Receptor-Mediated Endocytosis and Intracellular Trafficking of Insulin and Low-Density Lipoprotein by Retinal Vascular Endothelial Cells

Alan W. Stitt, Heather R. Anderson, Thomas A. Gardiner, Janice R. Bailie, and Desmond B. Archer

**Purpose.** The authors investigated the receptor-mediated endocytosis (RME) and intracellular trafficking of insulin and low-density lipoprotein (LDL) in cultured retinal vascular endothelial cells (RVECs).

**Methods.** Low-density lipoprotein and insulin were conjugated to 10 nm colloidal gold, and these ligands were added to cultured bovine RVECs for 20 minutes at 4°C. The cultures were then warmed to 37°C and fixed after incubation times between 30 seconds and 1 hour. Control cells were incubated with unconjugated gold colloid at times and concentrations similar to those of the ligands. Additional control cells were exposed to several concentrations of anti-insulin receptor antibody or a saturating solution of unconjugated insulin before incubation with gold insulin.

**Results.** Using transmission electron microscopy, insulin gold and LDL gold were both observed at various stages of RME. Insulin-gold particles were first seen to bind to the apical plasma membrane (PM) before clustering in clathrin-coated pits and internalization in coated vesicles. Gold was later visualized in uncoated cytoplasmic vesicles, corresponding to early endosomes and multivesicular bodies (MVBs) or late endosomes. In several instances, localized regions of the limiting membrane of the MVBs appeared coated, a feature of endosomal membranes not previously described. After RME at the apical PM and passage through the endosomal system, the greater part of both insulin- and LDL-gold conjugates was seen to accumulate in large lysosome-like compartments. However, a small but significant proportion of the internalized ligands was transcytosed and released as discrete membrane-associated quanta at the basal cell surface. The uptake of LDL gold was greatly increased in highly vacuolated, late-passage RVECs. In controls, anti-insulin receptor antibody and excess unconjugated insulin caused up to 89% inhibition in gold-insulin binding and internalization.

**Conclusion.** These results illustrate the internalization and intracellular trafficking by RVECs of insulin and LDL through highly efficient RME, and they provide evidence for at least two possible fates for the endocytosed ligands. This study outlines a route by which vital macromolecules may cross the inner blood–retinal barrier. Invest Ophthalmol Vis Sci. 1994;35:3384–3392.

The nonfenestrated endothelial cells of the retinal circulation and their tight junctions together form a highly selective barrier between blood constituents and the retinal neuropile. The restricted permeability of this blood–retinal barrier has been demonstrated ultrastructurally through the use of various protein tracers, such as horseradish peroxidase. It has been shown that although intravenous horseradish peroxidase does not penetrate the endothelial tight junctions, it does appear in the endothelial cytoplasm within endocytotic organelles. Similar results have been reported with horseradish peroxidase in the endothelium of the structurally analogous blood–brain barrier.
Horseradish peroxidase has been shown to enter the endothelial cytoplasm by virtue of its weak binding to the anionic glycoalx. Thus, during plasma membrane (PM) recycling or pinocytosis, this marker will appear in pinocytic vesicles and other related organelles. It is clear, therefore, that unconjugated horseradish peroxidase only serves as a marker of PM internalization and is liable to occur as a contaminant in specific receptor-mediated endocytotic pathways. Alternatively, ferritin or gold conjugated to specific macromolecules has provided a valuable tool for studying the receptor-mediated endocytosis and intracellular trafficking of specific macromolecular ligands.

It has been demonstrated in many cell types that macromolecules such as transferrin, insulin, low-density lipoprotein (LDL), and immunoglobulins are internalized through a highly efficient, receptor-mediated endocytosis mechanism. This process allows specific, extrinsic macromolecules to be concentrated at the level of the PM in specialized clathrin-coated pits, which invaginate to form coated vesicles within the cytoplasm. Internalization initiates a specific endocytotic pathway that permits recycling of the receptors back to the PM and subsequent passage of ligand to the lysosomal system or, as in the case of certain transporting epithelia, transcytosis to the opposite side of the cell.

If intravitreal passage from the ciliary epithelium can be disregarded, passage through the retinal pigment epithelium or the endothelium of the retinal vessels represents the only possible route for the delivery of blood-borne macromolecules to the extracellular space of the retina. It is already known that sugars, amino acids, and other low molecular weight solutes, require specific facilitated or active transport mechanisms to traverse these barrier cells. In the present investigation, the intracellular trafficking of insulin and LDL conjugated to colloidal gold was examined in cultured retinal vascular endothelial cells (RVECs). RVECs are known to express insulin receptors, and, although they do not require insulin for glucose transport, this hormone has marked growth-promoting effects on these cells in vitro. It was also considered that the uptake and intracellular trafficking of LDL by RVECs would be of interest because the retina undoubtedly requires cholesterol for such synthetic processes as membrane genesis. The uptake of LDL has been recognized in vascular endothelial cells from other tissues but has not been studied in RVECs. We sought to establish whether RVECs internalize these molecules by a receptor-mediated endocytotic pathway. Their fate, once internalized, was also to be determined.

**MATERIALS AND METHODS**

**Cell Culture**

Primary cultures of pure retinal endothelial cells were established using a method described by Wong et al. Briefly, bovine eyes were transported from a local abattoir on ice, and the retinas were removed and washed free of retinal pigment epithelium in Dulbecco's minimal essential medium (MEM). The neural retina was then homogenized in MEM and filtered through an 87-μm filter. The trapped microvessels were digested at 37°C for approximately 20 to 30 minutes in phosphate-buffered saline containing 200 μm/ml pronase, 200 μg/ml DNAase, and 50 μg/ml collagenase. The filtrate was microscopically examined to determine the end point for maximum retrieval of endothelial cells. Vessel fragments were then trapped in a 53-μm filter and resuspended in Dulbecco's modified Eagle's medium (DMEM) containing antibiotics (0.2 mg streptomycin sulphate, 0.12 mg benzyl penicillin, and 0.2 mg kanamycin), fungizone (2.5 μg/ml), and 15% fetal calf serum. This mixture was seeded into 25 cm² Falcon flasks and maintained at 37°C in a mixture of 5% CO₂ and air. Endothelial cell growth was supported by DMEM containing 7.5% human platelet-poor, plasma-derived serum (prepared as described by Laughton), 5 μg/ml insulin, and antibiotics. All subsequent experiments were performed on confluent monolayers of cells derived from passages 1 to 4 and plated onto multiwell dishes.

**Colloidal Gold Labeling of Insulin and LDL**

Gold conjugates were prepared according to a method outlined by De Bruyn and Cho. In the current investigation, commercially available 10 nm gold colloid was used (Agar Scientific, Essex, England).

**Insulin.** Bovine insulin (0.4 mg/ml) was suspended in water and dissolved by dropwise addition of 0.1 N HCl (pH 2.5). The colloidal gold solution, adjusted to pH 3.2 with 0.2 M H₃PO₄, was stirred vigorously, and insulin was added at a concentration of 13.3 μg/ml (minimum protecting amount). Stirring was continued for 2 minutes, and the pH of the mixture was raised to 9.0 with 0.2 N K₂CO₃ and 10% bovine serum albumin (pH 9.0) added to a concentration of 1%. The mixture was centrifuged at 4°C and 12,000 rpm for 70 minutes, and the pellet of gold insulin was resuspended in phosphate-buffered saline at the required concentration.

**LDL.** Human LDL (Sigma, St. Louis, MO) was added to colloidal gold solution (pH 5.5) at a concentration of 11.1 μg/ml (minimum protecting amount), and the mixture was stirred vigorously for 2 minutes. The pH of this mixture was slowly adjusted to 9.0 with 0.2 N K₂CO₃ and 10% bovine serum albumin added to a concentration of 1%. The gold conjugate mixture was centrifuged through a 25 mm Diaflo XM 50 membrane (Amicon, Danvers, MA) in an Amicon Ultrafiltration System until the required volume was reached.
TABLE 1. Percentage of Individual Ligand Gold Particles Appearing in Various Cell Regions

<table>
<thead>
<tr>
<th>Region</th>
<th>0 Minutes</th>
<th>1 Minute</th>
<th>2 Minutes</th>
<th>10 Minutes</th>
<th>20 Minutes</th>
<th>30 Minutes</th>
<th>I Hour</th>
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<tbody>
<tr>
<td>Uncoated membrane</td>
<td>76.31/76.10</td>
<td>25.64/35.80</td>
<td>6.66/4.17</td>
<td>5.80/18.19</td>
<td>1.47/-</td>
<td>-/4.71</td>
<td>-/0.99</td>
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<tr>
<td>Coated membrane</td>
<td>23.69/23.90</td>
<td>61.54/46.30</td>
<td>42.22/15.00</td>
<td>50.43/9.09</td>
<td>7.35/21.95</td>
<td>1.64/20.01</td>
<td>0.35/0.97</td>
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<tr>
<td>Coated vesicle</td>
<td>12.82/17.90</td>
<td>40.01/66.67</td>
<td>17.39/21.21</td>
<td>12.52/26.94</td>
<td>3.84/5.82</td>
<td>1.32/20.95</td>
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<td>Uncoated vesicle</td>
<td>-/—</td>
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<td>0.62/-</td>
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<td>Endosome</td>
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<td>MVB (compact)</td>
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<td>MVB (expanded)</td>
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<td>2° Lysosome</td>
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<td>—</td>
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<tr>
<td>Subcellular space</td>
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</table>

Insulin gold values are presented first, followed by LDL gold values. — = Absence of gold particles in all sections.

Gold Labeling Experiments

Before the experiments, the cell cultures were starved of serum and insulin for 24 hours. They were then incubated at 4°C for 5 minutes before the addition of DMEM containing insulin gold and LDL gold at concentrations of 10 and 100 μl/ml, respectively. In both cases, the cells were incubated at 4°C for a further 20 minutes (allowing the ligands to bind to surface receptors without being internalized) before being returned to 37°C, a procedure first described by Anderson et al.25 Several incubation times were employed, from 30 seconds to 1 hour. Some monolayers were fixed in 2.5% glutaraldehyde at time 0 hours. In the remainder, the gold conjugate solution was replaced with DMEM before being placed in the 37°C incubator. Unconjugated gold colloid controls were performed using cells incubated for 30 seconds and 1 hour. Additional control cells were preincubated at 4°C for 20 minutes in three concentrations of anti-insulin receptor polyclonal antibody (20, 4.0, and 0.4 μg/ml) (Upstate Biotechnology, Lake Placid, NY) or in basal medium containing a 100-fold higher concentration than normal (500 μg/ml) of unconjugated insulin. These cells were then exposed to insulin gold as described above.

RESULTS

Insulin. Endothelial cells fixed immediately, after a 20-minute incubation at 4°C (0 minutes), had insulin gold bound to their apical PM. Much of the binding occurred as nonclustered, individual gold particles on flattened regions of the membrane (76%); however, a small proportion of particles (24%) were observed on dense, clathrin-coated regions (see quantitative results, Table 1). Occasionally, the gold insulin appeared clustered in discrete aggregates within clathrin-coated, marginally indented regions of the membrane (Fig. 1). After a 1-minute incubation at 37°C, 26% of the insulin-gold particles were bound to undifferentiated apical PM, although the largest proportion appeared clustered in clathrin-coated regions that were often invaginated in pit-like formations (Table 1, Fig. 2). After a 2-minute incubation, gold conjugate was located within deep clathrin-coated pits. Moreover, some of the pits had become almost completely invaginated and only remained in contact with the cell exterior by a narrow neck.

FIGURE 1. 0 Minutes. Insulin-gold particles (small arrow) are bound diffusely at the apical plasma membrane (apm) of a cultured endothelial cell. Others are bound at a slightly indented region of coated membrane (large arrowhead). Original magnification, ×40,000.
Densely coated vesicles close to the surface, but completely detached from apical PM, contained 40% of the insulin gold (Fig. 3). It was apparent that gold label was never internalized within uncoated pits or caveolae, although 11% of the internalized particles occurred in smooth cytoplasmic vesicles similar in size to the coated vesicles. Similarly, insulin gold was never observed within the intercellular junctions between contiguous endothelial cells.

After a 10-minute incubation, in addition to the coated and uncoated vesicles described in the earlier time periods, internalized insulin gold was observed in larger, vacuole-like organelles. These larger structures, which appeared uncoated and electron lucent, were identified as endosomes, and they had gold bound to discrete portions of the internal faces of their limiting membranes. Internalized insulin gold was particularly evident close to the apical PM, in areas of cytoplasm rich in Golgi cisternae and associated vesicles (Fig. 4). After incubation with insulin gold for 20 minutes, the endosomes described after 10 minutes were similarly represented. At this time, however, a large proportion of the ingested gold (38%) now resided in large, darkly staining organelles filled with densely packed membranous material (Fig. 5). These organelles had the features of multivesicular bodies (MVBs), and, according to the plane of section, the membranous contents could appear vesicular or tubular in nature. The gold particles within MVBs were dispersed throughout the bodies, attached to the matrix side of the internal membranes. Often slender, finger-like projections were observed protruding from these organelles, although they never contained gold probe (Fig. 5).
In endothelial cells incubated in DMEM for 30 minutes (after initial exposure to insulin gold), MVBs contained by far the greater proportion of the internalized gold conjugate. In addition, larger MVB-like bodies also contained insulin gold after 30 minutes (36%). Some of these structures were more swollen in appearance, yet they still contained membranous vesicles (Fig. 6). Also, many showed densely stained material on defined regions of the cytoplasmic face of the limiting membrane, resembling clathrin-coated areas of the PM (Fig. 6). After 1 hour at 37°C, the dense MVBs, described at 20 and 30 minutes, still contained a significant proportion of gold particles (Table 1). However, most of the gold conjugate at this stage resided in large, clear vacuoles thought to represent secondary (2°) lysosomes. Many of these presumptive 2° lysosomes contained dense accumulations of gold particles, representing 42% of the total gold labeling. Typically, the gold was condensed within the matrix of the organelles, often in association with aggregated lysosomal debris (Fig. 7). Cultures incubated for 1 hour also showed discrete aggregates of gold particles lying free in the extracellular matrix at the basal cell surface (Fig. 8) or in association with the basal PM. Approximately 23% of the gold-insulin conjugate was present in this subcellular region at 1 hour.

LDL. The intracellular distribution of LDL gold was essentially similar to that described for insulin gold. At 0 minutes, LDL gold showed diffuse binding to the apical PM before clustering and internalization in coated pits and coated vesicles at 1 to 2 minutes (Fig. 9, Table 1). However, it was apparent that binding of LDL gold was greatly enhanced in late passage endothelial cells (e.g., passage 5) and especially in cells that had grossly swollen and vacuolated appearances. In these cell types, the amount of coated membrane and the number of coated pits were exaggerated in comparison with the remainder of the population, and, accordingly, gold labeling was extensive. After a 2-minute incubation, many coated pits were filled with gold particles (15%), as were coated and uncoated vesicles within the cytoplasm (67% and 14%, respectively) (Fig. 9). Many of these structures assumed unusual configurations, with bulbous extensions occurring from some coated pits (Fig. 10). Similarly, multilobed coated vesicles were occasionally observed, with gold probe present in each lobe. After a 20-minute incubation, endosomes, similar to those described with insulin gold, contained the LDL probe. As with the insulin gold, LDL-gold conjugates tended...
to be localized to discrete regions of the limiting membrane, whereas the remainder of the body appeared empty (Fig. 11). The proportion of LDL gold in endosomes was somewhat greater than in insulin gold-treated cells at the same incubation time (46% versus 30%, Table 1). Similarly, the larger, swollen MVBs contained a smaller proportion of the ingested gold. As in the insulin study, many of the large MVBs showed discrete regions of coated membrane (Fig. 12). Gold-labeled lysosome-like bodies were also present in LDL-treated cells at 1 hour. In normal early passage cells, it was obvious that LDL was taken up in smaller quantities than insulin. However, the proportion of labeling in the 2nd lysosome and subcellular space after 1 hour of incubation was similar to that of the equivalent insulin-gold incubation (Table 1).

**Controls.** In all controls made with unconjugated gold colloid, there was little binding to the apical PM of RVECs and no internalization. No gold was observed in the intercellular tight junctions between endothelial cells, and few gold particles appeared in the matrix region between the basal PM and the culture flask. The small quantity of gold colloid observed in this region tended to occur as single particles. The anti-insulin receptor antibody caused a significant and concentration-dependent inhibition of gold-insulin binding to the apical plasma membrane. A 100-fold increase in concentration of unconjugated insulin before exposure to the gold insulin caused a 74% inhibition of ligand binding to the apical plasma membrane (Table 2).

**DISCUSSION**

Using colloidal gold conjugates, this investigation has demonstrated the intracellular trafficking of insulin...
and LDL in RVECs through a highly efficient receptor-mediated endocytic pathway. Furthermore, there appears to be a selective sorting of internalized ligands, allowing them to be processed through more than one intracellular route.

Receptor-mediated endocytosis of various protein ligands has been described in many cell types, including intestinal epithelial cells, fibroblasts and smooth muscle cells. Endocytosis of insulin has also been reported in nonretinal capillary endothelial cells, demonstrating a receptor-mediated uptake, receptor recycling, and transcytosis of this hormone. Our observations confirmed this sequence of events rather than that proposed by Pastan and Willingham, who suggested that smooth vesicles are pinched off the side of the clathrin-coated pit. We never observed noncoated invaginations of the apical PM, other than occasional caveolae and it was apparent that ligands needed to cluster in clathrin-coated pits before endocytosis. Therefore, insulin internalization in RVECs appears to differ from that in bovine pulmonary artery endothelial cells, in which both clathrin-dependent and clathrin-independent uptake have been described.

The infrequency of smooth, uncoated vesicles in RVECs suggests that such organelles are relatively short lived, with formation of an early endosome occurring quickly after internalization. Recently, a steady state model for the endosomal processing of ligands internalized through RME has been proposed, in which populations of smooth vesicles shuttle molecules to and from preexisting endosomal compartments. If such a system were true for endosomal processing in RVECs, we would have expected a relatively large proportion of uncoated “shuttling” vesicles, especially in the longer time periods (>20 minutes). However, in the current study, only a small proportion of the gold particles were observed in small uncoated vesicles, which favors the maturation model of endocytic processing in which endosomes undergo a gradual remodeling and transformation, eventually forming dense lysosomes.

Endosomal processing of macromolecules internalized by RME has been reported in many cell types. In the present study, labeled endosomes were observed after 10 minutes of incubation with both insulin and LDL gold. These organelles have unique characteristics that permit a sorting of the incoming macromolecules according to their destinations and facilitate recycling of dissociated receptor proteins. In early endosomes, the gold-ligand complexes were invariably associated with the internal face of the limiting membrane, although in MVBs, which are thought to represent late endosomes, the gold was seen to decorate the matrix side of the internal tubules and vesicles. It was of interest that gold particles were never observed within the finger-like projections often present at the MVB stage. These projections represent
Receptor-Mediated Endocytosis in Endothelial Cells

a distinct compartment of the endosomal system associated with uncoupling of receptor–ligand complexes and subsequent recycling of the receptors to the apical PM. This compartment, which has been named the compartment of uncoupling of receptor and ligand,\(^{11,45}\) concentrates uncoupled receptors that then pinch off the endosome and return receptor proteins to the apical PM. Uncoupling of receptor–ligand complexes occurs at low pH within the endosome compartment and has been demonstrated for a number of macromolecules, including LDL.\(^{10}\) Because RME appears remarkably consistent in all cell types studied to date, it is likely that insulin and LDL receptors are recycled by RVECs.

The large vesicles containing membranous structures that were described after 20 to 30 minutes are identical to MVBs described elsewhere,\(^{2}\) although there may be variations of these structures between cultured cells and cells in vivo. MVBs have often been described as simply a transformation product of late endosomes.\(^{11}\) However, in this study, there is considerable variation between these organelles, both in terms of size and content. The gold conjugates first appeared in MVBs that were endosome-sized and densely packed with vesicles and tubules, but later the gold probes were seen in MVBs that were considerably larger with more dispersed contents. As a general rule, gold label was located at the internal aspect of the limiting membrane or was bound to the outside of the vesicular contents. It may be significant that some MVBs had a clathrin-like coat in small regions of their limiting membrane. The origin and function of such a coated membrane is uncertain, although it could represent a remnant of fusion with coated vesicles from the trans-Golgi network. Clathrin-coated vesicles are known to pinch off the trans-Golgi network,\(^{46}\) and it has been speculated that primary lysosomes derived from this organelle bind to MVBs, thereby converting this “late” endosome compartment into a 2° lysosome.\(^{47}\) Alternatively, the coated membrane of the MVB may indicate the presence of a retrieval system, analogous to the initial stages of RME at the PM, for ligands destined for fates other than the lysosome.

The uptake of LDL was generally lower than insulin, with the exception of swollen, vacuolated, late-passage cells. It is likely that such cells would require more cholesterol for synthesis of the extensive vacuole membranes. Increased RME of LDL has also been reported in osmotically swollen fibroblasts.\(^{46}\)

Transcytosis, whereby ligands internalized at the apical PM are redirected from the endosome to the basal PM, is well documented.\(^{7,11}\) In the current investigation, we suggest that in addition to a high level of lysosomal degradation, a small yet significant route exists through RVECs for gold ligands to be exocytosed at the basal PM.

Key Words

retinal vascular endothelial cells, receptor-mediated endocytosis, low-density lipoprotein, insulin, insulin receptor, colloidal gold conjugates, electron microscopy

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References


15. Rodewald R. Selective antibody transport in the proximal


