

Receptor Pattern Formation as a Signal for the Capture of Lipoproteins

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A critical step in the uptake of dietary cholesterol by the liver is the binding of remnant lipoprotein particles to receptors in the space of Disse. We have found that increases in the cholesterol content of hepatocyte membranes reduces the binding of β -very low density lipoproteins (β -VLDL) and decreases internalization. This increase in membrane cholesterol of human hepatoma cells (HepG2) produces a similar effect on binding to primary human fibroblasts. However, receptor-negative familial hypercholesterolemic (FH) fibroblasts lack the ability to respond to membrane cholesterol modification. A polyclonal antibody directed against the C-terminus region of the apo-B,E-(LDL) receptor importantly affects the internalization process, suggesting that protein-protein interactions consolidate the pattern formation of receptors, a process that triggers lipoprotein internalization. We propose that cholesterol interferes with this pattern formation by affecting the lateral movement and organization of the receptors. © 1996 Academic Press, Inc.

Several steps have been involved in plasma clearance of remnant lipoproteins (1-4). Initially remnants are sequestered within the space of Disse in the liver where apo-E interacts with the remnants and thereafter bind to heparan sulfate proteoglycans (HSPG) (5). The LDL receptor mediates part of the remnant lipoprotein internalization, whereas the lipoprotein related receptor protein or LRP has been implicated as the main receptor for remnant (apo-E) particles as β -VLDL lipoproteins (6).

Recently it was established that the uptake of remnant lipoproteins involves the initial interaction of lipoproteins with HSPG followed by an apparent transfer of the remnants to the receptor for internalization (7). The major proteins involved in remnant catabolism including apo-E, lipoprotein lipase and hepatic lipase have all been shown to bind to HSPG. A major goal in this area of research is to elucidate the fundamentals of receptor-mediated endocytosis involving the interaction of apo-E β -VLDL with the LDL receptor. The purpose of the present study has been to determine if the plasma membrane itself might modulate the internalization process of cholesterol loaded β -VLDL particles. Since cholesterol by changing the physical properties of membranes has been implicated in the modulation of the activity of several molecules (8), we studied if the modulation of the concentration of cholesterol in the plasma membrane of cells in culture affects the binding and internalization properties of the receptors involved in β -VLDL recognition.

EXPERIMENTAL PROCEDURES

Lipoprotein isolation. Rabbit β -VLDL were isolated from New Zealand White rabbits fed a high cholesterol diet for 10 days (9). The rabbit β -VLDL (d=1.006g/ml) were isolated by ultracentrifugation as described elsewhere (9). The β -VLDL were iodinated by the method of Bilheimer et al. (10). Enrichment of β -VLDL with human apo-E3 was accomplished by incubating the β -VLDL with apo-E3 at 37°C for 1 h prior to use (11). The 4°C

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binding assays of ^{125}I - β -VLDL + apo-E ($4\mu\text{g}$ of protein/ml + $6\mu\text{g}$ of human apo-E3) were performed for 4 h as described elsewhere (11).

Cell culture. Human hepatoma (HepG2) cells were previously incubated at 37°C for 6 h with fresh DMEM containing 10% LPDS and 2% liposomes formed either with phosphatidylcholine or phosphatidylcholine/cholesterol (7:3). The medium was then removed and the cells washed three times with PBS containing 0.2% bovine serum albumin at 4°C . Nonspecific binding was determined in the presence of a 100-fold excess of unlabeled ligand. After incubation, the cells were washed and dissolved in 0.1N NaOH, and the radioactivity measured with a gamma counter and protein determined by the method of Lowry et al. (12). Liposomes were formed by placing DMPC or DMPC/cholesterol dissolved in chloroform in a glass conical centrifuge tube. After drying under a stream of N_2 , the residue is redissolved with benzene, frozen with liquid nitrogen and lyophilized. When used, the lyophilized lipid mixtures were hydrated with PBS, sonicated for 3×2 min and centrifuged at room temperature. The uptake of ^{125}I - β -VLDL + apo-E particles was performed under identical conditions as for the binding experiments except that the cells were incubated at 37°C HepG2. Cells were previously incubated also at 37°C for 6 h with fresh DMEM containing 10% LPDS and 2% liposomes and washed in the same way as performed with the 4°C binding experiments.

When heparinase was used the enzyme was dissolved in sterile PBS immediately prior use. The enzyme was added to the specific tissue culture media and then added to the cells previously treated for membrane cholesterol enrichment. After treatment at the appropriate times, cells in culture were washed three times with media to remove the enzyme and the specific binding assay performed. The cells were treated with 6 units/ml of heparinase for 2 h at 37°C . After washing, binding assays were performed for 4 h at 4°C using ^{125}I - β -VLDL ($4\mu\text{g}$ of protein/ml) or ^{125}I - β -VLDL + apo-E ($4\mu\text{g}$ of protein/ml + $6\mu\text{g}$ of human apo-E3). Cholesterol liposomes treated cells contained $71\mu\text{g}$ cholesterol/mg protein. Control DMPC liposome treated cells contained $32\mu\text{g}$ cholesterol/mg protein.

Antibody assays. HepG2 cells pretreated at 37°C for 6h with fresh DMEM containing 10% LPDS were washed in the same way as performed for 4°C binding and 37°C uptake experiments. Pretreated cells were trypsinised in the presence of DMEM containing 10% LPDS, 0.5% albumin. Different amounts of the antibodies were added to the mixture and subjected to electroporation employing a Gene Pulser System by Bio Rad ($800\mu\text{Fi}$, 200-300 mV). After the treatment, the cells were placed in 6 mm plates for 6 h with fresh DMEM and washed. Control wells were used for viability counts, and uptake experiments started with the addition of ^{125}I - β -VLDL + apo-E. Results were adjusted in accordance to viability counts after transfection.

RESULTS AND DISCUSSION

HepG2 cells, primary fibroblasts and familial hypercholesterolemic (FH) fibroblasts were pre-exposed to cholesterol-containing phosphatidyl choline vesicles and then to ^{125}I - β -VLDL + apo-E particles at 4°C . At this temperature the cells bind the particles, but do not internalize them. Figure 1a shows that cells preincubated with cholesterol bound less β -VLDL + apo-E particles than cells exposed to vesicles lacking cholesterol. A Scatchard plot of the binding data, shows that both the B_{max} and K_d decrease at higher membrane cholesterol levels (inset, Fig. 1a). Measurement of the internalization of the β -VLDL + apo-E particles shows that increased cholesterol levels in the membranes also lower uptake (Fig. 1b).

Experiments on NBP15 cells (not shown) and primary fibroblasts, gave similar results when a lower binding was found in cells containing higher membrane cholesterol concentrations (Fig. 2a,b). Interestingly, FH-fibroblasts lacking the presence of the functional LDL receptor, but that contain active LRP, did not respond to high cholesterol levels in their plasma membranes (Fig. 2c,d). Cholesterol insensitivity in FH cells might be interpreted as the inability of membrane cholesterol to affect the binding of β -VLDL to LRP, a molecule several times bigger than the LDL receptor, and in which most of its mass lies in the extracellular space. Although LRP effectively binds the particles, slight changes in the physical state of the membrane do not seem to interfere with β -VLDL binding. As previously shown by Ji et al. (5), heparinase treatment decreased β -VLDL + apo-E binding to HepG2 cells, whereas binding of β -VLDL (without added apo-E) did not. In our hands HepG2 cells containing cholesterol enriched plasma membranes decreased β -VLDL + apo-E binding. In these cells, heparinase decreased β -VLDL + apo-E binding to values lower than those observed in the controls (Fig. 2e). Although heparin sulfate proteoglycans are involved in the initial binding of the apo-E-enriched remnants, membrane cholesterol may impede the right alignment and positioning of the LDL receptors in accordance to the apo-E pattern given by the number of copies and

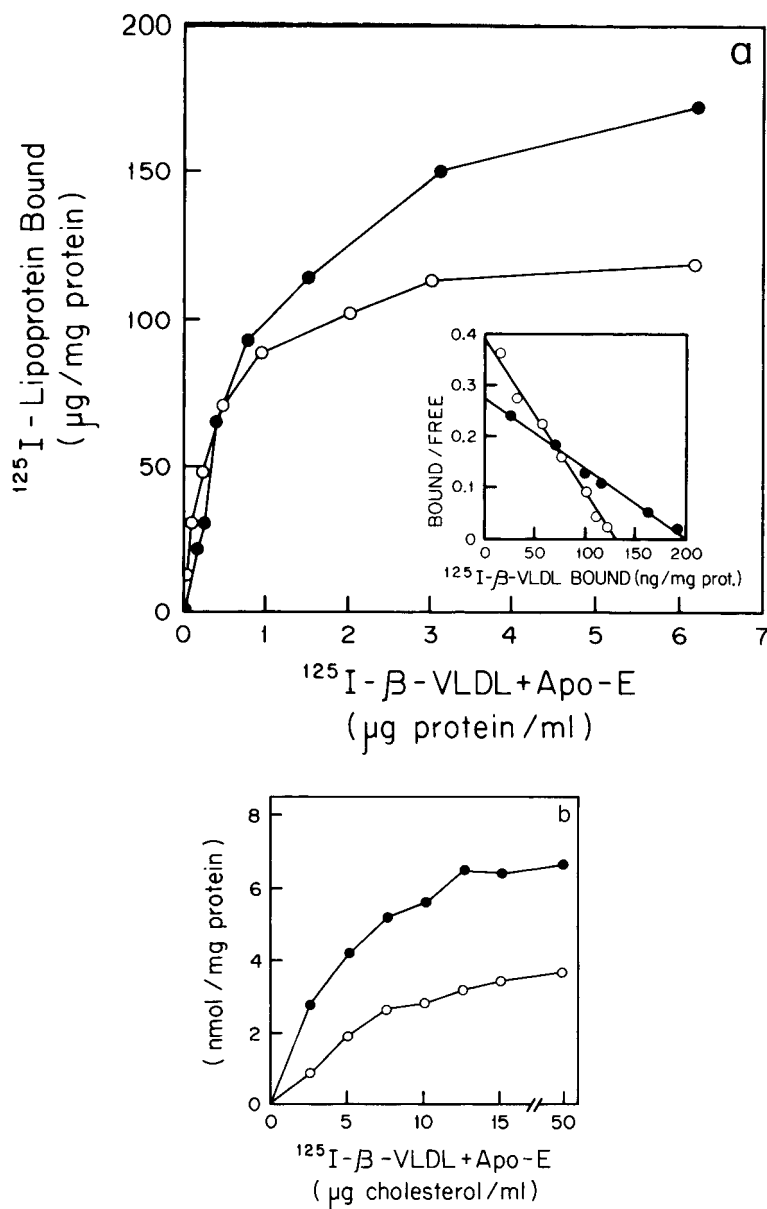


FIG. 1. Cholesterol loaded membranes affect binding and internalization of very low density lipoproteins to HepG2 cells. Phosphatidylcholine liposome treated cells (membrane cholesterol $46\mu\text{g/mg protein}$) (●). Phosphatidylcholine/cholesterol liposome treated cells (membrane cholesterol $88\mu\text{g/mg protein}$) (○). a) binding and b) internalization of ^{125}I - β -VLDL + apo-E particles to HepG2 cells. Inset: Scatchard plots of data. Results represent the mean of three independent experiments where the calculated S.D. corresponds to less than 10% of the total values.

position of apo-E in the lipoprotein particle, independently of the presence of HSPG on the membrane surface. Interestingly, HepG2 cells transfected with an antibody prepared against part of the C-terminus region of the apo-B,E (LDL) receptor ($\text{H}_2\text{N-KNWRLKNINSINFDN-PVYQK-COOH}$), presented lower internalization rates than cells transfected with an antibody prepared against a random peptide (Fig. 3). This result supports the thesis that the association receptor tail-cytoskeleton through protein-protein recognition is of paramount importance in

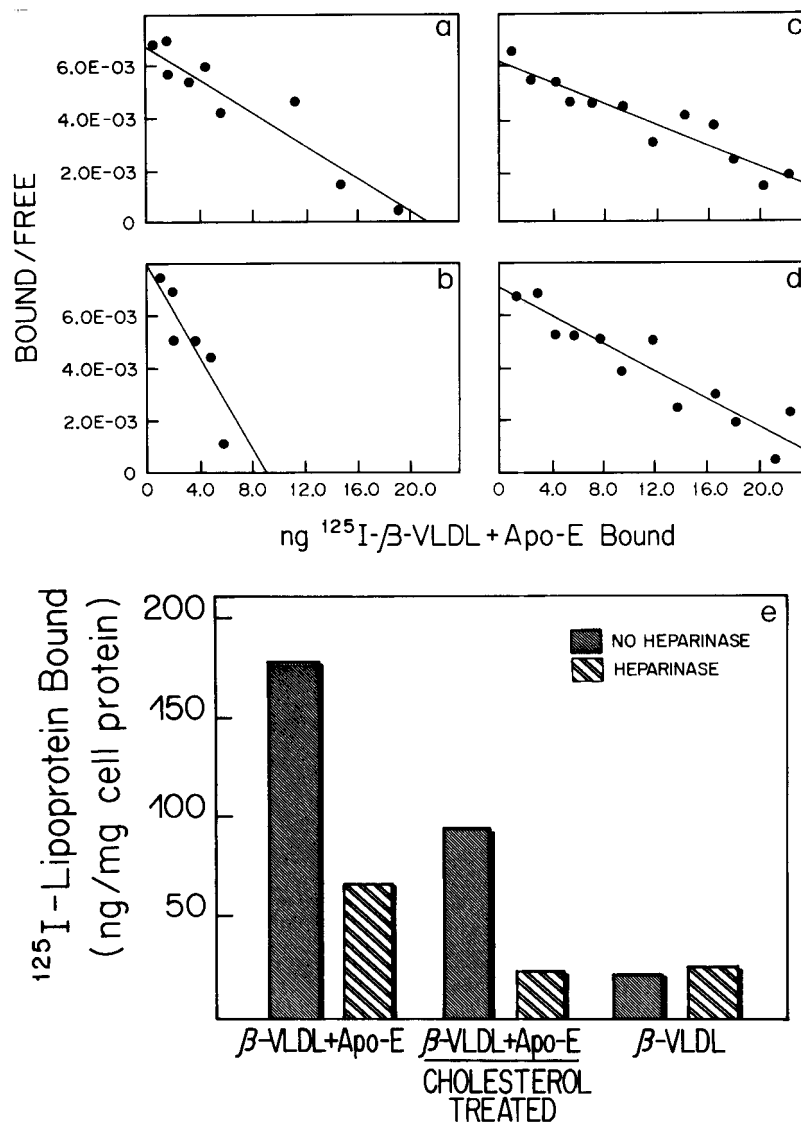


FIG. 2. Very low density lipoprotein binding to human primary fibroblasts is affected by high cholesterol concentrations in their plasma membranes whereas FH-fibroblasts show not to be affected by this increase in cholesterol concentration. Scatchard plots for β -VLDL binding using phosphatidylcholine liposome treated cells (a,c), and phosphatidylcholine/cholesterol liposome treated cells (b,d). Both primary fibroblasts (a,b) and FH-fibroblasts (c,d) grown and treated as described in experimental procedures HepG2 cells treated with heparinase (e). Membrane cholesterol concentration: a) $18\mu\text{g}/\text{mg}$ protein; b) $58\mu\text{g}/\text{mg}$ protein; c) $26\mu\text{g}/\text{mg}$ protein; d) $52\mu\text{g}/\text{mg}$ protein. a) $B_{\text{max}} 21.46$, $K_d 93.18\mu\text{g}/\text{ml}$. b) $B_{\text{max}} 93$, $K_d 10.84\mu\text{g}/\text{ml}$. c) $B_{\text{max}} 27.62$, $K_d 98.24\mu\text{g}/\text{ml}$. d) $B_{\text{max}} 25.9$, $K_d 97.14\mu\text{g}/\text{ml}$. Representative experiment in a series of three.

the process of lipoprotein internalization. The antibody most probably is interfering with pattern formation of receptors substantially lowering internalization.

High cholesterol levels in membranes are known to affect membrane function (13) and the lateral organization of membrane proteins by creating cholesterol rich phospholipid domains which enhance the probability for protein aggregation (14). If we believe a unique 2-dimensional LDL-receptor pattern is central for the internalization of β -VLDL particles, it follows

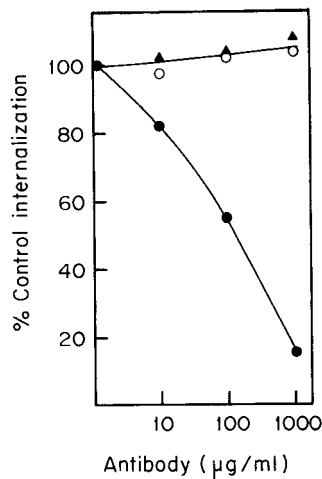


FIG. 3. β -VLDL uptake experiments using cells transfected with an antibody raised against the apo-B,E (LDL) receptor tail. Specific antibody anti-H2N-KNWRLKNINSINFDNPVYQK-COOH (●), control electroporation (○), random antibody anti-H2N-VSIYQLSKDAPDRIEAVSKD-COOH (▲). Representative experiment in a series of two.

that disrupting membrane organization by cholesterol or by blocking the receptor tails with an antibody would indirectly interfere with the binding of β -VLDL thereby inhibiting internalization. This was based on the following model for transmembrane signaling by single-crossing receptors. We define "networking proteins" as those proteins that function via protein-protein binding. Similarly to cytoskeletal proteins, they have kinase, ATPase, and phosphatase activities, and therefore form a subclass of networking proteins. Single-crossing receptors are networking membrane proteins that extend from the cytoskeleton through the plasma membrane. In the model these proteins form either dimers or a polymeric network structure. There are two types in this model, dimerizing and pattern-forming. Human growth hormone receptor may be considered as an archetype of a dimeric networking membrane protein, whereas the receptors for β -VLDL particles may be archetypal pattern-forming networking membrane proteins. We suggest that pattern-forming membrane networking proteins switch on receptor-mediated endocytosis through the formation of a 2-dimensional pattern on the cytosol side of the membrane. Such a pattern would nucleate a cytoskeletal structure, in this case a coated pit (15) with the participation of clathrin and AP-2 (16). The formation of the pattern would require the receptor molecules to: diffuse laterally in the plane of the bilayer and form a unique 2-dimensional pattern. Such pattern formation may be impeded by cationic amphiphilic class of drugs (17,18).

Proteins that span the membrane several times perform their various functions by folding up their long sequence of amino acids in specific patterns. An extremely slight rearrangement of how the protein is folded is sometimes all that is necessary to alter the protein from performing its function (19). Interactions between protein and phospholipid affect this fine relationship and small environmental changes due to an increase in the phospholipid/cholesterol ratio might alter protein function, as shown for the plasma membrane (Ca^{2+} , Mg^{2+})-ATPase which spans the membrane ten times (8). However, networking proteins like the LDL and LRP receptors which only cross the membrane once, do not present these characteristics, and therefore binding and internalization changes not easily explained by conformational alterations. Our findings may be a mechanism physiologically relevant by which receptor cross-linking caused by the interaction between a lipoprotein particle with at least two receptors, form a geometrical array sensed by the cytoskeleton. Networking proteins by binding a signal

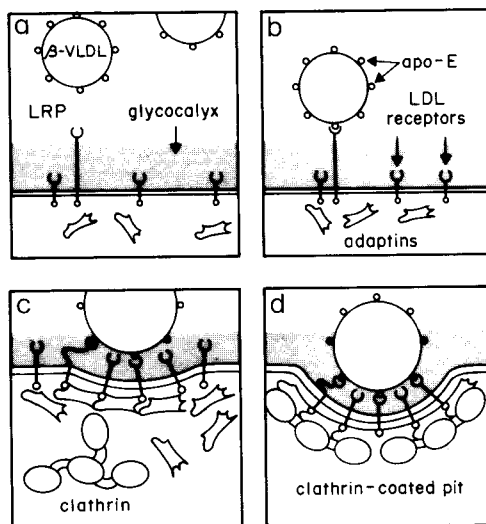


FIG. 4. Lateral diffusion of receptors on the plasma-membrane of cells in close association with the cytoskeleton promotes the formation of a pattern that creates coated pits. The presence of a lipoprotein containing several copies of the apoprotein in close contact with the membrane. (a) LRP extends beyond the glycocalyx of the space of Disse as β -VLDL particles approach the surface. Many other proteins are omitted. (b) LRP binds an apo-E on the particle tethering it to the membrane. Adaptins are monomers in the cytosol. (c) Once tethered, LRP with putative "hinges" brings the particle into contact with laterally diffusing LDL-receptors. As each receptor binds an apo-E, the "nearest neighbor" pattern of apo-E's on the particle arrays the receptors so that their endolayer domains form the same pattern. This pattern facilitates the binding of adaptins to the endolayer domains of the receptors. Once aligned, the adaptins also bind to each other. (d) Arrayed adaptins bind clathrin which introduces the curvature to form the pits and later the coated vesicles.

molecule on the extracellular side of the membrane might be arraying unique binding sites on the cytoplasmic side that would pass the information through the membrane in the form of a distinctive pattern recognized by the cytoskeleton and associated proteins (Fig. 4). With as few as two receptors occupied by two apo-E molecules associated to a β -VLDL particle a specific pattern signal could be formed. We suggest that large amounts of membrane cholesterol or the presence of an antibody against the receptor tail inhibit step (c) by interfering with the formation of the receptor pattern.

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