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Association of the low-density lipoprotein receptor with caveolae in hamster and rat liver☆

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Abstract

The association of the low-density lipoprotein (LDL) receptor with detergent resistant hepatic membranes was investigated using discontinuous sucrose gradients. In liver homogenates from both hamsters and rats, the fractions with the highest concentrations of LDL receptor coincided with the location of caveolin-1, a marker of the cholesterol-rich caveolae. Feeding the animals diets enriched in cholesterol slightly shifted both LDL receptor and caveolin-1 to positions of lower density. The cholesterol content of the caveolae fractions was increased 2-fold in animals fed cholesterol-supplemented diets. In homogenates of CHO cells, fractionated in the same manner, the LDL receptor was absent from the caveolae fractions but was present in denser fractions near the bottom of the gradient. Addition of caveolin-1 antibody to solubilized caveolae from liver coimmunoprecipitated the LDL receptor. These observations suggest that in liver, the LDL receptor is mainly located in caveolae. This location contrasts with the clathrin-coated pit location observed in fibroblasts and CHO cells.

Keywords: LDL receptor; Caveolae; Caveolin-1; ARH; Liver; CHO cells

The LDL receptor plays a critical role in cholesterol homeostasis. It binds, internalizes, and thereby removes LDL from serum [1,2]. The finding that liver transplantation of a child with homozygous familial hypercholesterolemia lowered the patient's plasma LDL cholesterol level by over 80% underscored the importance of hepatic LDL receptors in controlling LDL cholesterol levels [3]. It has been estimated that the liver contains at least 70% of the body's LDL receptors [4]. Yet most studies of the LDL receptor have been done with fibroblasts, lymphocytes or CHO cells [5,6].

The expression of LDL receptors is under feedback regulation by cholesterol [5]. Expression is increased when LDL is removed from the culture media. This transcriptional response is mediated through the binding of transcription factors to the sterol regulatory element-1 (TCACCCCACT) in the LDL receptor promoter [5]. These transcription factors are called sterol regulatory element-binding proteins [5]. They are generated by proteolytic cleavage of membrane-bound precursor proteins by the action of site-1 and site-2 proteases in response to cholesterol depletion.

In contrast with the observations obtained with cultured cells, feeding animals diets supplemented with cholesterol does not result in decreases in either hepatic LDL receptor protein [7] or mRNA [8]. Also levels of the mature nuclear forms of sterol regulatory element-binding proteins-1 or -2 are unaffected [8]. However, it would seem that LDL receptor activity should be decreased. When animals treated with an HMG-CoA reductase inhibitor were given diets supplemented with cholesterol, transcription of the hepatic LDL receptor was significantly reduced along with mRNA levels [8]. This agrees with the findings made in studies of cells cultured in the presence of a reductase inhibitor such as compactin [9]. Thus, reductase inhibitors appear to

☆ *Abbreviations:* LDL, low-density lipoprotein; CHO, Chinese hamster ovary; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; DTT, dithiothreitol; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; EGTA, (ethylene-bis[oxyethylenenitrilo])tetraacetic acid; EGF, epidermal growth factor; ARH, autosomal recessive hypercholesterolemia.

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unmask transcriptional regulation of the hepatic LDL receptor that is not normally observed [8].

Animals treated with statins, such as lovastatin, parvastatin, fluvastatin or cerivastatin, which are potent cholesterol lowering agents, exhibit increased hepatic LDL receptor mRNA levels [8]. Surprisingly hepatic LDL receptor protein levels are not increased. A possible explanation for this inconsistency was provided with the finding that the half-life of the receptor protein was substantially decreased in livers from statin treated rats. Thus, it was proposed that the statins acted to cause increases both in the rate of synthesis of hepatic LDL receptors and in their rates of degradation [8]. Since it has been demonstrated that the LDL receptor is recycled [5], it was proposed that statins act to increase this cycling process thus accounting for the increased uptake of LDL from the serum and the increased rate of degradation of the receptors since a fixed number of receptors would be degraded in each cycle [5]. Thus, the rate of synthesis of hepatic LDL receptors is increased as a result of increased mRNA levels. Additionally, the rate of degradation is increased since the half-life is decreased. This provides a novel means to increase LDL receptor function without an increase in LDL receptor protein level [8].

The cycling of the LDL receptor from the plasma membrane to endosomes to recycling vesicles and back to the plasma membrane is well established [10]. It seems reasonable that the rate of cycling would depend on the fluidity of the membranes in which the receptor resides. Subdomains of the plasma membrane rich in cholesterol and sphingolipids and referred to as caveolae have been described [11]. These could slow the rate of cycling of the LDL receptor if the receptor resided, in part, in these domains. Thus, we wished to determine whether the hepatic LDL receptor was ever associated with caveolae and whether cholesterol feeding might increase the portion of the LDL receptor in this structure. In fibroblasts, the LDL receptor is clustered in coated pits associated with clathrin [5]. Much to our surprise the major portion of hepatic LDL receptor in both hamsters and rats appeared to be located in caveolae. In marked contrast, this was not observed in CHO cells.

Materials and methods

Animals. Male Sprague–Dawley rats weighing 100–125 g and male 7- to 8-week-old Golden Syrian hamsters were purchased from Harlan. The animals were housed in 12 h/12 h reverse cycle light-controlled rooms at a temperature of 21 ± 1 °C and humidity of 45–55%. They were allowed free access to Harlan Teklad 22/5 Rodent Chow, which contains 22% protein, 5% fat and 4.5% fiber, and water. In some experiments, the diet was supplemented with 1% cholesterol. The animals were killed at the fourth hour of the dark period and livers were rapidly removed and homogenized for the preparation of caveolae using discontinuous sucrose gradients.

Materials. Antisera to a C-terminal sequence of the rat LDL receptor were generated in rabbits as previously described [12]. An affinity-purified rabbit polyclonal antibody raised against a N-terminal peptide of human caveolin-1 was purchased from Santa Cruz Biotechnology. A goat polyclonal antibody to a C-terminal peptide of the clathrin heavy chain was also purchased from Santa Cruz. The ECL Western blotting kit was purchased from Amersham.

Isolation of caveolae. Caveolae were isolated essentially as described [13]. A 400 mg piece of liver was homogenized in 2 ml MBS buffer (25 mM morpholinoethane sulfonic acid, pH 6.5, 0.15 M NaCl, 1% Triton X-100, and 1 mM PMSF) using a motor-driven Teflon glass Thomas homogenizer, which has a serrated pestle. Two ml of 80% sucrose in MBS without Triton X-100 was then added and the homogenate was placed in a 3 1/2 in. × 9/16-in. polyallomer centrifuge tube. This was carefully overlaid with 4 ml of 20% sucrose in MBS without Triton X-100 and 4 ml of 5% sucrose in MBS without Triton X-100. The discontinuous gradients were centrifuged at 39,000 rpm for 16 h in a SW41 rotor at 4°. The gradients were fractionated into 1 ml fractions. For Western blotting, 1 and 2 ml and 3 and 4 ml were combined and referred to as fractions 1 and 2 because of the very low level of protein in these fractions. The amount of cholesterol in each fraction was determined by the cholesterol oxidase method using the INFINITY Cholesterol Reagent from Sigma. The protein in these fractions was precipitated by the addition of 4 volumes of methanol and centrifugation at 9000*g* for 10 min [14]. The pellets were resuspended in minimal volumes of 1% SDS depending on the protein concentration of each fraction. The concentration of protein in each fraction was determined by the BCA protein assay from Pierce.

Immunoblotting analysis. Aliquots of each fraction, 15 μg of protein, were subjected to SDS–PAGE electrophoresis on 7.5% gels. Molecular weight markers were applied to one lane. The separated proteins were electrophoretically transferred to PVDF membranes. LDL receptor and caveolin-1 were detected using the ECL kit as previously described [15].

Coimmunoprecipitation. The caveolae fraction, 5–7 ml, was pooled. Aliquots of 15 μg of protein were solubilized in 300 μl of 20 mM CHAPS. Caveolin-1 antibody, 1 μl, was added and the mixture was incubated at room temperature for 1 h. Protein-A–Sepharose, 2.86 mg, was added and the mixture was placed on a rotating wheel at room temperature for 1 h and then centrifuged in a microfuge for 1 min. The pellet was washed four times in 50 mM Tris–HCl, pH 7.5, 0.15 M NaCl, 2 mM EGTA, 1 mg/100 of leupeptin, and 1 mm NEM. The pellet was then washed in sterile water and dissolved in 30 μl of 8 M urea containing 20 mM DTT. LDL receptor was detected by immunoblotting analysis.

Results

To assess the distribution of hepatic LDL receptor in hamsters, liver homogenates were fractionated on sucrose gradients followed by Western blotting analysis of the gradient fractions. As shown in Fig. 1A, the highest levels of LDL receptor are located in fractions 4–6. This is also where caveolin-1, a marker for caveolae, was found. Progressively lower levels of LDL receptor protein were observed in fractions 7–10. Western blotting analysis of these liver fractions with the clathrin antibody did not yield discernable bands. When the hamsters were fed a diet supplemented with cholesterol, LDL receptor protein was shifted slightly upward starting with fraction 3 (Fig. 1B). This would be consistent with increased lipid content in the caveolae

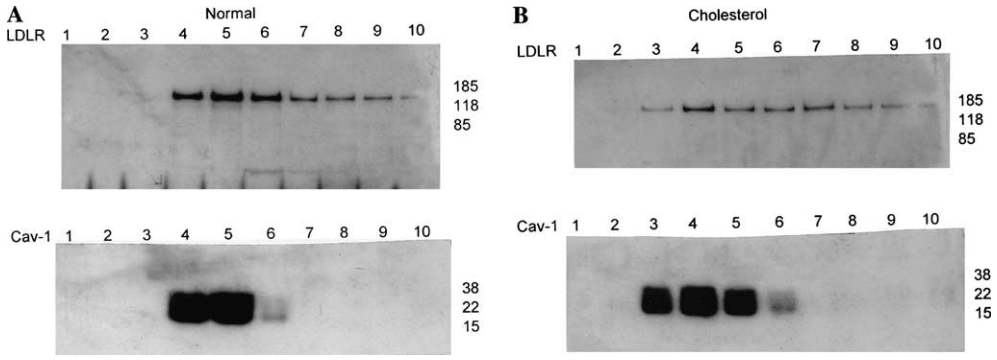

Fig. 1. Western blotting analysis of hamster liver LDL receptor and caveolin-1. (A) Liver fractions from hamsters fed normal chow diets were probed for LDL receptor and caveolin-1. (B) Fractions from cholesterol fed hamsters were analyzed. Fractions 1 and 2 contained material from pooled 2 ml fractions. The others had 1 ml each.

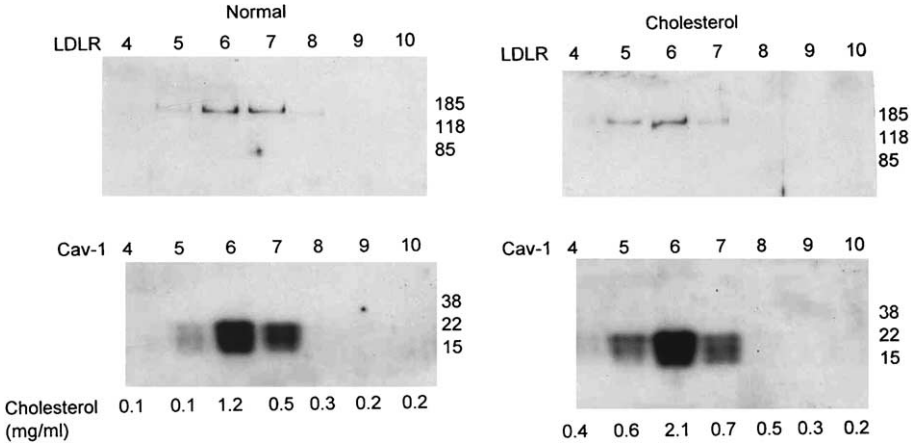

Fig. 2. Western blotting analysis of LDL receptor and caveolin-1 in livers from Brown–Norway rats. The fractions containing 4–10 ml of each gradient were analyzed for LDL receptor and caveolin-1. Livers from rats fed normal chow or chow supplemented with 1% cholesterol were fractionated. The concentration of cholesterol in each fraction is also presented.

membranes from animals fed cholesterol-supplemented diets. A corresponding shift was seen in the position of caveolin-1. Again LDL receptor protein trailed into the higher, non-caveolae fractions.

Similar fractionation experiments were done using Brown–Norway rats. Strikingly, nearly all of the LDL receptor protein in livers from these animals was located in the caveolae fractions (Fig. 2). Cholesterol supplementation had very little effect; only a slight increase in fraction 5 and slight decrease in fraction 7. However, the amount of cholesterol in the caveolae fractions was increased about 2-fold. This would be expected to decrease membrane fluidity and slow the rate of cycling of the LDL receptor.

As a further comparison, CHO cells were also fractionated and subjected to Western blotting analysis. As seen in Fig. 3, LDL receptor protein is located in fractions 7–10 while caveolin-1 is mainly located in fractions 3 and 4. Thus, in CHO cells the LDL receptor is not located in caveolae.

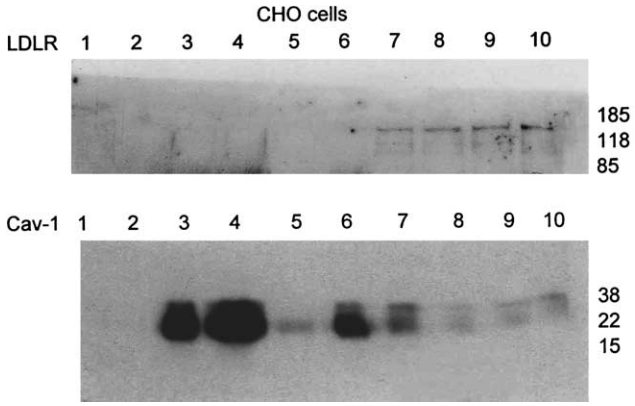

Fig. 3. Western blotting analysis of the LDL receptor and caveolin-1 in CHO cells. Fractions 1 and 2 each contained 2 ml because of low levels of protein present. The other fractions contained 1 ml each.

The question of whether the hepatic LDL receptor associates with caveolin-1, the major structural protein of caveolae, was investigated by performing

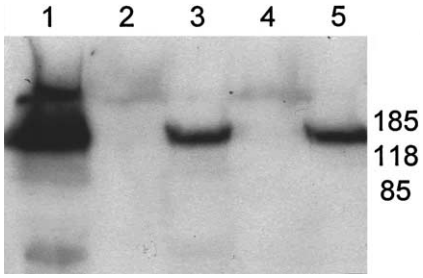

Fig. 4. Co-immunoprecipitation of LDL receptor and caveolin-1. A Western blot developed with LDL receptor antibody is presented. Lane 1 contains 30 μg of caveolae fraction from hamster liver. In lanes 2–5, 15 μg of caveolae fraction was solubilized in 20 mM CHAPS. Samples for lanes 2 and 3 were from hamster liver while those in lanes 4 and 5 contained caveolae from rat liver. To the samples for lanes 3 and 5, 1 μl of caveolin-1 antibody was added.

co-immunoprecipitation experiments. As shown in Fig. 4, addition of caveolin-1 antibody to CHAPS solubilized caveolae fractions from either hamster or rat liver pulled down LDL receptor protein. This suggests physical association of the LDL receptor with caveolin-1, indicating that a portion of hepatic LDL receptors is located in caveolae.

Discussion

Much to our surprise, we found that the majority of LDL receptors in livers from both hamsters and rats appear to be located in caveolae fractions. This is in contrast with previous reports demonstrating the localization of LDL receptors in clathrin-coated pits in fibroblasts [10]. Localization in liver has not been addressed previously. Yet the liver is the major organ expressing LDL receptors.

Several receptors have been reported to be located in caveolae. These include the HDL receptor (SR-BI) in studies with transfected CHO cells [16], the insulin receptor in rat adipocytes [17], epidermal growth factor receptor (EGFR) in quiescent fibroblasts [18], and a long list of other receptors and proteins as recently reviewed [19]. To this list, LDL receptor in liver is now added.

Caveolae are involved in the uptake of a large variety of molecules and also bud off from the cell surface and interact with the endocytic compartment [20]. Although originally described in endothelial cells, caveolae have been demonstrated in hepatocytes [20].

A recent study [21] identified a 60-amino acid-long sequence located in the juxtamembrane region on the extracellular portion of the EGF receptor, as sufficient to target the receptor to caveolae. This is in contrast with the NPVY sequence in the cytoplasmic tail of the LDL receptor that directs proteins to clathrin-coated pits [22–24]. Perhaps the hepatic LDL receptor contains a caveolae targeting sequence that is not present on the CHO cell receptor due to alternative splicing. Alternatively, a chaperone protein may be expressed specifically in liver, which interacts with LDL receptor and targets the receptor to caveolae. As shown in Fig. 3, the LDL receptor in CHO cells is in the heavier fractions (non caveolae) of the gradient. This agrees with a recent report showing that the apolipoprotein E receptor, but not the LDL receptor, is located in caveolae in CHO cells.

Recently, the defect in autosomal recessive hypercholesterolemia (ARH) has been identified as caused by mutations in the phosphotyrosine-binding domain of an adaptor protein that interacts with the NPVY sequence in the cytoplasmic tail of the LDL receptor [25]. LDL receptor activity in livers of ARH patients appears to be markedly impaired, based on a decreased rate of LDL clearance from plasma and decreased uptake of LDL by liver [26]. In contrast, LDL receptor activity in fibroblasts from these patients is normal. In Fig. 4, an interaction between caveolin-1, a tyrosine-phosphorylated protein [27], and the hepatic LDL receptor is demonstrated. Interactions of the LDL receptor with both caveolin-1 and ARH may be required for normal receptor function in liver. The localization of the hepatic LDL receptor in caveolae, demonstrated in the present study, as compared with the association of this receptor with clathrin-coated pits in fibroblasts may provide an explanation for the normal LDL receptor activity in fibroblasts in the face of severely impaired LDL receptor function in livers of ARH patients [26].

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