intact LDL (perhaps reflecting a high-affinity receptor-mediated process), one wonders whether they can deliver enough cholesterol to the luteal cells to account for the amount of progestins synthesized. Calculations[2] based on the information given in Table II show this to be an unlikely possibility: that is, the amount of cholesterol which could be released from these "internalized" particles accounts for no more than 2% of that necessary to sustain the observed steroid hormone production. Several other observations confirm this impression. For one, although ~10% of the luteal cell-associated grains appear over the cytoplasm of the cells in in situ experiments, they do not show specific association with cytoplasmic organelles (endosomes, lysosomes, Golgi bodies), which are normally associated with the LDL endocytic process. Second, if a high-affinity LDL receptor pathway were to exist, then even low concentrations of perfused LDL should elicit a progesterone response: our results show that perfusion concentrations of 10–50 μg of LDL protein/ml did not result in measurable progestin release, and that 100–500 μg of LDL protein/

2. This calculation is based on a perfusion delivery of 100 μg of LDL protein/ml for 60 min. Given an average uptake of 3.0 μg protein/ovary and assuming that all bound radioactivity is, in fact, associated with the luteal cells of the tissue and that LDL protein to cholesterol mass ratio is 1:1, then the luteal cells of the ovary receive 3.0 μg of cholesterol from the 1-h perfusion. If 10% of this cholesterol is internalized, and assuming 100% conversion, ~300 ng of cholesterol is available to account for the 600 ng of progestins produced (Table II). However, in other luteal cell systems studied the steroid-producing cells are able to convert only 0.1–4.0% of the available cholesterol to steroid hormones (39, 40) and if these conversion figures are used, then ony 3–12 ng of cholesterol would be available to the luteal cells for production of the 600 ng of progestins.

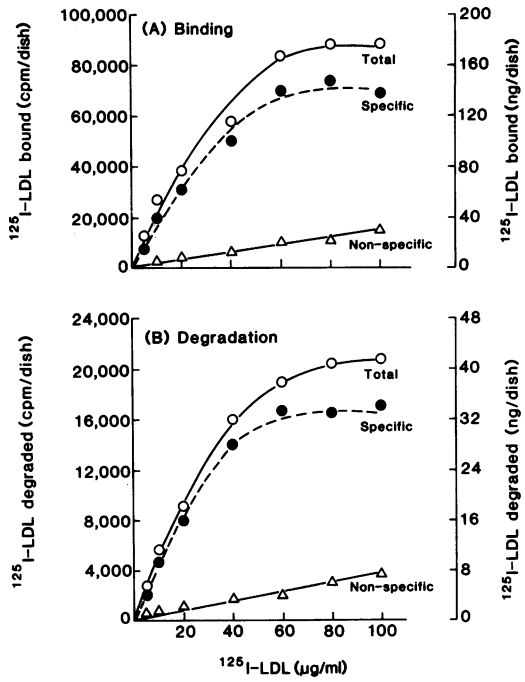

Figure 11. Typical granulosa cell uptake (*A*) and degradation (*B*) pattern with hLDL used in these studies. Cells (2×10^6 cells per dish) were incubated for 3 h with increasing concentrations of ^{125}I-hLDL in the absence (○) or presence (△) of 10-fold excess unlabeled LDL. Binding (*A*) is the difference in LDL uptake in the absence and presence of excess LDL (●). Degradation (*B*) is determined by measuring the appearance of trichloroacetic acid-soluble radioactivity in the media. Each point represents the mean of three determinations.

ml resulted in a progressively increasing, but submaximal response, as compared with the maximal response elicited with 1,000 μg of LDL protein/ml. Moreover, the percentage of exposed grains within the cells did not vary in ARGs prepared from animals perfused with different concentrations of LDL protein. Although these data suggest that our current procedures do not mask a more subtle endocytic process, the issue is clearly not closed and further experimentation with high resolution techniques will be required to settle the point.

We realize, too, that the conclusions of this report are in conflict with earlier studies by Steinberg and colleagues (41–43), in which the systemic administration of LDL covalently linked wiht nondegradable tags (e.g., sucrose) resulted in an accumulation of label within various tissues of the rat including those of the ovary. These results have been interpreted to mean that LDL internalization occurs in steroidogenic cells in vivo (43). Several technical differences in the experimental approach to the problem by our two laboratories may account for the observed incongruent findings. For instance, in the experiment carried out with the sucrose-tagged LDL, the ligand circulated in the blood stream for 24 h before the tissues were examined; if there is a very low rate of internalization of LDL (as discussed above), then it is possible that results not evident in our 1-h in vivo study might be fully apparent by the 24-h time point used in the studies of Steinberg et al. (41–43). On the other hand, changes in ligand are certain to occur during 24 h of ligand circulation through the various organs of the body, and it is not clear how much nonspecific material is being processed by the steroidogenic cells during this prolonged experimental period. Additionally, one wonders how certain it is that steroidogenic cells in vivo degrade LDL and accumulate the sucrose label in the same manner as the test cells (fibroblasts) did in the original studies (44, 45). Perhaps degradation of the lipoprotein occurs only at the surface membrane of the steroidogenic cells in inverted microvillar pockets [as proposed in an earlier report (4)] and the sucrose tag remains trapped in these sites. Given the differences noted between the in vitro and in vivo LDL uptake behavior of steroidogenic cells in the present study, it is conceivable that such an event could occur—and it could explain the conflicting points of view between the sucrose-tagged LDL studies and our own.

Additional, nonmorphologic evidence in support of the notion that rat luteal cells in situ can utilize LDL-cholesterol without internalizing the intact particle was provided by the fact that no perceptible LDL protein degradation occurred during the 1-h period of perfusion of the luteinized ovary with ^{125}I-labeled hLDL. Moreover, if the animals are "stimulated" with hCG 1 h prior to experimentation, their plasma progesterone levels increased sixfold over saline-injected rats, but effluent perfusion samples from ovaries of such rats failed to show measurable evidence of protein degradation. In contrast, granulosa cells in culture actively bind and degrade ^{125}I-hLDL provided from the same preparation. These biochemical data are consistent with the notion that LDL are handled differently by cells in situ and in vitro and support the conclusions of the morphologic studies that suggest that luteal cells in vivo do not need to degrade LDL particles intracellularly in order to obtain cholesterol for steroidogenesis.

How then can one explain that cells in vitro internalize the labeled LDL protein, whereas the in situ maintained cells do not? The issue is not simply a function of differing needs for exogenous cholesterol, because in both the in vivo and in vitro situations these differences can be demonstrated even when the cells are exposed to a lipoprotein-deficient environment (for in situ cells, the environment of a 4-APP-treated animal; for isolated cells, media prepared with lipoprotein-deficient serum). It is curious also, that freshly isolated granulosa cells (which are obtained simply by squeezing pierced ovarian follicles) show some internalization of the labeled ligand even within the first 2 h of their separation from the intact tissue: the amount of ligand that is internalized and the number of cells within any preparation that participate in this process merely increase with time in culture. The explanation for the functional difference between cells in vivo and in vitro is not yet at hand. It would seem that cells separated from their natural environment, even under the most gentle conditions, are structurally or functionally modified and may develop an accessory pathway for the uptake of cholesterol which is more in keeping with the endocytic-degradative pathway usually associated with LDL binding and uptake. Clearly this phenomenon requires further experimentation.

Finally, it should be emphasized that most of the morphology and virtually all of the biochemical observations of this study were made on the hormonally stimulated luteinized ovary of the pseudopregnant, immature, rat. The ovarian tissue of the pseudopregnant rat is highly stimulated and highly specialized, and although it is certain that the tissue utilizes both HDL and LDL cholesterol for steroidogenesis (46), it is not clear whether this tissue is unique in its processing of lipoproteins: we see no evidence of LDL uptake by the classical endocytic pathway;

moreover, it is not known whether this tissue is capable of endocytosis of any ligand. Questions relating to the more general nature of these findings in other rat tissues, and in other species, will be explored in subsequent studies.

Acknowledgments

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