Hepatic Binding and Internalization of Low Density Lipoprotein-Gold Conjugates in Rats Treated with 17α-Ethinylestradiol

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ABSTRACT Receptor-mediated hepatic uptake of low density lipoproteins (LDL) conjugated to colloidal gold was studied by perfusion of livers from rats treated for 5 d with 17α-ethinylestradiol. Estrogen treatment resulted in a marked decrease in serum lipid and lipoprotein concentrations. After 15 min of perfusion the conjugate was bound to the hepatic microvilli of both control and estrogen-treated rats; the estrogen-treated rats showed an 8- to 11-fold greater number of membrane-bound conjugates. The conjugates were bound to the membrane receptor by the LDL particle because the gold granules were regularly displaced from the membrane by 20 ± 3.2 nm, the diameter of LDL. Internalization of the conjugate, evident by gold particles in multivesicular bodies, occurred at coated pits at the base of the microvillus where coated vesicles containing a single gold-LDL conjugate were released. After 1 h of perfusion, the livers from the estrogen-treated rats showed all phases of endocytosis and incorporation into multivesicular bodies of the conjugate. After 2 h of perfusion, there was congregation of gold-labeled lysosomes near the bile canaliculi. Gold-LDL conjugates were also observed to bind and be internalized by Kupffer cells and sinusoidal endothelium.

These findings indicate that estrogen treatment induces hepatic receptors for LDL. The catabolic pathway of binding and endocytosis of the conjugate is similar to that seen in fibroblasts, although slower. Because gold-LDL conjugates were also present in the Kupffer and endothelial cells, the uptake of LDL by the liver involves the participation of more than a single cell type.

The catabolism of low density lipoproteins (LDL) has been studied in cultured human fibroblasts (3) and other extrahepatic tissues (7). The binding of LDL to cells is mediated by a high-affinity membrane receptor (2, 4), after which the LDL is internalized and catabolized in the lysosome (7). Because the liver plays a central role in lipoprotein metabolism, one question of importance is whether a similar process of binding, endocytosis, and lysosomal catabolism of LDL occurs in the liver.

The perfused rat liver is a useful system to study hepatic uptake and catabolism of lipoproteins. However, the liver does not express a large number of LDL receptors (24), and reproducible measurements of binding constants and other parameters of LDL-receptor interactions are difficult to obtain (8). Recently, 17α-ethinylestradiol was shown to stimulate hepatic lipoprotein catabolism, presumably by increasing the number of LDL receptors on the parenchymal cell membrane (8). Results from studies of perfused livers of estradiol-treated rats (8) and isolated membranes from livers of treated rats (24) suggest that the enhanced catabolic activity is attributable to an increased number of hepatic receptors (three- to tenfold) for LDL. These estradiol-induced receptors exhibit several properties analogous to the LDL receptor system as described for fibroblast cells (42) and are believed responsible for the enhanced in vivo uptake of LDL by livers of estradiol-treated rats (8). Although there are similarities between the estradiol-induced LDL receptor in parenchymal cells and the fibroblast LDL receptor, the cellular mechanisms related to hepatic binding and catabolism of LDL are unknown.

We have developed a new ultrastructural probe, a colloidal gold-LDL conjugate, to study receptor-mediated uptake and degradation of LDL by cultured human fibroblasts (18).
resulting conjugate consists of eight biologically active LDL molecules that are clustered around a central 19-nm gold particle (17). The gold particles are nondegradable, electron-dense, and easily visualized by transmission electron microscopy. In this study, we have used this conjugate in perfusions of rat livers of estradiol-treated animals to investigate the binding, endocytosis, and catabolism of LDL. Quantitative biochemical results of estradiol treatment on serum lipoproteins and electron micrographs detailing the time-dependent account of hepatic internalization of the gold-LDL conjugate are presented. These results provide correlative evidence that the enhanced hepatic catabolism of LDL resulting from 17α-ethinylestradiol administration may involve an increased number of membrane receptors for this lipoprotein.

MATERIALS AND METHODS

Materials

17α-ethinylestradiol and bovine serum albumin (Fraction V) were obtained from Sigma Chemical Co. (St. Louis, Mo.).

Preparation of LDL

LDL (d, 1.019–1.063 g/ml) was isolated from human serum by differential ultracentrifugation (20). The LDL was washed by recentrifugation at the higher density limit and dialyzed against 0.05% EDTA at pH 7.0.

Preparation of Colloidal Gold-LDL

Colloidal gold was prepared by the method of Frens (15) and conjugated to LDL, as previously described (18). For perfusion studies, 100 ml of colloidal gold was conjugated to 1.5 mg of LDL protein. This volume was condensed by Biogel dehydron to final volume of 20 ml, which contained ~7.5 × 10¹ⁱ conjugates/ml. Before perfusion, the conjugate was dialyzed against Krebs-Ringer bicarbonate buffer.

Chemical Analysis

Protein was determined by a modification of the method of Lowry et al. (25). Cholesterol was assayed by the method of Abell et al. (1). Lipids were extracted according to the method of Folch et al. (14) for the determination of triglycerides and phospholipids. Triglycerides were measured by the procedure of Van Handel and Zilversmit, as modified by Van Handel (35). Phospholipids were determined by the method of Zilversmit and Davis (45).

Liver Perfusion

The animals used were male Sprague-Dawley rats (Charles River, Wilmington, Mass.) weighing 200–250 g and fed standard Purina Chow ad libitum. One group of rats was treated with 17α-ethinylestradiol (5 mg/kg body weight) dissolved in 0.2 ml of propylene glycol, subcutaneously injected daily for 5 d. A second group was sham-injected with 0.2 ml of propylene glycol. The rats were anesthetized by an intraperitoneal injection of sodium pentobarbital (5 mg/100 g body weight). An aliquot of blood was obtained from the tail vein for analysis of serum cholesterol concentration. After opening the pericardial cavity, the common bile duct was cannulated, followed by rapid cannulations of the portal vein and inferior vena cava. Livers were perfused in situ in a constant temperature cabinet at 37°C, using a modification of the method of Mortimore (27) with a membrane oxygenator (16). The perfuse contained washed bovine erythrocytes at a 22% hematocrit in Krebs-Ringer bicarbonate buffer with 100 mg/dl glucose and 1 g/dl bovine serum albumin, equilibrated with 95% O₂ and 5% CO₂ at pH 7.4. The livers were flushed with 100 ml of perfusate and then perfused in a recirculating system with 80 ml of perfusate. Flow was 1–2 ml/min/g liver with a hydrostatic pressure of 12–14 cm water. For uptake studies, 20 ml of the colloidal gold-LDL was added to the reservoir after a 20 min of perfusion. The final perfusate (100 ml volume) contained 15 μg LDL protein/ml that was conjugated to 1.5 × 10¹ⁱ conjugates/ml. Recirculating perfusion was continued for 15 min for control livers and 15, 60, and 120 min for the estradiol-treated livers. Viability of the liver was monitored by measurements of arterio-venous O₂ differences during the perfusion with the use of an Instrumentation Laboratory blood gas analyzer. A-2 ml aliquot of perfusate was removed from the reservoir every 15 min during the perfusion and cleared of red blood cells by centrifugation. The relative amount of gold-LDL remaining in the perfusate was calculated by measuring absorbance at 525 nm (21) and relating it to a standard curve obtained by diluting perfusate samples at time = 0 h. The remaining amount of LDL (μg protein/ml) was calculated from the amount of remaining gold and converting this spectrophotometric measurement to represent remaining conjugated LDL.

Several competitive binding studies were conducted with livers from estrogen-treated rats using a 20-fold excess of unlabelled LDL (300 μg LDL protein/ml) in the presence of the gold-LDL conjugate (15 μg LDL protein/ml). Recirculating perfusion was continued for 15 and 60 min, after which the livers were prepared for electron microscopy (below).

Electron Microscopy

Perfusion fixation (0.5% glutaraldehyde in 0.1 M Na-cacodylate, pH 7.38, 382 mosm) was performed immediately after the specific perfusion time by injection into the portal vein cannula at a flow rate and pressure equal to those used during the perfusion study. Good fixation was indicated by a homogeneous discoloration of the liver and a concomitant hardening of the tissue (within 20–40 s). After an initial fixation for 5 min, the perfusion fixative was changed to buffered 3% glutaraldehyde and 1% osmium (540 mosm) for 1 h. The entire liver was removed, cut into 1-mm cubes, and rinsed with several changes of buffer. Immersion postfixation (2 h) was done in 1% OsO₄ in cacodylate buffer containing 0.1% ruthenium red that had been allowed to stand for 3 h before use (19). During this 3-h aging, ruthenium red is oxidized to a ruthenium nitrosyl compound (12) which we have found to impart superior contrast to the LDL molecules of the conjugate positioned at the surface receptor (18). Tissue agitation was used to minimize a fixation gradient. The samples treated with oxidized ruthenium red were en bloc stained with 2% uranyl acetate for 2 h, dehydrated, and embedded in Spurr’s resin (32). In each experiment, several representative tissue samples from normal and estrogen-treated animals were removed and prepared for ultrastructural viability tests using ruthenium red (6) or horseradish peroxidase (22) as electron-dense tracers to identify cellular membrane damage. Thus, after postfixation in a freshly prepared OsO₄-ruthenium red solution, cellular viability was determined on the basis of membrane exclusion of the ruthenium stain (6). Likewise, after postfixation in 1% OsO₄, the specimen was immersed in a 0.1% horseradish peroxidase solution. Diaminobenzidine cytochemistry was used to observe ultrastructural localization of the enzyme. As both methods are based on exclusion of the tracers, intracellular accumulation of either ruthenium red or horseradish peroxidase (evidenced by increased overall cell density) was interpreted to indicate loss of cellular integrity. Thin sections (40 nm) were contrasted with 6% uranyl magnesium followed by lead citrate and viewed under a Zeiss 9S electron microscope at 60 kV. Determination of the surface density of gold-LDL conjugates was evaluated from several samples from each perfusion study. The density is expressed as the number of gold particles/μm² length of external hepatocyte surface, where the external surface is considered as a straight line delimiting the cell boundary. This form of analysis does not include the surface of the microvillus as part of the external hepatocyte surface. The relative contribution of the microvillus surface is considered as equal for both control and experimental livers and is not tabulated. Final magnification was calibrated using a replica line grating (Polysciences, Inc., Warrington, Pa.) and 20 micrographs were counted from each sample.

RESULTS

Effect of Estrogens on Serum and Liver Lipids

Estradiol administration for 5 d resulted in a marked decrease in serum cholesterol, triglyceride, and phospholipid concentrations (Table 1), accompanied by an 85% decrease in serum very low density lipoprotein (VLDL) concentration and an 83% decrease in HDL protein concentration. No LDL could

| TABLE 1 | Effect of Estradiol Treatment on Serum Cholesterol, Triglyceride, and Phospholipid Concentrations |
|---------|---------------------------------|
| Cholesterol | Triglyceride | Phospholipid |
| mg/dl | mg/dl | mg/dl |
| Untreated | 37.9 | 74.6 | 96.3 |
| Estradiol-treated* | 9.7 | 3.9 | 39.4 |

Values represent pooled aliquots of serum from six rats.

* Rats were treated with estradiol (5 mg/kg) daily for 5 d.
be detected in the estradiol-treated group. After 5 d of estrogen treatment, the rats lost 5–15 gm body weight and liver weight increased slightly. Estradiol administration resulted in a 60% increase in liver cholesterol concentration.

**Cellular Structure of the Estradiol-treated Liver**

Liver samples from estrogen-treated animals before perfusion showed no alterations in cellular architecture from that established for the normal liver (22). The sinusoidal cavity is lined by Kupffer and endothelial cells that delimit the peri-sinusoidal space of Disse and the microvillus structures of the parenchymal cells. Occasional collagen bundles are evident and several bile canaliculi, located between hepatic cells, show adjacent right-junctional complexes. The polyhedral-shaped hepatic parenchymal cells contain numerous mitochondria, lysosomes, parallel arrays of rough endoplasmic reticulum, and a centrally disposed nucleus. Golgi complex bodies and smooth endoplasmic reticulum are also evident.

Viability studies based on the exclusion of ruthenium red (Fig. 1) or horseradish peroxidase (results not shown) reveal that the parenchymal cells, areas of cell-to-cell junctions, and endothelial cells were impermeable to the tracers after ethinylestradiol treatment. At the microvillus, the ruthenium red stain was observed to penetrate and fill apparently free vesicles and tubular structures, indicating luminal continuity out of the section plane. In the absence of ruthenium staining, it becomes difficult to quantitate coated pits from apparently free-coated vesicles that have released from the luminal membrane. We did not observe separation of hepatocyte junctions or the anomalous microvilli at lateral cell borders, as has been reported to occur after estrogen treatment (9). The viability tests were applied to liver samples from all perfusion studies and no generalized loss of cellular integrity was observed. However, the intense staining of ruthenium red and horseradish peroxidase obscured visualization of the receptor-bound gold-LDL conjugate. In other hepatocyte receptor studies employing ferritin as a tag, ruthenium red staining is reported to obscure surface-associated ferritin (37). Consequently, this staining protocol was used only in viability tests, not for ultrastructural studies of conjugate binding.

**Initial Events of Receptor Binding (15 min)**

After 15 min of perfusion, most of the conjugates are observed bound to the microvillus membranes of both normal (Fig. 2) and estradiol-treated (Figs. 3a and b) tissues. Although qualitatively similar, the estradiol-treated livers showed 33.0 ± 1.1 conjugates/μm length of membrane, compared to only 3.6 ± 1.1 conjugates/μm membrane length in the control perfused livers. The structure of the conjugate seen by negative staining (Fig. 4a) is similar to the thin-section image of the receptor-bound conjugates (Fig. 4b). With the use of preoxidized ruthenium red staining, the LDL moiety of the conjugate can be directly visualized at the membrane surface (Fig. 4b). Conjugates were observed as diffusely distributed along the entire microvillus surface. The conjugate was consistently bound to the cell receptor by the LDL molecule as the gold granule was separated from the membrane by a distance of 20 ± 3.2 nm, which is equal to the diameter of LDL. Due to the convoluted profiles of the microvillus, receptor-bound conjugates may appear excessively removed from or unusually close to the microvillus. However, in most of these cases, the microvillus membrane is not resolvable as a bilayer but appears as a diffuse membrane image. This results from oblique sectioning of the bilayer, and such areas were excluded from measurements of gold-membrane separation. Small coated pits were observed only at the base of the microvillus, not at the apex or slopes of the microvillus. After 15-min perfusion, ~65–70% of the conjugates were seen at the microvillus surface. During internalization, 100-nm diameter coated vesicles (Fig. 4c) were observed to pinch off from the microvillus. Receptor-bound conjugates usually occupy a central position within the 100-nm vesicle, which has a symmetrical radius of curvature and is clearly effaced with an outer cytoplasmic aspect of clathrin material. The majority (80%) of the vesicles contain only one gold-LDL conjugate. Delivery of the conjugate was made to subcellular structures that resemble elementary multivesicular bodies and possess a complete delimiting external membrane (Fig. 5). Often, the gold shows peripheral clustering along the inner aspect of the multivesicular bodies (MVB) membrane, suggesting continued receptor-ligand interaction after initial lysosomal delivery. After 15-min perfusion, 80% of the gold-LDL (12–13 μg LDL protein/ml) remained in the perfusate.

In the presence of a 20-fold excess of unlabeled LDL, there was a virtual absence of conjugate binding. Spectrophotometric measurements showed that nearly 99% of gold-LDL conjugate remained in the perfusate after 15 and 60 min of recirculating perfusion. Besides a lack of surface-associated conjugates, ultrastructural examination of liver tissues from estrogen-treated animals showed a trace number of internalized gold-LDL conjugates (results not presented).

**Steady-state Hepatic Internalization (1 h)**

After 1-h perfusion, the ethinylestradiol-treated livers showed all phases of endocytosis and cellular internalization of the gold-LDL conjugate. The elementary multivesicular bodies seen at 15 min develop into complex MVB (26), which contain greater numbers of gold particles and vesicles (Figs. 6a and b). Degradation of internalized vesicles leads to the formation of organelles having an electron-dense amorphous appearance in which the gold is randomly distributed (Fig. 6c).

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**FIGURE 1** Hepatocyte exclusion of ruthenium red stain after 15-min perfusion of the estradiol-treated liver. Intense staining confined to the external hepatocyte membrane (H) and endothelium (E) confirms the viable status of these cells and demonstrates bilayer integrity. The intense staining obscures visualization of receptor-bound gold-LDL conjugates. SC, sinusoidal cavity. × 10,000.

**FIGURE 2-3** Hepatic internalization of gold-LDL after 15-min perfusion in control and estrogen-treated livers. The conjugates (arrowheads), visible only by the central gold particle, are bound to the microvillus membranes of both normal (Fig. 2) and estrogen-treated rats (Fig. 3a). The gold particles are randomly distributed at the microvillus (MV) and shown in the enlarged (asterisks) view (Fig. 3b). The estrogen-treated liver bound an 8- to 11-fold greater number of conjugates than the control sample. Fig. 2, × 20,000; Fig. 3a, × 20,000, and b, × 75,000.
The distribution of gold particles as random pools, rather than associated with the inner aspect of the membrane, is interpreted to indicate a disruption of the conjugate-receptor complex. As the gold-LDL conjugate is resistant to acidic dissociation (18), the dissociation of the conjugate from the inner membrane aspect may represent proteolytic digestion of the LDL molecule, which is known to begin 15–30 min after endocytosis (8). After 1 h of perfusion, ~45% of the gold-LDL (6–7 μg LDL protein/ml) remained in the perfusate.

**Long-term Hepatic Internalization (2 h)**

Hepatic perfusion in estradiol-treated livers for 2 h is accompanied by congregation of organelles containing gold in the proximity zones of the bile canaliculi (Fig. 7). These organelles show a dense granular matrix, suggesting that they are lysosomes. Although all phases of cellular incorporation of the conjugate are evident (including multivesicular, amorphous, or electron-transparent organelles), the gold particles are easily identified in each structure (Fig. 7). After 2-h perfusion, the surface density of gold-LDL (29 ± 7.5 conjugates/μm length of membrane) is similar to that observed after 15-min perfusion of the treated livers, indicating that significant inhibition of receptor activity occurred after the addition of LDL. Approximately 25% of the gold-LDL (2.5–3.8 μg LDL protein/ml) remained in the perfusate.

**Nonparenchymal Cell Involvement**

Although Kupffer cells do not normally express LDL receptors (7), administration of estrogens is known to increase Kupffer cell phagocytic activity (38), which is an uptake system related to clearance of acetylated forms of LDL (24). The gold-LDL conjugates were observed within the stellate-shaped Kupffer cells at all times of perfusion, but only in the estradiol-treated animals (Fig. 8). Lysosomes (identified on a morphological basis) containing gold were seen infrequently in the control Kupffer cells. Although we were not able to observe a clear pattern of time-dependent internalization, in many cases fusion of coated vesicles with the lysosome was indicated by patches of coated membrane material integrated into the lysosomal membrane (Fig. 9). Interpretation of these vesicular structures as intracellular, rather than invaginations of the plasma membrane, was based on the absence of ruthenium red stain, which can be used as a tracer of regions continuous with the extracellular space (19). In addition, the gold often appeared peripherally clustered along the inner aspect of the lysosome, suggesting continued ligand-receptor interaction after lysosomal incorporation. These observations indicate an endocytic mechanism of uptake involving clathrin-effaced vesicles. However, we did not observe the formation of either elementary or complex MVB.

The endothelial cells at the sinusoidal cavity showed gold-LDL at the cell surface and significant accumulation of gold within lysosomes after 2 h of perfusion (Fig. 10). Receptor-bound conjugates were evidenced at coated pit regions, and delivery to lysosomes involved coated vesicles. The majority of the lysosomes were present at the central portion of the cell body, rather than at the peripheral fenestrated cell extremes. MVB were not observed.

**DISCUSSION**

Our results show that the normal and estradiol-treated livers of rats possess binding sites at the hepatocyte microvilli that recognize LDL labeled with gold. Colloidal gold is a nontoxic (44), electron-dense tracer that is easily identified by electron microscopy (17, 18). In agreement with previous reports (8, 24, 42), estrogen treatment resulted in marked hypolipidemia with decreased concentrations of VLDL, LDL, and high density lipoprotein (HDL) (5). Estradiol-treated livers (Fig. 2) bind an 8- to 11-fold greater number of gold-LDL conjugates than the normal liver (Fig. 3). These results are consistent with biochemical studies showing a 3- to 10-fold increase in LDL binding sites from isolated hepatic membranes (24, 42) and a 10-fold increase in the catabolic rate in the perfused liver (8). Our morphological evidence indicates that livers from both control and estrogen-treated rats possess LDL receptors that are randomly distributed on the microvilli. In the control liver, no clear indication of the distribution of surface-associated conjugates (diffuse vs. clustered at coated pits) could be assessed due to the small number present at the microvilli. In the estradiol-treated liver, >90% of the receptor-bound conjugates were diffusely distributed over the entire microvilli surface. This is in contrast to the localized binding of LDL at the coated pit regions in cultured fibroblasts (2–4). In view of the 3- to 10-fold increase in the number LDL receptors after estrogen treatment (8, 24, 42), the altered pattern of receptor distribution may reflect an inability of the coated-pit regions to accommodate the large increase in receptor complexes. Perhaps, by physical necessity, redistribution into a diffuse pattern may be required in the estrogen-augmented system. The gold particle is separated from the membrane surface by a distance 20 ± 3 nm, which is equivalent to the diameter of LDL. Receptor binding consistently involved the LDL moiety of the conjugate (Fig. 4b). Although gold-LDL is observed bound along the entire length of the microvillus, internalization via coated vesicle formation takes place only at the base of the microvillus (Fig. 4c). Receptor-mediated endocytosis involving coated-pit

![Figure 4](Visualization of receptor-bound conjugates after 15-min perfusion in the estrogen-treated liver, made possible after staining with oxidized ruthenium red (b), shows that the thin-section profile is similar to the conjugate structure observed by negative staining (a) before perfusion. Preoxidation of ruthenium red before staining is necessary to reduce the intense deposition of the stain that would otherwise obscure visualization of the conjugate (compare with Fig. 1). Endocytosis is accomplished by coated vesicles that form and release from the microvillus base (c). X 100,000.)

![Figure 5](Internalization of gold-LDL after 15-min perfusion in the estrogen-treated liver. Formation of elementary MVB, showing peripheral clustering of the gold at the inner aspect of the lysosomal membrane (arrowheads). Internalized vesicles (V) appear to lack a clathrin coat. X 100,000.)

![Figure 6](After 1-h perfusion in the estrogen-treated liver, complex MVB (a–b) having large numbers of gold particles and vesicles are seen near the bile canaliculi (8C). Also evident are amorphous organelles, in which the vesicular contents appear to be degraded (c). Frequently, Golgi apparatus bodies (G) are observed in the near vicinity. a, X 27,000; b, X 85,000; and c, X 85,000.)
clustering of randomly distributed ligands has been reported for epidermal growth factor (26) and a2 macroglobulin (39–41). This preferential site may indicate that randomly distributed receptors need to migrate to the microvillus base before endocytosis can begin. The necessity for receptor migration before endocytosis may account for the longer time period of hepatic internalization of LDL (up to 15 min), as compared to the shorter time sequence observed with fibroblasts (within 5 min) (2, 4) and other cultured cell types (7) which show preferential binding of LDL at the coated pit regions.

The 100-nm endocytic vesicles of the liver usually contain 1 gold-LDL conjugate, in contrast to the endocytic vesicles of the fibroblast which may contain up to 6 conjugates (18). Endocytic vesicles of a similar diameter have been described in the vesicular mechanism related to secretion of biliary proteins from hepatic parenchymal cells (29), and observed in parenchymal cell ingestion of nondegradable colloidal carbon particles (11, 30) and endocytosis of asialoglycoproteins (33, 37). Perhaps the coated vesicles, formed at a coated region at the microvillus base, represent a more generalized form of vesicular transport in the liver. Thus, the coated-pit region and the 100-nm coated vesicle of the liver cell may be the morphological equivalent of the coated pit–coated vesicle mechanism detailed for the fibroblast (3).

In endocytic vesicles, the gold is separated from the delimiting membrane by 20 ± 3 nm. This spatial relationship suggests concomitant internalization of the surface receptor with the ligand LDL. However, it is difficult to measure the gold–membrane relationship in the vesicles because the small radius of vesicle curvature reduces the chances of observing transverse membrane profiles. Consequently, measurements of gold–vesicle and gold–lysosome interactions during the formation of MVB (Fig. 5) were selected from areas with a clear bilayer delineation. The close apposition of the LDL moieties of the conjugate to the vesicle bilayer during internalization is similar to the cellular events occurring during endocytosis of epidermal growth factor (EGF) by cultured human carcinoma cells (26). Although the EGF was not actually visualized, the direct observation of LDL in our studies confirms the close association to the receptor during endocytosis. Delivery during 15-min perfusion is marked by formation of elementary MVB, which contain a small number of gold particles and vesicles. The genesis of MVB is thought to occur by fusion of endocytic vesicles, followed by vesicle eversion and internalization (26).

This mechanism of MVB-vesicle fusion is reported to maintain the polarity of interacting membranes (26). The gold is often observed preferentially clustered along the inner aspect of the MVB after initial vesicle fusion (Fig. 5). This mechanism of fusion and vesicle incorporation, which has been demonstrated in other receptor-mediated uptake systems (26), may account for the observed distribution of the gold granules. This mechanism of endocytosis suggests that the receptors are internalized with the ligand (26) and degraded in the lysosome as a form of down regulation. However, we did not observe any decrease in the number of receptor-bound conjugates during the time periods (15, 60, and 120 min) of perfusion. This observation, in agreement with biochemical studies of LDL uptake in estrogen-treated livers, may indicate a dissociation of the receptor complex from the LDL ligand after delivery to the MVB. As the endocytic vesicles fuse to form the MVB rather than fusing with the lysosome, it may be that the accumulation of the ligand-receptor complex to form the MVB allows for recycling of the receptor. The absence of the clathrin coat from vesicles within the MVB would suggest dissociation of this component that is related to cellular endocytosis. These results agree with the hypothesis that coated vesicles dissociate from their clathrin cage after fusion with specific internal organelles (28). The MVB share several ultrastructural characteristics reported from receptosomes, a recently described organelle that is believed to a special organelle that is derived from internalized coated vesicles (39–41). Functionally, receptosomes avoid early fusion with lysosomes to protect potentially recyclable receptors from lytic enzymes (41). It is perhaps from the receptosome (or MVB) that receptors are recycled and clathrin shed from vesicles before migration to and fusion with lysosomes. If such is the case, then the clathrin coat may prevent fusion of endocytic vesicles with lysosomes to prevent recyclable receptors or physiologically important ligands from proteolytic breakdown (39).

After 1-h perfusion, steady-state endocytosis of the conjugate is observed within the estradiol-treated livers (Figs. 6 a–c). An increased number of coated vesicles have been internalized to form complex MVB (Fig. 6 a and b). During this time, there is also the formation of organelles that have an amorphous lumen containing randomly distributed gold (Fig. 6c). This type of organelle may represent a later stage of complex MVB, in which internalized vesicular contents have been degraded so that membrane material is no longer resolvable. The distribution of gold as free pools within the lumen is interpreted to indicate degradation of the gold-LDL conjugate after disruption (recycling) of the receptor complex.

After perfusion for 2 h, the estradiol-treated livers continue to show all stages of binding, internalization, and lysosomal accretion of the gold-LDL conjugate (Fig. 7). The surface density of the conjugate is similar to that found at earlier liver perfusion times, an observation that is consistent with other reports of estradiol-treated livers, where hepatic tissues show a decreased rate of cholesterol synthesis (13), increased content of cholesterol esters (10), and continued and enhanced activity of the LDL receptor (8). The lysosomes have increased in number and changed from a random subcellular distribution to become clustered around the bile canaliculi after 2-h perfusion. The majority of lysosomes near the bile canaliculi are either amorphous or electron-transparent organelles. The electron transparency may indicate a mature status of the lysosome, in which amorphous material has reached an end stage of degradation. As such, the lysosomal contents may have been exported to the Golgi bodies for conversion into bile. Alter-

**FIGURE 7**  After 2-h perfusion of the estrogen-treated liver, gold particles are seen as free pools within organelles resembling lysosomes (L) located near the bile canalicular regions. Complex MVB and amorphous organelles (seen at 1 h) are also evident. × 33,000.

**FIGURES 8–9**  After 2-h perfusion, the gold-LDL conjugates (arrowheads) are seen within Kupffer cells (K), often peripherally clustered along the inner aspect of electron-transparent organelles (Fig. 8). Coated patches of membrane material are integrated into the delimiting membrane (Fig. 9, arrowheads). Fig. 8, × 14,000; and Fig. 9, × 37,000.
natively, the degraded vesicle membranes and LDL molecules may have been reduced to a state that no longer interacts with the strains used to impart contrast to lipid structures. The frequency of lysosomes having gold distributed as free pools indicates catabolism of luminal contents and separation of gold from the conjugated LDL (Fig. 7). However, no gold was observed in the bile canaliculi or in Golgi bodies. This may reflect the observations that bile flow in the livers of estradiol-treated animals is reduced by ~75% (8) or that nondegradable substances (colloidal carbon, 70-nm diameter) remain in parenchymal cell lysosomes up to 6 h after endocytosis (30). The uptake of gold-LDL evidenced in Kupffer cells (Figs. 8–9) may also be a direct consequence related to estradiol treatment (38). As Kupffer cells are able to initiate phagocytosis of foreign colloidal particles within minutes after injection into the circulation (31, 43), we were not able to differentiate between receptor-independent phagocytosis of the conjugate and receptor-mediated endocytic mechanism. The abundance of lysosomes is consistent with the large endocytotic capacity of the Kupffer cell (36, 43). The endothelial cell internalization of the conjugate (Fig. 10) may also reflect the ability of nonparenchymal cells to participate in uptake of serum lipoproteins (24, 34). Thus, the cellular response of the liver to estradiol can be interpreted as a concerted enhancement of LDL clearance from the circulation.

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REFERENCES

1. Abell, L. L., B. B. Levy, B. B. Brodie, and F. E. Kendall. 1950. A simplified method for the estimation of total cholesterol in serum and demonstration of its specificity. J. Biol. Chem. 185:357-366.

2. Anderson, R. G. W., J. L. Goldstein, and M. S. Brown. 1976. Localization of low density lipoprotein receptors on plasma membranes of normal human fibroblasts and their absence in cells from familial hypercholesterolemia homozygote. Proc. Natl. Acad. Sci. U. S. A. 73: 2434-2438.

3. Anderson, R. G. W., M. S. Brown, and J. L. Goldstein. 1977. The role of the coated endocytotic vesicle in the uptake of receptor bound low density lipoproteins in the human fibroblast cell. Cell. 10:351-364.

4. Anderson, R. G. W., E. Vacile, R. J. Mello, M. S. Brown, and J. L. Goldstein. 1978. Immunocytochemical visualization of coated pits and vesicles in human fibroblasts: relation to low density lipoprotein receptor distribution. Cell. 15:919-933.

5. Arbeeny, C. M., and H. A. Eder. 1980. Effects of 17a-ethinyl estradiol administration on serum lipoproteins of cholesterol-fed diabetic rats. J. Biol. Chem. 255:10547-10550.

6. Babai, F. 1978. An ultrastructural method for the study of cell viability using ruthenium red. Proceedings International Congress of Electron Microscopy. 2:304-305.

7. Brown, M. S., and J. L. Goldstein. 1979. Receptor-mediated endocytosis: insights from the lipoprotein receptor system. Proc. Natl. Acad. Sci. U. S. A. 76:3350-3357.

8. Chao, Y-S., E. E. Windler, G. C. Chen, and R. J. Havel. 1979. Hepatic catabolism of rat and human lipoproteins in rats treated with 17a-ethinyl estradiol. J. Biol. Chem. 254:11360-11366.

9. Cole, F. M., and G. D. Sweeney. 1980. Changes in rat hepatocyte plasma membranes caused by synthetic estrogens. Lab. Invest. 42:225-231.

10. Davis, R. A., R. Showalter, and F. Kern. 1978. Reversal by Triton WR-1339 of ethinylinduced hepatic cholesterol esterification. Biochem. J. 174:45-51.

11. De Bruin, W. C., J. P. M. Schebons, J. M. H. Van Bruitenen, and J. Van Der Meulen. 1980. X-ray microanalysis of colloidal gold-labelled lysosomes in rat liver sinusoidal cells after incubation for acid phosphatase activity. Histochemistry. 66:137-148.

12. Dietrichs, R. 1979. Ruthenium red as a stain for electron microscopy. Some new aspects of its application and mode of action. Histochemistry. 64:171-188.

13. Fewster, M. E., R. E. Pirrie, and D. A. Turner. 1967. Effect of estradiol benzoate on lipid metabolism in the rat. Endocrinology. 80:262-271.

14. Fulch, J. M., Leen, and G. H. Slomoe-Stanley. 1937. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. 226:497-507.

15. Frens, G. 1973. Controlled nucleation for the regulation of the particle size in monodisperse gold solutions. Nature Physical Sciences. 241:20-22.

16. Hamilton, R. L., M. N. Berry, M. C. Williams, and E. M. Severinghaus. 1980. A simple and inexpensive membrane "lum" for small organ perfusion. J. Lipid Res. 15:182-186.

17. Hando, D. A., C. M. Arbeeny, and S. Chien. 1980. Colloidal gold-low density lipoprotein conjugates as membrane receptor probes. Circulation. 62:214.

18. Hando, D. A., C. M. Arbeeny, L. D. Witte, and S. Chien. 1981. Colloidal gold-low density lipoprotein conjugates as membrane receptor probes. Proc. Natl. Acad. Sci. U. S. A. 78:368-371.

19. Handley, D. A., and S. Chien. 1981. Oxidation of ruthenium red for use as an intracellular tracer. Histochemistry. 71:249-258.

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

21. Horabaggar, M. 1980. Evaluation of colloidal gold as a cytochemical marker for transmis-

22. Handley, D. A., and S. Chien. 1981. Oxidation of ruthenium red for use as an intracellular tracer. Histochemistry. 71:249-258.

23. Hamburgh, M. 1980. Evaluation of colloidal gold as a cytochemical marker for transmis-

24. Kovanen, P. T., M. S. Brown, and J. L. Goldstein. 1979. Increased binding of low density lipoprotein to liver membranes from rats treated with 17a-ethinyl estradiol. J. Biol. Chem. 254:11367-11373.

25. Kohler, B., and G. Geyer. 1978. A peroxidase exclusion test for cell viability. Areu. Histochem. 62:261-264.

26. Kovanen, P. T., M. S. Brown, and J. L. Goldstein. 1979. Increased binding of low density lipoprotein to liver membranes from rats treated with 17a-ethinyl estradiol. J. Biol. Chem. 254:11367-11373.

27. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275.

28. McKirnan, J. A., H. T. Haiger, and S. Cohen. 1978. Hormone receptor topology and dynamics: morphological analysis using ferritin-labeled epidermal growth factor. Proc.
27. Mortizan, G. E. 1961. Effect of insulin on potassium transfer in isolated rat perfusion. Am. J. Physiol. 200:1315-1319.
28. Pearse, B. 1980. Coated vesicles. Trends Biochem. Sci. 112:151–154.
29. Benston, R. H., A. L. Jones, W. D. Christiansen, and G. T. Hradek. 1980. Evidence for a vesicular transport mechanism in hepatocytes for biliary secretion of immunoglobulin A. Science (Wash. D. C.). 208:1276–1278.
30. Schellens, J. P. M. 1974. Aging of mouse liver lysosomes. An experimental study using indigestible marker substances. Cell Tiss. Res. 155:455–473.
31. Seno, S., A. Tanaka, M. Urita, K. Hirata, H. Nakatsuika, and S. Yamamoto. 1975. Phagocytic response of rat liver capillary endothelial cells and Kupffer cells to positive and negative charged ion colloid particles. Cell Struct. Funct. 1:119–127.
32. Spurr, A. R. 1969. A low-viscosity epoxy resin embedding medium for electron microscopy. J. Ultrastruct. Res. 26:31–43.
33. Stockert, R. J., H. B. Haimer, A. G. Morell, P. M. Novikoff, A. B. Novikoff, N. Quintana, and J. Sternlieb. 1980. Endocytosis of asialoglycoprotein-enzyme conjugates by hepatocytes. Lab. Invest. 43:556–563.
34. Van Berkel, T. J. C., and A. Van Tol. 1979. Role of parenchymal and nonparenchymal rat liver cells in the uptake of cholesterol ester labeled serum lipoproteins. Biochim. Biophys. Acta 550:1077–1081.
35. Van Handel, E. 1961. Suggested modifications of the microdetermination of triglycerides. Clin. Chem. 7:249–251.
36. Wake, K. 1980. Perisinusoidalstellate cells (fat-storing cells, interstitial cells, lipocytes), their related structure in and around the liver sinusoids, and vitamin A-storing cells in extrahepatic organs. Int. Rev. Cytol. 66:303–355.
37. Wail, D. A., G. Wilson, and A. L. Hubbard. 1980. The galactose-specific recognition system of mammalian liver: the route of ligand internalization in rat hepatocytes. Cell. 21:75–93.
38. Widmann, J. J., and H. D. Fahimi. 1976. Proliferation of endothelial cells in estrogen-stimulated rat liver. Lab. Invest. 34:141–149.
39. Willingham, M. C., F. R. Maxfield, and I. H. Pastan. 1979. Receptor-mediated endocytosis of alpha-macroglobulin in cultured fibroblasts. J. Cell Biol. 82:614–625.
40. Willingham, M. C., F. R. Maxfield, and I. H. Pastan. 1980. Receptor-mediated endocytosis of alpha-macroglobulin in cultured fibroblasts. J. Histochem. Cytochem. 28:818–822.
41. Willingham, M. C., and I. Pastan. 1980. The receptosome: an intermediate organelle between endocytosis and the plasma membrane. Cell. 21:67–77.
42. Windler, E. E. T., P. Kovanen, Y-S. Chao, M. S. Brown, R. J. Havel, and J. L. Goldstein. 1980. The estradiol-stimulated lipoprotein receptor of rat liver. J. Biol. Chem. 255:10464–10471.
43. Wisse, E. 1977. Ultrastructure and function of Kupffer cells and other sinusoidal cells in the liver. In Kupffer Cells and Other Liver Sinusoidal Cells. E. Wisse and D. L. Knook, editors. North Holland Biomedical Press, Amsterdam. 33–60.
44. Yarom, R., H. Stein, P. D. Peters, S. Slavin, and T. A. Hall. 1975. Nephrotoxic effect of parenteral and intraarticular gold. Arch. Pathol. 99:36–43.
45. Zilversmit, D. B., and A. K. Davis. 1950. Microdetermination of plasma phospholipids by trichloroacetic acid precipitation. J. Lab. Clin. Med. 35:155–160.