Biochemical and Biophysical Research Communications 445 (2014) 422–427

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Distribution of the LDL receptor within clathrin-coated pits and caveolae in rat and human liver

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ARTICLE INFO

Article history:
Received 2 February 2014
Available online 12 February 2014

Keywords:
LDL receptor
Clathrin
Caveolin-1
Rat liver
Human liver
Human hepatic cells

ABSTRACT

Several findings suggest that the low-density lipoprotein (LDL) receptor may internalize different lipoprotein particles via diverse pathways. Using a combination of discontinuous sucrose gradients and Triton solubilization studies, we demonstrated that the LDL receptor could be located simultaneously in clathrin-coated pits and caveolae in rat and human liver and in human hepatocyte-like C3A cells. Treatment with the cholesterol biosynthesis inhibitor, zaragozic acid A, shifted the distribution of the LDL receptor to clathrin containing fractions, whereas treatment with cholesterol or LDL shifted the receptor distribution towards caveolin-1 containing fractions. The LDL-dependent shift of the LDL receptor to caveolae coincided with a reduction in internalization of Bodipy-LDL. Redistribution within plasma membrane microdomains in response to specific treatments resulting in changes in LDL receptor function represents a novel paradigm that could be exploited in the development of a new class of therapeutic drugs.

1. Introduction

The primary function of the low density lipoprotein (LDL) receptor is the removal of highly atherogenic LDL particles from the circulation [1]. The LDL receptor pathway mediates at least 60–75% of LDL turnover in rats [2], 67% in rabbits [3], and 56–80% in man [4]. Since the liver contains about 70% of the total LDL receptors in the body [5], a better understanding of the regulatory mechanisms that control the hepatic expression of this receptor is essential.

Several findings suggest that the LDL receptor may internalize different lipoprotein particles via diverse pathways. First, the acid-dependent mechanism of lipoprotein release in the endosome has been shown to be required for LDL but not for uptake of very low density lipoprotein (VLDL) remnants [6]. Second, hepatocytes prepared from mice lacking the LDL receptor adaptor protein-1 (LDLRAP1) are able to internalize VLDL but not LDL [7]. These results correlate with the finding that although the defects in clearing LDL from the circulation in autosomal recessive hypercholesterolemic (ARH) patients is comparable to that of patients with homozygous defects for the LDL receptor (familiar hypercholesterolemia-1; FH1), ARH patients have lower LDL cholesterol levels and develop symptomatic coronary heart disease significantly later in life [7]. The severe hypercholesterolemia in FH1 results from an increase in LDL production and a decrease in LDL clearance [7]. In ARH, however, if VLDL clearance is preserved as in LDLRAP1 deficient mice, LDL production would be decreased explaining the lower LDL levels as compared to FH1 patients [7]. Interestingly, mice lacking caveolin-1 have elevated levels of VLDL but not of LDL [8].

Based on these findings, it is possible that removal of VLDL, which might require a slower process to allow the lipoprotein to escape the receptor and deliver its cargo to extrahepatic tissues, is carried out via internalization by the LDL receptor into caveolae. On the contrary, removal of LDL, which requires a more efficient/rapid process to supply the liver with enough cholesterol, is achieved by directing the LDL receptor to the clathrin-dependent internalization pathway. In agreement, the LDL receptor has been independently located within clathrin-coated pits [9] and in caveolae [10]. Herein, we examined whether the hepatic LDL receptor

Abbreviations: LDL, low density lipoprotein; VLDL, very low density lipoprotein; LDLRAP1, LDL receptor adaptor protein-1; CH, cholesterol; ZA, zaragozic acid A; HC, heavy chain; TS, Triton soluble; TI, Triton insoluble.

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http://dx.doi.org/10.1016/j.bbrc.2014.02.019
0006-291X/

could be localized simultaneously in both plasma membrane microdomains and whether this distribution could be influenced by specific treatments.

2. Materials and methods

2.1. Animals

Normal male Sprague–Dawley rats weighing 125–150 g were purchased from Harlan Industries (Madison, WI). Experiments with rats were carried out according to the regulations and oversight of the University of South Florida Institutional Animal Care and Use Committee, protocol #3571. Rats were housed in a light-controlled reversed cycle room with 12 h light/12 h darkness and had free access to water. Animals were fed Harland Teklad 22/5 rodent chow. Some rats received 2% cholesterol (CH) mixed with their chow for 2 days, whereas others were injected with zaragozic acid A (ZA) subcutaneously at a dose of 2 mg/kg of body weight, 16 h before euthanization. Animals were euthanatized at the 4th h of the dark period by an isofluorane overdose. Liver portions were quickly removed to be employed in the different experiments.

2.2. Materials

ZA was from Merck (Whitehouse Station, NJ). The rabbit antibody against the rat LDL receptor was generated as previously described [11]. The antibody against LDLRAP-1 was kindly provided by Dr. Helen H. Hobbs (Department of Molecular Genetics and Internal Medicine, University of Texas Southwestern Medical Center, Dallas, TX). The antibodies against caveolin-1 and the BD™ MITO+ serum extender (MITO+) were from BD Biosciences (San Jose, CA). Antibodies specific for clathrin heavy chain (HC) and actin, and all the horseradish peroxidase (HRP)-labeled secondary antibodies were from Santa Cruz Biotechnology (Dallas, TX). The antibody against the inducible degrader of LDL receptors (IDOL), Lane Marker Reducing Sample Buffer, the BCA protein assay, and the SuperSignal West Pico chemiluminescence substrate were from Pierce (Rockford, IL). The mouse antibody against proprotein convertase subtilisin/kexin-9 (PCSK9) was from Cayman Chemicals (Ann Arbor, MI). The human hepatocyte-like C3A cell line was obtained from the American Type Culture Collection (Manassas, VA). Low glucose (5.55 mM) Dulbecco's modified Eagle's medium (D-MEM), fetal bovine serum (FBS), precast 4–20% SDS–PAGE gels, unlabeled LDL, and Bodipy-LDL were purchased from Invitrogen (Carlsbad, CA). The Pointe Scientific Cholesterol Oxidase Assay was from Fisher Scientific (Pittsburgh, PA).

2.3. Discontinuous sucrose gradients

Fractionation of rat liver homogenates in discontinuous sucrose gradients was performed as previously described [10]. About 400 mg of rat liver was homogenized in 2 mL of MBS buffer (25 mM morpholinoethane sulfonic acid, pH 6.5, 0.15 M NaCl, 1% Triton X-100, and protease/phosphatase inhibitors). 2 ml of 80% sucrose in MBS without Triton X-100 was mixed with the homogenate. The mixture was transferred into an ultracentrifuge tube and carefully overlaid with 4 ml of 20% sucrose in MBS without Triton X-100 followed by 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 °C. 1 ml fractions (12 fractions total and a 13th fraction from resuspending the remaining pellet in 500 μl of homogenization buffer) were collected from the top of the gradient. Cholesterol levels in each fraction were determined using the Pointe Scientific Cholesterol Oxidase Assay, whereas protein concentrations were determined using the BCA protein assay. Equivalent amounts of proteins from each fraction were precipitated with 4 volumes of ice-cold methanol and centrifugation at 9000*g* for 10 min [12]. The pellets were resuspended in 1X Lane Marker Reducing Sample Buffer prior to electrophoresis and Western blotting analysis as described later.

2.4. Preparation of post-mitochondrial fractions and microsomes from rat liver

Rat liver pieces were homogenized in 10 volumes of ice cold 0.25 M sucrose. The homogenate was centrifuged at 16,000*g* for 15 min. The supernatant was transferred to a fresh tube and re-centrifuged. The top 10 ml of the supernatant was removed with a pipette and saved as lysosome-free post-mitochondrial supernatants, or centrifuged at 100,000*g* for 1 h to pellet the microsomes.

2.5. Preparation of microsomes and cytosolic fractions from human liver S9 samples

Pooled human liver S9 fractions were purchased from Xeno-Tech, LLC (Lenexa, KS; conc: 20 mg/ml; mixed gender). After diluting with 20 volumes of cold 0.25 M sucrose, the liver samples were centrifuged at 100,000*g* for 1 h to separate microsomes (pellet) from cytosolic fractions (supernatant).

2.6. Cell culture and Bodipy-LDL binding and internalization studies

Human hepatocyte-like C3A cells were maintained at a density of 10^7 cells per 75-cm^3 flask in DMEM medium supplemented with 10% FBS and antibiotics at 37 °C under humidified atmosphere and 5% CO_2. For Triton solubilization experiments, 8×10^6 viable cells were set per 160-cm^3 flask in maintaining medium. 24 h later, the medium was changed to a medium where the FBS has been replaced with 1 ml/L of BD™ MITO+ serum extender (MITO+ medium). The MITO+ serum extender is composed of minimal levels of growth factors and hormones (i.e., epidermal growth factor, human transferrin, insulin, endothelial cell growth supplement, hydrocortisone, triiodothyronine, progesterone, testosterone, estradiol-17β, selenious acid, and O-phosphorylethanolamine) that allow cells to grow under a serum-free condition [13]. No morphological and/or growth changes were observed in these cells when incubated in MITO+ medium (data not shown). The MITO+ medium was used as a lipid/cholesterol deficient medium (alternative to delipidated medium; cholesterol levels in the sera: Mito = 0.2625 mg/dL; FBS = 18.99 mg/dL). Unlabeled LDL (5 μg/ml) was added to some of the flasks 24 h before lysing the cells. For Bodipy-LDL binding and internalization studies, 8×10^5 viable cells were plated per well in 6-well plates in maintaining medium. After treating with MITO+ medium ± unlabeled LDL for 24 h, cells were incubated on ice for 30 min to stop cycling of the LDL receptor. At that point, 5 μg/ml of Bodipy-LDL was added to some wells. After incubating for 1 h at 4 °C to allow Bodipy-LDL binding, plates were transferred to 37 °C and incubated for exactly 30 min to allow internalization of Bodipy-LDL. Cells were washed twice with ice-cold PBS and suspended by gently scraping into PBS/2 mM EDTA. Bodipy-LDL binding and internalization data was collected using the BD FACSAria Cell Sorter system (BD Biosciences). This flow cytometer is equipped with a 488 nm laser (exciting probe) and a FITC emission channel, FL1 (505 LP, 530/10 BP), which were used to detect Bodipy. Percentage fluorescent cells were defined as the percentage of cells within each subpopulation with fluorescence intensity exceeding that of the maximum level of autofluorescence of unlabeled cells in the same subpopulation. The difference between Bodipy-LDL labeled and unlabeled cells was 189-fold (data

not shown). Mean fluorescence intensities were recorded for 10,000 events for each experiment.

2.7. Preparation of basolateral membranes from control and LDL-treated C3A cells

Basolateral membrane fractions were prepared by a modification of a previously reported method [14]. Briefly, cells were washed twice with ice-cold PBS and then gently scrapped into 5 ml of cold-PBS/2 mM EDTA. Cells were sedimented at 500*g* for 10 min and then homogenized in 500 µl of a buffer containing 150 mM NaCl, 5 mM EDTA, and 25 mM Tris–HCl, pH 7.4. The homogenates were centrifuged at 500 rpm for 5 min to remove unbroken cells, nuclei and debris. Supernatants were supplemented with 50 µl of 100 mM calcium chloride solution (1/10th dilution). After mixing by pipetting up and down, the samples were incubated for 30 min on ice followed by microcentrifugation at maximum speed for 15 min to pellet the basolateral membranes.

2.8. Triton X-100 solubilization studies

Rat and human microsomal pellets and basolateral membrane pellets from C3A cells were used in the preparation of Triton soluble (TS) and Triton insoluble (TI) proteins following a modification of a previously described method [15]. Briefly, the pellets were homogenized in 5 volumes of ice-cold Triton solubilization buffer (0.25 M sucrose; 0.2% Triton X-100; protease/phosphatase inhibitors) and rapidly microcentrifuged at 12,000*g* for 10 min at 4 °C. Supernatants were carefully removed and saved as "TS proteins". The pellets were washed twice in cold PBS + protease inhibitors, followed by centrifugation after each wash, and then homogenized in 5 volumes of ice-cold RIPA buffer (25 mM Tris–HCl, pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, and protease/phosphate inhibitors). After incubating for 15 min on ice, samples were centrifuged at 12,000*g* for 10 min at 4 °C. The supernatant was removed and saved as "TI proteins".

2.9. Western blotting analysis

Protein concentrations were determined using the BCA protein assay. Equivalent amounts of proteins were denatured in Lane Marker Reducing sample buffer at 70 °C for 5 min and subjected to electrophoresis on 7% gels or precast 4–20% SDS–PAGE. Electrophoresis, electroblotting onto nitrocellulose membranes, blocking with 5% non-fat dry milk-TTBS were carried out using standard methods. Incubations with antibodies against the LDL receptor (diluted 1:2000), LDLRAP1 (diluted 1:1000), caveolin-1 (diluted 1:500), clathrin HC (diluted 1:100), IDOL (diluted 1:500), PCSK9 (diluted 1:1000), and actin (diluted 1:200) were performed overnight at 4 °C. Immunoreactive proteins were visualized using a 1:10,000 dilution of HRP-conjugated secondary antibody and the SuperSignal West Pico Chemiluminescence Substrate. All the antibodies were diluted in blocking solution. Multiple exposures ranging from 0.5 s to 20 min were made using a Kodak Image Station 4000R Pro Imaging System (New Haven, CT). Quantitation of the Western blot signals was performed using the Imaging software. Actin was used as the internal control for the post-mitochondrial supernatant and cytosolic samples.

2.10. Statistical analysis

Data from the individual parameters for at least three independent measurements ($n = 3$) were compared by analysis of variance (ANOVA) followed by Student–Newman–Keuls multiple comparison test or *t*-test when applicable, using the GraphPad Prism 5 software (La Jolla, CA). A $p < 0.05$ was considered significant for all tests.

3. Results

Rat liver homogenates were separated in discontinuous sucrose gradients, and the resulting gradient fractions were analyzed using Western blotting. The highest caveolin-1 signal, as a marker of

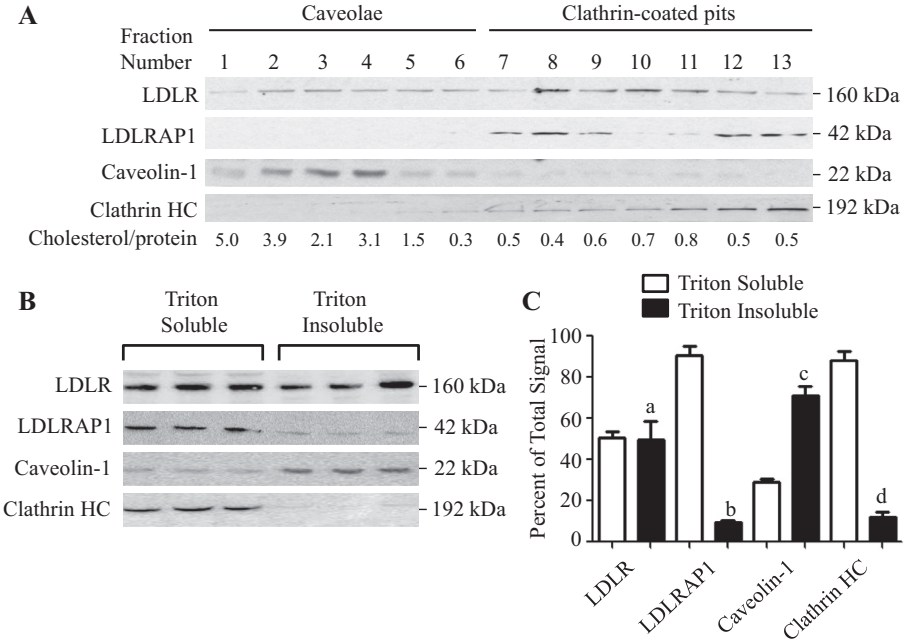

Fig. 1. Protein expression of the rat LDL receptor, LDLRAP1, caveolin-1 and clathrin HC. (A) Rat liver homogenates separated using discontinuous sucrose gradients were analyzed using Western blotting. Measured cholesterol and protein levels are presented as cholesterol/protein ratio for each fraction. This experiment was repeated four times. (B) Triton solubilization studies on rat liver microsomes followed by Western blotting analysis. (C) Data from Triton solubilization studies are presented as percentage of total signal (addition of the signal from TS and TI fractions for the same sample) mean ± SEM for $n = 3$. "a" ($p = 0.9215$), "b" ($p < 0.0001$), "c" ($p = 0.001$), and "d" ($p = 0.0001$) were obtained when comparing TS and TI for the same protein.

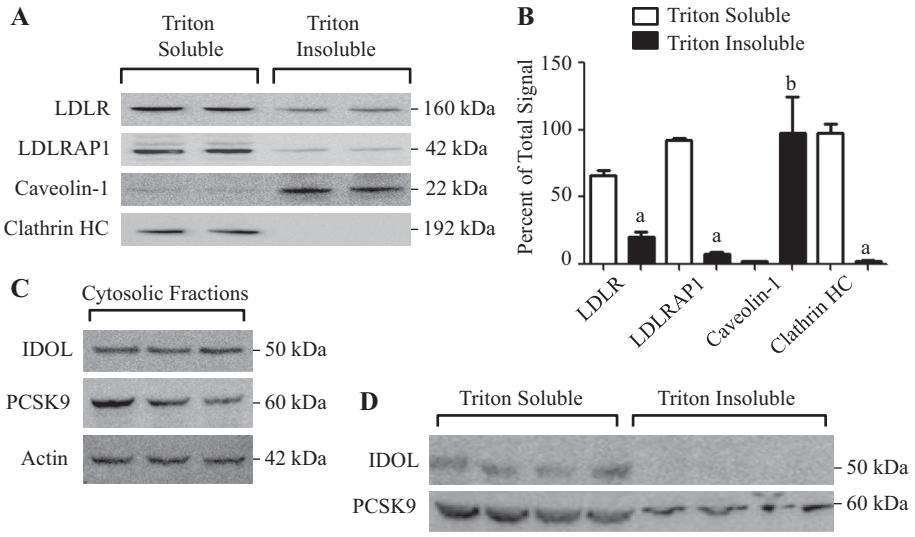

Fig. 2. Protein expression of the human LDL receptor, LDLRAP1, caveolin-1 and clathrin HC. (A) Representative Western blots showing TS and TI fractions. (B) Data are presented as percentage of total signal mean ± SEM for $n = 8$. "a" ($p < 0.0001$) and "b" ($p = 0.0051$) were obtained when comparing TS and TI for the same protein. (C) Protein expression of PCSK9 and IDOL in human cytosolic fractions. Actin was the internal control for this experiment. (D) Expression of PCSK9 and IDOL in TS and TI fractions. This experiment was repeated three times.

caveolae, was detected in fractions 1–6, which also correlated with the fractions containing the highest cholesterol/protein ratio (Fig. 1A). The highest signal for clathrin protein, corresponding to clathrin-coated pits, was detected in fractions 7–13 (Fig. 1A). LDL receptor protein was detected in all the fractions, but the majority of LDLRAP1 was detected in the clathrin containing fractions. Similar results were obtained using Triton solubilization studies (Fig. 1B and C). The LDL receptor distribution among the two Triton fractions was nearly identical (50.51% in TS vs. 49.49% in TI; $p = 0.9215$; Fig. 1C).

Triton solubilization studies were also carried out using human liver microsomes. Fig. 2A and B illustrate that the distribution of the human LDL receptor was mostly towards the TS fractions (66.15%; $p < 0.0001$). As in rat liver, the human LDLRAP1 was mainly detected within TS fractions (92.75%, $p < 0.0001$; Fig. 2A and B). We also examined the distribution of the two degraders of the LDL receptor, PCSK9 and IDOL [16,17]. PCSK9 is the convertase that interacts with the LDL receptor at the surface of hepatic cells leading to degradation of the receptor within the lysosomes [16]. IDOL works by enhancing the ubiquitination of the LDL receptor, which also results in receptor degradation within the lysosomes [17]. As expected, high levels of PCSK9 and IDOL were detected in human cytosolic fractions (Fig. 2C). Surprisingly, PCSK9 was detected in both TS (64.9%) and TI fractions (35.1%; $p = 0.0009$), but IDOL was only detected in TS fractions (Fig. 2D).

To determine if this LDL receptor distribution within plasma membrane microdomains could be altered, rats treated with either cholesterol (CH; 2% for 2 days) or with zaragozic acid A (ZA; 2 mg/kg of body weight for 16 h). Microsomes were prepared from liver samples followed by Triton solubilization studies. These treatments are known to cause alterations in the LDL receptor protein degradation without affecting the steady-state levels of the receptor [18]. The results in Fig. 3A confirmed that neither treatment affected the expression of the LDL receptor in rat post-mitochondrial supernatants. However, the receptor distribution within plasma membrane microdomains was significantly altered by these treatments (Fig. 3B). CH shifted the distribution of the rat LDL receptor in the direction of the caveolin-1 containing fractions (TI; 85%; $p = 0.001$), whereas ZA shifted the receptor distribution towards clathrin containing fractions (TS; 73.66%; $p = 0.0051$) (Fig. 3B). In

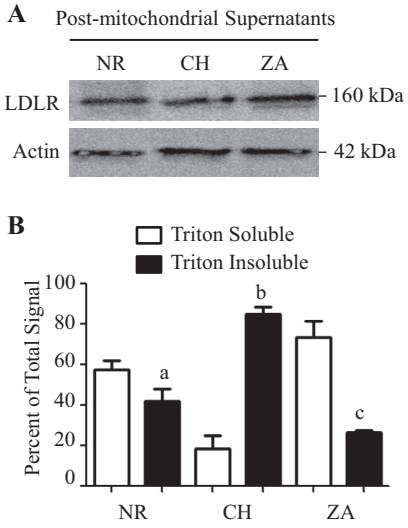

Fig. 3. Effects of cholesterol (CH) and zaragozic acid A (ZA) treatment on the LDL receptor distribution within plasma membrane microdomains in rat liver. NR refers to control (untreated) rats. (A) Representative Western blots for LDL receptor and actin (internal control) in post-mitochondrial supernatants. (B) Data from Triton solubilization studies are presented as percentage of total signal mean ± SEM for $n = 3$. "a" ($p = 0.1047$), "b" ($p = 0.0051$) and "c" ($p = 0.001$) were obtained when comparing TS and TI for the same treatment group.

these experiments, the LDL receptor distribution in control (NR) rats was 58% in TS vs. 42% in TI fractions ($p = 0.1047$; not significant).

To investigate whether similar results could be obtained in human cells, the hepatocyte-like C3A cell line, a highly selected subclone of HepG2, was employed. We used C3A instead of HepG2, because C3A possesses many properties of normal human hepatocytes that HepG2 cells do not [19]. These properties are strong contact inhibition of growth at confluency, high expression of albumin, and an ability to grow in glucose deficient medium [19]. Cells were incubated for 24 h in the presence or absence of 5 μg/ml of LDL in MITO+ medium (a cholesterol deficient medium). Basolateral membranes were prepared and used in Triton solubilization

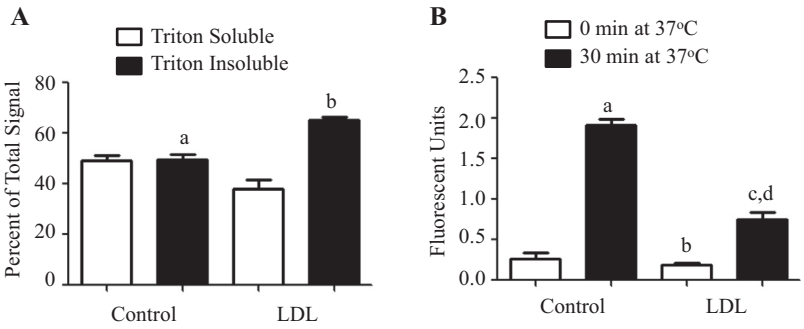

Fig. 4. Effects of LDL treatment on the LDL receptor distribution within plasma membrane microdomains in human hepatocyte-like C3A cells. (A) Data from Triton solubilization studies are presented as percentage of total signal mean ± SEM for $n = 4$. "a" ($p = 0.8106$) and "b" ($p = 0.0003$) were obtained when comparing TS and TI for the same treatment group. (B) Data from Bodipy-LDL binding and internalization studies are presented as percentage of total fluorescent unit mean ± SEM for $n = 3$. "a" ($p = 0.0001$), "b" ($p = 0.3496$), and "c" ($p = 0.0007$) were obtained when comparing to control at the "0 min" time point. "d" ($p = 0.0036$) was obtained when comparing the "0 min" and "30 min" time points for the LDL condition.

experiments. As in the CH treated rats, treating C3A cells with LDL resulted in a shift in the LDL receptor distribution towards caveolin-1 containing fractions (TI; 65.16%; $p = 0.0003$) (Fig. 4A). The LDL receptor distribution in control C3A samples was 49.17% in TS vs. 50.83% in TI fractions ($p = 0.8106$; not significant).

To examine whether the function (internalization rate) of the LDL receptor could be affected in correlation with this shift to caveolin-1 containing fractions, Bodipy-LDL binding and internalization studies were performed in C3A cells treated ± unlabeled LDL. Bodipy-LDL binding ("0 min" time point) was comparable in both treatment groups (0.267 in control vs. 0.192 in LDL; $p = 0.3496$; not significant) (Fig. 4B). This correlated with Western blotting results showing no significant differences in the steady-state levels of LDL receptor protein in control and LDL treated cells (data not shown). Treating with unlabeled LDL prior to the internalization studies resulted in a 61.32% percent reduction ($p = 0.0007$) in internalization of Bodipy-LDL by the receptor as compared to the internalization seen in control (untreated) cells (Fig. 4B).

4. Discussion

To internalize a lipoprotein, like LDL, the receptor must undergo endocytosis within a plasma membrane microdomain. Internalization via clathrin-coated pits is a well-known pathway for internalization via the LDL receptor [1,9], but the finding that this receptor is also present in caveolae in hamster and Brown-Norway rat livers [10] is indicative of alternative internalization pathways, which may be influenced by species-specific factors. Herein, we showed that in human and Sprague–Dawley rat livers, and in human hepatocyte-like C3A cells, the LDL receptor was localized in clathrin and caveolin-1 containing fractions simultaneously. In addition, we demonstrated that this distribution could be shifted in response to treatments like cholesterol or LDL (towards caveolae) and the cholesterol biosynthesis inhibitor, ZA, (towards clathrin-coated pits). Thus, there is a possibility that, *in vivo*, the LDL receptor could migrate between different plasma membrane microdomains depending on genetic, dietary, and/or hormonal factors. In agreement with our findings, the study using hamsters and Brown-Norway rats reports that cholesterol feeding enhances the localization of the LDL receptor into caveolae [10]. Interestingly, migration between plasma membrane microdomains has been reported for the LDL receptor related protein-1 in response to insulin treatment [20]. Thus, it is possible that a similar mechanism governs localization of the LDL receptor.

It could be possible that the ability of the LDL receptor to migrate between plasma membrane microdomains may influence how species adapt to dietary factors. Due to the lack of LDLRAP1, any LDL receptor molecule located within caveolae could have a diminished ability to internalize LDL, or any other lipoprotein, resulting in an accumulation of cholesterol in the serum. On the contrary, if the receptor is present within clathrin-coated pits, where LDLRAP1 is available to enhance the receptor function, LDL internalization becomes more efficient resulting in a reduction in plasma LDL levels. Here we also reported that an enhancement in LDL receptor distribution towards caveolae coincided with a reduction in Bodipy-LDL internalization supporting the link between receptor plasma membrane distribution and function.

We also reported that PCSK9 was detected in both clathrin and caveolin-1 containing fractions, while IDOL was only detected in clathrin containing fractions. Our finding about PCSK9 is in agreement with reports showing that degradation of the LDL receptor in response to PCSK9 can occur in the absence of LDLRAP1 although at a lower rate [21,22]. IDOL, on the other hand, enhances internalization of the LDL receptor via a clathrin- and caveolae-independent pathway [23,24], which disagrees with our results on IDOL. Further studies are required to confirm the specific plasma membrane microdomains involved in the degradation of the LDL receptor by PCSK9 and IDOL.

In summary, our studies demonstrated that the LDL receptor can be located simultaneously within clathrin-coated pits and caveolae in hepatic cells. Furthermore, internalization studies showed that treatment with LDL decreased the internalization of Bodipy-LDL while shifting the receptor distribution towards caveolae. These findings suggest a novel regulatory mechanism to control the function of the LDL receptor directly at the plasma membrane that could be further explored to design more efficient drugs to fight hypercholesterolemia.

Acknowledgments

This project was supported by funds from the State of North Carolina, The Golden LEAF Foundation, BRITE, and Dr. Li-An Yeh.

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