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# Chylomicron- and VLDL-derived Lipids Enter the Heart through Different Pathways

## *IN VIVO EVIDENCE FOR RECEPTOR- AND NON-RECEPTOR-MEDIATED FATTY ACID UPTAKE**

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**Lipids circulate in the blood in association with plasma lipoproteins and enter the tissues either after hydrolysis or as non-hydrolyzable lipid esters. We studied cardiac lipids, lipoprotein lipid uptake, and gene expression in heart-specific lipoprotein lipase (LpL) knock-out (hLpL0), CD36 knock-out (*Cd36*$^{-/-}$), and double knock-out (hLpL0/*Cd36*$^{-/-}$-DKO) mice. Loss of either LpL or CD36 led to a significant reduction in heart total fatty acyl-CoA (control, 99.5 ± 3.8; hLpL0, 36.2 ± 3.5; *Cd36*$^{-/-}$, 57.7 ± 5.5 nmol/g, $p < 0.05$) and an additive effect was observed in the DKO (20.2 ± 1.4 nmol/g, $p < 0.05$). Myocardial VLDL-triglyceride (TG) uptake was reduced in the hLpL0 (31 ± 6%) and *Cd36*$^{-/-}$ (47 ± 4%) mice with an additive reduction in the DKO (64 ± 5%) compared with control. However, LpL but not CD36 deficiency decreased VLDL-cholesteryl ester uptake. Endogenously labeled mouse chylomicrons were produced by tamoxifen treatment of *β-actin-MerCreMer*/*LpL*$^{flox/flox}$ mice. Induced loss of LpL increased TG levels >10-fold and reduced HDL by >50%. After injection of these labeled chylomicrons in the different mice, chylomicron TG uptake was reduced by ~70% and retinyl ester by ~50% in hLpL0 hearts. Loss of CD36 did not alter either chylomicron TG or retinyl ester uptake. LpL loss did not affect uptake of remnant lipoproteins from ApoE knock-out mice. Our data are consistent with two pathways for fatty acid uptake; a CD36 process for VLDL-derived fatty acid and a non-CD36 process for chylomicron-derived fatty acid uptake. In addition, our data show that lipolysis is involved in uptake of core lipids from TG-rich lipoproteins.**

Under normal physiological conditions, myocardial energy demands are predominantly met by fatty acid (FA)[3] oxidation (>70%) with the remaining energy provided by glucose, lactate, and ketones. The majority of the lipid entering cardiac cells is diverted toward FA utilization (1) with some being stored or used for structural requirements. FAs are delivered to the heart from two sources: (*a*) FAs esterified as triglyceride (TG) contained in circulating lipoproteins and liberated by lipoprotein lipase (LpL)-mediated lipolysis, and (*b*) non-esterified FA, referred to as free FAs (FFAs), bound to serum albumin. The mechanisms responsible for the uptake of FAs by the heart, or any organ, are incompletely understood. Specifically, the importance of receptor-mediated uptake *versus* diffusion of FAs across membranes is under debate. Studies in cardiomyocytes (2–4) and other cells (5, 6) have suggested that uptake of FAs by cultured cells can occur via two pathways (7, 8). A low capacity but high affinity uptake pathway thought to represent receptor-mediated uptake is operative at FFA/albumin ratios normally found in the plasma (5). At higher FFA concentrations, uptake occurs via a lower affinity non-saturable process (9, 10); this has been studied in synthetic membranes and thought to represent non-receptor uptake also referred to as "flip-flop" (11–13).

Several proteins including fatty acid translocase (FAT/CD36), plasma membrane fatty acid-binding protein, and fatty acid transport protein (14) are postulated to mediate FFA transport across cell membranes (15). Until 2 decades ago CD36 was best known as the thrombospondin receptor (16) but since then has been shown to bind several ligands including modified lipoproteins (17), collagen (18), and plasmodium parasite-infected erythrocytes (19, 20). Studies in adipocytes identified a cell surface protein capable of transporting FFAs into cells that was subsequently shown to be identical to CD36 (21, 22). Reduced uptake of FFAs into heart, skeletal muscle, and adipose tissue of mice with a genetic deletion of this multiligand receptor (23) strongly supports a role for CD36 as a FFA transporter (for reviews, see Refs. 24–28). CD36 knock-out (*Cd36*$^{-/-}$) mice also exhibit increased serum FFA levels (29).

LpL-mediated lipolysis of TG-rich chylomicrons (CMs) and VLDL produces non-esterified FAs that can enter the cells by processes similar to the albumin-associated FFAs, thereby competing with each other when studied in isolated perfused hearts (30). The importance of LpL in lipoprotein metabolism and lipid delivery to tissues is well established. Total LpL dele-

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[3] The abbreviations used are: FA, fatty acid; FFA, free fatty acid; TG, triglyceride; CM, chylomicron; RE, retinyl ester; CE, cholesteryl ester; ApoE, apolipoprotein E; LpL, lipoprotein lipase; DKO, double knock-out; TC, total cholesterol.

tion in mice results in severe hypertriglyceridemia and neonatal death (31), probably due to hypoglycemia (32). We have previously shown that LpL-mediated TG hydrolysis is a major source of FFAs for the heart *in vivo* (33). Heart-specific LpL knock-out (hLpL0) mice develop cardiac dysfunction with age (30) and are unable to tolerate stress from acute pressure overload (30) and chronic hypertension (34) despite the compensatory increase in glucose metabolism.

In addition to FFAs, the heart requires other lipids such as cholesterol and retinoids for normal function. *De novo* cholesterol biosynthesis in the heart is minimal compared with the liver and adrenal gland (35). Moreover, the heart obtains very little cholesterol from circulating LDL (36). Fielding (37) showed that CMs deliver cholesterol into isolated perfused hearts; the cholesterol uptake was not via LDL receptors. Therefore, in the isolated heart, cholesterol can be delivered in concert with metabolism of TG-rich lipoproteins. A similar process might allow for uptake of other core lipids including retinyl ester (RE) from TG-rich lipoproteins.

By studying lipid uptake into hearts that have deficiencies of LpL, CD36, and both we have obtained *in vivo* data supporting a role for both receptor- and non-receptor-mediated transport of FFAs to the heart. In addition, we provide *in vivo* data illustrating a route for heart acquisition of both cholesterol and retinoid.

## EXPERIMENTAL PROCEDURES

*Animals*—All procedures involving animals were approved by the Institutional Animal Care and Use Committee at Columbia University. hLpL0 mice (38) were backcrossed 10 times into the C57Bl/6J background. $Cd36^{-/-}$ mice (23) were crossed with hLpL0 mice to obtain the different genotypes. Mice were maintained under appropriate barrier conditions and received food and water *ad libitum* unless otherwise stated. All animals were maintained on standard laboratory chow diet (LabDiet). For all gene expression and heart lipid measurements, the different groups of mice were fasted for 4 h, following which the hearts were harvested, flash frozen in liquid nitrogen, and stored at −80 °C until further use.

*RNA Isolation and Gene Expression Analysis*—Total RNA was extracted from the heart using the phenol guanidine isothiocyanate method (TRIzol kit, Invitrogen) as per the manufacturer's instructions. Total RNA (0.4 μg) was reverse transcribed for 1 h at 50 °C with the THERMOSCRIPT RT-PCR system (Invitrogen). The reverse transcribed cDNA was then amplified using an iCycler (Bio-Rad) with the Brilliant SYBR Green QPCR master mix (Stratagene). Relative quantification of gene expression was performed using the standard curve method with *18S* rRNA as the housekeeping gene. Real time PCR primer sequences are provided under supplemental Table S1.

*Plasma and Cardiac Lipid Measurements*—Blood was collected from mice fasted for 4–5 h for measurement of plasma TG, total cholesterol (TC), FFA, and glucose. TG and TC were measured enzymatically using an Infinity kit (ThermoFisher Scientific Inc.) and FFA was measured by a NEFA kit (Wako Pure Chemical Industries). Plasma glucose was measured by Autokit Glucose (Wako Chemicals). Heart fatty acyl-CoA measurements were performed by the LC-MS/MS technique described in detail by Neschen *et al.* (39).

*Heart LpL Enzyme Activity*—LpL enzyme assay was performed as previously described (40) using hearts obtained from mice fasted for 4–5 h.

*Preparation of Double-labeled VLDL Particles and in Vivo Clearance*—Double-labeled human VLDL particles were obtained by a method previously described for LDL (41). Briefly, VLDL was isolated from healthy human plasma by ultracentrifugation at $d = 1.006$ g/ml for 22 h at 39,000 rpm. Isolated VLDL was added to a glass tube evenly coated with 40 μCi of [$^{14}$C]triolein ([$^{14}$C]TG) or 400 μCi of [$^{3}$H]cholesteryl oleate ([$^{3}$H]CE) (PerkinElmer Life Sciences). For every 2.2 ml of VLDL, 1 ml of the 1.21 bottom fraction (obtained from sequential ultracentrifugation of the human plasma at $d = 1.21$ g/ml) as a source of cholesteryl ester transfer protein was added to the VLDL-radioisotope mixture and incubated overnight with shaking at 37 °C. This was followed by re-isolation of the now radiolabeled VLDL by ultracentrifugation. The VLDL preparation was extensively dialyzed against buffer containing 150 mM sodium chloride and 0.24 mM EDTA, and filtered through 0.45-μm syringe filters prior to use for turnover experiments. The TG/TC ratio of the VLDL preparation was 6.3 ± 0.5. The 4 groups of mice were injected with a mixture of the labeled VLDL such that each mouse received $5 \times 10^5$ dpm of [$^{14}$C]TG-VLDL and $5 \times 10^6$ dpm of [$^{3}$H]CE-VLDL (0.33 mg of TG and 0.06 mg of TC per mouse) via the tail vein. Blood was collected 0.5, 5, 10, and 30 min after injection. At 30 min, mice were perfused with cold PBS, hearts were harvested, flash frozen in liquid nitrogen, and stored at −80 °C until further use. Radioactivity was determined in 10 μl of plasma and 100 μl of heart homogenate on a LS 6500 multipurpose scintillation counter (Beckman Coulter). VLDL kinetic studies were performed in two different sets of mice each with 4–5 animals per group.

*Preparation of Double-labeled CMs and in Vivo Clearance*—β-Actin-driven tamoxifen-inducible-Cre (Mer/Cre/Mer) transgenic mice on a C57Bl/6J background obtained from Jackson Laboratory were crossed with floxed LpL mice (38) to obtain the *β-actin-MerCreMer/LpL$^{flox/flox}$* offspring designated as $LpL^{-/-}$ mice. After confirming genotype by tail DNA, the $LpL^{-/-}$ mice were given an intraperitoneal injection of 1 mg of 4-hydroxytamoxifen (Sigma) in peanut oil for 5 consecutive days. Plasma TG was determined 2 weeks after the last tamoxifen injection. $LpL^{-/-}$ mice with tamoxifen-induced LpL deletion exhibited a significant increase in plasma TG levels (Fig. 4*A*). The $LpL^{-/-}$ mice with the elevated plasma TG levels were used to prepare endogenously radiolabeled CMs. 32 μCi of [$^{14}$C]TG and 250 μCi of [$^{3}$H]retinol (PerkinElmer Life Sciences) along with 84 μg of unlabeled all-*trans*-retinol (Sigma) were dried under a nitrogen stream and then dissolved in 1.4 ml of peanut oil. 0.1 ml of the mixture per $LpL^{-/-}$ mouse previously fasted for 4 h was given by gavage. 4–5 h after gavage, CMs were obtained by ultracentrifugation of plasma ($d = 1.006$ g/ml) at 39,000 rpm for 45 min. The TG/TC ratio of the now radiolabeled CM preparation was 17 ± 2. Endogenously radiolabeled CMs were then injected into the 4 groups of mice via the tail vein at time 0, such that each mouse received $2 \times 10^5$ dpm of [$^{14}$C]TG-CMs and $6 \times 10^5$ dpm of [$^{3}$H]retinol-labeled CMs

(2.4 mg of TG and 0.12 mg of TC per mouse). Blood was collected at 0.5, 5, and 15 min after injection. At 15 min, mice were perfused with cold PBS, hearts were harvested, flash frozen in liquid nitrogen, and stored at −80 °C until further use. Radioactivity was determined in 10 μl of plasma and 100 μl of heart homogenate on a LS 6500 multipurpose scintillation counter (Beckman Coulter). CM kinetic studies were performed in two different sets of mice each with 4–5 animals per group.

*Preparation of $^{125}$I-Tyramine Cellobiose-conjugated VLDL Particles and in Vivo Clearance*—Human VLDL was isolated from plasma as described above. $^{125}$I-labeled tyramine cellobiose ($^{125}$I-TC) was prepared by using 50 μg of IODO-GEN (1,3,4,6-tetrachloro-3α,6α-diphenyl-glycouril) (Thermo Scientific), 0.2 μmol of TC (obtained from Dr. Franz Rinninger, University of Hamburg), and 3 mCi of $^{125}$I (PerkinElmer), incubated for 1 h followed by activation with 18 μg of cyanuric chloride (Sigma). The $^{125}$I-TC was then covalently linked to human VLDL (1 mg of protein), eluted on a desalting column (Bio-Rad), and extensively dialyzed. $^{125}$I-TC-conjugated VLDL was incubated with unlabeled HDL (15 mg of protein) obtained by sequential ultracentrifugation of human plasma at $d = 1.21$ g/ml to exchange the non-ApoB-conjugated proteins on VLDL. $^{125}$I-TC-conjugated VLDL was re-isolated by ultracentrifugation and stored at 4 °C until further use. [$^3$H]CE-labeled VLDL was prepared as described above using the cholesteryl ester transfer protein method. The two VLDL preparations were mixed and intravenously injected into the different groups of mice such that each mouse received a final dose of $4 \times 10^5$ cpm of $^{125}$I-TC-conjugated VLDL and $3.5 \times 10^5$ cpm of [$^3$H]CE-VLDL at time 0. Blood was collected at 0.5, 5, 10, and 30 min after injection. At 30 min, mice were perfused, hearts were harvested, flash frozen in liquid nitrogen, and stored at −80 °C until further use. Radioactivity was determined in 10 μl of plasma and 100 μl of heart homogenate on a LS 6500 multipurpose scintillation counter (Beckman Coulter) for the $^3$H counts. $^{125}$I counts were measured in a γ-counter (WALLAC 1470 Wizard).

*Preparation and in Vivo Clearance of [$^3$H]CE-labeled Remnant Particles*—6–8 *ApoE*$^{-/-}$ mice were fasted for 4 h and each gavaged with $8.7 \times 10^6$ cpm of [$^3$H] retinol and 6 micrograms of unlabeled retinol in corn oil. Plasma isolated 4 h after gavage was ultracentrifuged at $d = 1.006$ g/ml for 22 h at 39,000 rpm to isolate the radiolabeled remnant fraction. The TG/TC ratio of the remnant preparation was 0.29 ± 0.04. Mice were injected with this remnant tracer such that each mouse received $5 \times 10^4$ cpm of [$^3$H]RE remnants (0.2 mg of TG and 0.56 mg of TC) via the tail vein. Blood was collected at 0.5, 5, and 20 min after injection. At 20 min, mice were perfused with cold PBS, hearts were harvested, flash frozen in liquid nitrogen, and stored at −80 °C. Radioactivity was determined in 10 μl of plasma and 100 μl of heart homogenate.

For all turnover studies, radioactivity per gram of tissue was normalized to the respective 30-s plasma counts (injected dose). The initial slope of each decay curve was determined and used to calculate fractional decay in pools per min.

*Statistical Analyses*—All data are expressed as mean ± S.E. Data were analyzed using SigmaStat and analysis of variance was used to analyze between group differences, followed by Holm-Sidak for multiple comparisons when appropriate. Data were considered statistically significant at $p < 0.05$.

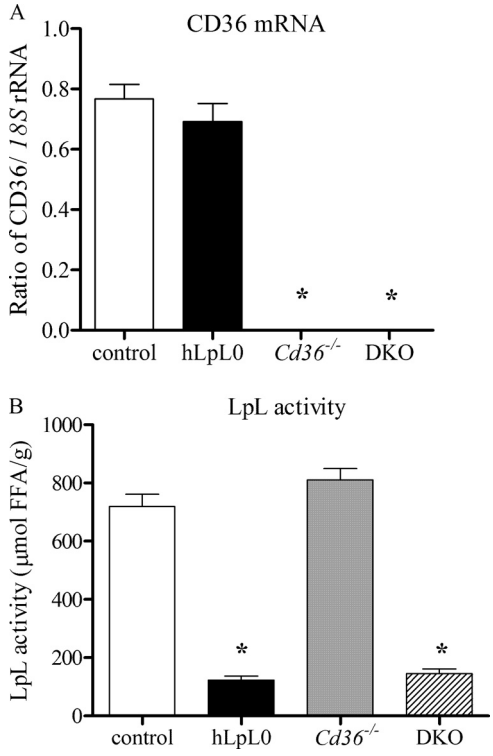


FIGURE 1. **Characterization of LpL/CD36 double knock-out mice.** The LpL/CD36 DKO on C57Bl/6J background were generated by a breeding scheme as described under "Experimental Procedures." 14-Week-old male mice were fasted for 4–5 h before harvesting tissues for all measurements. *A*, cardiac CD36 mRNA expression normalized to *18S* rRNA expression measured by real time RT-PCR. *B*, cardiac LpL enzyme activity per g wet weight of heart. $n = 4–6$ per group; *, significantly different from control, $p < 0.05$.

## RESULTS

*Characterization of LpL/CD36 Double Knock-out Mice (DKO)*—The *Cd36*$^{-/-}$ mice were crossed with the hLpL0 group, both on the C57Bl/6 background, to obtain four different genotypes: homofloxed controls, hLpL0, *Cd36*$^{-/-}$, and DKO. In addition to tail genotyping, we measured mRNA levels for CD36 in the hearts of these mice. CD36 deletion was observed in the *Cd36*$^{-/-}$ and DKO groups (Fig. 1*A*). LpL enzyme activity was reduced 6-fold in the hLpL0 and DKO hearts compared with the control but was unchanged in the *Cd36*$^{-/-}$ hearts (Fig. 1*B*).

*Plasma and Heart Lipid*—Body weight was unchanged among the different groups studied at 14 weeks of age. Heart weight normalized to body weight was significantly increased in only the *Cd36*$^{-/-}$ mice compared with control by Student's *t* test ($p < 0.05$) (Table 1). Plasma TG levels in the hLpL0 mice were significantly higher than control ($p = 0.01$) (Table 1). DKO showed an ~2.5-fold increase in plasma TG compared with control and *Cd36*$^{-/-}$ ($p < 0.05$, Table 1). Plasma TC and FFA were significantly elevated in both the *Cd36*$^{-/-}$ and DKO groups compared with control (Table 1). Increased cholesterol and non-esterified fatty acids have been previously reported in *Cd36*$^{-/-}$ mice (29).

Total fatty acyl-CoA content in the hearts measured by mass spectroscopy was reduced to 36 ± 9 and 58 ± 5% of control in

**TABLE 1**
**Heart weight and fasting plasma measurements**

| | Control | hLpL0 | *Cd36*$^{-/-}$ | DKO |
|---|---|---|---|---|
| BW[a] (g) | 24.8 ± 0.7 | 26.7 ± 0.9 | 26.0 ± 0.3 | 25.0 ± 0.5 |
| HW/BW ratio | 4.6 ± 0.1 | 4.5 ± 0.2 | 5.1 ± 0.2[b] | 4.7 ± 0.1 |
| TG (mg/dl) | 37.2 ± 6.0 | 86.8 ± 16.8 | 33.6 ± 4.5 | 91.4 ± 16.2[b,c] |
| TC (mg/dl) | 68.9 ± 4.0 | 66.9 ± 2.9 | 90.9 ± 2.5[b,d] | 90.4 ± 4.8[b,d] |
| FFA (mEq/liter) | 0.4 ± 0.0 | 0.3 ± 0.0 | 0.5 ± 0.1[b,d] | 0.6 ± 0.1[b,d] |
| Glucose (mg/dl) | 153.7 ± 11.1 | 176.0 ± 3.7 | 166.0 ± 15.8 | 143.9 ± 10.1 |

[a] BW, body weight; HW, heart weight; TG, triglyceride; TC, total cholesterol; FFA, free fatty acids. $n = 4–6$ per group; $p < 0.05$.
[b] Different from control.
[c] Different from *Cd36*$^{-/-}$.
[d] Different from hLpL0.

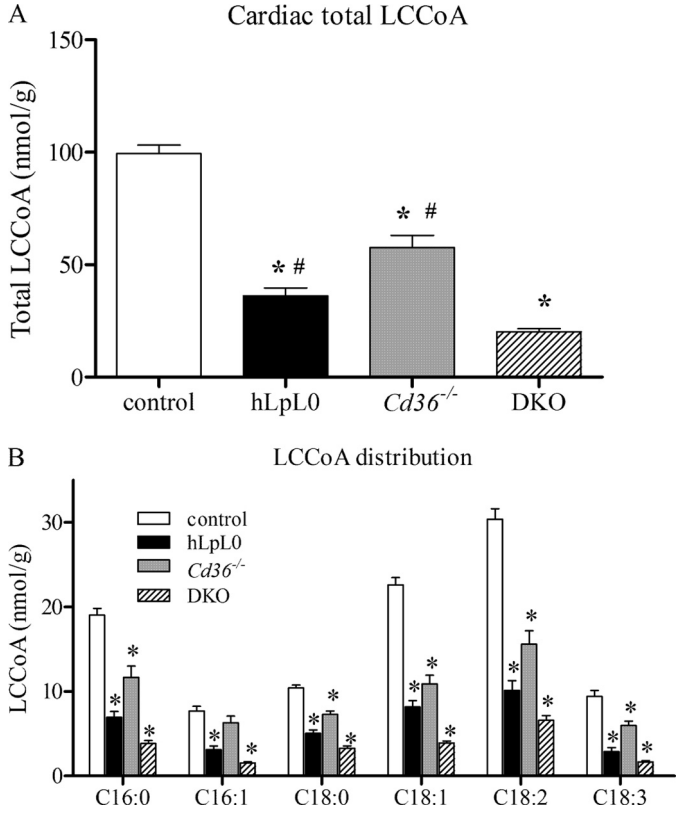


FIGURE 2. **Cardiac total long chain fatty acyl-CoA (*LCCoA*) content.** 12–13-Week-old male mice were fasted for 4–5 h before harvesting the hearts for measurement of cardiac fatty acyl-CoA content by the LC-MS/MS technique described under "Experimental Procedures." *A*, cardiac total LCCoA content. *B*, distribution of LCCoA species in the heart. $n = 5–8$ per group; *, significantly different from control; #, significantly different from DKO, $p < 0.05$.

the hLpL0 and *Cd36*$^{-/-}$ groups, respectively; the DKO group had an additive effect and was reduced to 20 ± 1% of control ($p < 0.05$, Fig. 2*A*). The reduction in fatty acyl-CoAs was seen in all fatty acyl-CoA species; long and medium chain, saturated and unsaturated, and omega 3 polyunsaturated fatty acyl-CoAs (Fig. 2*B*). Therefore loss of CD36 and LpL did not preferentially affect a single class of FAs.

We previously reported the effects of LpL deficiency on cardiac gene expression (30) and others reported the effects of loss of CD36 on cardiac gene expression (42, 43). The DKO mice had a reduction in acyl-CoA oxidase (AOX/ACO) to 69 ± 3% of control. Although this was lower than in the single knock-outs (78 ± 8 and 83 ± 15% of control), this further decrease was not statistically significant. Pyruvate dehydrogenase kinase 4 (PDK4) was the most robustly reduced gene and was 9 ± 1, 5 ± 0.6, and 4 ± 0.7% of control in hLpL0, *Cd36*$^{-/-}$, and DKO, respectively.

*Cardiac VLDL-TG Uptake*—Plasma clearance for VLDL-TG was not different between the genotypes (Fig. 3*A*). VLDL-TG uptake into the heart was similarly reduced in the hLpL0 (31 ± 6%) and *Cd36*$^{-/-}$ (47 ± 4%) mice compared with controls ($p < 0.05$, Fig. 3*B*). VLDL-TG uptake in the DKO hearts was further reduced by 64 ± 5%, which was different from control and hLpL0 mice ($p < 0.05$, Fig. 3*B*). The reduction compared with *Cd36*$^{-/-}$ hearts was not significant. Some residual uptake in the hLpL0 group might be attributed to peripheral or lung lipolysis via LpL and subsequent uptake via CD36. Isolated perfused heart studies looking at VLDL-TG uptake have also observed ~50% residual uptake with pharmacologic LpL inhibition (44); this suggests that there are LpL and non-LpL-mediated pathways for uptake of VLDL-TG.

*Cardiac VLDL-CE Uptake*—Heart is not an active site for cholesterol biosynthesis or LDL uptake (35, 36), therefore it requires an alternative cholesterol acquisition pathway(s). Cholesteryl esters within lipoproteins other than LDL could serve as a source of this lipid. No differences in plasma clearance for VLDL-CE were observed in the groups (Fig. 3*C*). Uptake into the heart is only a small percentage of injected dose as compared with other tissues such as liver (radioactivity/g of tissue normalized to the injected dose in liver is ~10-fold higher than that in the heart). Therefore, the minor contribution of the heart knock-out would not be expected to lead to changes in plasma decay curves for the two labels.

But VLDL-CE uptake into the heart was reduced in hLpL0 mice (81 ± 2%) compared with control (Fig. 3*D*) and was unchanged in the *Cd36*$^{-/-}$ mice. The DKO group mirrored the hLpL0 in exhibiting a 75 ± 4% reduction in VLDL-CE uptake into the heart ($p < 0.05$, Fig. 3*D*). Thus, one source of heart CE is via the LpL-mediated metabolism of VLDL. Hence, the additional decrease in VLDL-TG uptake seen in the DKO hearts might reflect a reduction in the particle uptake component as well. Nonetheless, there is a preference for LpL-mediated TG lipolysis rather than whole particle uptake as is evident from the cardiac uptake in Fig. 3, *B* and *D*. In addition, these data prove that CD36 does not function as an uptake receptor for VLDL or VLDL remnants.

*Generation and Characterization of the Inducible LpL Knock-out Mouse Model*—Floxed LpL mice previously created in the laboratory (38) were crossed with *β-actin-MerCreMer* transgenic mice to obtain *LpL*$^{-/-}$ offspring on a C57Bl/6J background. TG measured 2 weeks after tamoxifen injection to activate the Cre demonstrated a 4–20-fold increase in fasting plasma TG levels as indicated by the milky plasma (Fig. 4*A*). Due to the variability observed in plasma TG in response to tamoxifen, for subsequent studies we used mice with TG > 400 mg/dl (66% of the mice) to create chylomicrons (Fig. 4*B*). In addition, LpL deletion was associated with 64 ± 3% decrease in plasma HDL cholesterol levels compared with controls (control, 84 ± 8; *LpL*$^{-/-}$, 31 ± 4 mg/dl) ($p < 0.001$). The increase in plasma TG was observed in the VLDL fraction with no changes in the LDL-TG and HDL-TG fractions (Fig. 4*C*). Thus, despite the lack of cholesteryl ester transfer

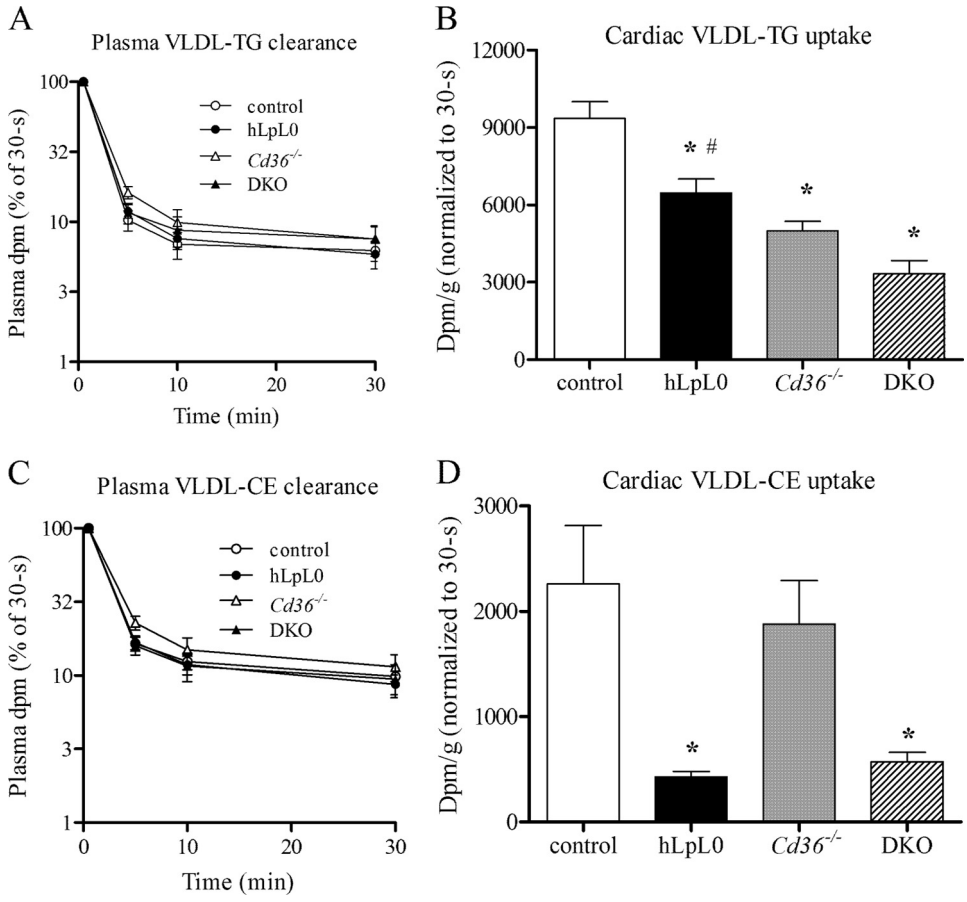


FIGURE 3. **Cardiac VLDL-TG and VLDL-CE uptake.** 13–14-Week-old male mice fasted for 4–5 h were administered double-labeled human VLDL particles by tail vein. *A*, plasma decay curves for VLDL-TG. *B*, cardiac VLDL-TG uptake 30 min after tracer injection. *C*, plasma decay curves for VLDL-CE. *D*, cardiac VLDL-CE uptake 30 min after tracer injection expressed. $n = 4–5$ per group; *, significantly different from control; #, significantly different from DKO, $p < 0.05$.

protein in the mouse, LpL deletion resulted in a decrease in LDL and HDL cholesterol (Fig. 4*D*).

*CM-derived TG Uptake into the Heart*—The plasma CM-TG clearance rate was reduced in hLpL0 and DKO groups, whereas clearance in control and *Cd36*$^{-/-}$ mice was identical (average slopes: control, 0.14; hLpL0, 0.06; *Cd36*$^{-/-}$, 0.12; DKO, 0.09 pools/min, $p < 0.05$) (Fig. 5*A*). The residual counts in the plasma after the initial 5 min are likely due to circulating remnants whose decay is relatively slower. hLpL0 and DKO mice showed a 70–80% reduction in CM-derived TG uptake into the heart compared with the control group ($p < 0.05$, Fig. 5*B*). Despite the observed reduction in VLDL-TG uptake, *Cd36*$^{-/-}$ hearts had no defect in CM-TG uptake (Fig. 5*B*). Therefore, unlike heart uptake of VLDL-TG that was equally reduced by deficiency of LpL and CD36, CM-TG uptake was specifically affected by LpL alone.

*Core Lipid (RE) Delivery to the Heart*—CMs contain a number of non-lipolyzed diet-derived lipids including CE and fat-soluble vitamins. RE has been used as a CM tracker and is also an essential vitamin. CM-RE plasma clearance was delayed in the hLpL0 and DKO groups compared with the control group (average slopes: control, 0.09; hLpL0, 0.03; *Cd36*$^{-/-}$, 0.07; DKO, 0.04 pools/min, $p < 0.05$) (Fig. 5*C*). CM-derived RE uptake into the heart was reduced in the hLpL0 (54 ± 12%) and DKO groups (58 ± 7%) compared with control ($p < 0.05$, Fig. 5*D*). Loss of CD36 did not affect cardiac CM-RE uptake.

The predominant pathway for retinoid delivery to tissues is via retinol-binding protein (or retinol-binding protein 4) (45). However, because CMs are major lipid sources for the heart; dietary retinoids packaged into these particles can also serve to deliver retinoids to the heart. Because cardiac LpL deficiency reduced CM-RE uptake into the heart, we measured cardiac retinol and RE levels by HPLC. We found no significant differences in the total retinol concentrations among the 4 genotypes (retinol: control, 2.0 ± 0.2; hLpL0, 1.5 ± 0.1; *Cd36*$^{-/-}$, 1.6 ± 0.1; DKO, 1.8 ± 0.1 nmol/g of tissue) (RE: control, 11.2 ± 1.2; hLpL0, 8.3 ± 1.0; *Cd36*$^{-/-}$, 9.1 ± 1.1; DKO, 7.6 ± 0.8 nmol/g of tissue). In addition, the expression of the cytochrome P450 family member *Cyp26B1*, a retinoic acid responsive gene that catabolizes excessive retinoic acid, was not altered among the 4 groups (control, 1.00 ± 0.24; hLpL0, 1.02 ± 0.27; *Cd36*$^{-/-}$, 0.54 ± 0.20; DKO, 0.99 ± 0.26, expressed as ratio of *18S* rRNA gene expression normalized to the control group).

*VLDL ApoB and ApoE Knock-out Remnant Uptake into the Heart*—We next explored the possible pathways by which LpL could mediate heart uptake of non-hydrolyzable lipids. LpL-mediated uptake of VLDL-CE could have occurred either via selective uptake of core lipid or via uptake of whole lipoproteins or their remnants. To investigate this, we studied cardiac uptake using $^{125}$I-TC conjugated to the protein moiety and CE-labeled VLDL particles. There were no differences between the two genotypes for both VLDL-CE (average slopes: control, 0.05; hLpL0, 0.04 pools/min) and VLDL-ApoB (average slopes: control, 0.02; hLpL0, 0.01 pools/min) plasma decay curves (Fig. 6*A*). hLpL0 mice exhibited a 91% reduction in heart VLDL-CE uptake associated with a 57% reduction in ApoB particle uptake into the heart ($p < 0.05$, Fig. 6*B*). This suggested that in addition to TG lipolysis, LpL also mediates cardiac uptake of core lipid via a whole particle uptake mechanism. However, because the decrease in VLDL-ApoB particle uptake did not account for the entire 91% reduction in VLDL-CE uptake, some LpL-mediated selective lipid uptake must occur.

To further probe the mechanism of LpL-mediated core lipid uptake we used ApoE knock-out mice to endogenously radiolabel the core lipid in remnant particles with [$^3$H]RE. Plasma decay curves were not different between the groups (average

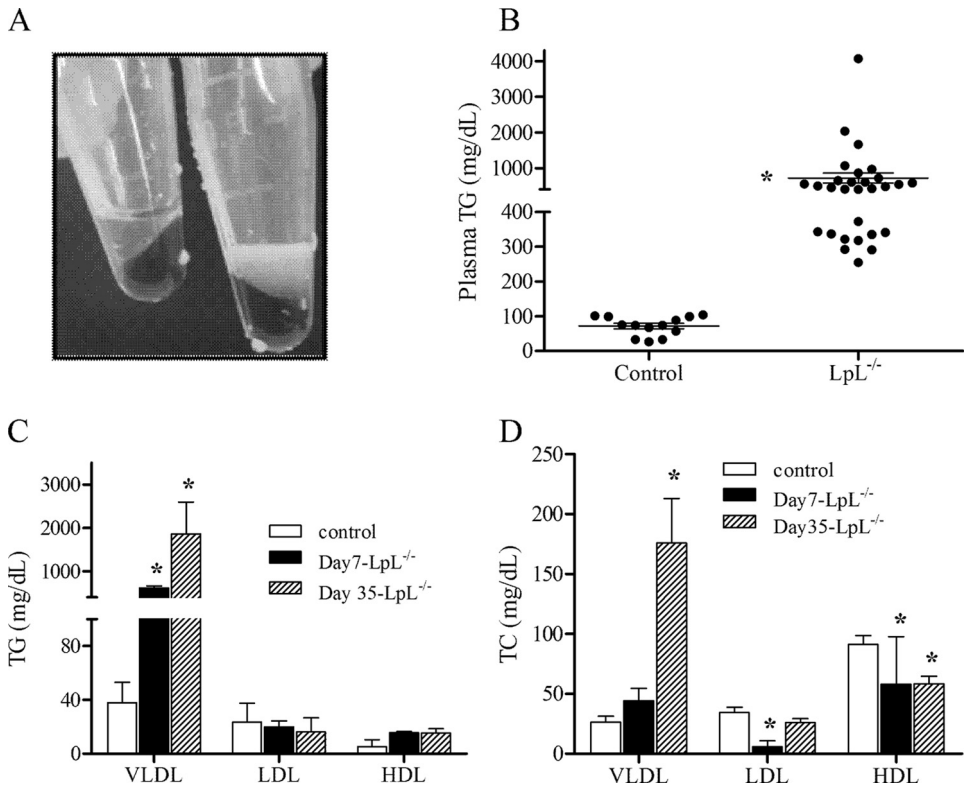


FIGURE 4. **Characterization of plasma lipids in tamoxifen-induced LpL deletion mouse model (*LpL*$^{-/-}$).** *A*, representative plasma from control and LpL-depleted mice 2 weeks after tamoxifen injection. *B*, plasma TG measured 2 weeks after tamoxifen injection. Mice with plasma TG > 400 mg/dL were used to make endogenously labeled CMs in subsequent studies. Lipoprotein TG (*C*) and TC (*D*) in control and *LpL*$^{-/-}$ mice 7 and 35 days after tamoxifen injection. VLDL refers to $d < 1.006$ fraction that contains both VLDL and CMs. *, significantly different from control, $p < 0.05$.

slopes: control, 0.05; hLpL0, 0.03 pools/min) leading to ~40% of the tracer removal by 20 min (Fig. 6*C*). LpL deletion did not affect remnant uptake into the heart (Fig. 6*D*). This suggests that LpL is involved in generation of remnants but does not directly mediate uptake of these particles.

## DISCUSSION

The goal of our current studies was to compare the role(s) of LpL and CD36 in lipid uptake into the heart and assess whether these two proteins function in conjunction with or exclusive of each other *in vivo*. Moreover, we explored whether both TG-rich lipoproteins use identical lipid uptake pathways. Our results highlight the roles of LpL and CD36 in lipid uptake and we believe that we have resolved the long-standing conflict on the routes of FFA uptake into the heart. Our data show the following: 1) LpL and CD36 deficiency reduced uptake of VLDL-TG-derived FFAs. There was a further reduction in the DKO. 2) Uptake of lipoprotein core lipid, represented by CE and RE, was decreased only by loss of LpL. Therefore, LpL participates in a pathway for acquisition of these two essential lipids by the heart. 3) LpL loss reduced both ApoB and CE uptake, but the effects on CE were greater. Thus, some CE uptake includes lipid that dissociates from the ApoB remnant. 4) We developed a new method to create endogenously labeled mouse CMs. 5) We then showed that LpL but not CD36 contributed to uptake of CM-TG-derived FFAs. Thus, using lipoproteins of different TG abundance, we demonstrated two pathways for FA uptake *in vivo*.

*TG Delivery to the Heart*—TG-derived FA from VLDL can be acquired by tissues either after the TG is lipolyzed to FFA or as a component of nascent or remnant lipoproteins. Our data show that loss of either CD36 or LpL resulted in an equivalent reduction in VLDL-TG-derived FA uptake. One interpretation of these data is that the LpL and CD36 function in conjunction with each other to mediate lipoprotein-derived TG uptake, *i.e.*, LpL creates FFAs that then require CD36 for cellular uptake. Our data are consistent with studies in *Cd36*$^{-/-}$ mice showing that these animals have a 50–80% reduction in heart FFA uptake, especially when studied using low FFA concentrations (23). CD36 deficiency reduced VLDL-TG-derived FFA uptake into the heart by about 50%, further emphasizing the importance of CD36 downstream of LpL-mediated lipoprotein-TG hydrolysis. The simultaneous loss of LpL and CD36 resulted in an additional reduction, which might reflect loss of CD36-mediated uptake of both local and systemically produced FFAs.

We were surprised to discover that heart uptake of CMs and VLDL was not identical. Although CD36 was required for delivery of VLDL-TG-derived FAs, it did not play a role in CM-TG delivery to the myocardium. In contrast, LpL was critical to both VLDL and CM-derived lipid uptake into the heart. Thus, we postulate that lipolysis of VLDL-TG by LpL generates a local FFA concentration that is suitable for subsequent CD36-mediated transport. But, lipolysis of CM particles by LpL generates a higher local FFA concentration that saturates CD36-mediated transport, a low $K_m$ and high affinity process, and leads to uptake of the majority of FFAs by passive diffusion, a high $K_m$ low affinity pathway (Fig. 7*A*). Non-CD36-mediated oleate transport was also observed *in vitro* by Duncan *et al.* (42), indicating pathways independent of CD36 for cardiac FFA delivery. We cannot rule out the possibility of a role for other known or unknown transport proteins in this process. Nonetheless, our *in vivo* results are consistent with *in vitro* studies of cardiomyocytes (46) and other cells (9, 47, 48) that suggest two possible pathways for FFA uptake: a saturable and high affinity protein-mediated uptake and a lower affinity non-saturable process.

Our data are also consistent with previous studies in isolated perfused hearts that showed that CM-TG is a superior source of cardiac TG than VLDL-TG (44). CMs are a better substrate for LpL than VLDL (49). Moreover, larger more lipid-rich lipopro-

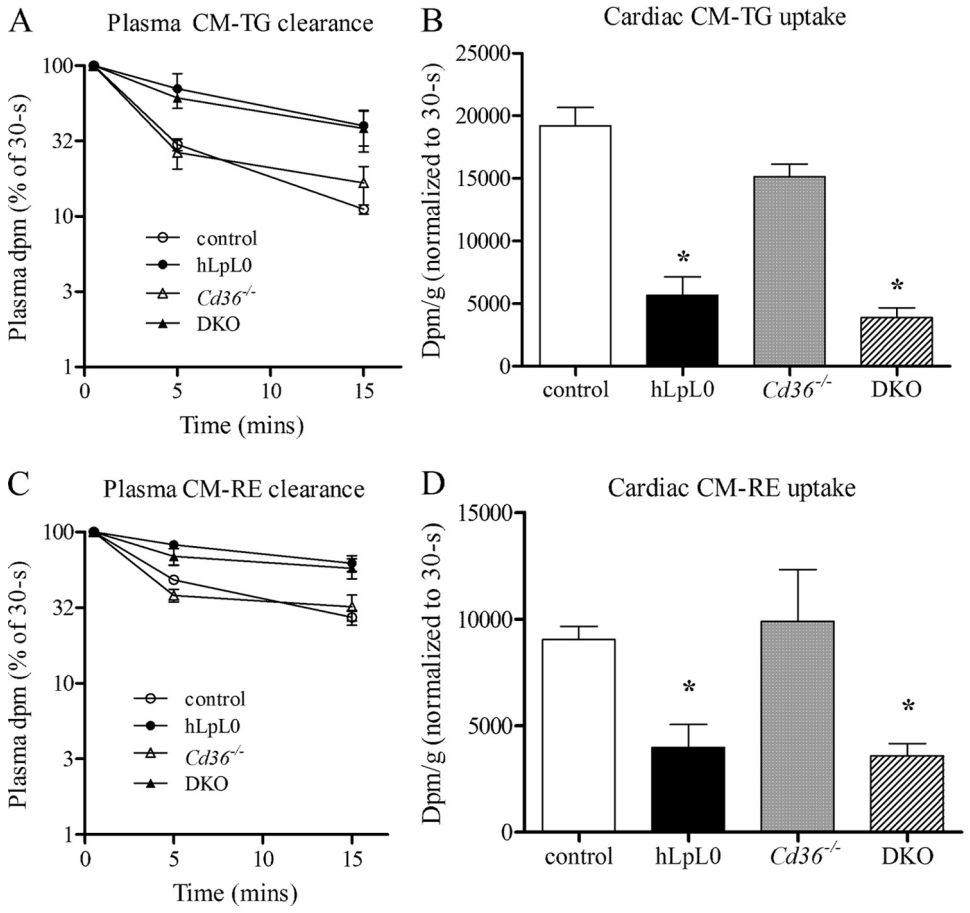


FIGURE 5. **Cardiac CM-TG and CM-RE uptake.** 10–12-Week-old male mice fasted for 4–5 h were administered double-labeled mouse CMs by tail vein. *A*, plasma decay curves for CM-TG. *B*, cardiac CM-TG uptake 15 min after tracer injection. *C*, plasma decay curves for CM-RE. *D*, cardiac CM-RE uptake 15 min after tracer injection. $n = 5–7$ per group; *, significantly different from control, $p < 0.05$.

teins that have greater surface area are more fluid (50); this might allow CMs to "flatten" along the surface of endothelial cells therefore facilitating lipolysis and direct non-receptor movement of FFAs across the membrane. Therefore, the mode of cellular FFA uptake is dependent on both the amount of LpL and the type of TG-rich lipoproteins.

*Non-hydrolyzable Core Lipid Delivery to the Heart*—Cholesterol delivery to the heart has not been extensively studied. The mouse heart is among the least active tissues for cholesterol biosynthesis (35) and takes up little circulating LDL to meet its cholesterol needs (36). Therefore, the heart ought to use other pathways for cholesterol uptake. In addition to TG hydrolysis, LpL has also been shown to mediate core lipid uptake from lipoproteins, attributed both to LpL enzymatic activity (51) and non-enzymatic bridging action (32). The majority of these effects of LpL have been studied *in vitro* or in liver tissues. Our current *in vivo* data indicate a role for heart LpL in core lipid uptake from lipoprotein particles, VLDL and CMs. CE and RE core lipid uptake could occur either as whole particle or selective lipid uptake. We observed that heart-specific LpL deficiency significantly reduced uptake of CE from VLDL and RE from CM particles. Because the heart is such a metabolically active tissue for TG-rich lipoproteins, we propose that LpL-mediated CE uptake is sufficient to supply its cholesterol needs. Our data are totally consistent with studies three decades ago by Fielding *et al.* (37) showing that CMs can supply CE to the heart by a pathway that is not via LDL receptors. In contrast, our data do not support an *in vivo* role for CD36 to mediate heart uptake of core lipids.

Uptake of core lipid from TG-rich lipoproteins could occur via three LpL-mediated processes (Fig. 7*B*): 1) LpL could serve as a cell surface receptor for these particles allowing their interaction with other receptors or uptake via recycling of the cell membrane, especially its proteoglycan components. 2) LpL might be required to create remnants that are internalized as whole particles. 3) Core lipids associated with surface apoproteins are likely to be shed during LpL-mediated lipolysis at the endothelial surface. This shedding process creates non-ApoB-associated lipid and results in an uptake process that has also been termed selective lipid uptake. We postulate that lipid shedding is akin to the lipolysis-mediated step that leads to transfer of TG-rich lipoprotein lipids to HDL (52).

By simultaneously labeling VLDL ApoB and CE and following uptake of both labels by the heart, we were able to show that heart LpL deficiency reduced both CE and ApoB uptake. The reduction in ApoB uptake probably reflects a decrease in receptor-mediated uptake of LDL or VLDL remnants because LpL-mediated VLDL conversion to LDL may have occurred within the heart. In this experiment the decrease in CE uptake was much greater than that of ApoB. Thus, we clearly have evidence for a dissociation of CE from the remainder of the ApoB-containing lipoprotein.

We next studied remnants prepared from $ApoE^{-/-}$ mice and were surprised that loss of LpL did not alter uptake of these particles by the heart. Thus LpL was only important for uptake of the non-hydrolyzable core lipid from TG-rich lipoproteins, *i.e.*, LpL bridging was not important for cardiac uptake of $ApoE^{-/-}$ remnants that are a poor substrate for LpL (53, 54). It should be noted that ApoE lipoproteins are unusual; they are cholesterol enriched and lack ApoE. However, their rapid turnover in these mice, as opposed to the slow turnover of CM-RE in ApoE knock-out mice (55), suggests that the particles have acquired ApoE from their recipients. Thus, our data strongly imply that much of the lipoprotein core lipid uptake into the heart occurs via lipid shedding.

All tissues require retinoids for gene expression and vitamin A-dependent functions. Like FAs, retinoids circulate in the blood in two forms: non-esterified retinol bound to retinol-binding protein and esterified retinol, *i.e.*, RE within CMs. We

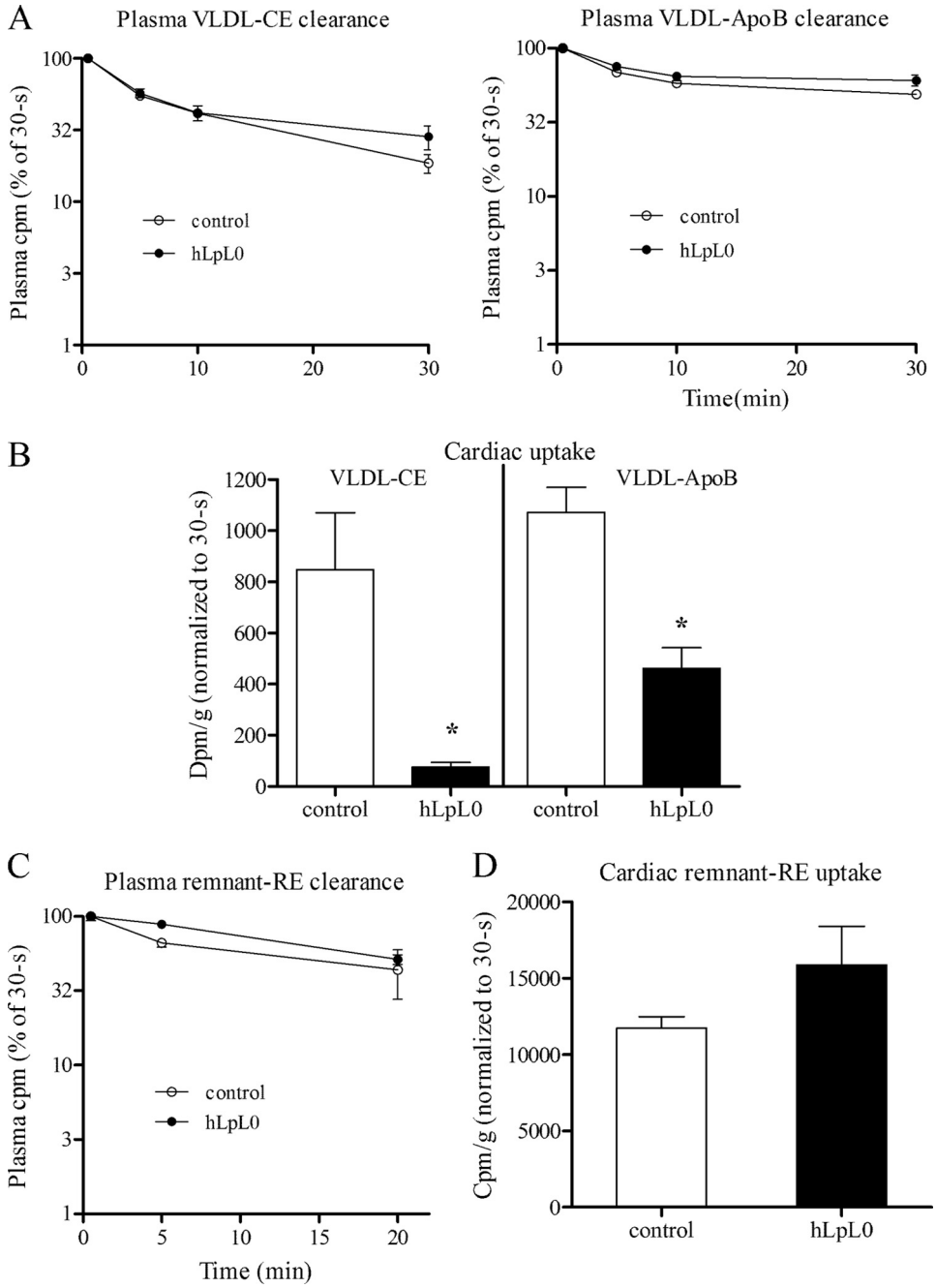


FIGURE 6. **Cardiac uptake of VLDL-CE, VLDL-ApoB, and remnants.** 12–13-Week-old male mice fasted for 4–5 h were administered $^{125}$I-TC-conjugated human VLDL particles along with [$^{3}$H]CE-labeled VLDL via the tail vein. *A*, plasma decay curves for VLDL-CE (*left panel*) and VLDL-ApoB (*right panel*). *B*, cardiac uptake 30 min after tracer injection, $n = 5–7$ per group. 12–14-Week-old male mice fasted for 4–5 h were administered [$^{3}$H]RE-labeled remnant particles via the tail vein. *C*, plasma decay curves for ApoE$^{-/-}$ remnant particles. *D*, cardiac uptake of tracer 20 min after injection, $n = 3–5$ per group; *, significantly different from control, $p < 0.05$.

observed a decrease in RE uptake from CMs in the absence of heart LpL, an effect specific to loss of LpL but not CD36. Our data suggest a role for LpL in retinoid delivery to the heart. The reduced CM-RE uptake into the heart was not reflected in the cardiac retinoid levels or in downstream genes modulated by tissue retinoids. Because retinoids are essential for normal cellular function, lack of one retinoid uptake pathway leads to compensatory mechanisms required for maintenance of function as we have found in other locations such as the eye (56) and milk (57).

*Method for Endogenous Production of Labeled Chylomicrons*—An important methodological advance was our development of a method to endogenously label mouse CMs. Whole body LpL deletion is associated with severe hypertriglyceridemia and consequent neonatal death in mice (31). In the current work, we achieved LpL deletion in the adult mouse by tamoxifen-inducible Cre expression driven by the $\beta$-actin promoter. LpL deletion resulted in a marked elevation in plasma TG levels; over 66% of the injected mice developed TG levels >400 mg/dl. This enabled us to gavage the hypertriglyceridemic mice with labeled lipids, thereby generating double-labeled CMs.

Humans (58) with LpL deficiency have reduced HDL levels and acute inhibition of LpL in primates led to rapid reduction in HDL associated with TG enrichment and rapid HDL turnover (59). Faster HDL turnover was associated with cholesteryl ester transfer protein modification of HDL and its subsequent hydrolysis by enzymes such as hepatic lipase (60) and endothelial lipase (61), and clearance in the kidney (62). Our data show that LpL-mediated lipolysis of CM and VLDL particles, a process that dissociates lipids and surface apoproteins that transfer to HDL (52, 63), is a major pathway for HDL production. Thus, both creation of HDL as well as delivery of core lipids is likely to involve a similar LpL-dependent shedding of lipids. It is important to note that tamoxifen can exert several side effects including those on plasma lipids. Poirot and colleagues (64) showed that tamoxifen is an acyl-CoA:cholesterol *O*-acyltransferase (ACAT) inhibitor in macrophages and hepatocytes, thereby exhibiting atheroprotective effects. We have previously shown that the ability of tamoxifen to raise plasma TG was only transient and returned to normal in 2 weeks (65). Furthermore, in the current report, reduction in plasma HDL was seen early on (4 days) and stayed low even 3 months after tamoxifen when the effects on TG had dissipated. Therefore, the reduction in plasma HDL in the current studies

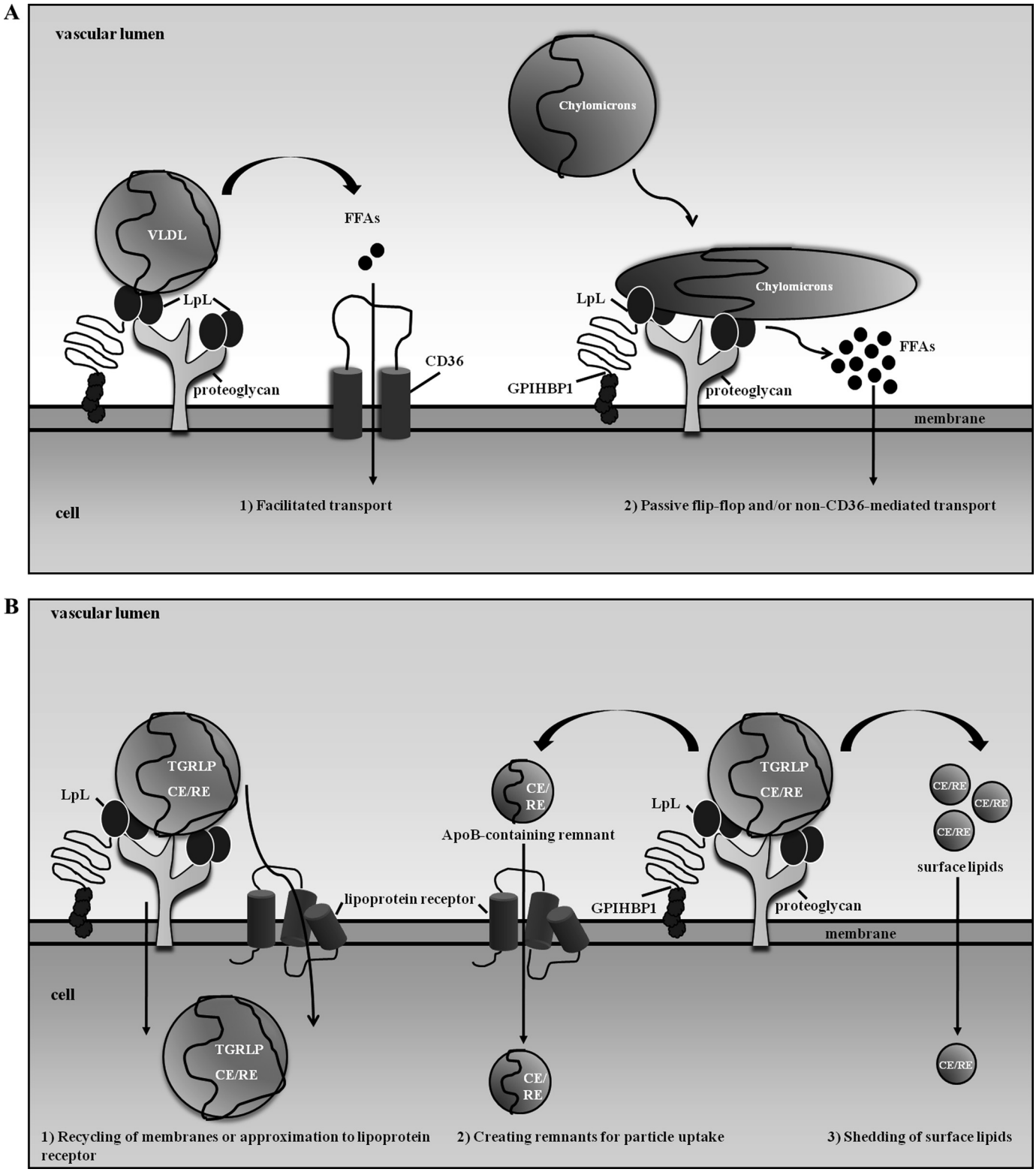


FIGURE 7. *A*, schematic model for VLDL- and CM-derived TG uptake pathways. 1) Hydrolysis of VLDL particles generates FFAs that are subsequently transported into cells via the fatty acid transporter CD36 by the facilitated transport process. LpL is shown attached to the cell surface via binding to proteoglycans and its binding partner glycosylphosphatidylinositol-anchored high density lipoprotein-binding protein 1 (*GPIHBP1*). 2) Lipolysis of TG-rich CM particles generates a higher local concentration of FFAs most of which enter cells by a passive flip-flop and/or non-CD36-mediated mechanism. *B*, schematic model for LpL-mediated uptake of core lipid. 1) LpL approximates TG-rich lipoproteins (*TGRLP*) to the cell membrane and increases uptake of particles either via recycling of membranes or along with lipoprotein receptors. 2) LpL creates ApoB-containing remnants that are better ligands for lipoprotein receptors. 3) During lipolysis, apoproteins and lipids, including core lipid esters such as CE and RE, are shed as surface lipids and subsequently transferred to underlying cells.

is predominantly the consequence of the loss of LpL rather than tamoxifen *per se*.

In conclusion, we provide *in vivo* evidence that FAs are transported across membranes both passively and via carrier proteins. Our data show that pathways for FFA transport depend on the lipoprotein source and therefore on nutritional status. VLDL-TG-derived FFAs utilize CD36 for FFA transport; this is most important in fasting. CMs utilize a non-CD36-mediated FFA uptake pathway that is primarily important in the postprandial state. In addition, we provide data on pathways for the uptake of non-hydrolyzable core lipids by the heart. The cellular requirement for FAs, CE, and RE by tissues appears to have led to the development of multiple lipid uptake processes.

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