Uptake of Dietary Retinoids at the Maternal-Fetal Barrier

IN VIVO EVIDENCE FOR THE ROLE OF LIPOPROTEIN LIPASE AND ALTERNATIVE PATHWAYS

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Dietary retinoids (vitamin A and its derivatives) contribute to normal embryonic development. However, the mechanism(s) involved in the transfer of recently ingested vitamin A from mother to embryo is not fully understood. We investigated in vivo whether lipoprotein lipase (LPL) facilitates the placental uptake of dietary retinyl ester incorporated in chylomicrons and their remnants and its transfer to the embryo. We examined the effects of both genetic ablation (MCK-L0 mice) and pharmacological inhibition (P-407) of LPL by maintaining wild type and MCK-L0 mice on diets with different vitamin A content or administering them an oral gavage dose of [3H]retinol with or without P-407 treatment. We showed that LPL expressed in placenta facilitates uptake of retinoids by this organ and their transfer to the embryo, mainly through its catalytic activity. In addition, through its "bridging function," LPL can mediate the acquisition of nascent chylomicrons by the placenta, although less efficiently. Quantitative real-time PCR and Western blot analysis showed that placental LPL acts in concert with LDL receptor and LRP1. Finally, by knocking out the retinol-binding protein (RBP) gene in the MCK-L0 background (MCK-L0-RBP−/− mice) we demonstrated that the placenta acquires dietary retinoids also via the maternal circulating RBP-retinol complex. RBP expressed in the placenta facilitate the transfer of postprandial retinoids across the placental layers toward the embryo.

Vitamin A, a fat-soluble vitamin, is an essential nutrient obtained from food. Regardless of its dietary origin (retinol, retinyl ester, provitamin A carotenoids), the ingested vitamin A is packaged within the enterocytes as retinyl esters into chylomicrons, which are lipoprotein particles containing triglycerides, fat soluble vitamins, cholesterol, and proteins. Once secreted into the lymphatic system, the chylomicron particle reaches the general circulation, where its triglyceride core is hydrolyzed by the enzyme lipoprotein lipase (LPL) on the surface of capillary endothelial cells in extrahepatic tissues such as adipose and muscle (1, 2). This hydrolysis leads to the formation of smaller particles called chylomicron remnants, which still contain retinyl esters (3). A large portion of the retinyl ester-containing chylomicron remnants are rapidly cleared by the liver, the major site of vitamin A storage in the body. However, −25% of them are taken up by extrahepatic tissues (4, 5), including embryonic and extra-embryonic tissues (6).

Retinoids (vitamin A and its derivatives) are vital for reproduction and embryogenesis (7–9). The mammalian embryo relies on retinoids circulating in the maternal bloodstream for its supply of vitamin A. Within the maternal circulation, retinol bound to its sole specific carrier retinol-binding protein (RBP, also known as RBP4) is the most abundant retinoid form in the fasting state (10, 11). In the fed state, retinyl esters packaged in chylomicrons and their remnants may account for the majority of circulating retinoids. Fetal development can exclusively rely on postprandial retinoids, at least up to a certain extent, if the retinol-RBP pathway is impaired (6, 12). Hence, retinyl esters within chylomicrons and/or chylomicron remnants must be taken up by the maternal-fetal barrier, i.e. placenta and yolk sac, and transferred to the embryo. However, the exact mechanisms that mediate this process are poorly understood. LPL is a major enzyme in lipid metabolism responsible for the hydrolysis of the core triglycerides in chylomicrons and very low density lipoprotein and subsequent release of free fatty acids (13). LPL has also been shown in vitro and in vivo to mediate the uptake of chylomicrons and their remnants containing retinyl esters in adipocytes, skeletal muscle (14–16), heart (17), and mammary gland (18), not only by facilitating the formation of chylomicron remnants but also by directly hydrolyzing retinyl ester within these particles (14). LPL is present in both human and mouse placental tissues (19–21). However, the placenta not only takes up and processes retinoids, like adult tissues, but it also transfers the vitamin A to the developing embryo. Therefore, because of this unique function of the maternal-fetal barrier, whether and how placental LPL would play a role in this process was not clearly known.

The current study addressed this issue in vivo by examining the effects of both the genetic ablation (MCK-L0 mice (22)) and pharmacological inhibition (Poloxamer-407 (23)) of LPL on placental and embryonic uptake of dietary retinoids. We...
showed that placental LPL mediates the acquisition of recently ingested vitamin A and its transfer to the embryo acting in concert with the lipoprotein receptors LDLR and LR1. Both the systemic and local (i.e. within the placenta) catalytic function of this enzyme contribute to this process. We also provided evidence that the placenta can take up not only chylomicron remnants but also nascent chylomicrons, likely through the bridging function of LPL, which involves facilitating the interaction of these particles with lipoprotein receptors or their uptake via recycling of the cell membrane (proteoglycan components) (17, 24). Finally, the placenta may also acquire dietary retinoids via the maternal circulating complex RBP-retinol. RBP expressed in the placenta is involved in the transfer of retinoids toward the embryo for normal development.

**EXPERIMENTAL PROCEDURES**

**Animals**—Wild type (WT) and MCK-L0 (ML0) mice were used for our studies. LPL knock-out mice (L0) die 18 h after birth (25), probably because of hypoglycemia (26, 27). However, the overexpression of the human LPL gene in their skeletal muscle rescues the lethality (22). Mice lacking RBP in the ML0 background were also used for a subset of studies, and they were generated by crossing RBP−/− males with ML0 females. The F1 double heterozygous mice expressing the transgene were then crossed to generate MCK-L0-RBP−/− (ML0RBP−/−) animals that were obtained in a Mendelian ratio. Genotypes of the mice were determined as previously published (12, 22). All mice employed in this study were from a mixed genetic background, C57/BL6 × 129sv. For all the studies, unless indicated differently, both diet and water were available to the animals on an ad libitum basis. Mice were maintained on a 12:12 light/dark cycle with the period of darkness between 7 p.m. and 7 a.m. All experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (28) and were approved by the Rutgers University Institutional Committee on Animal Care.

**Nutritional Manipulation**—WT and ML0 female mice were maintained on a standard nutritionally complete vitamin A-sufficient chow diet (vitamin A, 25–28 IU/g of diet; manufactured by TestDiet (W. F. Fisher and Son, Inc., NJ)) until approximately three months of age when they were mated with their respective males. At the time of a vaginal plug detection (set as 0.5 days post coitum (dpc), the onset of gestation), females were randomly assigned to one of the following purified diets: vitamin A-sufficient (25–28 IU of vitamin A/g of diet) or vitamin A-excess diet (220 IU of vitamin A/g of diet) until the day of sacrifice (14.5 dpc). These diets are otherwise nutritionally complete, providing nutritionally sufficient levels of all other macro- and micronutrients. Although the vitamin A-excess diet contained 10-fold the concentration of retinoids of the vitamin A-sufficient diet, these levels are not considered toxic. By using this latter diet, we wanted to investigate the response of the placenta to an extra postprandial load of vitamin A, although still within a physiological range. This range was chosen based on guidelines of the World Health Organization for a range of recommended daily allowances for vitamin A during pregnancy that would avoid possible teratogenic effects during embryogenesis. The World Health Organization indeed recommends an intake of 900 IU/day and a maximum of 8000 IU/day (almost a 10-fold range) for pregnant women (29). All animals were euthanized by CO2 inhalation between the hours of 9 a.m. and 11 a.m. Maternal serum and tissues as well as placenta and embryos were collected frozen and stored at −80 °C until further analyses.

**1H Retinol Gavage and Poloxamer-407 (P-407) Injection**—Ninety-day-old WT, ML0, and ML0RBP−/− female mice maintained on a standard nutritionally complete vitamin A-sufficient chow diet (vitamin A, 25–28 IU/g of diet) were mated with their respective males. At 0.5 dpc females continued to be maintained on a vitamin A-sufficient diet until day 13.5 dpc. At this stage females were randomly grouped to be injected in the evening with P-407 (1 g/kg body weight), a lipase inhibitor (23, 30), or PBS (vehicle). All female mice were fasted overnight and the following morning were given an oral bolus dose of all-trans-[3H]retinol (2 × 106 cpm all-trans-[3H]retinol and 6 μg of unlabeled all-trans-retinol) in 100 μl of peanut oil (31). Mice were sacrificed 4 h later. Blood was collected from the vena cava, and tissues were then perfused with PBS also via the vena cava to remove residual blood. Maternal tissues were collected. The uterus was cut open, and embryos, placentas, and yolk sacs were collected separately. All tissues were frozen and kept at −80 °C until further analyses.

**HPLC**—Reverse-phase HPLC analysis was performed to measure serum and tissue retinol and retinyl esters levels (32). Tissues (100–200 mg) were homogenized in PBS using a PRO200 homogenizer (Oxford, CT). Retinoids were separated on a 4.6 × 250 mm UltraspHERE C18 column (Beckman Instruments) preceded by a C18 guard column (Supelco Inc., Bellefonte, PA) using acetonitrile, methanol, and methylene chloride (70:15:15, v/v) as the mobile phase flowing at 1.8 ml/min. A Dionex Ultimate 3000 HPLC system and a computerized data analysis work station with Chromelone software were used. Retinol and retinyl esters were identified by comparing retention times and spectral data of experimental compounds with those of authentic standards. The concentrations of retinoids were determined by comparing peak integrated areas for unknowns against those of known amounts of purified standards. Loss during extraction was accounted for by adjusting for the recovery of retinyl acetate, the internal standard added immediately following homogenization of the tissues. In addition, fractions corresponding to retinol, retinyl linoleate, palmitate, and stearate HPLC peaks were collected in scintillation vials, and samples were measured in a Beckman LS 1800 liquid scintillation counter.

**RNA Extraction, cDNA Synthesis, and Quantitative Real-time PCR (QPCR)**—Total RNA was extracted from individual placentas using the RNA Bee kit (Tel-test Inc.) according to the manufacturer’s instructions. RNA concentrations were measured by using the Nanodrop 1000, and quality was ascertained by the 260/280 ratio and by a formaldehyde gel. This step was followed by DNase I treatment (Roche Diagnostics). One
microgram of the DNase-treated RNA was reverse-transcribed to cDNA using the instructions and reagents (random hexamer primers were used) from the Transcriptor First Strand cDNA Synthesis kit (Roche Diagnostics) in a final volume of 20 µl. A no-reverse transcriptase control was included. The LightCycler 480 SYBR Green I Master kit (Roche Diagnostics) was used for QPCR. A validation test (calibration curve) was carried out for each primer pair using a pool of control samples to determine PCR efficiency, and reactions were performed according to the protocol of the manufacturer with 500 nM of each primer (final concentration), cDNA, and the SYBR Green I. All the analyzed amplicons of interested genes encompass at least one intron. For the QPCR experiments, 500 nM concentrations of each primer (final concentration) (see supplemental Table 1 for the primer list and amplicon size) together with an amount of cDNA for each sample equivalent to 7.5 ng of the total RNA input in a final volume of 15 µl was used. Clear plates were used for the reaction in the Lightcycler 480 machine (Roche Diagnostics). All samples were run in triplicate, and each experiment was run in duplicate. Included within each experiment were the no-reverse transcriptase control and the non-template control. After enzyme activation (10 min, 95 °C), 35–40 PCR amplification cycles were performed: 10 s at 95 °C, 20 s at 58 °C, and 30 s at 72 °C. At the end of each run samples were heated to 95 °C with a temperature transition rate of 0.1 °C/s to construct dissociation curves. For each gene, several samples were tested on a 2% agarose gel.

From the instrument, quantification cycles were obtained for each sample for each gene of interest. To determine changes in gene expression, the normalization of quantification cycle values obtained for the genes of interest were calculated for each sample using the geNORM method as outlined by Vandesompele et al. (33). This was first achieved by determining the quantitative value of the reference genes, β-actin (Actb), peptidyl-prolyl cis-trans isomerase A (Ppia), and glyceraldehyde-3-phosphate dehydrogenase using the average of the control cDNA samples. Second, the average expression stability measure, M, was calculated for each of these reference genes with the geNorm software (33). Actb and Ppia were the most stable reference genes, with M values of 0.592 and 0.610, respectively. Third, one set of normalization factors was obtained by geNorm with the reference genes. The normalization factors together with the PCR efficiency of each gene of interest and the quantitative value of each sample was used for all the analyzed genes. All groups were expressed as -fold change from the control group (WT on the vitamin A-sufficient diet).

Western Blot Analysis—Placental content of LDL receptor-related protein 1 (LRP1), low density lipoprotein receptor (LDLR), and RBP and serum RBP levels were analyzed by Western blot. 25 µg of placental protein was run on an SDS-PAGE gel (8% for LDLR and LRP1; 15% RBP4). A rabbit monoclonal LRP1 (Epitomics, CA), a goat polyclonal anti-mouse LDLR (R&D Systems, MN), and a rabbit polyclonal anti-mouse RBP4 (Adipogen, Incheon, Korea) were used for immunodetection. Signals were detected by using a Bio-Rad Chemidoc XRS Molecular Imager System. β-Actin, detected by a mouse monoclonal anti-β-actin antibody (Sigma) was used as a loading control for tissue samples, whereas albumin, detected by a rabbit polyclonal anti-albumin antibody (Abcam), was used for serum RBP levels analysis. The molecular weight of each detected protein is as follows: LRP1, 85 kDa; LDLR 150 kDa; RBP, 21 kDa; β-actin, 42 kDa. The quantification of the membranes was completed by densitometry analysis with Quantity One Program (Bio-Rad).

Statistical Analyses—Normality of the data was determined using the Kolmogorov Smirnov test. Data that did not show a normal distribution were log-transformed. Data were analyzed by an analysis of variance test with a correction for multiple comparisons using the Fisher’s least significant test or a t test. Analyses were performed with SPSS statistical software (SPSS 11.0 for Windows Student Version, 2001; SPSS Inc. IL). A p value <0.05 was used to establish statistical significance.

RESULTS

Effects of the Lack of Tissue-specific LPL on Placental Retinoid Uptake and Its Transfer to the Embryo—To gain insight into the role of LPL in mediating the transfer of maternal circulating postprandial retinoids across the placenta to the embryo, we used LPL knock-out mice overexpressing human LPL under the muscle-creatine kinase promoter (ML0) and WT mice as control. ML0 mice do not express mouse or human LPL in placenta (Ref. 21 and supplemental Fig. 1A). Therefore, they are a suitable model to address the role of placental LPL specifically in mediating the transfer of postprandial retinoids across this tissue. Ninety-day-old WT and ML0 females raised on the vitamin A-sufficient diet were mated with their respective males and maintained either on the vitamin A-sufficient or -excess diet from the time a vaginal plug was observed (0.5 dpc). At 14.5 dpc, dams were sacrificed, and maternal serum as well as the embryos and placenta were collected to measure retinoid levels by reverse phase HPLC (32). Maternal serum retinol and retinyl ester levels increased upon vitamin A-excess feeding in all strains in comparison to mice of the same genotype fed the vitamin A-sufficient diet as expected (Table 1). Circulating levels of RBP were not increased on the vitamin A-excess diet irrespective of the genotype, suggesting that this excess retinol could be transported bound to lipoproteins (supplemental Fig. 2). Similar litter size, number of unsuccessful pregnancies, and resorbed embryos is noted for all groups (supplemental Table 2). In addition, the embryos appeared grossly morphologically normal regardless of their genotype and maternal diet (data not shown).

| Table 1 | HPLC analysis of maternal serum retinol and retinyl ester levels in WT and ML0 dams fed the vitamin A-sufficient or -excess diet during pregnancy |
|---------|--------------------------------------------------------------------------------|
| Genotype | Maternal serum retinol and retinyl ester levels | Vitamin A-sufficient | Vitamin A-excess |
|         | Retinol | Retinyl ester | Retinol | Retinyl ester |
|         | µg/dl   | µg/dl           | µg/dl   | µg/dl           |
| WT   | 7.6 ± 1.6 | 3.1 (0.5–6.5) | 18.7 ± 6.7 | 27.9 (5.9–70.7) |
| ML0  | 5.4 ± 1.2 | ND | 11.9 ± 2.5 | 18.3 | 5.4–44.2 |

*p < 0.05 versus sufficient group of the same genotype. Retinol data are presented as the mean ± S.D. Retinyl ester data are presented as the geometric mean (95% confidence interval); 5–9 serum samples were analyzed.

Notes:
- ND: Not detectable (limit of detection 0.1 ng/dl).

Placental and Embryonic Uptake of Dietary Retinyl Ester
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HPLC analysis of the placenta showed that the maternal regimen of dietary vitamin A excess increased the levels of retinol and retinyl esters in this tissue irrespective of the mouse genotype (Table 2). Furthermore, comparable levels of placental retinoids were detected in WT and ML0 mice on any dietary regimen (Table 2). Similar to the placenta, embryonic retinoid levels increased when the dams were fed the vitamin A-excess diet compared with embryos from dams fed the vitamin A-sufficient diet regardless of the genotype (Table 2). However, irrespective of the diet, retinyl ester levels were lower in ML0 embryos compared with WT (Table 2), and retinol levels were lower in the ML0 embryos compared with WT under the vitamin A-excess regimen (Table 2). Overall our data show that the transfer of retinoids from the maternal circulation to the embryo is impaired in the ML0 strain (i.e. when LPL is absent in placenta).

**Effects of Inhibition of LPL Activity on Postprandial Retinoid Uptake by the Placenta and Its Transfer to the Embryo—ML0 mice make chylomicron remnants because of the muscle specific expression of human LPL (22). Therefore, to discriminate between the local (i.e. within the placenta) versus the whole body systemic role of LPL in the postprandial uptake of retinoids by the maternal-fetal barrier, we employed the total lipase inhibitor P-407 (23, 30) to prevent hydrolysis and clearance of chylomicrons generated after a gavage dose of [3H]retinol in peanut oil. Ninety-day-old WT and ML0 females raised on the vitamin A-sufficient diet were mated with their respective males and maintained on the same diet during pregnancy. At 14.5 dpc they were injected with P-407 (1 g/kg of body weight) and fasted overnight. The following morning (14.5 dpc) they were given an oral bolus dose of all-trans-[3H]retinol (2 × 10⁶ cpm all-trans-[3H]retinol and 6 µg of unlabeled all-trans-retinol) in 100 µl of peanut oil and sacrificed 4 h later to collect maternal serum and liver as well as embryo and placenta. Groups of dams injected with vehicle were used as controls. As expected, the pharmacological inhibition of LPL with P-407 resulted in milky white serum with increased total [3H]retinoid cpm compared with vehicle-treated animals regardless of the genotype (Refs. 23 and 31 and supplemental Fig. 3A). Accordingly, hepatic uptake of [3H]retinol cpm was reduced in mice injected with P-407 compared with vehicle treated animals regardless of the genotype (supplemental Fig. 3B). When the distribution of [3H] cpm into the maternal serum retinol and retinyl ester was analyzed by HPLC analysis followed by fraction collection, we confirmed that the inhibition of the chylomicron remnant clearance increased serum [3H] cpm retinol and retinyl ester regardless of the genotype (supplemental Table 3).

We next analyzed the effect of the inhibition of LPL activity on placental and embryonic [3H]retinoid levels. Placental [3H]retinol and -retinyl esters levels were reduced in ML0 placenta compared with WT in the absence of P-407 treatment (Fig. 1, A and B). This suggests that LPL expression in placenta facilitates the uptake of maternal circulating retinoids in this tissue. In the embryo of vehicle-treated dams, a reduction in [3H]retinyl ester levels was observed in ML0 mice compared with WT without changes in [3H]retinol (Fig. 1, C and D). This result indicates that the absence of LPL in placenta also affects the amount of retinoids that reach the embryo from the maternal bloodstream.

When chylomicron remnant formation was inhibited by giving P-407 to WT dams, placental [3H]retinyl ester levels were increased compared with vehicle-injected WT (Fig. 1B). Embryonic [3H]retinol and -retinyl ester levels were significantly reduced compared with WT embryos from dams administered the vehicle (Fig. 1, C and D). In ML0 dams, P-407 treatment resulted in reduced placental [3H]retinyl ester levels compared with vehicle (Fig. 1A). Although a trend toward an increased [3H]retinyl ester levels was observed in the placenta (Fig. 1B), this did not reach statistical significance. Also, [3H]retinol levels (both retinol and retinyl ester) in placenta from ML0 P-407-treated dams were significantly lower that those from WT dams injected with the inhibitor (Fig. 1, A and B). Finally, in P-407-treated ML0 dams, the amount of retinoids reaching the embryos was the lowest between these two strains (Fig. 1, C and D). Together these data suggest that placenta can take up large nascent chylomicrons even in the absence of placental LPL, albeit to a lesser degree. However, the retinoid content of these chylomicrons does not seem to be efficiently transferred to the embryo.

**The Contribution of LPL-independent Pathways—Hepatic secretion of recently ingested vitamin A in the form of retinol bound to RBP has been shown to occur as early as 2 h after a gavage dose of radiolabeled retinol (34). We knocked out the RBP gene in the ML0 background (ML0RBP−/− mice). ML0RBP−/− mice are viable and fertile when maintained on the vitamin A-sufficient diet. The embryos appear grossly morphologically normal, and no litter size difference could be observed in this strain when compared with WT and ML0 animals (data not shown). In this strain, LPL systemic activity is still present, as the mice overexpress human LPL in muscle. Therefore, to evaluate the contribution of RBP and/or other LPL-independent pathways in delivering recently ingested retinoids at the maternal-fetal barrier, we performed the same gavage experiment described in the previous section by inhibiting LPL catalytic activity with P-407 administration in ML0RBP−/− females mated with males of the same genotype. Interestingly, both placental and embryonic [3H]retinol and -retinyl esters levels were reduced in ML0RBP−/− mice compared with both WT and ML0 strains in the absence of P-407 treatment (Fig. 1). This suggests that the RBP pathway also contributes to the delivery of recently ingested vitamin A to the placenta.

**TABLE 2**

HPLC analysis of placental and embryonic retinol and retinyl esters levels from WT and ML0 dams fed the vitamin A-sufficient or -excess diet during pregnancy

|                | Placental and embryonic retinol and retinyl ester levels | Vitamin A-sufficient | Vitamin A-excess |
|----------------|----------------------------------------------------------|----------------------|-----------------|
|                | Retinol Retinyl ester | Retinol Retinyl ester |
| Placenta       | ng/g ng/g | ng/g ng/g |
| WT             | 67 ± 6 47 ± 20 | 146 ± 46 153 ± 59 |
| ML0            | 67 ± 11 33 ± 2 | 184 ± 53 127 ± 37 |
| Embryo         | ng/g ng/g | ng/g ng/g |
| WT             | 111 ± 19 297 ± 48 | 199 ± 32 823 ± 223 |
| ML0            | 101 ± 29 188 ± 30 | 156 ± 27 504 ± 149 |

*p < 0.05 versus sufficient diet group of the same genotype.

*p < 0.05 vs. WT within the same diet group. Retinoid data are presented as mean ± S.D.; 4–6 placenta and 5–9 embryos analyzed per group.
placenta and to its transfer to the embryo. When P-407 was
given to ML0RBP−/− dams, placental and embryonic [3H]reti-
non levels did not change (Fig. 1, A and C). Placental and embry-
onic [3H]retinyl ester levels, however, showed a pattern similar
to that observed in WT and ML0 mice when compared with
their respective vehicle-injected groups (Fig. 1, B and D). These
results confirm the ability of placenta to take up nascent chylo-
microns and suggest that alternative LPL- and RBP-indepen-
dent mechanisms may still deliver recently ingested retinoids to
the placenta and the embryo.

Additional Mediators of Dietary Vitamin A Uptake at the
Maternal-Fetal Barrier—We analyzed by QPCR the expression
levels of a number of receptors, enzymes, and transporters pre-
viously shown to mediate lipoprotein particle uptake in adult
peripheral tissues. No changes were observed in the placental
mRNA levels of endothelial lipase or hormone sensitive lipase
in the ML0 mice regardless of dietary regimen (Fig. 2, A and B).
These results confirm the ability of placenta to take up nascent chylo-
microns and suggest that alternative LPL- and RBP-independent
mechanisms may still deliver recently ingested retinoids to
the placenta and the embryo.

![Figure 1](image1.png)

FIGURE 1. Placental and embryonic [3H] cpm associated with retinol and retinyl ester fractions from WT and ML0. Shown are placenta (A and B) and embryos (C and D) from dams given vehicle (gray bars) or P-407 (black bars) the evening before sacrifice (14.5 dpc) followed by an oral gavage of [3H]retinol. Samples were analyzed by reverse phase HPLC followed by fractions collection (see “Experimental Procedures”). [3H] cpm associated with retinol (A and C) and retinyl ester (B and D) fractions are shown. Data are presented as the mean ± S.D. Placenta and embryos were obtained randomly from 3–5 dams per group. *, p < 0.05 versus vehicle within genotype; #, p < 0.05 versus WT of same treatment; $, p < 0.05 versus WT and ML0 of same treatment. Only one embryo from the ML0 + P-407 group showed retinyl ester counts (D).

In agreement with the QPCR results, Western blot analysis
showed a similar pattern of placental LDLR and LRP1 protein
expression (Fig. 3). Specifically, protein expression for both
LDLR and LRP1 was greater in the placenta of ML0 mice com-
pared with WT irrespective of diet (Fig. 3). Taken together,
these results suggest a role for LRP1 and LDLR in ensuring
adequate placental retinoid uptake from the maternal blood-
stream in the absence of or in addition to LPL, especially under
conditions of excessive maternal dietary vitamin A intake.

The Role of Placental RBP—Maternal RBP, the sole specific
carrier for retinol, has been shown not to cross the placenta
(12), implying a role for RBP expressed in this organ in facilitat-
FIGURE 2. QPCR analysis of placental expression of endothelial lipase (EL) (A), hormone-sensitive lipase (HSL) (B), LDLR (C), LRP1 (D), CD36 (E), scavenger receptor class B-1 (SRB1) (F), GPIHBP1 (G), and RBP (H). Analysis of placenta from WT and ML0 dams fed a vitamin A-sufficient diet (Suff; black bars) or vitamin A-excess diet (Exc; gray bars) for 14 days during gestation. Placenta from WT dams fed a vitamin A-sufficient diet was set as a calibrator at 1. Data are presented as the mean ± S.D. -fold change from WT-sufficient. Placenta were randomly chosen from 4–5 dams per group. *, p < 0.05 versus vitamin A-sufficient diet within the same genotype; #, p < 0.05 versus WT of same diet. Values were normalized using normalization factors calculated from expression of Actb and Ppia housekeeping genes, as indicated under “Experimental Procedures.”
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ing the movement of retinol across the maternal-fetal barrier toward the embryo. We, therefore, analyzed mRNA and protein levels of RBP within the placenta. Although the vitamin A-excess maternal dietary regimen significantly up-regulated placental RBP mRNA levels in WT mice compared with dams of the same genotype on the vitamin A-sufficient diet (Suff, black bars) or vitamin A-excess diet (Exc, gray bars) for 14 days during gestation is shown. A, representative Western blots of repeated experiments are shown. Quantitative measurement of LDLR (B) and LRP1 (C) from Western blot analysis was completed by densitometry analysis with Quantity One Program (Bio-Rad). Placenta from WT dams fed a vitamin A-sufficient diet was set as a calibrator at 1. Data are presented as the mean ± S.D. -fold change from WT-sufficient. Placentas were randomly chosen from 4–5 dams per group. #, p < 0.05 versus WT of same diet. Values were normalized to β-Actin.

DISCUSSION

Most of the current knowledge regarding the uptake of chylomicrons, LPL could generate remnants that are internalized via a receptor-mediated mechanism. Importantly, LPL has also been shown to hydrolyze chylomicron retinyl ester after most of the triglyceride has been hydrolyzed, thus enhancing the uptake of chylomicron retinoids by the tissues (14). In addition, LPL could facilitate the uptake of retinyl ester from chylomicrons and/or chylomicron remnants through its bridging function.

Despite the well known importance of retinoids for embryonic development (8) and the significant contribution of dietary retinoids in meeting the elevated retinoid demand of the embryo (6), the mechanisms of dietary vitamin A uptake and transfer at the maternal-fetal barrier, i.e. placenta, have remained elusive along with the role of LPL in these processes. In mammals the exchange of gas and nutrients between mother and fetus occurs within the placenta. In the mature placenta, which in mouse is formed at about 10.5 dpc (35), this exchange takes place at the level of the microvillus and basal membranes that separate the maternal blood from the fetal circulation. In mice, this barrier is composed of a bilayer of syncytiotrophoblasts adjacent to the fetal endothelium and a layer of trophoblast cells that lines the maternal blood sinuses (35, 36) (see also the scheme in Fig. 4). LPL expression has been detected in the syncytiotrophoblast cells of the placenta (21, 37, 38), which are of embryonic origin (35).

Uptake of Postprandial Retinoid by the Placenta—By using a radioactive vitamin A tracer in the bolus dose given to the mice, we showed that LPL expressed in placenta facilitates the uptake of dietary retinoids by this organ. Indeed, mice lacking placental LPL (ML0 and ML0RBP−/−) had reduced [3H]retinoid levels compared with WT in the absence of P-407 treatment (Fig. 1). Furthermore, we interpret the increased placental [3H]retinyl ester levels observed upon P-407 administration in the strains analyzed as the result of impaired local retinyl ester hydrolysis (i.e. within the placenta), suggesting a major role played by the LPL catalytic activity in ensuring adequate placental processing of postprandial retinoids. The gavage experiments in combination with the total lipase inhibitor also confirmed that placenta, like other tissues (39), can take up newly formed chylomicrons containing retinyl esters even in the absence of placental LPL, although to a lesser extent (Fig. 1B). We speculate that the LPL bridging function, supposedly still present only in the placenta of WT mice treated with P-407, could play a predominant role in the uptake of the whole nascent chylomicrons. In agreement with previous studies showing that steady state retinoid content of adult tissues was not different between WT and ML0 mice despite reduced chylomicron remnant uptake (15, 17), steady state placental retinoid levels were not statistically different between WT and ML0 mice in our study (Table 2). Hence, in the absence of placental LPL expression, alternative pathways may exist to facilitate the uptake of postprandial retinoids by the placenta. Because the reduction of placental [3H]retinoid levels was exacerbated in ML0RBP−/− mice vehicle-injected (Fig. 1), we provide evidence that recently ingested vitamin A rapidly re-secreted from the liver, as retinol-RBP (34) also delivers postprandial retinoids to the placenta. However, as the uptake of recently ingested vitamin A was not completely abrogated in the ML0RBP−/− mice injected with the lipase inhibit-
tor, additional compensatory LPL- and RBP-independent pathways to acquire postprandial retinoids at the placenta barrier may exist and/or be up-regulated in this strain. This is in agreement with the crucial role of retinoids in ensuring normal embryonic development.

Additional Mediators of LPL Action at the Maternal-Fetal Barrier—It has been demonstrated that LPL can promote binding of lipoproteins to receptors in tissues (16, 40). LDLR and LRP1 are two key receptors that facilitate the uptake of chylomicrons and their remnants in adult tissues (41, 42). Both receptors are expressed in mouse placenta at 14.5 dpc (Ref. 43 and Fig. 2, C and D). Placental mRNA levels of LRP1, the so-called remnant receptor (44), were increased in WT placenta of mice maintained on the vitamin A-excess diet, implicating LRP1 as the main molecular player acting in concert with LPL and activated to respond to changes in vitamin A content of the...
maternal diet. Interestingly, the up-regulation of both LRP1 and LDLR mRNA and protein levels in the placenta of the ML0 strain (Figs. 2 and 3) suggest that a receptor-mediated uptake of chylomicrons and their remnants can contribute to the placental uptake of dietary vitamin A even in the absence of LPL. This could represent an additional LPL-independent placental pathway of retinoid uptake that might partly explain the similar steady state retinoid levels between WT and ML0 (Table 2). The fatty acid transporter, CD36, and the HDL receptor, scavenger receptor class B-1, are also expressed in human and mouse placenta (45–48) and have been implicated in the uptake of lipoproteins. In agreement with Bharadwaj et al. (17), who showed that the cardiac uptake of chylomicrons containing retinyl esters was not different from control in mice lacking CD36, we found no difference in CD36 mRNA expression levels between ML0 and WT mice regardless of the dietary regimen. We show that only when ML0 were fed the vitamin A–excess diet, scavenger receptor class B-1 expression was up-regulated in placenta (Fig. 2F). Because both placental LDLR and LRP1 were increased in ML0, perhaps to a limit, then an additional pathway such as SR-B1 may be needed when there is an extra load of postprandial vitamin A reaching the placenta. This concept is similar to the study by Hu et al. (49), who show that SR-B1 takes up chylomicrons when LDLR and LRP1 are absent in the liver. Finally, we showed for the first time expression of GPIHBP1 mRNA in mouse placenta (Fig. 2G). GPIHBP1 transports LPL across endothelial cells, making LPL available to bind and/or hydrolyze chylomicrons (50). The lack of changes in its mRNA levels suggests that GPIHBP1 is not a key player in the regulatory mechanism(s) that controls the uptake of recent dietary retinoids at the placental barrier. We also tested the hypothesis that other lipases could be involved in this uptake process. In contrast to early studies by Lindegaard et al. (21), who showed that endothelial lipase mRNA was up-regulated in the placenta of the ML0 mice, we did not detect significant differences in endothelial lipase placental mRNA expression levels between genotypes nor diets (Fig. 2A). This discrepancy could be because of the different embryonic stage we analyzed (14.5 versus 18.5 dpc in Ref. 21).

Transfer of Dietary Vitamin A from Placenta to Embryo—We have provided evidence to support the hypothesis that LPL expressed in placenta plays a crucial role in mediating the transfer of dietary chylomicron retinyl ester from the maternal bloodstream to the embryonic tissues. First, steady state retinoid levels were reduced in ML0 embryos compared with WT irrespective of the maternal dietary regimen (Table 2). Second, and similar to the steady state levels, embryonic [3H]retinyl ester levels in the ML0 strain were also significantly lower compared with WT embryos in vehicle-injected mice (Fig. 1D). Third, when placental and whole body systemic LPL activity was inhibited (i.e. P-407 treatment) in the WT strain, the levels of both embryonic [3H]retinol and [3H]retinyl ester (Fig. 1, C and D) were reduced. In placenta, retinyl ester hydrolysis into retinol seems to be a crucial step to allow for an efficient transfer of vitamin A to the embryo in the form of retinol bound to RBP synthesized within the syncytiotrophoblast layer (51). Our data support the hypothesis that LPL synthesized within the same placental layers (21, 37, 38) is the main enzyme involved in the generation of retinol. Interestingly, we showed that even in the absence of placental LPL activity, both nascent chylomicrons and remnants can be taken up by the placenta and transferred to the embryo, although less efficiently. This suggests that other retinyl ester hydrolases may be involved in this process. The lack of changes in placenta mRNA levels of hormone-sensitive lipase, a placental lipase (52) that acts as a retinyl ester hydrolase (53) (Fig. 2B), does not rule out a role for this enzyme. Furthermore, additional retinyl ester hydrolases like carboxylesterase have been described in rat placenta (54, 55) and in the microsomal fraction of the human amniotic epithelium (56, 57), implying their potential role in this process.

The dramatic reduction of embryonic [3H]retinoids levels in the ML0RBP+/− mice vehicle-injected (Fig. 1C) likely reflects the limited placental retinoid uptake in this strain because of the lack of both placental LPL and the maternal retinol-RBP pathway. Also, the reduced embryonic [3H]retinoid levels confirmed the importance of RBP synthesized within the syncytiotrophoblast in moving retinoids across the placental layers toward the embryo. In contrast to WT and ML0 mice, inhibition of the systemic LPL activity (i.e. P-407 administration) in the ML0RBP+/− strain did not affect embryonic [3H]retinol levels (Fig. 1C), likely because they were already minimal due to the lack of RBP. Although this latter experiment highlights the importance of RBP in facilitating the transfer of retinoids across the placenta to the embryo, homeostatic levels of retinoids within the developing tissues are not reached by regulating placental RBP protein levels.

In conclusion, as summarized in Fig. 4, we propose that 1) the catalytic activity of LPL expressed in placenta per se is important to acquire recently ingested retinoids within chylomicrons or chylomicron remnants generated by systemic LPL. In particular, the ability of LPL to hydrolyze retinyl ester into retinol is essential for the transfer of retinoids to the embryo. We cannot rule out that other placental enzymes able to hydrolyze retinyl ester are involved in this process. However, their contribution appears to be minor. 2) LPL expressed in placenta can mediate the placental acquisition of nascent chylomicrons through its bridging function, although further processing of these particles to make retinoids available to the embryos seems to be rather inefficient. 3) Placental LPL acts in concert with LDLR and LRP1. 4) Upon uptake by the maternal liver, postprandial vitamin A can also be rapidly re-secreted as retinol-RBP to be delivered to the placenta. 5) RBP expressed in the syncytiotrophoblast layer of the placenta is also crucial to mediate the transfer of recently ingested retinol (either acquired as such or generated through retinyl ester hydrolysis) toward the embryo. Overall, the data and the proposed model provide a significant mechanistic advance to the field of retinoid metabolism during embryogenesis.

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