DURING THE LAST TRIMESTER OF PREGNANCY, there is a high fetal demand for fatty acids (FA), as they are critical for normal neural and vascular development. The fetus is able to synthesize saturated and monounsaturated FAs from glucose and ketone bodies (35) but depends entirely on placental transport for its supply of the essential fatty acids (EFA) linoleic and α-linolenic acid. The EFAs and their derivatives the long-chain polyunsaturated free fatty acids (LC-PUFAs) arachidonic (ARA) and docosahexaenoic (DHA) are essential constituents of membranes, act as precursors of cellular signaling molecules, and are particularly important for the developing brain and retina (16).

Triglycerides (TG) are transported in the bloodstream as lipoproteins (22). TGs must be hydrolyzed into free fatty acids (FFA) before they can be transferred across the syncytiotrophoblast of the human placenta. This transporting epithelium is a multinucleated syncytium, polarized with the apical or microvillous plasma membrane (MVM), bathed in maternal blood, and the basal plasma membrane (BM), oriented toward the fetal capillary. At least two types of TG hydrolases have been identified in MVM, a placenta-specific hydrolase and lipoprotein lipase (LPL) (58, 59). LPL has been suggested to be the most important hydrolase in the initial step for transfer of TG-derived FA, as the placenta-specific TG hydrolase is inhibited by serum (57, 58, 59). Subsequent to TG hydrolysis, the FFA are available for transport, and once they reach the syncytiotrophoblast they are bound to intracellular cardiac or liver fatty acid binding proteins (C- and L-FABPs) (11). Thereafter, the FFA are guided to various intracellular sites for esterification, β-oxidation, and prostaglandin production or they are directly transported to the fetal circulation (14) by facilitated diffusion across the BM or through production of lipoproteins in the syncytiotrophoblast (40), which are subsequently secreted into fetal blood.

In other tissues, such as adipose, skeletal, and heart muscle, LPL is synthesized by underlying parenchymal cells and then transported to the luminal surface of the capillary endothelium. LPL is anchored to heparan sulfate proteoglycans (HSPG) (24) and is easily displaced by heparin (3). LPL is regulated in a complex manner, either at the transcriptional or at the posttranscriptional level, and mechanisms of LPL regulation include effects on mRNA stability, glycosylation, interaction with activating proteins (apo-CII), and monomer/dimer activation (5, 19, 43). LPL is regulated in a tissue-specific manner by fasting, feeding, and insulin (38), and alterations in LPL activity appear to correlate with local tissue requirements of FFA (22). FFA in excess have been shown to lower LPL activity due to competition with the LPL/HSPG-binding site (54), whereas in mice FFA and TG deficiency results in a higher LPL activity in adipose and muscle tissue (26). However, mechanisms responsible for placental LPL regulation remain unclear (9, 43).
We have previously demonstrated that LPL activity in the MVM was upregulated in pregnancies complicated by type 1 diabetes and associated with fetal overgrowth, whereas a decreased placent al LPL activity was observed in preterm pregnancies complicated by intrauterine growth restriction (IUGR) (41). These data are compatible with the hypothesis that alterations in placental LPL activity contribute to changes in FFA delivery to the fetus and, as a consequence, fetal growth (41). Therefore, information on the mechanisms of regulation of placental LPL will advance our understanding of the pathophysiology of common pregnancy complications.

Insulin, TG, and FFA have been shown to regulate LPL in other tissues and are altered in pregnancies complicated by diabetes (34, 45, 47, 50, 53) and IUGR (13, 18, 49, 52), respectively. Therefore, the objective of the present study was to investigate the effect of insulin, TG, and FFA on LPL activity and expression in cytotrophoblast cells isolated from human term placenta of uncomplicated pregnancies. LPL activity and expression in placental tissue from a patient suffering from LPL deficiency and giving birth to normal sized healthy babies near term was also investigated.

MATERIALS AND METHODS

Materials. The monoclonal LPL antibody (SD2) was kindly provided by Dr. John Brunzell (University of Washington, Seattle, WA). The L-FABP monoclonal antibody (Abcam, Cambridge, UK) cross-reacts with human and rat L-FABP. All chemicals were purchased from Sigma, unless otherwise noted.

LPL-negative patient. A 36-yr-old woman with primary LPL deficiency was followed rigorously during two pregnancies and was delivered at 36 wk of gestation by cesarean section in both. The condition of this patient has been described in detail (21) and represents typical chylomicronemia due to lack of significant extracellular LPL activity. The LPL defect in this patient has been characterized as a compound heterozygote carrying one allele that does not produce LPL mRNA and another that results in synthesis of a catalytically active but defectively transported form of LPL (21).

Tissue collection. The collection of human placental tissue was approved by the Göteborg University Committee for Research Ethics and conducted with informed consent. Villous tissue was dissected from the placenta and rinsed in physiological saline before further processing.

MVM preparation. MVM vesicles were prepared according to methods previously described (31), with minor modifications (41) and stored at -80°C until use. Alkaline phosphatase activity 10-fold greater than homogenate was used as an enrichment marker for MVM. Protein concentrations were determined by the Bradford method (8).

Isolation of cytotrophoblasts. Isolation of cytotrophoblasts from term human placenta was carried out using a method described previously (32). Ten micrograms of MVM protein per minute or picomoles per milligram wet weight per minute of Intralipid-supplemented cell culture medium. Cell culture medium was supplemented with linoleic acid, oleic acid, or a combination of linoleic, oleic, and arachidonic acid (100, 200, or 400 μM) containing 100 mg/ml bovine serum albumin (BSA). The supplemented medium was added to cells 18 h after plating and incubated for 24 h. Control cells were incubated in medium containing an equivalent volume of 100 μg/ml FA-free BSA. All cultures were quickly and carefully washed twice with standard cell culture medium before harvesting of cells.

Inulin-supplemented cell culture medium. At 24 and 3 h prior to LPL assay, cell cultures were incubated with insulin (final concentrations 36.25, 145, or 290 ng/ml)-supplemented cell culture medium. The insulin was dissolved in a stock buffer consisting of 0.1% BSA and 1.6% glycerol in sterile water. Control cells were given an equal amount of insulin stock buffer. The LPL activity was determined after 90 h in culture.

LPL activity measurement. The activity of LPL was measured by hydrolysis of [14C]trioleate incorporated into Intralipid micelles as described previously (42, 58, 59), with minor modifications. The LPL assay solution is optimized at pH 8 and contains 0.5 mg/ml FA-free BSA (dissolved in 10 mM Tris·HCl, pH 7.0, 1.0 mM glycerol trioleate, 0.1% blocking reagent) (9, 10, 11), and 7.5% fetal calf serum. Cytotrophoblasts (150 μg total protein) were combined with the assay buffer and incubated for 60 min at 37°C. LPL activity in fresh villous tissue was measured after heparin release, as described in detail previously (42). Termination of the reaction (for both cytotrophoblasts and villous tissue) was accomplished by the addition of 3.25 ml of stop solution (methanol-chloroform-heptane, 1:4:1.25:1.0 vol/vol). One milliliter of borate-carbonate (pH 10.2) was added, and the samples were forcefully vortexed for 30 s and centrifuged at 2,200 rpm at 4°C for 20 min. The upper aqueous phase was removed and transferred to scintillation vials for counting. Individual standard curves were made for all assays. LPL activity is expressed as picomoles mono(9,10-n)-[14C]trioleate released per milligram of total cell protein per minute or picomoles per milligram wet weight per minute for fresh villous tissue.

Western blotting. Western blotting was performed according to a previously published protocol (32). Ten micrograms of MVM protein or 15 μg of cytotrophoblast cell homogenate protein was loaded and separated on a 10% SDS-PAGE for LPL detection. Rat cardiac muscle served as a positive control. For L-FABP, 40 μg of total protein from placental homogenates were loaded onto a 12% SDS-PAGE. Rat liver was used as a positive control. Primary antibodies (5D2, 1:250, or L-FABP, 1:100) were incubated for 1 h and peroxidase-labeled horse-anti-mouse IgG (1:1,000) for 1 h. The immunolabeling was made visible using ECL detection (Amersham Biosciences).

Quantification of mRNA expression. Total RNA was extracted using RNA STAT-60 (Tel-Test, Friendswood, TX) according to the
RESULTS

Lipid status and placental LPL activity and protein and mRNA expression in the LPL-deficient mother. During both pregnancies, the patient was placed on a fat-restricted diet with additional ω-3 fish oil tablets. The clinical management of the first pregnancy also included plasmaphoresis performed repeatedly to reduce the plasma TG levels, which varied between 820 and 4,200 mg/dl. This resulted in a transient reduction in TG levels. During the last four months of the second pregnancy TG values varied between 2,000 and 9,000 mg/dl.

The results from the placentas from the LPL-deficient mother and term controls are given in Table 1. The LPL activity was fourfold lower in villous tissue from the first placenta of the LPL-deficient mother and 50% lower in the second placenta compared with mean LPL activity in control placentas at term (n = 7). The placental expression of LPL protein in MVM was 50% lower in the LPL-deficient mother than in term control placentas (n = 6). One band at 37 kDa was detected in Western blot, and the results are shown in Table 1. The L-FABP expression in placental homogenates was 23% lower in the LPL-deficient mother compared with controls (n = 8). The protein migrated at 15 kDa, as previously described (41). When the protein expression data were being analyzed, the values for the control samples were arbitrarily assigned a density value of 1 to simplify comparisons between groups. The mRNA expression was threefold higher in the placenta from the LPL-deficient mother compared with normal term controls (n = 4).

LPL activity and protein and mRNA expression in cytotrophoblasts. No detectable LPL activity was measured in the cell culture medium when randomly tested in all treatment groups (data not shown).

Assay efficiency for LPL activity in cytotrophoblasts. The efficiency of oleic acid extraction for all LPL assays performed was 71.9 ± 0.27% (n = 663, total no. of test tubes) and the intra-assay coefficient of variation was 0.98 ± 0.06%. The coefficient of variation for interassay efficiency was 5.92%.

Time in culture. LPL activity was present at all of the times. No significant difference in LPL activity was observed between the time points (Fig. 1, n = 6, except at 90 h n = 5). We measured a significant rise in human chorionic gonadotropin production, a marker for syncytialization, between 48 and 66 h in culture (data not shown). One band at 37 kDa was detected by Western blot for LPL. No significant differences were detected in LPL protein expression (18 h: 1.00 ± 0.11; 42 h: 0.93 ± 0.05; 66 h: 0.97 ± 0.10; 90 h: 0.80 ± 0.04; 18 h was arbitrarily assigned a value of 1; n = 6 for all groups) by Western blot or in LPL mRNA expression (18 h: 0.56 ± 0.20; 42 h: 0.32 ± 0.15; 66 h: 0.24 ± 0.11; 90 h: 0.31 ± 0.12; relative values, n = 6) by real-time PCR.

Intralipid treatment. Cytotrophoblast cells incubated in 40 and 400 mg/dl Intralipid for 24 h showed a 28–39% decrease in LPL activity compared with control (n = 10–14, P < 0.05, one-way ANOVA; Fig. 2A). LPL protein was observed as a single band at 37 kDa, and a 16% higher expression was seen after incubation with 400 mg/dl Intralipid compared with control (n = 6, P < 0.05, t-test; Fig. 2B). LPL mRNA expression was measured after 24-h Intralipid incubation, but no significant differences were detected compared with controls (control: 0.74 ± 0.19; 4 mg/ml: 0.30 ± 0.13; 40 mg/ml: 0.18 ± 0.04; 400 mg/ml: 1.03 ± 0.45; control n = 10, Intralipid-treated groups n = 5).

FFA treatment. Incubation with 400 μM linoleic acid and 400 μM combined linoleic-oleic-arachidonic acid for 24 h reduced LPL activity by 37 and 34%, respectively (n = 10, P < 0.05, repeated-measures ANOVA; Fig. 3). LPL protein

Table 1. LPL activity and expression in fresh villous tissue from LPL-deficient vs. normal-term women

<table>
<thead>
<tr>
<th></th>
<th>LPL Activity, pmol/ mg wet wt/min</th>
<th>LPL Protein Expression</th>
<th>LPL mRNA Expression</th>
<th>L-FABP Expression</th>
</tr>
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<tbody>
<tr>
<td>LPL-deficient</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mother</td>
<td>1) 0.77</td>
<td>0.49</td>
<td>3.01</td>
<td>0.75</td>
</tr>
<tr>
<td>Normal-term</td>
<td>2) 1.55</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n = 7</td>
<td>n = 6</td>
<td>n = 4</td>
<td>n = 8</td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>3.06</td>
<td>1.00</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>95% CI</td>
<td>2.36–3.75</td>
<td>0.86–1.14</td>
<td>0.53–1.42</td>
<td>0.67–1.33</td>
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</table>

Data in the control group are presented as means and 95% confidence interval (CI). Lipoprotein lipase (LPL) activity was assessed in placentas from 2 pregnancies of the same LPL-deficient mother. L-FABP, liver fatty acid-binding protein. When protein and RNA expression data were analyzed, normal-term values were arbitrarily assigned a density value of 1 to simplify comparisons between groups.
was detected as a single band at 37 kDa, and no significant alterations in LPL protein expression (control: 1.00 ± 0.08; 400 μM linoleic acid: 0.98 ± 0.10; 400 μM linoleic-oleic-arachidonic acid: 0.86 ± 0.02; 400 μM oleic acid: 1.03 ± 0.09; the control group was arbitrarily assigned a value of 1; n = 6 for all groups) were observed after 24 h of incubation with FFA.

**Insulin treatment.** We found no change in LPL activity or protein expression after 3 or 24 h of incubation with various concentrations of insulin (Table 2) in the trophoblast cultures. Similarly, no effect was found on LPL protein expression after 24 h of incubation (control: 1.00 ± 0.11; 290 ng/ml insulin: 0.87 ± 0.13; n = 4).

**DISCUSSION**

There are several novel findings in the present study. First, we found that LPL activity was reduced in placenta of an LPL-deficient mother with hypertriglyceridemia in pregnancy, as expected if the developing fetus has inherited the nonfunctional allele. Furthermore, we demonstrate that TG and/or FFA may be regulators of human placental LPL activity. We show for the first time that LPL is expressed and active in cultured cytotrophoblast cells. These findings strongly support that placental LPL is produced by the trophoblast cells.

Lipases in the apical membrane of the placenta provide a mechanism for release of FFA from maternal lipoproteins. Placental LPL prefers hydrolyzing circulating TG-containing FFA, which are more unsaturated (48). In the third trimester of pregnancy, maternal plasma LC-PUFAs are mainly esterified and associated with circulating lipoproteins rather than bound to albumin (27). Therefore, the major source of LC-PUFAs transported to the fetus is derived from maternal circulating lipoproteins, further supporting the importance of LPL in placental lipid transport and fetal LC-PUFA supply.

In an extreme example of hypertriglyceridemia experienced by a pregnant woman with nonfunctional extracellular LPL, two babies (different pregnancies) of normal weight were delivered near term. We demonstrated that the activity and protein expression of placental LPL were markedly reduced and mRNA expression was threefold higher compared with control placentas at term. In a previously published paper, this patient was carefully characterized and found to possess one allele that probably does not produce LPL mRNA and another that results in synthesis of a catalytically active but defectively transported form of LPL (21). The fetuses and placentas are therefore likely to be heterozygotic for LPL if the mutations in the mother did not affect only somatic cells. Our data suggest that the reduction in placental LPL activity is likely...
due to inheritance of one functional allele from the father and one defective allele from the mother. In one case, the placenta LPL activity met the expected 50% reduction, whereas in the other it was lower than predicted (25% of control). Protein expression was in the expected range predicted by an obligate heterozygote. We also found that the expression of L-FABP was decreased, suggesting a reduction in FFA handling capacity in the cytoplasm of the syncytiotrophoblast. Whether this is a regulatory step to reduce transfer of FFA to the fetus or in response to the reduction in LPL activity is currently unknown.

The LPL-deficient patient in our study gave birth to babies of normal birth weight, in agreement with previously reported pregnancies (1, 33, 56), indicating that placental FFA transfer was sufficient to allow for normal fetal growth. Placental FFA transfer in the LPL-negative patient may have been maintained in two ways despite a markedly decreased MVM LPL activity. In addition to LPL, placenta-specific lipase (58, 59) provides an additional mechanism for hydrolysis of TG to release FFA. The protein has not been cloned, and little is known about its source, regulation, or in vivo function. Since the pH optimum of the placental specific lipase is 6.0, the activity of this lipase was not assessed in our experiments. In a recent study, Lindegaard et al. (37) demonstrated mRNA and protein expression of endothelial lipase (EL) in syncytiotrophoblasts and mainly in endothelial cells along with LPL in placental syncytiotrophoblasts. In other tissues, EL mainly hydrolyzes phospholipids in high-density lipoproteins (23). A study in mice showed that EL can compensate for a lack of LPL in adipose tissue and mediates some of the FFA taken up by the tissue (39). Indeed, a stronger expression of EL was detected in the placenta from LPL-deficient mice (37). EL may be upregulated in the placenta of the LPL-deficient patient.

The source of placental LPL has not been definitively established. LPL production by fetal macrophages has been suggested (7), and recently, using in situ hybridization, LPL RNA was found in trophoblast cells (37). To our knowledge, this study is the first to demonstrate LPL activity and protein and mRNA expression in isolated cytotrophoblasts. The activity and expression were relatively stable over 90 h in culture. These data suggest that both cytotrophoblasts and syncytiotrophoblasts are able to synthesize LPL. In adipose and muscle, LPL is produced in underlying parenchymal cells and is transported, by mechanisms that are not completely clear, to the capillary endothelial surface where it is bound to HSPG and has its primary function. The mechanism in which LPL is translocated to MVM in syncytiotrophoblast is not clear and the syncytial nature of this epithelium, lacking intercellular spaces, would make transfer of LPL from underlying cytotrophoblasts a complex process. Furthermore, the number of cytotrophoblasts present in the placenta decreases, and the syncytiotrophoblast mass increases continuously during the second half of pregnancy, a time when placental LPL expression and activity increases (29, 42). Therefore, we propose that the syncytiotrophoblast is the primary source of placental LPL.

To test the hypothesis that maternal lipid status regulates placental LPL activity, we isolated cytotrophoblasts and measured LPL activity and expression in response to incubation with TG, FFA, or insulin. We found that LPL activity was significantly inhibited by incubation with 40 or 400 mg/dl Intralipid, concentrations that are within the physiological range in late pregnancy (204–265 mg/dl) (2, 10, 30). Intralipid was chosen as a VLDL/chylomicron substitute, as it contains a good source of EFAs (61%). The finding that physiological levels of TG are inhibitory to LPL activity was not expected. We predicted that pathological levels as were seen in the LPL-deficient patient would be inhibitory. Because residual Intralipid associated with the cells would alter the results, we carefully monitored LPL activity after short incubations with Intralipid to ensure that an adequate washing procedure was developed. However, the possibility remains that residual Intralipid alters the findings of the assay. Another possible explanation is that LPL was eluted from the cells and bound to Intralipid during the 24-h incubation. We did not test the medium after Intralipid incubation for LPL activity. However, control medium was periodically tested and no LPL activity was detected.

We were interested in whether FFA alone or in combination would be sufficient to alter LPL activity in cultured trophoblast cells. We found an inhibiting effect of 400 μM FFA. The total plasma FFA content in late pregnancy is in the range of 300–600 μM (30, 55). In other tissues LPL is known to respond to rapid changes in nutrient status and is regulated by fasting/feeding and exercise/rest in muscle and adipose tissues (4, 20, 38, 51). Much of this appears to be due to differential responses to metabolic hormones such as insulin, cortisol, and epinephrine (9, 19). In a recent study (42), we demonstrated that hyperglycemic medium together with insulin increased LPL activity in freshly isolated placental villous tissue after 3 h of incubation. In the current study, we found no effect of insulin on LPL activity or expression in cultured trophoblasts. Whether this was due to the fact that placental LPL does not respond to insulin or to the relatively low insulin receptor number in late pregnancy (15) is not clear. In a recent study in human placental choriocarcinoma (BeWo) cells where cells were incubated with various FFA and insulin, no insulin effect on FFA uptake was seen (17).

As LPL is a key enzyme in lipoprotein metabolism, it has been implicated in many pathological conditions involving hypertriglycerideremia, including chylomicronemia, obesity, insulin resistance, and diabetes (43). Any of these conditions during pregnancy, which is normally associated with insulin resistance and a fourfold increase in maternal plasma TG (28, 29), could lead to pathological plasma lipid concentrations. Obesity is strongly correlated with elevated TG and VLDL in pregnancy, both in the mother and in the macrosomic newborn (46). A better understanding of the mechanisms whereby placental lipid transfer is regulated in pregnancy is critically needed.

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