JACC: BASIC TO TRANSLATIONAL SCIENCE VOL. 5, NO. 6, 2020
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PRECLINICAL RESEARCH

Lipoprotein(a) Cellular Uptake Ex Vivo and Hepatic Capture In Vivo Is Insensitive to PCSK9 Inhibition With Alirocumab

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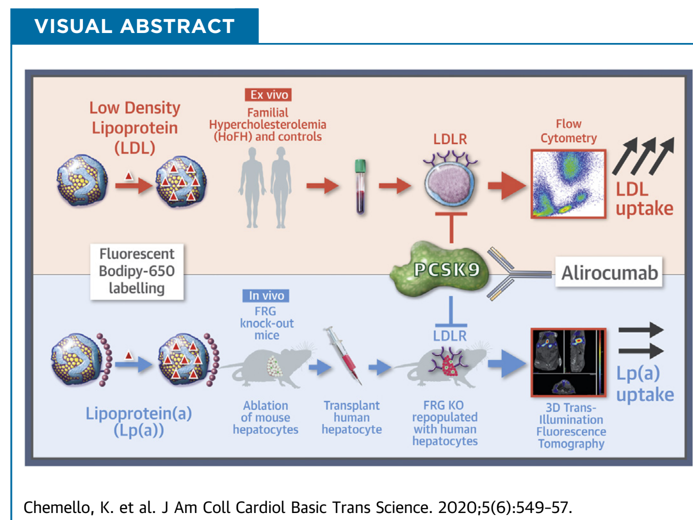
VISUAL ABSTRACT



Chemello, K. et al. J Am Coll Cardiol Basic Trans Science. 2020;5(6):549–57.

HIGHLIGHTS

- **Modulating LDL receptor expression genetically (in familial hypercholesterolemia) or pharmacologically (using statins or the PCSK9 inhibitor alirocumab) does not alter the cellular uptake of Lp(a) in primary human lymphocytes.**
- **Lp(a) hepatic capture is not modulated by PCSK9 inhibition with alirocumab in liver-humanized mice.**
- **LDLR does not appear to play a significant role in mediating Lp(a) plasma clearance in vivo.**

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The authors attest they are in compliance with human studies committees and animal welfare regulations of the authors' institutions and Food and Drug Administration guidelines, including patient consent where appropriate. For more information, visit the *JACC: Basic to Translational Science* author instructions page.

Manuscript received December 26, 2019; revised manuscript received March 11, 2020, accepted March 11, 2020.

ISSN 2452-302X https://doi.org/10.1016/j.jacbts.2020.03.008

ABBREVIATIONS AND ACRONYMS

3D = 3-dimensional

apoB100 = apolipoprotein B100

AU = arbitrary unit

bodipy = boron dipyrromethene

BSA = bovine serum albumin

ELISA = enzyme-linked immunosorbent assay

FCR = fractional catabolic rate

FRG = Fah(−/−)Rag2(−/−) Il2rg(−/−)

HoFH = homozygous familial hypercholesterolemia

LC-MS/MS = liquid chromatography tandem mass spectrometry

LDL = low-density lipoprotein

LDL-C = low-density lipoprotein cholesterol

LDLR = low-density lipoprotein receptor

Lp(a) = lipoprotein(a)

MFI = mean fluorescence intensity

PBS = phosphate-buffered saline

PBMC = peripheral blood mononuclear cell

PCSK9 = proprotein convertase subtilisin/kexin type 9

rPCSK9 = recombinant proprotein convertase subtilisin/kexin type 9

SUMMARY

Lipoprotein(a) (Lp[a]) is the most common genetically inherited risk factor for cardiovascular disease. Many aspects of Lp(a) metabolism remain unknown. We assessed the uptake of fluorescent Lp(a) in primary human lymphocytes as well as Lp(a) hepatic capture in a mouse model in which endogenous hepatocytes have been ablated and replaced with human ones. Modulation of LDLR expression with the PCSK9 inhibitor alirocumab did not alter the cellular or the hepatic uptake of Lp(a), demonstrating that the LDL receptor is not a major route for Lp(a) plasma clearance. These results have clinical implications because they underpin why statins are not efficient at reducing Lp(a). (J Am Coll Cardiol Basic Trans Science 2020;5:549-57) © 2020 The Authors. Published by Elsevier on behalf of the American College of Cardiology Foundation. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

Elevated lipoprotein(a) (Lp[a]) is the single most common genetically inherited risk factor for cardiovascular disease and calcified aortic valve stenosis (1). Elevated Lp(a) is common; approximately 25% of the general population has Lp(a) levels in the atherogenic range (i.e., above 30 to 50 mg/dl or 75 to 125 nmol/l) (2). Lp(a) is a low-density lipoprotein (LDL)-like particle secreted by the liver. Its major structural difference with LDL is that Lp(a) contains a second large protein, apolipoprotein(a) (apo[a]), bound to the apolipoprotein B100 (apoB100) moiety of a LDL particle by a single disulfide bond (1).

The liver represents the main route for Lp(a) clearance from the circulation, and various receptors have been proposed to mediate Lp(a) cellular uptake (3). Given the structural similarity between LDL and Lp(a), the LDL receptor (LDLR) has received the most attention as a candidate receptor for Lp(a). However, statins, which increase LDLR expression and reduce LDL, do not lower the circulating levels of Lp(a) in humans (4). On these premises, it had not been anticipated that proprotein convertase subtilisin/kexin type 9 (PCSK9) inhibitors, which increase the cell surface expression of LDLR via an inhibition of LDLR intracellular degradation, would not only lower LDL but also reduce Lp(a) plasma levels (5).

This observation has led to a flurry of research aimed at investigating the roles of PCSK9 and LDLR in Lp(a) plasma clearance. Thus, in HepG2 cells and primary human fibroblasts, PCSK9 was shown to reduce the binding and cellular uptake of Lp(a) via LDLR (6). LDLR inhibition with PCSK9 or LDLR blockade using antibodies targeting the extracellular domain of the receptor reduced Lp(a) binding to HepG2 cells (7). These results were confirmed in HuH7 hepatoma cells and primary murine hepatocytes (8). In contrast, we and others have reported no significant role of LDLR in mediating Lp(a) cellular uptake in primary human hepatocytes or in fibroblasts and HepG2 cells (9,10).

The incorporation of stable isotopes in apo(a) allows the determination of Lp(a) kinetic parameters in vivo, but studies conducted in humans also yielded opposite conclusions regarding the role of LDLR and the effects of PCSK9 inhibition on Lp(a) clearance. For instance, the Lp(a) fractional catabolic rate (FCR) was similar in control individuals and homozygous familial hypercholesterolemia (HoFH) patients who lack LDLR function (11). In contrast, the PCSK9 inhibitor alirocumab was shown to increase (albeit not significantly) the FCR of Lp(a) in 1 study (12), whereas the PCSK9 inhibitor evolocumab in monotherapy did not alter Lp(a) FCR. However, combined with a statin, evolocumab did increase Lp(a) FCR in that study (13). We have recently reported that alirocumab does not significantly modulate Lp(a) FCR in nonhuman primates (14). Therefore, the role of the LDLR in mediating Lp(a) plasma clearance remains a matter of considerable debate.

Lp(a) is only found in humans, old-world monkeys, and hedgehogs. None of the common animal models naturally presents the Lp(a) trait, which severely complicates functional in vivo analysis (2). Using an original mouse model repopulated with human hepatocytes (15) combined with transillumination tomography imaging techniques as well as primary human lymphocytes (16,17) and flow cytometry to track fluorescent lipoproteins, we provide new evidence that LDLR is not a significant contributor to Lp(a) clearance ex vivo and in vivo.

METHODS

LP(A) AND LDL FLUORESCENT LABELING. Plasma from an anonymous male donor with Lp(a) levels >75 nmol/l (with a mean number of 22 kringle IV

domains determined by liquid chromatography tandem mass spectrometry [LC-MS/MS]) was purchased from Bioreclamation IVT (Westbury, New York). Lp(a) was isolated by sequential ultracentrifugation ($1.050 < d < 1.125$ g/ml) at 40,000 g. Lp(a) fraction was dialyzed against phosphate-buffered saline (PBS) (137 mmol/l NaCl, 2.7 mmol/l KCl, 8 mmol/l Na_2HPO_4, and 2 mmol/l KH_2PO_4) and purified by fast performance liquid chromatography on a Lysine Sepharose 4 FF column (GE Healthcare, Velizy-Villacoublay, France). Lp(a) was subsequently dialyzed against PBS. Native purified human LDL samples were purchased from Alfa Aesar (Haverhill, Massachusetts). Lp(a) and LDL were fluorescently labeled with boron dipyrromethene (bodipy 650/665-X, Thermo Fisher Scientific, Waltham, Massachusetts), and the nonconjugated dye was removed by extensive dialysis against PBS. The absence of free label was checked by high performance liquid chromatography on Acquity UPLC Columns (200 Ang, 1.7 μm, 4.6 mm × 150 mm) from Waters (Saint Quentin, France).

PERIPHERAL BLOOD MONONUCLEAR CELL ISOLATION FROM PATIENTS AND HUMAN VOLUNTEERS. Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll Paque Plus (Sigma-Aldrich, St Louis, Missouri) as previously described (16,17) from healthy volunteers (12 men and 12 women, age 31 ± 7 years [range 22 to 58 years]; LDL cholesterol [LDL-C]: 2.6 ± 0.8 mmol/l [range 1.1 to 4.6 mmol/l], and Lp[a]: 28.2 ± 4.8 nmol/l [range 6 to 95 nmol/l]) and from 1 patient with negative HoFH (a 25-year-old woman with genetically confirmed compound heterozygote for LDLR mutations E92X and E387A treated with rosuvastatin 20 mg/d + ezetimibe 10 mg/d + lipoprotein apheresis every fortnight, LDL-C: 5.2 mmol/l, Lp(a): 30 nmol/l [on treatment before apheresis]). The project was approved by the Human Research Ethics Committee of the University of Cape Town Health Sciences Faculty. All patients provided written informed consent for genetic analysis and further research. PBMCs were subsequently frozen at −80°C in RPMI culture medium (Life Technologies, Saint Aubin, France) containing 70% fetal calf serum and 10% dimethyl sulfoxide until use.

LDLR EXPRESSION, LP(A), AND LDL UPTAKE IN HUMAN PRIMARY LYMPHOCYTES. Freshly thawed PBMCs were seeded in flat bottom 96-well plates (2.10^5 cells/well) in RPMI containing 10 mmol/l hdroxy ethyl piperazine ethane sulfonic acid (HEPES), 1 mmol/l sodium pyruvate, and 0.5% fetal calf serum for 2 h at 37°C. The culture medium was subsequently supplemented with 0 or 10 μg/ml mevastatin (Sigma-Aldrich) for 24 h. Recombinant gain of function PCSK9-D_{374}Y (0 or 600 ng/ml) (Cyclex Co., Nagano, Japan) was added to the medium for the final 4 h of the incubation time. In a subset of experiments, alirocumab (Sanofi, Chilly-Mazarin, France) was added concomitantly into the wells at a final concentration of 19.2 μg/ml (16–18).

For cell surface LDLR expression determination, lymphocytes were washed twice in ice-cold PBS containing 1% bovine serum albumin (BSA) and incubated with an allophycocyanin-conjugated antibody against the human LDLR (clone 472413) or an immunoglobulin G1 (clone 11711) isotype control (R&D Systems, Lille, France) at 0.625 μg/ml for 20 min at room temperature in the dark. Lymphocytes were then washed twice in ice-cold PBS-1% BSA and once in ice-cold PBS. Cells were analyzed on a Cytoflex flow cytometer (Beckman Coulter, Indianapolis, Indiana). Forward scatter versus side scatter gates were set to include only viable lymphocytes. A minimum of 5,000 lymphocytes was analyzed using CytExpert software (Beckman Coulter). The mean fluorescence intensity (MFI) of cells incubated with the isotype control fluorescent antibody (nonspecific binding) was subtracted from the MFI of cells incubated with a specific anti-LDLR fluorescent antibody to determine specific MFI levels (ΔMFI) of LDLR cell surface expression (16,17).

For fluorescent LDL and Lp(a) uptake assessment, LDL-bodipy650 or Lp(a)-bodipy650 was added to the medium at a 10-μg/ml final concentration for the final 3 h of incubation time. In a subset of experiments, an excess of unlabeled Lp(a) (200 μg/ml) was added 5 min before the addition of fluorescent Lp(a) (9). In another subset of experiments, Lp(a) uptake was performed in the presence of 0.2 mol/l epsilon aminocaproic acid (6). Cells were washed twice in ice-cold PBS-1% BSA, once in ice-cold PBS, and resuspended in ice-cold PBS supplemented with 0.2% trypan blue (Sigma-Aldrich) to quench cell surface-bound fluorescent LDL or Lp(a) before flow cytometry analysis, exactly as described previously. Background fluorescence was measured in lymphocytes incubated without fluorescent lipoproteins. The MFI of the cells incubated without fluorescent lipoproteins (autofluorescence) was subtracted from the MFI of cells incubated with fluorescent lipoproteins to determine the specific MFI levels (ΔMFI) of LDL and Lp(a) uptake in those cells, respectively (16,17). The ΔMFI is expressed in arbitrary units (AUs) throughout. All measurements were performed at least 3 times.

In a subset of experiments, primary lymphocytes were incubated either with 10 μg/ml of native (i.e., unlabeled) Lp(a) or fluorescent Lp(a)-bodipy as described earlier. Cells were washed intensively, and their apo(a) cellular content was measured by enzyme-linked immunosorbent assay (ELISA) using the STA-359 ELISA kit (Cell Biolabs, San Diego, California). To ascertain that the integrity of fluorescent Lp(a)-bodipy was maintained during uptake experiments, Lp(a) diluted in culture medium before and after incubation with primary PBMCs was subjected to Western blot analysis for apoB100 under reducing and nonreducing conditions using the AF3260 anti-human apoB100 antibody (Bio-Techne, Rennes, France), as described previously (9,19).

CHARACTERIZATION OF THE CHIMERIC FUMARYLACETOACETATE HYDROLASE (FaH) (-/-) RECOMBINATION ACTIVATING GENE 2 (Rag2) (-/-) INTERLEUKIN-2 RECEPTOR GAMMA (Il2rg) (-/-) (FRG)MOUSE MODEL. In vivo studies were performed in agreement with European Union directives for the standard of care and use of laboratory animals and approved by the animal care and use committee of Sanofi R&D. Chimeric liver-humanized male mice (referred to as Fah^{−/−}Rag2 [−/−]Il2rg^{−/−} FRG mice) were engineered (15,20,21) and provided by Yecuris Corporation (Portland, Oregon). These animals were housed in a pathogen-free facility under a standard 12-h light/12-h dark cycle with free access to water and fed ad libitum a PicoLab high-energy 5LJ5 chow diet (Ssniff Spezialdiäten, Soest, Germany) with 55%, 20%, and 25% of calories from carbohydrates, proteins, and fats, respectively. The chimera FRG mouse model was initially characterized by assessing the concentration of human and mouse apoB100, apo(a), and apo(a) kringle IV number using a validated multiplexed assay involving trypsin proteolysis and subsequent analysis of proteotypic peptides (Supplemental Table 1) by LC-MS/MS (14). The limit of detection of this assay is 1 nmol/l. Serum lipoproteins were resolved using a fast performance liquid chromatography Äkta system (GE Healthcare) and cholesterol measured in the eluted fractions using the Amplex Red Cholesterol Assay Kit (Life Technologies) (14). Serum samples were analyzed for direct LDL-C on a Pentra 400 biochemical analyzer (Horiba ABX, Montpellier, France) using standard colorimetric assays, for apo(a) using the STA-359 ELISA kit (Cell Biolabs) with a limit of detection of 0.1 pmol/l, and for human apoB100 using the EA7001-1 ELISA kit (Assaypro, Saint-Charles Missouri). The total PCSK9 concentrations were determined using the Quantikine SPC900 ELISA (R&D Systems).

LP(A) AND LDL HEPATIC UPTAKE IN FRG MICE. Animals were prepared for imaging studies by skin depilation of the liver area. During imaging, mice were maintained anesthetized with 2% isoflurane in oxygen, and body temperature was monitored. After baseline imaging capture, mice were injected with alirocumab or immunoglobulin G1 placebo control (Regeneron, Tarrytown, New Jersey) (200 mg/kg, subcutaneously) 18 h before infusion of the Lp(a)-bodipy650 or LDL-bodipy650 tracers (1 mg apoB per kg, intravenously). Repeated fluorescence recordings were performed 15, 30, and 45 min after fluorescent lipoproteins injections. After a washout period, mice were randomly assigned to a new group for paired injections with alirocumab or placebo 18 h before infusion with Lp(a)-bodipy650 or LDL-bodipy650 in a crossover protocol. Repeated fluorescence recordings were performed. Three-dimensional (3D) fluorescence imaging was performed using the IVIS Spectrum CT (PerkinElmer, Villebon sur Yvette, France), allowing fluorescence measurement combined with x-ray imaging (6 transillumination points in the liver area at Excitation: 640 nm, Emission: 680 nm, prone position). Living Image 4.5 software (PerkinElmer) was used to reconstruct 3D fluorescent tomographic analysis for each animal from 2-dimensional optical and X-ray data; 3D fluorescence volumetric pixels were quantified inside the region of interest (30 × 20 × 20 mm in hepatic area) and expressed in AUs throughout.

STATISTICAL ANALYSES. Statistical analyses were performed with Prism 6.01 (GraphPad, La Jolla, California). Data distribution was tested using the D'Agostino-Pearson normality test. Normally distributed variables are presented as mean ± SEM, and non-normally distributed variables are presented as median (25th to 75th percentile). Cell treatment comparisons among LDLR cell surface expression levels were assessed by analysis of variance followed by the Tukey post hoc test for multiple pairwise comparisons. Comparisons between groups of mice were performed using the Student's *t*-test for normally distributed variables (LDL-C and apoB) or the Mann-Whitney test for non-normally distributed variables (apo[a] and fluorescence volumetric pixels). Correlation analyses were performed using the Spearman rank correlation test. A value of $p < 0.05$ indicates statistical significance.

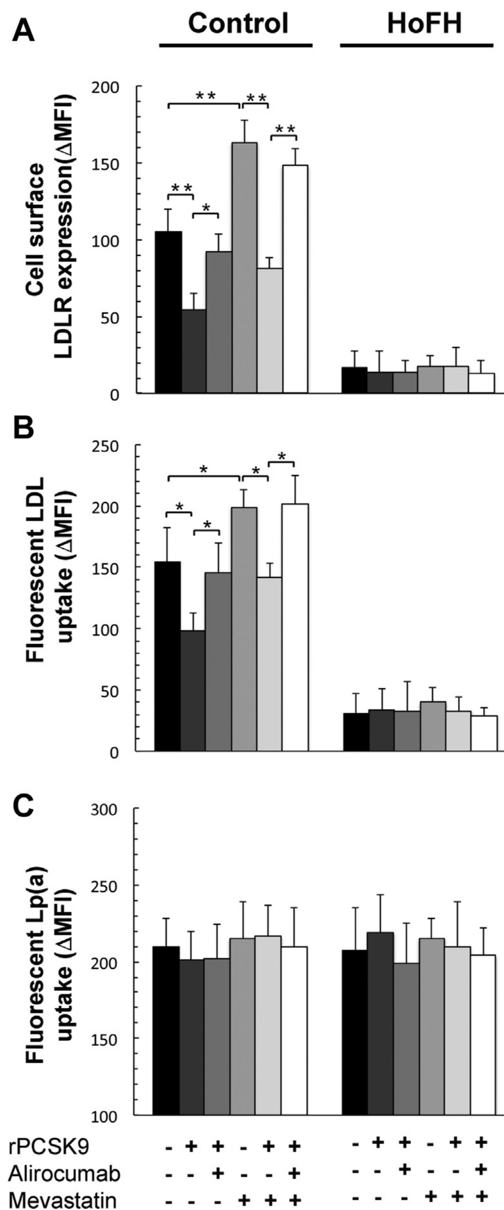
RESULTS

Primary lymphocytes isolated from a representative control volunteer and an HoFH patient were

incubated sequentially with mevastatin, recombinant human PCSK9 (rPCSK9), and the PCSK9 inhibitor alirocumab. Baseline LDLR expression assessed by flow cytometry at the surface of control lymphocytes was found at ΔMFI of 104 ± 16 AU and at ΔMFI of 19 ± 10 AU at the surface of HoFH lymphocytes. Mevastatin increased, whereas rPCSK9 reduced LDLR cell surface expression in lymphocytes from the control donor. Alirocumab restored LDLR cell surface expression in control lymphocytes treated with rPCSK9. In contrast, neither mevastatin nor rPCSK9 significantly modulated LDLR cell surface expression in HoFH lymphocytes (Figure 1A). We then assessed the cellular uptake of fluorescent LDL in these cells. Paralleling the levels of LDLR cell surface expression, LDL uptake by control lymphocytes was found at ΔMFI of 153 ± 19 AU and at ΔMFI of 30 ± 15 AU in HoFH lymphocytes. Mevastatin increased, rPCSK9 reduced, and alirocumab restored LDL uptake in control lymphocytes. In contrast, neither mevastatin nor rPCSK9 significantly altered LDL uptake in HoFH cells (Figure 1B). We next assessed the cellular uptake of fluorescent Lp(a) in lymphocytes from these individuals. In sharp contrast with LDL uptake, Lp(a) cellular uptake was similar in lymphocytes isolated from the control volunteer (ΔMFI 208 ± 20 AU) and from the HoFH patient (ΔMFI 205 ± 29 AU). Mevastatin, rPCSK9, and alirocumab treatments did not significantly alter Lp(a) cellular uptake in the control and HoFH lymphocytes (Figure 1C). We ascertained cellular Lp(a) uptake by measuring in parallel the cellular content in apo(a) after incubations with native Lp(a) or fluorescent Lp(a)-bodipy (Figure 2A). We also ascertained by Western blot under denaturing and nondenaturing conditions that fluorescently labeled Lp(a) particles remained intact during the incubation process (i.e., that apo[a] and apoB100 proteins remained covalently attached over the time course of the cellular uptake experiments) (Figure 2B). Finally, to validate the specificity of fluorescent Lp(a) cellular uptake in primary lymphocytes, we verified that Lp(a)-bodipy uptake was reduced by the addition of 20-fold excess unlabeled Lp(a) into the culture medium as well as in the presence of epsilon aminocaproic acid, a lysine analog known to reduce binding of Lp(a) to cell-surface lysines (Figure 2C). Altogether these results demonstrate that unlike LDL uptake, Lp(a) cellular uptake is not responsive to genetic or pharmacological modulations of LDLR cell surface expression in primary human lymphocytes.

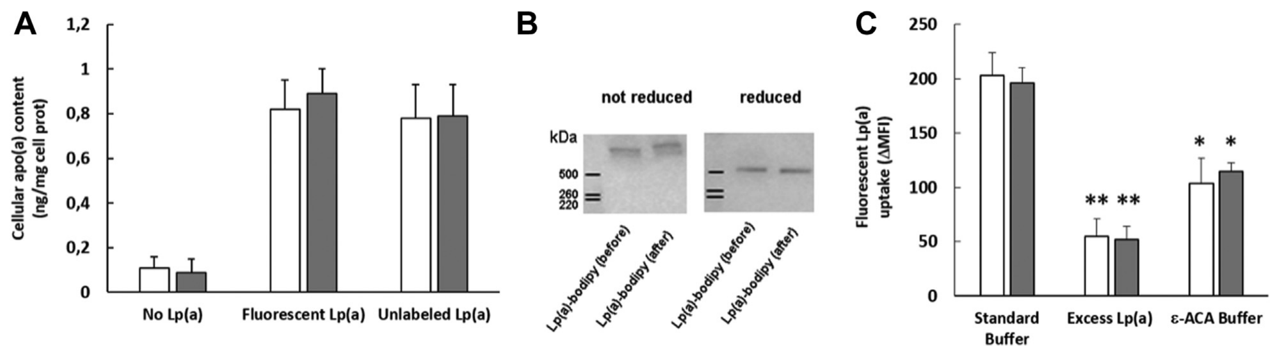
In line with these observations, we next investigated the relationship between LDLR and Lp(a) by correlating LDLR cell surface expression measured in

FIGURE 1 Lp(a) Cellular Uptake Is Not Modulated by Changes in LDLR Cell Surface Expression Ex Vivo



Peripheral blood mononuclear cells were plated for 24 h in serum-deprived medium with or without mevastatin (10 µg/ml) and supplemented or not for the last 4 h of the incubation with recombinant proprotein convertase subtilisin/kexin type 9 (rPCSK9) (600 ng/ml) with or without alirocumab (19.2 µg/ml) before flow cytometry analysis. **(A)** Cell surface low-density lipoprotein receptor (LDLR) expression, **(B)** low-density lipoprotein (LDL)–boron dipyrromethene (bodipy) uptake, and **(C)** lipoprotein(a) (Lp[a])-bodipy uptake in primary lymphocytes from a control volunteer and a homozygous familial hypercholesterolemia (HoFH) patient. Data are expressed in Δ mean fluorescence intensity. Histograms represent mean ± SEM of a minimum of 3 independent experiments performed in duplicates. Comparisons were made by analysis of variance followed by a Tukey post hoc test. *$p < 0.05$. **$p < 0.01$.

FIGURE 2 Lp(a) Cellular Uptake Is Not Modulated by Recombinant PCSK9

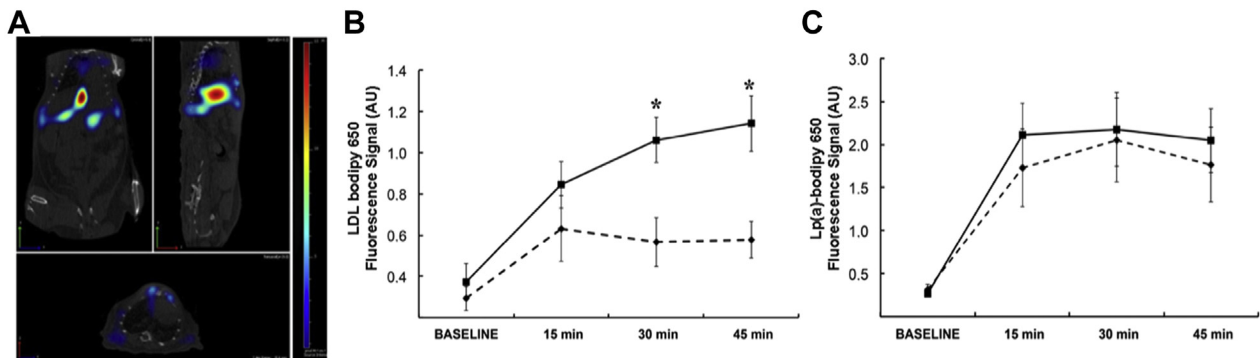


Peripheral blood mononuclear cells (PBMCs) treated with (solid bars) or without **(open bars)** recombinant proprotein convertase subtilisin/kexin type 9 (600 ng/ml) were incubated with 10 μg/ml fluorescent lipoprotein(a) (Lp[a]) or native (unlabeled) Lp(a) for 3 h. **(A)** Cellular Lp(a) uptake was determined by measuring the content of apo(a) in the cellular extracts. **(B)** Lp(a) diluted in culture medium before and after 3 h of incubation with PBMCs was subjected to Western blot analysis for apolipoprotein B100 (apoB100) under reducing and nonreducing conditions; apoB100 association with apo(a) was evidenced in nonreducing conditions. **(C)** Lp(a)-boron dipyrromethene (bodipy) uptake in control lymphocytes was assessed in the presence of a 20-fold excess of unlabeled Lp(a) or in the presence of 0.2 mmol/l epsilon aminocaproic acid. Comparisons were made by analysis of variance followed by a Tukey post hoc test. $^*p < 0.05$, $^{**}p < 0.01$ vs. standard conditions.

lymphocytes isolated from 24 control volunteers with their plasma lipoproteins concentrations. Baseline levels of LDLR expression measured at the surface of lymphocytes (i.e., without rPCSK9, mevastatin, or alirocumab) significantly and negatively correlated with the circulating levels of total cholesterol (Spearman rank correlation coefficient [rs] = −0.35, p = 0.046) and LDL-C (rs = −0.43, p = 0.019) measured in the plasma of these 24 individuals but not with their circulating levels of Lp(a) (rs = −0.26, p = 0.210), further indicating that LDLR is not a major physiological regulator of circulating Lp(a) levels in humans.

Next, we used the FRG chimeric mouse model in which mouse hepatocytes have been ablated and repopulated with human hepatocytes. We first verified that the lipoprotein profile of FRG mice is similar to that of humans because most of their plasma cholesterol is associated with LDL compared with control wild-type mice in which most of the cholesterol is in high-density lipoproteins (Supplemental Figure 1). In addition, these mice present with detectable concentrations of human apo(a)/Lp(a) with a mean number of 15.3 kringle IV domains determined by LC-MS/MS. This was ascertained by Western blot analysis (data not shown). These animals also express human apoB100 and human PCSK9 in their plasma. We determined that 72% of their total apoB100 was human and 28% murine, indicating a degree of hepatic chimerism close to 80% because a small amount of apoB100 can derive from the intestine in rodents (21). We next ascertained that FRG mice responded to alirocumab. Compared with controls, alirocumab reduced LDL-C levels (1.86 ± 0.17 mmol/l vs. 0.93 ± 0.11, respectively; p = 0.008), circulating human apoB100 (99 ± 11 vs. 64 ± 4 mg/dl, p = 0.012), and circulating apo(a)/Lp(a) (1.13 [0.96 to 1.43] vs. 0.57 [0.26 to 0.86] nmol/l; p = 0.031; n = 4-5 per group). It is noteworthy that human PCSK9 plasma levels remained unchanged in immunoglobulin G1-treated FRG mice but sharply increased in FGR mice treated with alirocumab (from 99 ± 17 to 801 ± 87 ng/ml [p < 0.001]), indicating the accumulation of alirocumab-trapped PCSK9 in the plasma of these animals. Chimeric FRG mice treated with alirocumab or immunoglobulin G1 control were subsequently intravenously infused with LDL-bodipy. Fluorescent LDL uptake was monitored by 3D trans-illumination fluorescence tomography for 45 min in the liver of these animals (**Figure 3A**). Background fluorescence in the region of interest (liver) at baseline (i.e., before LDL-bodipy infusion) was similar in FRG mice treated with alirocumab or immunoglobulin G1. The fluorescence signal in the region of interest increased significantly in the alirocumab and IgG1 treatment groups as soon as 15 min after LDL-bodipy infusion (**Figure 3B**). This increase was significantly more pronounced in FRG mice treated with alirocumab compared with FRG mice treated with immunoglobulin G1 at the 30-min time point

FIGURE 3 **Alirocumab Increases Fluorescent LDL But Not Fluorescent-Lp(a) Hepatic Uptake In Vivo**



After baseline imaging capture, Fah(−/−)Rag2 (−/−)Il2rg(−/−) (FRG) mice treated with alirocumab or immunoglobulin G1 were infused either with low-density lipoprotein (LDL)–boron dipyrromethene (bodipy) or lipoprotein(a) (Lp[a])-bodipy tracers and recordings of 3-dimensional (3D) transillumination fluorescence tomography imaging were performed 15, 30, and 45 min after tracer infusions. Fluorescence volumetric pixels were quantified in the region of interest and expressed in arbitrary units (AUs). **(A)** Representative recordings of 3D transillumination fluorescence tomography with fluorescence intensity scale bar. **(B)** Quantification of LDL-bodipy hepatic uptake in FRG mice treated with immunoglobulin G1 (**plain line**, n = 4) or alirocumab (**dotted line**, n = 6). **(C)** Quantification of Lp(a)-bodipy hepatic uptake in FRG mice treated with immunoglobulin G1 (**plain line**, n = 4) or alirocumab (**dotted lines**, n = 5). Comparisons between treatments were performed using the Mann-Whitney test. *$p < 0.05$ vs. immunoglobulin G1.

(1.06 ± 0.11 AU vs. 0.56 ± 0.12 AU; $p = 0.017$) as well as at the 45-min time point (1.14 ± 0.14 AU vs. 0.57 ± 0.09 AU; $p = 0.015$), demonstrating that alirocumab significantly enhanced fluorescent LDL uptake in the liver of these animals (Figure 3B). When FRG mice treated with alirocumab or immunoglobulin G1 were intravenously infused with Lp(a)-bodipy, the fluorescence signal in the hepatic region increased similarly 15 min after Lp(a)-bodipy infusions in both treatment groups. This increase in fluorescence was not significantly different in FRG mice treated with alirocumab compared with FGR mice treated with immunoglobulin G1 at the 30-min time point (2.18 ± 0.43 AU vs. 2.05 ± 0.49 AU, respectively; $p = 0.852$) and at the 45-min time point (2.04 ± 0.37 AU vs. 1.77 ± 0.44 AU, respectively; $p = 0.639$), demonstrating that alirocumab did not significantly modulate fluorescent Lp(a) hepatic uptake in humanized liver FRG mice (Figure 3C). It is noteworthy that fluorescence density of the Lp(a)-bodipy tracer was 2.3-fold higher than that of the LDL-bodipy tracer. Taken together, these results show that Lp(a) hepatic uptake is not responsive to pharmacological modulation of the LDLR by alirocumab in chimeric liver-humanized mice.

DISCUSSION

In this study, we showed that modulating LDLR expression genetically (in HoFH) or pharmacologically (with statins, rPCSK9, and alirocumab) does not alter the cellular uptake of Lp(a) in human lymphocytes and that LDLR expression does not correlate with circulating Lp(a). We also showed that Lp(a) hepatic uptake is not modulated by PCSK9 inhibition with alirocumab in liver-humanized mice. These combined results indicate that LDLR does not play a significant physiological role in mediating Lp(a) plasma clearance in vivo.

The cellular experiments of the present study clearly demonstrate a total absence of change in Lp(a) uptake in primary lymphocytes despite the important modulation of cell surface LDLR expression induced by statins, rPCSK9, and alirocumab treatments. In addition, the absence of functional LDLR at the surface of HoFH lymphocytes did not impact the ability of human lymphocytes to promote Lp(a) uptake. These results are in line with previous studies conducted in HoFH dermal fibroblasts, human primary hepatocytes, and various cell lines (9,10). Thus, irrespective of the cellular model tested (i.e., dermal fibroblasts, hepatocytes, and now primary lymphocytes), Lp(a) cellular uptake is not impacted by PCSK9 inhibitors, mevastatin treatment, or the combination of both drugs. However, the present results remain at odds with studies conducted in mouse primary hepatocytes, human skin fibroblasts, and cell lines by others (6,8). There is certainly an inherent limitation of using primary lymphocytes as a proxy for hepatocytes, but this cell type is easily accessible, and the LDLR pathway in

lymphocytes and hepatocytes is similar in that it requires the same endocytic machinery, in particular the LDLR adaptor protein 1 (17). Further advocating against a significant role for LDLR in mediating Lp(a) clearance is the absence of significant correlation between the levels of LDLR measured at the surface of lymphocytes and the levels of circulating Lp(a), an observation that we have also made in FH patients (16). The absence of modulation of Lp(a) cellular uptake observed here underlines that circulating Lp(a) levels are primarily regulated at the production rather than at the catabolism level (1).

The experiments of the present study conducted in chimeric FRG mice also indicate an absence of a significant role for LDLR in mediating Lp(a) hepatic uptake. These in vivo results are inambiguous in that they provide a direct visualization of fluorescent tracer accumulation in the livers of humanized mice, unlike stable isotope studies, which despite their merits rely on mathematical modeling and thus indirectly assess Lp(a) kinetic parameters (11–14). Our results provide a demonstration of an absence of an effect of PCSK9 inhibition with alirocumab on physiological Lp(a) uptake in human hepatocytes. Indeed, these cells are engrafted in a liver environment and not coated onto plastic with a collagen matrix, a material that has been proposed to nonspecifically bind human apo(a) (8). However, our in vivo study has the following limitations: 1) we have only tested FRG mice repopulated with human hepatocytes from a single donor; 2) these animals present with detectable but low plasma levels of Lp(a); 3) Lp(a) accumulation beyond the hepatic region was not assessed; and 4) the rate of chimerism of these mice is not 100%. In line with these observations, a recent study showed that chimeric FRG mice repopulated with human hepatocytes from 2 different donors also display low Lp(a) plasma levels, albeit on average twice higher than those measured in the present study (21). Despite their high cost, FRG mice are a powerful tool to assess human lipoprotein metabolism because they display a typical human lipoprotein profile with LDL as the predominant lipoprotein even on a normal chow diet (20). This has been recently ascertained by others on a nonobese diabetic background (21). In that respect, it would be extremely informative to perform similar in vivo studies in double transgenic mice that coexpress human apoB100 and apo(a) (22).

Taken together our ex vivo and in vivo results clearly indicate that modulation of LDLR expression with alirocumab does not alter the cellular nor the hepatic uptake of Lp(a). However, the exact mechanisms by which PCSK9 inhibitors reduce Lp(a) remain to be elucidated. In that respect, chimeric FRG mice repopulated with human hepatocytes from donors with elevated Lp(a) should prove instrumental to investigate whether PCSK9 and its inhibitors modulate apo(a)/Lp(a) production.

ACKNOWLEDGMENTS The authors thank Athanase Bayard, Fabrice Tirode, and Brice Nativel for excellent technical assistance.

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PERSPECTIVES

COMPETENCY IN MEDICAL KNOWLEDGE: Statins reduce LDL-C levels by increasing the expression of the LDL receptor. Monoclonal antibodies targeting PCSK9, a novel class of lipid-lowering drugs, also reduce LDL-C by decreasing the degradation of the LDL receptor. However, unlike statins, PCSK9 inhibitors also reduce the circulating levels of another class of atherogenic lipoproteins (i.e., Lp[a]). We now report that the LDL receptor is not significantly involved in Lp(a) plasma clearance ex vivo and in vivo. These results explain why, unlike statins, PCSK9 inhibitors reduce Lp(a) plasma levels in dyslipidemic patients.

TRANSLATIONAL OUTLOOK: Lp(a) is an LDL-like particle containing a peculiar signature protein, apo(a). Our study suggests that Lp(a) is not primarily regulated by its catabolism but rather by the production of apo(a) in the liver. This underpins the promising results obtained with novel therapies targeting apo(a) gene expression with antisense oligonucleotides or RNA interference currently under development.

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KEY WORDS lipoprotein(a), liver-humanized mice, low-density lipoprotein receptor, proprotein convertase subtilisin/kexin type 9

APPENDIX For a supplemental table and figure, please see the online version of this paper.