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Maternal loading with very low-density lipoproteins stimulates fetal surfactant synthesis

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Abstract

We examined whether administration of very low-density lipoproteins (VLDL) to pregnant rats increases surfactant phosphatidylcholine (PtdCho) content in fetal pre-type II alveolar epithelial cells. VLDL-triglycerides are hydrolyzed to fatty acids by lipoprotein lipase (LPL), an enzyme activated by heparin. Fatty acids released by LPL can incorporate into the PtdCho molecule or activate the key biosynthetic enzyme cytidylyltransferase (CCT). Dams were given BSA, heparin, VLDL, or VLDL with heparin intravenously. Radiolabeled VLDL given to the pregnant rat crossed the placenta and was distributed systemically in the fetus and incorporated into disaturated PtdCho (DSPtdCho) in pre-type II cells. Maternal administration of VLDL with heparin increased DSPtdCho content in cells by 45% compared with control ($P < 0.05$). VLDL produced a dose-dependent, saturable, and selective increase in CCT activity. VLDL did not significantly alter immunoreactive CCT content but increased palmitic, stearic, and oleic acids in pre-type II cells. Furthermore, hypertriglyceridemic apolipoprotein E knockout mice contained significantly greater levels of DSPtdCho content in alveolar lavage and CCT activity compared with either LDL receptor knockout mice or wild-type controls that have normal serum triglycerides. Thus the nutritional or genetic modulation of serum VLDL-triglycerides provides specific fatty acids that stimulate PtdCho synthesis and CCT activity thereby increasing surfactant content.

Keywords

phosphatidylcholine; triglycerides; cytidylyltransferase

The developing fetus is highly dependent on the availability of lipid substrates, such as fatty acids, that are utilized for the biosynthesis of phosphatidylcholine (PtdCho) (40). Fetal alveolar type II epithelial cells utilize fatty acids to synthesize disaturated phosphatidylcholine (DSPtdCho), the major surfactant lipid that lowers surface tension (40). Fatty acids serve as integral components of the DSPtdCho molecule and also directly activate CTP:phosphocholine cytidylyltransferase [(CCT) EC 2.7.7.15], the rate-regulatory enzyme involved in PtdCho biosynthesis. (31). Potential sources of fatty acids include de novo synthesis within the fetus or placenta, or transport of free fatty acids from the maternal circulation. However, the fetus cannot solely rely upon de novo fatty acid synthesis, as up to 50% of fatty acids are derived maternally (10). Thus a major pathway for free fatty acids to the fetus appears to be from maternal lipids carried within lipoproteins (20).

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Very low-density lipoproteins (VLDL) are triglyceride-enriched particles that are cleared from the microcirculation by a serine hydrolase, lipoprotein lipase (LPL), which is anchored to endothelial surfaces and the placenta by proteoglycans. LPL reacts with triglycerides in VLDL, generating monoacylglycerols and unesterified fatty acids. LPL also functions as a ligand for lipoprotein receptors by simultaneously binding lipoprotein particles and facilitating their cellular uptake by a receptor-mediated process. It has been demonstrated that a variety of proteins, including the low-density lipoprotein receptor (LDLR), the LDL receptor related-protein (LRP), VLDL receptor, and glycoprotein 330 (known as gp330 or LRP-2), all appear to participate in the cellular internalization of VLDL (47).

Studies suggest that maternal lipoproteins, such as VLDL, provide the free fatty acid substrate required for fetal surfactant synthesis *in vivo*, although to date this hypothesis has not been directly tested. In support of this hypothesis, the concentration of all lipoproteins, including VLDL, increases in maternal circulation during pregnancy (24). Although very low levels of VLDL are detected initially in the human and rat fetal circulation (3,25), these levels increase with development (3), achieving high concentrations postnatally (23). Furthermore, lipolytic products of maternally administered VLDL, such as fatty acids, cross the placenta after hydrolysis by maternal LPL (24), and a highly regulated placental VLDL receptor is expressed during later gestation (35). Concurrent with these changes in lipoprotein expression, pulmonary LPL content increases severalfold, developing high expression in interstitial cells and alveolar macrophages (15,26,37). Interestingly, LPL knockout mice have severe hypertriglyceridemia and die from neonatal respiratory distress, indicative of LPL's essential role in lipid catabolism (6). Collectively, these studies suggest that hydrolysis of maternally derived VLDL by LPL might provide fatty acid substrates utilized by the fetus. However, the destination of maternally derived VLDL fatty acids is not known, nor specifically whether they regulate surfactant DSPtdCho synthesis.

We previously published studies demonstrating that VLDL with LPL stimulates surfactant lipid synthesis in primary rat fetal type II cells and in a fetal alveolar type II cell line (29). In the present study, we investigated whether VLDL might upregulate surfactant lipid synthesis *in vivo*. After administering VLDL intravenously in the pregnant rat, we observed that these particles are distributed into the fetal lung and increase DSPtdCho content. Our studies reveal that the increase in DSPtdCho is secondary to a selective increase in fatty acids that are incorporated into the DSPtdCho molecule and regulate its biosynthesis.

MATERIALS AND METHODS

Materials

Intermediate-size VLDL with a sedimentation flotation rate of 60–100 (from the density <1.006 g/ml fraction of plasma), heparin, and bovine serum albumin (BSA) were from Sigma (St. Louis, MO). Silica LK5D (0.25 mm × 20 cm × 20 cm) thin-layer chromatography (TLC) plates were purchased from Whatman International (Maidstone, UK). The MRE-040 Micro-Rethane tubing was from Braintree Scientific (Braintree, MA). All radiochemicals were purchased from DuPont New England Nuclear Chemicals (Boston, MA). The radiolabeled tripalmitin [palmitic-1-¹⁴C] (specific activity, 55 mCi/mmol) was obtained from American Radiolabeled Chemicals (St. Louis, MO). Immunoblotting membranes were obtained from Millipore (Bedford, MA). The enhanced chemiluminescence (ECL) Western blotting detection system was from Amersham Pharmacia Biotech (Piscataway, NJ). Anti-CCT rabbit polyclonal antibody to synthetic peptide corresponding to residues 164–176 (22) was generated by Covance Research Products (Richmond, CA). Fatty acid standards were obtained from Nu Chek Prep (Elysian, MN) and Supelco (Bellefonte, PA). The free fatty acids, half-micro test was from Roche Diagnostics (Mannheim, Germany). Polyclonal antibody against LPL was generated in the chicken-against-bovine LPL antigen. The Harvard infusion pump was

purchased from Harvard Apparatus (Holliston, MA). The C57BL/6J mice, LDLR knockout mice, and apoprotein E (ApoE) knockout mice were obtained from Jackson Labs (Bar Harbor, ME).

Animals and cell preparation

Timed-pregnant Sprague-Dawley rats at *days 17–20* of gestation (day = 0, designated by presence of vaginal sperm plug) were obtained from Harlan Sprague Dawley (Indianapolis, IN). Animals were deeply anesthetized with ketamine (67 mg/kg ip) and xylazine (6.7 mg/kg ip). The anterior cervical area was shaved and prepped with betadine ointment, and under blunt dissection, the left external jugular vein was identified. The distal end of the vein was ligated with 4-0 silk suture, and the proximal visible end was cannulated with 22-gauge MRE-040 tubing. The suture was removed, and we confirmed catheter patency by flushing the catheter with 0.25 ml of heparinized saline. The surgical site was closed with 4-0 silk suture. The pregnant rats were monitored and allowed to recover overnight and fed ad libitum. The next morning, animals were administered BSA in normal saline (1 mg), heparin (2–10 units), VLDL (0.5–3.0 mg/ml), or heparin (2–10 units) in combination with VLDL (1 mg) intravenously over 3 h via a Harvard infusion pump. After infusions were terminated, animals were euthanized with phenobarbital (50 mg/kg ip), and fetal rats were delivered by Cesarean section from their dams. The fetal lungs were resected, pooled, and placed in calcium- and magnesium-free Hanks' balanced salt solution (HBSS) and subsequently used to isolate fetal pre-type II alveolar epithelial cells as previously described (33). The purity of isolates was >85% as determined by *Maclura pomifera* lectin binding. With regard to the mice, animals were fed ad libitum and euthanized as above. The tracheas were cannulated with 0.040-diameter plastic tubing and lavaged, and crude alveolar surfactant pellets were isolated as described (42). Lung supernatants were obtained by sequential centrifugation (27). These animal procedures were approved by the University of Iowa Animal Care and Use Committee.

Radiotripalmitination of lipoproteins

VLDL from normal human volunteers (protein concentration, 1.456 mg/ml) were radiolabeled with [^{14}C] tripalmitin as described (34). For triglyceride labeling, 75 μCi of [^{14}C]tripalmitin were dried under nitrogen gas, resuspended in medium containing 4 mg/ml BSA, and sonicated in a water bath for 30 min at 25°C. VLDL particles (2 mg protein) were added to the [^{14}C] tripalmitin solution, incubated at 37°C for 20 min, and then returned to room temperature. The final concentration of radiotripalmitinated VLDL was 75.3×10^6 disintegrations $\cdot\text{min}^{-1}\cdot\text{ng}$ protein $^{-1}$.

Phospholipid and DSPtdCho analysis

Lipids were extracted by the method of Bligh and Dyer (7). Lipids dried under nitrogen gas were spotted on silica LK5D plates and resolved in chloroform/methanol/petroleum ether/acetic acid/boric acid [40:20:30:10:1.8, vol/vol (13)] for resolution of phospholipids or chloroform/methanol/7 M ammonium hydroxide [65:35:5, vol/vol/vol (31)] for DSPtdCho analysis. Samples that comigrated with phospholipid standards as detected by iodine exposure were scraped from the silica gel. In the case of PtdCho, lipids were reacted with osmium tetroxide before a run in the second dimension to isolate DSPtdCho (29). For individual phospholipid determination, cellular PtdCho was not consistently resolved from phosphatidylinositol (PI), and thus these lipids were quantitated and reported together. Phospholipid content was quantitated by a phosphorus assay (9). In selected studies, spots corresponding to PtdCho were scraped from the gel and quantitated directly by scintillation counting.

Enzyme assays

The activities of choline kinase and choline-phosphotransferase were assayed exactly as described (28). We determined the activity of CCT by measuring the rate of incorporation of [methyl-¹⁴C]phosphocholine into CDP-cho-line by a charcoal extraction method (29). Assays were performed without the inclusion of a lipid activator, PtdChooleic acid, in the reaction mixture. All activities were assayed in total cellular lysates.

Immunoblot analysis

For immunoblot analysis, equal amounts of protein from pre-type II cell lysates were used. Each sample was adjusted to give a final solution of 60 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.1% bromphenol blue, and 5% mercaptoethanol, heated at 100°C for 5 min, electrophoresed through a 10% SDS-polyacrylamide gel, and transferred to polyvinylidene difluoride membrane. We probed for CCT or LPL mass with the ECL Western blotting detection system. The dilution factor for anti-CCT and anti-LPL antibodies was 1:1,000.

Fatty acid analysis

Lipids were extracted from cell lysates (1 mg protein) according to the method of Bligh and Dyer (7). Fatty acid methyl ester (FAME) was prepared by transmethylation in the presence of 10% boron trifluoride. The FAME derivatives were separated by gas liquid chromatography and detected by flame ionization. The gas chromatography column packing was 10% SP-2330 on 100/120 Chromosorb W AW (Supelco). The initial column temperature was held at 165°C for 8 min and then increased at a rate of 3°C/min to a final temperature of 210°C, which was maintained for 12 min. We identified individual fatty acids by comparing the retention times with those of known standards. Free fatty acid content was determined by a free fatty acids, half-micro test per the manufacturer's instructions with palmitic acid as a standard. Levels of fatty acids in extracts were within the linear range of testing.

Statistical analysis

The data are expressed as means $\pm$ SE. Unless otherwise stated, statistical analysis was performed by one-way ANOVA with the Bonferroni adjustment for multiple comparisons (41).

RESULTS

Distribution of radiolabeled VLDL

We first determined whether parenterally administered radiolabeled VLDL particles in the mother transfer across the placenta and become distributed systemically in fetal tissues. We were specifically interested in determining whether these lipoproteins accumulate in fetal lung. We also examined whether lipid components contained within lipoproteins serve directly as substrates used for fetal surfactant synthesis. To address this, we gave VLDL particles containing radiolabeled [¹⁴C]tripalmitin to the pregnant dams and measured radioactivity in various maternal and fetal organs and isolated alveolar type II epithelial cells (Fig. 1). In addition, we assayed radioactivity contained within the PtdCho fraction isolated from pre-type II cells, whole lung, and maternal lavage (Fig. 1, *insets*). As shown in Fig. 1, giving dams either radiolabeled VLDL or radiolabeled VLDL in combination with heparin resulted in variable levels of activity that was distributed systemically in both maternal and fetal organs. In the mother, the bulk of radioactivity was recovered in the lung and type II cells (Fig. 1A). This was an anticipated finding because radiolabeled VLDL was given via the external jugular vein. VLDL with heparin significantly increased activity in the lung compared with other maternal whole organs. In the fetus, [¹⁴C]tripalmitin VLDL particles were incorporated in several organs, including the fetal lung, although the highest activity tended to be observed in the fetal

kidney with coadministration of heparin (Fig. 1B). Perhaps more importantly, radiolabeled [^{14}C]tripalmitin particles were incorporated into the PtdCho fraction isolated from maternal and fetal type II cells and whole lung (Fig. 1 and Fig 2 and *insets*). Moreover, [^{14}C]tripalmitin activity was readily detected within the PtdCho fraction isolated from adult surfactant in alveolar lavage (Fig. 1A, *inset*). Collectively, these results provide evidence supporting transplacental distribution of native VLDL or its remnant or lipid components, with distribution to the fetal lung, where they are incorporated into surfactant lipid within fetal type II cells. Some of these effects also appear to be accentuated by heparin.

Phospholipid analysis

The maternal administration of lipoproteins significantly elevated PtdCho and DSPtdCho mass in fetal pre-type II cells. In head-to-head studies with animals receiving saline-BSA, heparin, VLDL, or VLDL plus heparin, VLDL with heparin increased DSPtdCho content by 45% ($P < 0.05$ vs. control). Infusion of VLDL increased DSPtdCho levels by 30%, although these effects did not reach statistical significance (Fig. 2). In comparison, maternal treatment with heparin did not alter DSPtdCho content in the fetus compared with control (Fig. 2). In addition to testing for these effects of VLDL in *day 20* fetuses, we infused lipoproteins in mothers earlier in gestation. Compared with results in gestationally matched controls, VLDL given maternally at *days 17, 18, and 19* of gestation increased DSPtdCho levels by 11, 15, and 78%, respectively. Thus these results suggest that effects of lipoprotein loading on DSPtdCho are most pronounced during the canalicular phase (*days 19–20*) of lung development. VLDL elevated total PtdCho-PI content by ~54%, and it did not change the composition of other major phospholipids (Fig. 2, *inset*), nor did it alter surfactant apoprotein (surfactant protein A) levels in preliminary studies (data not shown). Together, these results indicate that prenatal treatment with VLDL selectively increases DSPtdCho content, the major surfactant lipid. These effects of VLDL are enhanced by concurrent infusion with heparin and are most apparent during the later stages of gestation.

Enzyme assays

We next pursued studies to determine whether maternal VLDL loading increases DSPtdCho content in fetal lung by altering the activity of enzymes of the CDP-choline pathway, the principle pathway for *de novo* synthesis of DSPtdCho. Maternal VLDL treatment increased CCT activity, the rate-limiting enzyme for DSPtdCho synthesis (Fig. 3). The administration of heparin or VLDL with heparin also significantly increased CCT activity. However, maternal VLDL administration increased enzyme activity by nearly three- to fourfold compared with control, and effects were significantly greater than effects of heparin alone or VLDL with heparin ($P < 0.05$). Effects of VLDL were dose dependent, reaching a plateau for maximal enzyme activation at about threefold between 1 and 3 mg of administered lipoprotein (Fig. 4A). Concurrent administration of heparin significantly increased VLDL activation of CCT above control at all doses tested (Fig. 4B). As with DSPtdCho, we tested VLDL effects on CCT activity at other stages of fetal development. VLDL tended to produce stimulatory effects at *day 19* of gestation and significantly increased CCT activity in fetal pre-type II cells when administered to *day 20* dams. VLDL did not alter enzyme activity when given to mothers at *days 17 or 18* of gestation (Fig. 4C). Together, these results indicate that VLDL increases surfactant content by stimulating a key regulatory enzyme required for surfactant synthesis.

Finally, in contrast to effects on CCT, in three separate experiments VLDL particles had no significant effect on activities of choline kinase, the first committed enzyme of the CDP-choline pathway [$49 \pm 17 \text{ pmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ (control) vs. $45 \pm 5 \text{ pmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ (VLDL)], or cholinephosphotransferase, the terminal enzyme of the pathway [$503 \pm 131 \text{ pmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ (control) vs. $664 \pm 204 \text{ pmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ (VLDL)].

Immunoblot analysis

The observation that maternal lipoprotein administration stimulates CCT activity in fetal pre-type II cells led us to investigate whether this is secondary to an increase in the amount of enzyme (Fig. 5A). Overall, the levels of immunoreactive CCT remained unchanged compared with control in fetal pre-type II cells isolated from mothers given heparin or VLDL; VLDL in combination with heparin tended to increase levels of CCT mass, but these effects were not significant (Fig. 5, A and B). In contrast, immunoblot studies revealed that heparin in combination with VLDL increased LPL levels in maternal serum relative to VLDL alone without affecting levels in the fetus (Fig. 5C). These results suggest that VLDL-activation of CCT occurs by posttranslational mechanisms. Heparin, however, appears to regulate LPL activity by increasing its release into serum.

Mouse surfactant analysis

To further test our hypothesis that triglycerides carried within VLDL regulate surfactant synthesis, we assayed alveolar DSPtdCho levels and CCT activity in ApoE and LDLR knockout mice. We compared expression of these parameters with those in wild-type (C57BL/6J) controls. Unlike the wild-type and LDLR knockout mice, the ApoE knockout mice have increased VLDL-triglyceride levels (21,39). The results show nearly two- and three-fold increases in CCT activity in ApoE knockout mice compared with wild-type controls and LDLR knockout mice, respectively (Fig. 6A). In addition, ApoE knockout mice exhibited a similar, approximately twofold increase in levels of alveolar DSPtdCho content compared with mice with normal serum triglyceride levels (Fig. 6B). Overall, these studies suggest that modification of some genes involved in lipoprotein catabolism results in hypertriglyceridemia; these elevated lipids in turn appear to be associated with altered surfactant synthesis.

Fatty acid analysis

Palmitic acid is esterified to the glycerol backbone of DSPdCho, whereas other unsaturated fatty acids are potent activators of CCT (12,30). We determined whether maternal lipoprotein loading increases the availability of specific fatty acid species in pre-type II cells that could be used to synthesize surfactant. Treatment with VLDL and heparin increased the proportion of palmitic (16:0) and stearic (18:0) acids ($P < 0.05$) and tended to increase oleic acid (18:1, $P = 0.09$) compared with saline-BSA (Table 1). However, lipoprotein administration also decreased the relative amounts of the long-chain polyunsaturates, docosahexanoic (22:6) and arachidonic acids (20:4, $P < 0.05$ and $P = 0.07$, respectively). The results suggest that a predominant mechanism by which exogenous VLDL plus heparin increases surfactant lipid is by increasing the relative amounts of saturated species that are incorporated into the DSPdCho molecule. Maternal VLDL loading also produces a modest increase in oleic acid, a potent stimulator of CCT activity. We next extended our fatty acid analysis to wild-type and ApoE knockout mice (Table 2), the latter of which exhibit higher surfactant levels (Fig. 6). We observed that ApoE knockout mice compared with control mice had a significant increase in docosahexanoic (22:6) and arachidonic (20:4) acids vs. control ($P < 0.05$). These polyunsaturates are potent activators of CCT (31). Thus both exogenous VLDL loading in rats and elevated VLDL-triglycerides in mice harboring genetic defects in LDL catabolism exhibit increases in DSPdCho levels that may be due, in part, to the selective induction of key fatty acids required for CCT activation and surfactant lipid synthesis.

Finally, our present studies show that maternal VLDL treatment stimulates CCT activity to a greater extent than VLDL plus heparin (Fig. 3). VLDL with heparin, however, increases DSPtdCho levels more than VLDL alone in pre-type II cells (Fig. 2). One possible explanation for these results is that VLDL increases more free fatty acids that are available for CCT activation. The addition of heparin to VLDL might trigger hydrolysis of triglycerides, also releasing free fatty acids; however, these will be less abundant, resulting in lower CCT activity

but higher phospholipid content as they are rapidly esterified into the DSPtdCho molecule. To test this hypothesis, we assayed free fatty acids in the lung and observed that levels were 5.03 ± 0.9 nmol/mg protein in dams given VLDL alone vs. 2.42 ± 0.64 nmol/mg protein in dams administered VLDL plus heparin ($P < 0.05$, $n = 6$, Student's *t*-test). These results suggest that higher levels of CCT activity induced by VLDL may be attributed to greater availability of free fatty acid activators, whereas after heparin treatment these fatty acids originating from VLDL are promptly acylated as phosphoglycerides resulting in higher DSPtdCho levels.

DISCUSSION

The fate of maternally derived fatty acids with regard to their proposed role in the biogenesis of pulmonary surfactant in the fetus has not been investigated. Although fatty acids stimulate PtdCho synthesis *in vitro*, most circulating fatty acids are complexed to albumin or are present naturally as esterified lipids contained within lipoproteins. Thus in this study we tested whether maternal VLDL loading might regulate surfactant synthesis in fetal lung by elevating fatty acids. Indeed, we observed for the first time maternal-fetal transfer of VLDL components together with induction of surfactant lipids within fetal type II alveolar epithelia. Our radiolabeling studies provide compelling evidence that VLDL-triglycerides are hydrolyzed to fatty acids that in turn are incorporated into the DSPtdCho molecule and activate an important regulatory enzyme required for surfactant lipid synthesis. Studies with genetically altered mice exhibiting the ApoE-deficient phenotype, characterized by elevated circulating VLDL-triglycerides, were also observed to have increased surfactant synthesis. As with *in vivo* VLDL treatment to induce hyperlipidemia in rats, hypertriglyceridemia in mice selectively regulates the expression of key fatty acids in the lung (Table 1 and Table 2). These results suggest that VLDL triglycerides serve as substrates for synthesis of surfactant phospholipid. The results do not rule out VLDL effects on intraalveolar PtdCho clearance.

The current studies using VLDL stem from prior work that attempted to provide lipid administration to neonates or adults with the intent of improving lung function and reducing the severity of lung disease (4,5,14,17,38,43). The underlying hypothesis for this work has been that surfactant-deficient lung disease can be ameliorated by lipid loading, which increases the biosynthesis of DSPtdCho; alternatively, lipid loading might expand the pool of polyunsaturated lipids in the lung, which counteracts the effect of oxidants that induce cellular injury. However, in some of these studies, using free fatty acids or lipid emulsions showed no beneficial effects or was associated with neonatal lung toxicity (14,43). One explanation for these results is that when lipid emulsions are administered alone they may not be effectively delivered (44), may not be selective in increasing PtdCho composition (36), or may undergo oxidation (1,8,32). Furthermore, free fatty acids can also directly induce endothelial and epithelial injury (2).

Although lipoproteins may not ultimately be a realistic therapeutic option to stimulate surfactant synthesis in patients, we pursued a strategy providing lipids prenatally, using VLDL with heparin, to better understand their role in phospholipid metabolism. Structurally, VLDL particles (30–110 nm) contain a unique outer monolayer surface composed of PtdCho with free cholesterol and a major apoprotein (ApoB), which interacts with cell surface receptors. These properties allow for rapid solubility and sufficient access for lipases to act on the hydrophobic triglyceride core. Glycosaminoglycans, such as heparin, bind LPL, displacing it from the endothelium or the placenta. Thus heparin was administered concurrently with VLDL in these studies to promote intravascular LPL-mediated VLDL hydrolysis. The net effect of VLDL catabolism is the liberation of fatty acid substrates that might be utilized by the fetal lung. Presumably during this process, intermediate and low-density lipoprotein remnants are generated as byproducts of VLDL catabolism and are internalized by a receptor-dependent process in extrahepatic fetal or maternal tissues. In this regard, some catabolism probably

occurs in fetal type II cells because they express lipoprotein receptors (45). Because VLDL catabolism is highly regulated at the molecular level, lipoproteins could be administered in this manner without undue cell toxicity. Indeed, we observed that the mothers tolerated VLDL infusions without respiratory distress or gross evidence of pulmonary hemorrhage unlike in prior studies using lipid emulsions (43).

The current results suggest that VLDL transfer from the placenta to the fetus, although the predominant site(s) of lipolysis and pathways for dissemination of fatty acids requires further elucidation. Analysis of radiolabeled VLDL particles revealed that significant radioactivity was detected in the maternal system where levels of circulating LPL increased (Fig. 1 and Fig 5). Thus considerable lipolytic activity and VLDL processing appear to occur rapidly within the maternal compartment after lipoprotein infusions are initiated. After VLDL hydrolysis, the placenta can esterify free fatty acids directly or transfer maternal free fatty acids into fetal circulation by binding them to a placental fatty acid transporter protein (18). In fact, we observed that compared with transplacental delivery of VLDL apoprotein, incorporation of fatty acids into the fetus was relatively small in the fetus compared with the mother, which may be due to maternal-placental lipid catabolism. Fatty acids not utilized by the placenta may then be bound by albumin or α -fetoprotein for delivery to fetal sites (35). Alternatively, there is evidence for placental internalization of native lipoproteins by high-affinity VLDL receptors (19). Once within the fetal circulation, VLDL may be catabolized by fetal lung fibroblasts to triglycerides, as there is evidence for a fibroblast-epithelium lipid transfer process that drives surfactant synthesis (48). Overall, the present data suggest that intact VLDL, VLDL remnants, or complexed fatty acids originating in the mother ultimately transfer to fetal pre-type II cells.

VLDL-derived fatty acids within fetal type II cells can be acylated to lyso-PtdCho, yielding PtdCho or activate CCT, a key enzyme regulated by lipids. The data shown in Fig. 1, that maternal treatment with radiolabeled VLDL-tripalmitin results in activity recovered within the PtdCho fraction in fetal pre-type II cells, suggest that components of VLDL, such as palmitate or diacylglycerol, incorporate into this phospholipid. Because we used [^{14}C]tripalmitin in these studies, it is likely that the ^{14}C incorporation into PtdCho was representative of the surfactant-specific lipid dipalmitoylphosphatidylcholine (or DSPtdCho). Consistent with this result, VLDL with heparin significantly elevated palmitic acid in cells and elevated DSPtdCho mass by 45% compared with control (Fig. 2, Table 1). Although on the surface these changes appear to be modest, they are indeed significant, given the in vivo model system used and the fact that PtdCho content is tightly regulated within eukaryotic cells (49). The magnitude of changes in DSPtdCho mass in response to VLDL loading is in line with changes in cellular phospholipid content observed in in vitro systems (28,46,49,50). An interesting result from our work indicates that VLDL with heparin (7.5 units) is more effective in increasing DSPtdCho mass but less potently activates CCT compared with VLDL alone (Fig. 2 and Fig 3). However, cellular free fatty acid levels were lower in the VLDL-plus-heparin treatment group compared with the VLDL group, indicating that the addition of heparin liberates fatty acid species, such as palmitate (16:0), that rapidly incorporate into DSPtdCho thereby increasing its content (Table 1). In the absence of heparin, we observed higher levels of cellular CCT activity after maternal VLDL administration because more free fatty acids are available for enzyme activation. However, importantly, the effect of VLDL alone on the downstream product (i.e., DSPtdCho mass) was less than in combination with heparin, suggesting control at other metabolic check points that independently regulate DSPtdCho levels. Another possibility is that there may be differences in the kinetics of CCT activation and resultant DSPtdCho synthesis from the time of administration to analysis between the various groups that could partly explain our results.

Finally, in addition to providing exogenously administered VLDL to stimulate PtdCho synthesis, we examined whether modification of genes involved in lipoprotein catabolism in

mice also results in altered surfactant metabolism. Mice lacking functional LDL receptors exhibit a marked increase in serum LDL cholesterol with normal triglycerides, whereas mice defective in ApoE have increased levels of both VLDL-cholesterol and triglycerides (21,39). Thus these animal models were selected to test primarily whether stimulatory effects of VLDL on the surfactant system are driven by specific circulating lipids. Our results showing an increase in CCT activity, coupled with elevated alveolar DSPtdCho content in ApoE knockout mice compared with mice with normal circulating levels of triglycerides, support our hypothesis that VLDL-triglycerides, rather than cholesterol, regulate surfactant synthesis in vivo. Our results contrast with work by Hass and Longmore (16) demonstrating a precursor-product role for serum cholesterol and surfactant composition, and with studies by Davidson et al. (11), in which pharmacological inhibition of cholesterol synthesis impaired surfactant synthesis. Additional work using murine model systems with targeted disruption of genes involved in LDL or VLDL catabolism may help determine how these lipids are internalized and utilized for enzyme activation vs. incorporation into PtdCho in type II cells. The results might prove to be useful in devising lipid-based replacement therapies for surfactant-deficient lung disease.

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Fig. 1.

Distribution of maternally administered ^{14}C tripalmitinated very low-density lipoprotein (VLDL) particles into the mother and fetus. ^{14}C tripalmitinated VLDL particles were generated as described (20). Pregnant rats were administered intravenously [^{14}C]VLDL (75.3×10^6 disintegrations $\cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$) on day 20 of gestation with or without heparin (7.5 U) via an external jugular vein catheter over 3 h. Incorporation of ^{14}C VLDL into maternal (A) and fetal organs (B) and alveolar type II epithelial cells was then determined after isolation, homogenization, and scintillation counting. In addition, incorporation of radioactivity into phosphatidylcholine (PC) within type II cells, lavage, and whole lung was also assayed (*insets*). Values were corrected for background and normalized to protein concentration. The data are expressed as disintegrations per minute per milligram protein and as the means $\pm$ SE of 4 independent experiments. * $P < 0.05$ vs. other organs, by ANOVA.

Fig. 2.

Effect of VLDL administered to pregnant dams on phospholipids and disaturated phosphatidylcholine (DSPtdCho) content in fetal alveolar pre-type II epithelial cells. Pregnant rats (*day 20* of gestation) were administered BSA (1 mg) in normal saline, heparin (7.5 units), VLDL (1.0 mg), or heparin (7.5 units) in combination with VLDL (1 mg) intravenously. After infusions, animals were euthanized, and fetal pre-type II alveolar epithelial cells were isolated from fetuses. *Inset*: individual phospholipid content, of phosphatidylinositol (PI), phosphatidylglycerol (PG), phosphatidylethanolamine (PE), phosphatidylserine (PS), sphingomyelin (SM), and DSPtdCho, was determined after lipid extraction from cell lysates, separation by TLC, and quantification by the phosphorus assay. The data are expressed as means $\pm$ SE from 6 independent experiments. Each experiment consisted of pre-type II cells isolated from 1 pregnant rat/group. * $P < 0.05$ VLDL-heparin group vs. saline-BSA group, by ANOVA.

Fig. 3.

Effect of maternally administered VLDL on cytidylyltransferase (CCT) activity in fetal alveolar pre-type II epithelial cells. Pregnant rats (*day 20* of gestation) were administered BSA (1 mg) in normal saline, heparin (7.5 units), VLDL (1.0 mg), or heparin (7.5 units) in combination with VLDL (1 mg) intravenously. After infusions, animals were euthanized, and fetal pre-type II alveolar epithelial cells were isolated from fetuses. Assays for CCT activity were conducted in the absence of exogenous lipid in the reaction mixture. The data are expressed as means $\pm$ SE from 4 independent experiments. Each experiment consisted of 4 pregnant rats/group. * $P < 0.001$ vs. saline-BSA and + $P < 0.05$ vs. heparin or VLDL-heparin groups as determined by ANOVA.

Fig. 4.

Effects of VLDL, heparin, and gestation on ability of maternally administered VLDL to stimulate CCT activity. Pregnant rats (*day 20* of gestation) were administered VLDL (0.33–3.0 mg, *A*) or VLDL (1 mg, *B*) in combination with heparin (2–10 units) intravenously over 3 h. *C*: pregnant rats (*days 17–20* of gestation) were administered VLDL (1.0 mg) as above. After infusions, animals were euthanized, and fetal pre-type II alveolar epithelial cells were isolated from fetuses. Assays for CCT activity were conducted in the absence of exogenous lipid in the reaction mixture. The data are expressed as fold increase in CCT specific activity observed above control (saline-BSA) values. Each data point in each panel represents the mean $\pm$ SE from 3–4 independent experiments. *A*: $P < 0.05$ for 0.5- and 2-mg doses vs. saline-BSA control and $P \pm 0.001$ for 1- and 3-mg doses vs. saline-BSA control. *B*: $*P \leq 0.01$ for 2, 5, and 10 units of heparin vs. saline-BSA and $^+P = 0.05$ for 7.5 units heparin vs. saline-BSA. *C*: $*P < 0.05$ for *day 20* of gestation vs. gestationally matched saline-BSA control and *day 17–18* of gestation. Statistical analysis was performed by ANOVA.

Fig. 5.

Immunoblot analysis of CCT protein in fetal pre-type II alveolar epithelial cells. **A:** pregnant rats (*day 20* of gestation) were administered BSA in normal saline (1 mg), heparin (7.5 units), VLDL (1.0 mg), or heparin (7.5 units) in combination with VLDL (1 mg) intravenously. Fetal pre-type II alveolar epithelial cells were isolated from fetuses as described above. Cell lysates (300 μ g protein) were separated by SDS-10% polyacrylamide gel electrophoresis, transferred to nitrocellulose, and probed with an anti-CCT rabbit polyclonal antibody. **B:** densitometric analysis of the immunoblots showing means $\pm$ SE of arbitrary units from 4 independent experiments. **C:** similar methods were used to detect the immunoreactive content of lipoprotein lipase (LPL) in maternal and fetal serum after intravenous infusion of VLDL (1.0 mg) or heparin (7.5 units) in combination with VLDL. A polyclonal antibody against LPL generated in the chicken-against-bovine LPL antigen was used to detect the protein. The far *left* lane of each immunoblot shows purified rat liver CCT (42 kDa, **A**) or LPL (55 kDa, **C**). The data in **C** are representative of 3 independent experiments. S, saline; H, heparin; V, very low-density lipoprotein.

Fig. 6.

Surfactant synthesis in genetically altered mice. C57BL/6J mice (wild-type), low-density lipoprotein receptor knockout mice (LDLR KO), and apoprotein E knockout mice (ApoE KO) were euthanized and lavaged. *A*: whole lung supernatants were isolated for analysis of CCT activity. *B*: alveolar DSPtdCho content was determined after lipid extraction of lavage pellets, separation by TLC, and quantification by the phosphorus assay. Data in *A* are expressed as means $\pm$ SE with 3 mice/group. * $P = 0.05$ for LDLR KO vs. C57BL/6J groups and ** $P < 0.01$ in ApoE KO vs. C57BL/6J groups, by ANOVA. Data in *B* are expressed as means $\pm$ SE with 5 mice/group, except LDLR ($n = 3$ mice). * $P < 0.05$ ApoE KO vs. C57BL/6J groups.

Table 1
Effect of maternally administered VLDL particles on fatty acid composition in fetal alveolar type II epithelial cells

Condition	14:0	16:0	16:1	18:0	18:1	18:2	20:4	22:6
Saline-BSA	1.19 ± 0.3	28.94 ± 2.1	3.45 ± 0.7	15.52 ± 0.5	19.70 ± 0.8	9.78 ± 0.4	16.70 ± 1.2	4.68 ± 0.7
VLDL+heparin	1.47 ± 0.3	34.52 ± 0.8	3.84 ± 0.1	17.66 ± 0.1	24.38 ± 0.8	8.22 ± 0.3	8.77 ± 1.4	1.10 ± 0.3
P value	NS	<0.05	NS	<0.05	0.09	NS	0.07	<0.05

Data are expressed as means ± SE of percentage of total fatty acid composition. Pregnant rats (*day* 20 of gestation) were intravenously administered saline-BSA or very low-density lipoprotein (VLDL, 1 mg protein) with heparin (7.5 U) over 3 h. Fetal alveolar type II epithelial cells were isolated and lipids extracted from 1 mg of cellular protein. After generation of fatty acid methyl esters, fatty acid composition was determined by gas liquid chromatography. Data are representative of 3 separate experiments. Each experiment consisted of type II cells isolated from 1 control and 1 VLDL-heparin-treated pregnant rat. Statistical analysis was performed using the Student's *t*-test. NS, not significant.

Table 2

Fatty acid composition in mice

Condition	14:0	16:0	16:1	18:0	18:1	18:2	20:4	22:6
C57BL/6J	0.21 ± 0.02	37.7 ± 0.87	3.93 ± 0.34	12.79 ± 0.56	16.86 ± 0.3	15.50 ± 0.4	7.12 ± 0.21	5.85 ± 0.63
ApoE KO	0.19 ± 0.02	35.3 ± 0.57	3.73 ± 0.07	13.17 ± 0.21	16.53 ± 0.7	14.45 ± 0.4	8.07 ± 0.17	8.49 ± 0.53
P value	NS	NS	NS	NS	NS	NS	<0.05	<0.05

Data are expressed as means ± SE of percentage of total fatty acid composition. C57BL/6J mice (wild-type) and apolipoprotein E knockout mice (ApoE KO) were euthanized. Lung homogenates were isolated and lipids extracted from 1 mg of cellular protein. After generation of fatty acid methyl esters, fatty acid composition was determined by gas liquid chromatography. Data are representative of 3 separate experiments. Each experiment consisted of 1 control and 1 ApoE KO mouse. Statistical analysis was performed using the Student's *t*-test.