Uptake of Gold- and \[^{3}H\]Cholesteryl Linoleate–labeled Human Low Density Lipoprotein by Cultured Rat Granulosa Cells: Cellular Mechanisms Involved in Lipoprotein Metabolism and Their Importance to Steroidogenesis

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ABSTRACT We used electron microscopy, acid hydrolase cytochemistry, and biochemistry to analyze the uptake and metabolism of colloidal gold- and \[^{3}H\]cholesteryl linoleate–labeled human low density lipoprotein (LDL) by cultured rat granulosa cells. The initial interaction of gold-LDL conjugates with granulosa cells occurred at binding sites diffusely distributed over the plasma membrane. After incubation with ligand in the cold, 99.9% of the conjugates were at the cell surface but <4% lay over coated pits. Uptake was specific since it was decreased 93–95% by excess unconjugated LDL and heparin, but only 34–38% by excess unconjugated human high density lipoprotein. LDL uptake was related to granulosa cell differentiation; well-luteinized cells bound 2–3 times as much gold-LDL as did poorly luteinized cells. Ligand internalization was initiated by warming and involved coated pits, coated vesicles, pale multivesicular bodies (MVBs), dense MVBs, and lysosomes. A key event in this process was the translocation of gold-LDL conjugates from the cell periphery to the Golgi zone. This step was carried out by the pale MVB, a prelysosomal compartment that behaves like an endosome. Granulosa cells exposed to LDL labeled with gold and \[^{3}H\]cholesteryl linoleate converted \[^{3}H\]sterol to \[^{3}H\]progestin in a time-dependent manner. This conversion was paralleled by increased gold-labeling of lysosomes and blocked by chloroquine, an inhibitor of lysosomal activity. In brief, granulosa cells deliver LDL to lysosomes by a receptor-mediated mechanism for the hydrolysis of cholesteryl esters. The resulting cholesterol is, in turn, transferred to other cellular compartments, where conversion to steroid occurs. These events comprise the pathway used by steroid-secreting cells to obtain the LDL-cholesterol vital for steroidogenesis.

Cholesterol is an obligate intermediate in the synthesis of steroids. Cells generally obtain this sterol through de novo synthesis or from plasma lipoproteins. Over the past few years, it has become clear that steroid-secreting organs of several mammals use cholesterol carried by circulating lipoproteins in preference to sterol produced through de novo synthesis for the manufacture of steroid hormones (13). In particular, tissues that produce large quantities of steroid, such as luteinized ovaries, rely heavily on lipoproteins as a source of cholesterol. Indeed, de novo synthesis alone is incapable of providing sufficient substrate to support the metabolic requirements of rat ovarian cells in vivo or in vitro (25, 26, 30).
For ovaries of many species, low density lipoproteins (LDL) are the main source of cholesterol (13). In rodents, although high density lipoproteins (HDL) are the predominant lipoproteins in plasma, ovarian cells can use both LDL and HDL (13). Recent evidence indicates that LDL and HDL interact with steroid-secreting cells through binding sites (19) that differ in a number of ways, including lipoprotein specificity, sensitivity to protease treatment, requirement for divalent cations (6), and the ability of sulfated glycosaminoglycans to displace bound ligand (10). Not only do these lipoproteins use separate binding sites, but differences may also exist in how each is processed by cells (12, 23).

Neither the cellular mechanisms underlying the uptake and processing of lipoproteins nor their relationship to hormone production has been established for steroid-secreting cells. Non-steroid-secreting cells, such as fibroblasts, accumulate lipoprotein through receptor-mediated endocytosis. In this process, which has been clarified through the work of Brown, Goldstein, Anderson, and collaborators on the accumulation of LDL by cultured human fibroblasts (for reviews, see references 3 and 9), LDL binds to receptors at the cell surface, is internalized via coated pits, and undergoes hydrolysis within lysosomes, liberating free cholesterol. The fate of sterol obtained from circulating lipoproteins, however, is quite different in steroid-secreting and non-steroid-secreting cells. In steroidogenic cells, cholesterol is converted mainly to hormones, whereas in fibroblasts it becomes incorporated into cellular membranes.

In view of these major differences, there was a strong need to examine the pathway for the utilization of lipoproteins in steroidogenic tissues. Thus, the purpose of the present study was to determine the cellular basis for uptake and processing of LDL by steroid-secreting cells. We used primary cultures of rat granulosa cells since the steroidogenic function of these cells depends, in large part, on the availability of lipoproteins (25, 26, 30). Use of an in vitro system also permitted us to follow lipoprotein processing under controlled conditions. Our main probe, selected for its sensitivity, resolution, and specificity of the cholesteryl ester, was quantitated by the method of Rudel and Morris (27), who used the 1H-LDL conjugated with human chorionic gonadotropin (Sigma Chemical Co., St. Louis, MO). Ovaries were removed 4 h later, and primary cultures of rat granulosa cells were established as described previously (26, 30), except that the cells (≈250,000 viable cells/culture) were seeded onto collagen-coated culture dishes. Culture medium consisted of RPMI 1640 containing 4 mM glutamine, 25 mM HEPES, and 50 μg/ml gentamicin supplemented with 20% human male serum. Cells were cultured for 48 h, then washed with serum-free medium and placed into culture fluid supplemented with 50 mM lipoprotein-deficient serum (LPDS medium) for 24 h before use.

Conjugation of LDL to Gold: Colloidal gold particles, 20 nm in diameter, were prepared by reducing aqueous HCl/LDL with aqueous trisodium citrate according to the method of Brown et al. (2). LDL was diluted in 4.4-6.6 μg of LDL apoprotein/ml of gold solution) was used to prepare gold-LDL conjugates, as described by Handley (14), with slight modification; i.e., to remove unconjugated LDL, the final pellet was washed by centrifugation in polyethylene glycol (0.2 mg/ml in distilled water) at 10,000 rpm for 45-60 min. Gold-LDL conjugates, stained with 1% sodium phosphotungstic acid, were viewed in an electron microscope. Gold-LDL conjugates were used within 24 h of their preparation.

Uptake of Gold-LDL Conjugates by Granulosa Cells: On the day of the experiment, cultures were chilled to 4°C for 30 min and then incubated with 1.0 ml of cold LPDS medium containing gold-LDL conjugates (≈10^12-10^14 conjugates/ml) for 60-90 min at 4°C or 37°C. After washing six times with LPDS medium to remove unconjugated LDL, cultures were fixed for electron microscopy or incubated in warm LPDS medium (lacking gold-LDL conjugates) for 2-60 min at 37°C, then fixed and processed for electron microscopy.

To determine the fate of sterol carried by exogenous LDL, cultures were incubated simultaneously with gold-LDL conjugates and either (a) 0.1 μg of native LDL or HDL or (b) heparin (10 mg/ml; Sigma Chemical Co.), which displaces bound LDL but not HDL (6, 10). Other cultures were incubated with unconjugated gold.

Metabolism of [3H]Cholesteryl Linolate Carried by LDL: To determine the fate of sterol carried by exogenous LDL, cultures were chilled to 4°C for 30 min and then incubated with medium containing [3H]-cholesterol ester (600,000 dpm [3H]cholesteryl ester/dish) for 90 min at 4°C. After incubation in the cold, other cultures were warmed to 37°C for 30-180 min, with or without chloroquine (50 μM; Sigma Chemical Co.). Media were collected for identification of steroids, and the protein content of the cholesteryl ester was determined by the method of Bradford (2), using BSA as a standard. In parallel experiments, cultures were exposed to LDL labeled with both gold and 1H-cholesterol linolate. Cells were washed with cold LPDS medium, then washed and fixed. After collecting media for biochemical analysis, cultures were washed and processed for electron microscopy.

Other Methods: Radioactive 20α-hydroxyprogesterone-4-en-3-one was isolated by two-dimensional thin layer chromatography, using silica gel plates (SilG3-F-PA, J. T. Baker Chemical Co., Phillipsburg, N.J.) and a solvent system (chloroform/diethyl ether, 9:1, vol/vol). In each system, the radioactive products co-chromatographed with authentic 20α-hydroxyprogesterone.

Electron Microscopy: Cultures were fixed and processed for electron microscopy in the cold. Cultures incubated in the cold were fixed for 60 min in cold 1% paraformaldehyde-1% glutaraldehyde-0.01% trinitroresol in 0.1 M sodium cacodylate buffer, pH 7.4 (FGC-cacodylate) (22). Cultures incubated at 37°C were fixed for 15 min in warm FGC-cacodylate, then for 45 min in cold fixative. After washing in cold buffer, cultures were postfixed, stained en bloc, dehydrated, and embedded as described previously (22). Membrane structure was enhanced by mordanting cultures, after postfixation, with 1% tannic acid (No. 1764, Mallinckrodt Inc., St. Louis, MO) and 1% osmium tetroxide in 0.1 M sodium cacodylate, pH 7.4. After dehydration, cultures were embedded in resin (Ted Pella, Inc., Redding, CA). Thin sections were cut on a diamond knife, mounted on 100-mesh grids, and observed in a transmission electron microscope (Philips EM 300).

Electron Microscopy of Granulosa Cells: Culture of Granulosa Cells: Immature rats (Sprague Dawley, Charles River Breeding Laboratories, Wilmington, MA) were bred under a 14:10 light cycling regimen and killed at 3 days old. Ovaries were removed with 5 IU pregnant mare's serum gonadotropin (Organon Inc., West Orange, NJ) 60 h before receiving 25 IU human chorionic gonadotropin (Sigma Chemical Co., St. Louis, MO). Ovaries were removed 4 h later, and primary cultures of rat granulosa cells were established as described previously (26, 30), except that the cells (≈250,000 viable cells/culture) were seeded onto collagen-coated culture dishes. Culture medium consisted of RPMI 1640 containing 4 mM glutamine, 25 mM HEPES, and 50 μg/ml gentamicin supplemented with 20% human male serum. Cells were cultured for 48 h, then washed with serum-free medium and placed into culture fluid supplemented with 40 mM lipoprotein-deficient serum (LPDS medium) for 24 h before use.

Uptake of Gold-LDL Conjugates by Granulosa Cells: On the day of the experiment, cultures were chilled to 4°C for 30 min and then incubated with 1.0 ml of cold LPDS medium containing gold-LDL conjugates (≈10^12-10^14 conjugates/ml) for 60-90 min at 4°C or 37°C. After washing six times with LPDS medium to remove unconjugated LDL, cultures were fixed for electron microscopy or incubated in warm LPDS medium (lacking gold-LDL conjugates) for 2-60 min at 37°C, then fixed and processed for electron microscopy.

To determine the fate of sterol carried by exogenous LDL, cultures were chilled to 4°C for 30 min and then incubated with medium containing [3H]-cholesterol ester (600,000 dpm [3H]cholesteryl ester/dish) for 90 min at 4°C. After incubation in the cold, other cultures were warmed to 37°C for 30-180 min, with or without chloroquine (50 μM; Sigma Chemical Co.). Media were collected for identification of steroids, and the protein content of the cholesteryl ester was determined by the method of Bradford (2), using BSA as a standard. In parallel experiments, cultures were exposed to LDL labeled with both gold and 1H-cholesterol linolate. Cells were washed with cold LPDS medium, then washed and fixed. After collecting media for biochemical analysis, cultures were washed and processed for electron microscopy.

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Areas 1–2 mm square were cut from plastic-embedded cultures and attached to prepolymerized epoxy blocks. Thin sections (50–100 nm thick), cut perpendicular to or parallel to (en face) the bottom of the culture dish, were prepared, picked up on 0.3% formvar-coated slot grids, and stained with uranyl acetate (15–30 min) and lead citrate (3–5 min).

Quantitation of Gold-LDL Particles, Gold-LDL-Labeled Organelles, and Coated Vesicles: The number of gold-LDL particles or gold-labeled organelles was quantified in sections cut perpendicular to the bottom of the culture dish. A predetermined, nonrandom pattern was used to photograph cells to ensure that they were selected in a nonbiased manner; i.e., the first 20–50 consecutive cells (cellular profiles) were photographed in their entirety at a magnification of 7,000. The final magnification of the prints used for counting was 15,000. The data was expressed as gold particles per cellular profile, or as the percentage of gold particles at the surface, in the cell, or in various organelles. When percentages of gold-labeled organelles were determined, an organelle was scored as labeled if it contained one or more gold particles.

For quantitation of cultures exposed to LDL labeled with both gold and [3H]cholesterol ester, sections were obtained from at least three areas in each of two culture dishes; 26–46 cellular profiles were photographed and analyzed per dish for each time period. Gold-labeled organelles and gold particles associated with various cellular compartments were quantified, percentages calculated, and the mean of the two dishes determined for each category.

To determine whether gold-labeled organelles became associated with the Golgi complex or GERL (the Golgi zone), sections were cut en face, and cells selected and photographed as above. On the final prints, the region containing the Golgi zone was circumscribed with a pen; gold-labeled organelles inside the zone and those within 0.5 cm from the outer edge of the zone were scored as within the Golgi zone. Gold-labeled organelles beyond this region were scored as outside of the Golgi zone. The percentage of organelles within and beyond the Golgi zone was then calculated.

To determine if coated vesicles subjacent to the plasmalemma were free within the cytoplasm or if they represented tangential sections through coated pits, gold-labeled, “apparent” coated vesicles were studied in consecutive serial thin sections (25–35 nm thick). Section thickness was estimated by determining the number of sections in which profiles of the smallest diameter vesicles were present (24).

Cytochemistry: Cultures used for ACPase localization were exposed to gold-LDL conjugates for 60 min at 4°C, washed, and fixed with cold FGC-cacodylate for 30 min or warmed to 37°C for 2, 10, or 60 min, then washed and fixed. ACPase activity was localized in these cultures using methods described previously (22). Cultures used for arylsulfatase localization were exposed to gold-LDL conjugates for 60 min at 4°C, warmed to 37°C for 60 min, and washed and fixed for 2 min with FGC-cacodylate. They were then reacted for the localization of arylsulfatase activity according to methods previously published (22). Specificity of these cytochemical reactions was verified as detailed by Paavola (22).

RESULTS

Fine Structure of Granulosa Cells In Vitro

Most granulosa cells were well-luteinized, containing abundant smooth endoplasmic reticulum, occasional lipid droplets, and an extensive Golgi zone (Golgi cisternae and GERL-like structures) that occupied a perinuclear position in 89% of 150 cells (Fig. 1). Other noteworthy cytoplasmic structures included MVBs and lysosome-like dense bodies. MVBs were 0.2–0.5 μm in diameter, contained one or more small vesicles in a matrix that was electron lucent (pale MVBs) or dense.
(dysosome-like dense bodies were 0.3-0.7 μm in diameter, with a lucent to dense matrix in which myelin-like figures or other residua occurred (Fig. 1). Microvilli studded the surfaces of these cells. A modest number of granulosa cells were poorly luteinized, containing a well-developed Golgi zone, considerable rough endoplasmic reticulum, but little smooth endoplasmic reticulum. They were relatively flat, with few microvilli. Cells intermediate in morphology between these two types were present. Although the proportions varied, well-luteinized cells predominated; in five cultures dishes, they comprised 64–100% of the cells present, with a mean of 75 ± 6% (±SE).

**Uptake of Gold-LDL at 4°C and Specificity of Accumulation**

Granulosa cells cultured for 24 h in lipoprotein-free medium and exposed to gold-LDL conjugates in the cold avidly accumulated gold-LDL particles (Fig. 2a). Typically, 99.9% were at the cell surface (6,730 gold particles in 33 cellular profiles). Of these, <4% lay over coated pits. Most were over noncoated regions of the plasma membrane (Fig. 2a), particularly microvilli (Fig. 2, a and c), and gold-labeling of coated pits at the bases of microvilli was common (Fig. 2d, inset). Although the overall number of gold particles associated with an individual granulosa cell profile was 204 (n = 33 cellular profiles), well-luteinized cells accumulated more gold particles (295 per cellular profile; n = 19) than did poorly luteinized cells (85 gold particles per cellular profile; n = 14). Of the gold particles at the surfaces of poorly luteinized cells, 38% were associated with microvilli, whereas 78% were over microvilli of well-luteinized cells. The lipoprotein component of gold-LDL conjugates was visualized in thin sections of tissue stained en bloc with ruthenium red or treated with tannic acid (Figs. 3a and 4, a-d). Similar results were obtained when granulosa cells were cultured continuously in medium containing 20% serum. However, the number of gold particles per cellular profile was decreased, presumably due to the occupancy of LDL binding sites by LDL present in the serum and to a reduction in the number of lipoprotein receptors because cholesterol was available in the serum.

Uptake of gold-LDL by granulosa cells was lipoprotein specific. In cultures exposed to gold-LDL and excess unconjugated LDL, accumulation of gold particles was reduced by 92.2 ± 5.1% (±SE) in 103 cellular profiles from four experiments as compared with control cultures incubated with gold-LDL alone (Fig. 2b). In contrast, excess unconjugated HDL reduced uptake of gold-LDL by only 38 ± 10.9% (±SE) (n = 38 cellular profiles from three experiments) when incubated at 4°C, and by 34% when incubated at 37°C (n = 36 cellular profiles from one experiment). Cultures incubated with gold-LDL and heparin at 4°C (n = 31 cellular profiles) or 37°C (n = 15 cellular profiles) accumulated 97–99% fewer gold particles than did control cultures. The possibility that unconjugated gold was accumulated nonspecifically at gold-binding sites was ruled out by incubating cultures with unconjugated gold particles at 4°C, with or without warming to 37°C. Unwarmed cultures accumulated unconjugated gold at 6% of that for control cultures exposed to gold-LDL conjugates; warmed cultures took up 12% of that for control cultures.

Although the affinity of the LDL receptor on rat cells for human LDL is lower than that for homologous LDL (18), the cholesteryl ester it carries is used effectively in progestin synthesis by rat ovarian cells (25, 26, 30), and it has been used to quantitate LDL receptor activity in steroidogenic tissues of rats and mice (25). Hence, human LDL is a valid probe with which to map the pathway of LDL metabolism in rodent cells.

**Distribution of Gold-LDL among Intracellular Organelles after Warming to 37°C**

To determine the temporal sequence in which intracellular organelles involved in uptake and processing of LDL become labeled with gold, gold-containing organelles were quantified after exposure of granulosa cells to gold-LDL conjugates for 1 h in the cold and warming to 37°C for 2–60 min. After 2 min of warming, 98.8% of the gold particles were at the cell surface, with 1.2% over cytoplasmic structures (Table I). Our

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**TABLE I**

| Time (Min) | No. cellular profiles | Gold particles/ cellular profile | Percent at cell surface | Percent inside cell |
|------------|-----------------------|---------------------------------|------------------------|------------------|
| 0          | 27                    | 6,216                           | 230                    | 99.9             | 0.1  |
| 2          | 20                    | 3,265                           | 163                    | 98.8             | 1.2  |
| 4          | 19                    | 3,224                           | 170                    | 98.6             | 1.4  |
| 6          | 34                    | 4,027                           | 118                    | 98.9             | 1.1  |
| 8          | 34                    | 5,294                           | 161                    | 96.6             | 3.4  |
| 10         | 35                    | 6,881                           | 197                    | 93.6             | 6.4  |
| 12         | 53                    | 12,955                          | 244                    | 97.5             | 2.5  |
| 15         | 32                    | 9,530                           | 299                    | 93.8             | 6.2  |
| 20         | 34                    | 4,369                           | 128                    | 83.6             | 16.4 |
| 30         | 27                    | 3,554                           | 132                    | 74.6             | 25.4 |
| 60         | 52                    | 10,456                          | 201                    | 45.0             | 55.0 |

Granulosa cells, incubated for 24 h in LPDS medium, were exposed to gold-LDL conjugates for 1 h at 4°C and warmed to 37°C for 0–60 min. Quantitation was carried out on thin sections cut perpendicular to the bottom of the culture dish. Gold particles and gold-labeled organelles were quantified in 19–53 cellular profiles per time period as described in Materials and Methods.
 raw data indicated that 60% of the gold-labeled intracellular bodies were small (130 nm in diameter; usually coated) vesicles (Fig. 4d), and 40% were pale MVBs (Fig. 4e). Virtually all gold-labeled coated vesicles were near the plasma membrane. To ascertain if such structures were true vesicles or represented tangential cuts through coated pits, 70 “apparent” coated vesicles containing gold particles were examined in serial thin sections; 64% were coated pits since they were connected to the cell surface, and 36% were true vesicles, being detached completely from the plasma membrane. After correcting our raw figures for the percentage of apparent coated vesicles that were in reality coated pits, we found that pale MVBs comprised 74% of the gold-labeled intracellular bodies (Table II). Gold particles of pale MVBs lay beneath the limiting membrane (Fig. 3d) or were scattered throughout the matrix (Fig. 3e), and were encircled by round particles (20 nm in diameter), presumably LDL particles, in tissue treated with tannic acid (Fig. 3, d and e). Gold particles were absent from the tail-like extensions of MVBs (Fig. 3c). Another gold-labeled structure noted after warming was a double-walled electron-lucent vacuole (Fig. 3b).

It is important to note that even though the majority of gold particles were initially localized to noncoated regions of the plasma membrane (Fig. 4a), those particles in the first stages of internalization were consistently associated with slightly or deeply indented coated pits (Fig. 4, b and c).

The distribution of gold label between the cell surface and cytoplasm and among intracellular organelles after 4 and 6 min of warming was similar to that after 2 min (Table II). By 8 min, the percentage of gold particles at the surface decreased and for the first time dense MVBs contained gold (Table II; Fig. 4f). Between 8 and 15 min, the percentage of gold-labeled dense MVBs increased substantially (Table II). By 15 min, a modest number of lysosome-like dense bodies contained gold particles (Fig. 4, g and h), and as warming time increased, the percentage of gold-labeled lysosome-like dense bodies rose steadily, reaching 63% after 60 min at 37°C (Table II; Fig. 5). Individual lysosome-like dense bodies were often heavily labeled; some contained more than 300 gold particles. Between 15 and 60 min of warming, the percentage of gold particles associated with the plasma membrane fell from 94 to 45%, while that in cytoplasmic structures rose correspondingly (Table II). Regardless of the time period, none of the 69,771 gold particles in the 367 cellular profiles examined for this part of the study was observed in other organelles, including the cisternae of the Golgi complex or GERL.

The possibility that chilling modified the pattern of LDL uptake was assessed by incubating cultures with gold-LDL conjugates continuously at 37°C for 60 min. The distribution of gold particles among intracellular organelles in these cells was identical to that for cells exposed to gold-LDL at 4°C and then warmed.

To determine if ligand moved from the cell surface to the region of the Golgi complex, gold-labeled organelles within and beyond the Golgi zone were quantified in cultures exposed to gold-LDL conjugates and warmed for 0–60 min. In

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**TABLE II**

Percent of Gold-LDL-labeled Intracellular Organelles in Granulosa Cells at 4°C and after Various Periods of Warming to 37°C

| Min at 37°C | No. cellular profiles examined | No. labeled structures examined* | Labeled organelle | % | % | % | % |
|---|---|---|---|---|---|---|---|
| 0 | 27 | 4 | 100 | 0 | 0 | 0 | 0 |
| 2 | 20 | 22 | 26 | 74 | 0 | 0 | 0 |
| 4 | 19 | 26 | 32 | 68 | 0 | 0 | 0 |
| 6 | 34 | 26 | 40 | 60 | 0 | 0 | 0 |
| 8 | 34 | 72 | 31 | 66 | 3 | 0 | 0 |
| 10 | 52 | 121 | 20 | 66 | 13 | 1 | 0 |
| 12 | 53 | 97 | 21 | 67 | 12 | 0 | 0 |
| 15 | 32 | 79 | 15 | 49 | 30 | 6 | 0 |
| 20 | 34 | 115 | 12 | 43 | 23 | 21 | 0 |
| 30 | 27 | 98 | 13 | 29 | 13 | 44 | 0 |
| 60 | 52 | 470 | 9 | 15 | 12 | 63 | 0 |

Granulosa cells, incubated for 24 h in LPDS medium, were exposed to gold-LDL conjugates for 1 h in the cold and warming for various times. In tissue treated with tannic acid, it was apparent that the lipoprotein component of gold-LDL conjugates in pale MVBs was intact, suggesting that degradation of LDL does not occur in this compartment. Two such gold-LDL conjugates lie beneath the limiting membrane (arrow) of a pale MVB in d and one is at the center of another MVB in e. x 44,200. (a–e) Cultured for 24 h in LPDS medium before labeling with gold-LDL conjugates for 1 h in the cold and warming for various times. (b–e) Treated with tannic acid.
This series of micrographs shows the sequence in which structures involved in processing of gold-LDL conjugates became labeled with gold particles. a–c represent the likely sequence for the earliest phases of ligand accumulation. The initial interaction between gold-LDL conjugates and granulosa cells was at the plasma membrane, primarily along noncoated segments (a, arrowheads). Subsequent internalization of conjugates involved slightly (b, arrowheads indicate the coat) and deeply (c) indented coated pits and the formation of coated vesicles (d). Between 2 and 12 min of warming, MVBs were the most abundant gold-labeled organelle (e; arrowheads indicate dense patches along the limiting membrane). By 8 min of warming, dense MVBs contained gold particles (f) and after 15 min, lysosome-like dense bodies were labeled with gold (lysosome with electron-lucent [g] and electron-dense [h] matrix). (a–e) From tissue treated with tannic acid; thus, the lipoprotein component of the gold-LDL conjugate is visible. a–c, x 79,400; d, x 90,700; e–f, x 47,900; g, x 29,200; h, x 51,000. All were cultured for 24 h in LPDS medium and exposed to gold-LDL conjugates for 1 h in the cold and warmed to 37°C for various times.

Uptake and Metabolism of Gold- and \([1^\text{H}]\)Cholesteryl Linoleate–labeled LDL

Granulosa cell cultures were exposed to LDL labeled with \(^{3}\text{H}\)CE-LDL alone or with both gold and \(^{3}\text{H}\)cholesteryl ester (gold-\(^{3}\text{H}\)CE-LDL) to assess the fate of sterol carried by LDL. This approach provided a unique opportunity to follow lipoprotein processing simultaneously at the structural and functional level. In addition, it allowed us to determine if binding of gold to LDL altered the physiological behavior of the lipoprotein.

After incubation of granulosa cells with \(^{3}\text{H}\)CE-LDL for 90 min at 4°C and subsequent warming, the medium contained tritiated 20α-hydroxyprog-4-en-3-one. Conversion of sterol to progestin was time-dependent, and the time course and extent of conversion were virtually identical for cultures incubated with \(^{3}\text{H}\)CE-LDL or gold-\(^{3}\text{H}\)CE-LDL (Table V). Thus, conjugation of gold to LDL did not alter the kinetics of lipoprotein uptake or the ability of the lipoprotein to be used in steroid hormone formation. Conversion of \(^{3}\text{H}\)esterified sterol to \(^{3}\text{H}\)progestin was blocked by chloroquine (Table V).

Fine structural analysis of cultures exposed to gold-\(^{3}\text{H}\)CE-LDL showed that the kinetics of sterol to steroid conversion were accompanied by increasing percentages of (a) internalized gold particles, (b) gold-labeled lysosomes, which rose from 47% at 30 min of warming to 78% at 180 min, and (c) intracellular gold particles contained within lysosomes, which reached 96% at 180 min of warming (Table V). In addition, the number of lysosomes per cellular profile increased, peaking at 5.6 (Table V).

Numerous large, electron-lucent vacuoles occurred in the cytoplasm of cells exposed to chloroquine. We grouped these vacuoles with lysosomes since treatment with chloroquine is reported to cause enlargement and vacuolation of lysosomes (21). However, we cannot rule out the possibility that they might represent massively enlarged pale MVBs, since the MVB compartment of hepatocytes was markedly increased by chloroquine administration (17). Unlike lysosomes of control (nontreated) cells, vacuolated lysosomes of chloroquine-treated cells contained intact lipoprotein particles (Fig. 6).

Uptake of gold-LDL conjugates was reduced in chloroquine-treated cells (30% uptake versus 54% for nontreated cultures). However, the percentage of surface particles lying over coated pits was unaffected by drug treatment, remaining remarkably constant from 0–180 min of warming for all cultures (Table V). Similarly, chloroquine treatment did not markedly alter
FIGURE 5 Granulosa cell incubated for 24 h in LPDS medium, exposed to gold-LDL conjugates for 1 h at 4°C, and warmed to 37°C for 1 h. By 60 min of warming, the number of gold particles at the surface had declined markedly (arrowheads; compare with Fig. 2, a and c), whereas that in the cytoplasm had risen. Intracellular gold particles were associated primarily with lysosome-like dense bodies (long arrows); one in this field contains more than 100 gold particles. A gold-labeled pale MVB (short arrow) is also present. x 26,000.

the distribution of gold label among intracellular compartments, except that the number of gold-labeled dense MVBS per cellular profile was decreased by 10–20-fold (data not shown). 167,630 gold particles in 419 cellular profiles were analyzed for this portion of the study.

Localization of Acid Phosphatase and Arylsulfatase Activity

To identify gold-labeled intracellular structures belonging to the lysosomal system, organelles containing gold particles alone or both gold particles and ACPase reaction product were quantified in cells after exposure to gold-LDL conjugates and incubation for ACPase activity. In cultures warmed for 2 min, 19 of 20 gold-labeled pale MVBS present in 62 cellular profiles were negative for ACPase activity. None of the 39 gold-labeled small vesicles in these cells contained ACPase reaction product. At 10 min of warming, 63 of 66 (95%) gold-labeled pale MVBS in 37 cellular profiles were negative for ACPase activity (Fig. 7 d). None of the 33 gold-labeled small vesicles was reactive for ACPase.

At 2 and 10 min of warming, lysosomes lacked gold parti-
Granulosa cells were prepared as described in the Table II legend, except that thin sections were cut en face (parallel to the bottom of the culture dish). Gold-labeled organelles within and beyond the Golgi zone were quantified in 20-40 cellular profiles per time period as detailed in Materials and Methods.

* Includes apparent coated vesicles.
* Corrected for presence of apparent coated vesicles.

**Average protein content per dish = 17.8 + 4.34 \mu g/dish (SE, n = 12).**

**Corrected for presence of apparent coated vesicles.**

### TABLE III

**Percentage of Gold-LDL-labeled Intracellular Organelles within and beyond the Golgi Zone in Granulosa Cells at 4°C and after Warming to 37°C**

| Min at 37°C | No. cellular profiles examined | No. gold-labeled organelles examined | Percentage within Golgi zone | Percentage beyond Golgi zone |
|-------------|-------------------------------|-------------------------------------|-----------------------------|------------------------------|
| 0           | 27                            | 0                                   | 0                           | 0                            |
| 6           | 20                            | 84                                  | 16                          | 84                           |
| 8           | 25                            | 112                                 | 18                          | 82                           |
| 10          | 54                            | 259                                 | 24                          | 76                           |
| 12          | 28                            | 132                                 | 33                          | 67                           |
| 15          | 23                            | 135                                 | 20                          | 80                           |
| 20          | 20                            | 191                                 | 23                          | 77                           |
| 60          | 13                            | 254                                 | 24                          | 76                           |

**Granulosa cells were prepared as described in the Table II legend, except that thin sections were cut en face (parallel to the bottom of the culture dish). Gold-labeled organelles within and beyond the Golgi zone were quantified in 20-40 cellular profiles per time period as detailed in Materials and Methods.**

### TABLE IV

**Percent Distribution of Gold-LDL-labeled Organelles within the Golgi Zone of Granulosa Cells**

| Min at 37°C | Labeled vesicles | Labeled pale MVBs | Labeled dense MVBs | Labeled lysosomes |
|-------------|------------------|-------------------|-------------------|------------------|
| 0           | 0                | 0                 | 0                 | 0                |
| 6           | 6                | 11                | 0                 | 0                |
| 8           | 8                | 18                | 5                 | 95               |
| 10          | 10               | 57                | 2                 | 82               |
| 12          | 12               | 40                | 0                 | 90               |
| 15          | 15               | 26                | 0                 | 77               |
| 20          | 20               | 42                | 7                 | 52               |
| 60          | 60               | 3                 | 8                 | 10               |

**Granulosa cells were prepared as described in the Table II legend, except that thin sections were cut en face (parallel to the bottom of the culture dish). Gold-labeled organelles within and beyond the Golgi zone were quantified in 20-40 cellular profiles per time period as detailed in Materials and Methods.**

### TABLE V

**Metabolism of Gold- and \([^{3}H]\)cholesterol Linoleate-labeled Human LDL by Rat Granulosa Cells In Vitro: Quantitative Results**

| Min at 37°C | \([^{3}H]20\alpha\)-Hydroxy-pregn-4-en-3-one | \(^{3}HCE-LDL^*\) Gold particles | \(^{3}HCE-LDL^*\) Gold particles per cell profile | Gold particles at surface | Gold particles inside cell | Surface particles over coated pits | Labeled lysosomes* | Intracellular gold particles in lysosomes | Labeled no. gold particles per cell | Labeled no. gold particles per cell profile | Labeled no. gold particles examined |
|-------------|---------------------------------------------|----------------------------------|-----------------------------------------------|--------------------------|----------------------------|-----------------------------------|-----------------|------------------------------------------|-----------------------------------|---------------------------------------|-----------------------------------|
| 0           | 0                                           | 0                                | 65                                            | 16,760                   | 259                       | 99.9                              | 0.1             | 3.4                                      | 0                                 | 0                                     | 0                                 |
| 30          | 0.97                                        | 4.52                             | 73                                            | 23,988                   | 361                       | 83.1                              | 16.9            | 3.6                                      | 46.8                              | 62.3                                  | 1.5                               |
| 60          | 2.08                                        | 3.41                             | 67                                            | 40,572                   | 609                       | 74.7                              | 25.3            | 7.4                                      | 57.4                              | 77.0                                  | 3.7                               |
| 120         | 4.60                                        | 14.31                            | 76                                            | 33,542                   | 440                       | 58.3                              | 41.7            | 4.2                                      | 70.1                              | 89.3                                  | 5.6                               |
| 180         | 30.25                                       | 47.59                            | 74                                            | 23,685                   | 320                       | 46.4                              | 53.8            | 3.9                                      | 77.6                              | 93.6                                  | 3.0                               |
| 180 + chloroquine | 3.56                                        | 3.78                             | 64                                            | 29,083                   | 450                       | 69.9                              | 30.1            | 3.7                                      | 79.8                              | 96.4                                  | 3.2                               |

**Granulosa cells, incubated for 24 h in LPDS medium, were exposed to \(^{3}HCE-LDL^*\) gold-LDL conjugates. Each result is an average of two culture dishes for that time point.**

**Average protein content per dish = 17.8 \pm 4.34 \mu g/dish (SE, n = 12).**

### DISCUSSION

We have demonstrated for the first time the pathway by which steroid-secreting cells of rats take up and process gold- and \([^{3}H]\)cholesterol linoleate-labeled human LDL. Since lipoproteins bearing apolipoprotein E bind to the LDL receptor (4), the pathway we have described may be identical to that used by ovarian cells in situ to metabolize apolipoprotein E-containing HDL, which is abundant in rat plasma (33).

Accumulation of LDL by granulosa cells began with the binding of gold-LDL conjugates to the plasma membrane. Few conjugates were over coated pits, indicating that LDL binding sites were not typically preclustered before interaction with ligand, as they are in fibroblasts (1). Movement of gold-LDL conjugates into granulosa cells was mediated by coated pits and true coated vesicles. Although true coated vesicles are reported to occur in fibroblasts (24), others argue that they do not exist freely within the cytoplasm (35). After internalization, gold-LDL conjugates moved through a series of cellular compartments, beginning with small vesicles and ending with lysosomes (Fig. 8). Hence, delivery of LDL to lysosomes in steroid-secreting cells involves a pathway basically similar to that described for receptor-mediated endocytosis of this ligand by nonsteroidogenic cells (1, 5).

An important step in LDL uptake was the centripetal transport of ligand from the periphery of the granulosa cell to the perinuclear Golgi zone (Table III). Translocation to the Golgi region, although implied in the literature, has not been documented previously at the fine structural level. Gold-LDL...
FIGURE 6 Vacuolated lysosome in granulosa cell of chloroquine-treated culture. Vacuolated lysosomes bore a superficial resemblance to electron-translucent lysosomes of untreated cultures. However, in contrast to lysosomes of untreated cells, vacuolated lysosomes of chloroquine-treated cells contained intact (undigested) lipoprotein particles. The area indicated by the arrowhead is shown at higher magnification in the inset, x 51,000. (Inset) Note that the lipoprotein component (arrowhead) of the gold-LDL conjugates contained within the lysosome is identical in morphology to that of gold-conjugates at the cell surface (compare with Fig. 4, a-c). x 94,000. Culture was incubated in LPDS medium for 24 h before exposure to LDL labeled with both gold and [3H]cholesteryl linoleate for 90 min at 4°C and warming to 37°C for 180 min. The culture was treated with tannic acid.

conjugates did not actually enter Golgi or GERL cisternae of granulosa cells, as reported to occur during endocytosis of epidermal growth factor by other cell types (34). The pale MVB, a rapidly forming organelle, was responsible for carrying LDL to the Golgi zone (Table IV). Pale MVBs are distinct from lysosomes in that they contain intact (undigested) lipoprotein particles and lack lytic enzymes (Figs. 3 and 7). Indeed, pale MVBs may be the structural correlate of the nonlysosomal intermediate suggested by biochemical studies to transport ligand to lysosomes (20). The presence of tail-like projections on MVBs hints at a possible role of this structure in receptor recycling (8). As indicated by the temporal sequence in which they became labeled with gold particles, dense MVBs represent a transition stage between pale MVBs and secondary lysosomes. Fusion of structures involved in LDL processing is suggested by our findings that, on the average, coated vesicles contained 4, pale MVBs 8, dense MVBs 17, and lysosomes 29 gold particles (Paavola, L., unpublished data) and that individual lysosomes can contain hundreds of gold particles.

Our data firmly establish the key role of lysosomes in the utilization of lipoproteins by granulosa cells. The kinetics of [1H]cholesteryl linoleate conversion to [1H]progesterin were closely paralleled by quantitative changes in the gold-labeling of the lysosomal compartment and sterol to steroid conversion was prevented by inclusion of the medium of chloroquine, an inhibitor of lysosomal activity. Moreover, blockade of progesterin formation was accompanied by an accumulation of undigested lipoprotein particles in the vacuolated lysosomes of drug-treated cultures. Since the ester linkage of the lipid must be cleaved before cholesterol is free to enter the steroidogenic pathway, these data imply that hydrolysis of LDL-carried cholesteryl ester by granulosa cells occurs in lysosomes as it does in fibroblasts (4, 11).

Available data indicate that cultured granulosa cells of rats and cows degrade the protein component of [125I]LDL (25, 28) by a chloroquine-sensitive process (25, 29). After a 30-min lag, hydrolysis of LDL protein began and then continued at a linear rate for several hours (28). These data correlate closely with our finding that the proportion of gold-labeled lysosomes was modest before 30 min of warming but rose steadily thereafter. At 15 min of warming, we observed few gold-labeled lysosomes in the Golgi zone, where interaction of endosome and lysosome presumably initiates degradation of ligand. However, by 60 min, lysosomes were the most abundant gold-labeled organelle in this area. Herman and Albertini (16) recently reported a remarkably similar time course for the redistribution of lysosomes and endosomes to the perinuclear region in living granulosa cells. Thus, the lag between surface binding of LDL to granulosa cells and appearance of degradation products corresponds closely to the time required for gold-labeled LDL to reach lysosomes.

Our study documents important differences in how steroid-secreting cells and nonsteroid-secreting cells handle lipoproteins. The first and most striking difference is the fate of the sterol carried by lipoprotein. In fibroblasts, cholesterol liberated from LDL is used primarily in membranes and is not further processed once it exits the lysosome. In contrast, cholesterol obtained from LDL in granulosa cells is metabolized to progesterin. This requires transport of sterol from the lysosome to other cellular compartments where the remaining steps in sterol synthesis occur.

Secondly, the time course for the uptake of LDL differs radically for steroid- and non-steroid-secreting cells. Even
after 3 h of warming, considerable gold-LDL remained at the surfaces of granulosa cells, whereas internalization of ferritin-labeled human LDL by human fibroblasts was virtually complete within 10 min (1). The slower rate of LDL uptake by granulosa cells may reflect inherent differences in the kinetics of internalization for the two cell types. Most LDL receptors on fibroblasts are preclustered in coated pits, favoring rapid internalization of the ligand. In contrast, most LDL binding
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