Endothelial and lipoprotein lipases in human and mouse placenta

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Abstract Placenta expresses various lipase activities. However, a detailed characterization of the involved genes and proteins is lacking. In this study, we compared the expression of endothelial lipase (EL) and LPL in human term placenta. When placent al protein extracts were separated by heparin-Sepharose affinity chromatography, the EL protein eluted as a single peak without detectable phospholipid or triglyceride (TG) lipase activity. The major portion of LPL protein eluted slightly after EL. This peak also had no lipase activity and most likely contained monomeric LPL. Fractions eluting at a higher NaCl concentration contained small amounts of LPL protein (most likely dimeric LPL) and had substantial TG lipase activity. In situ hybridization studies showed EL mRNA expression in syncytiotrophoblasts and endothelial cells and LPL mRNA in syncytiotrophoblasts. In contrast, immunohistochemistry showed EL and LPL protein associated with both cell types. In mouse placentas, lack of LPL expression resulted in increased EL mRNA expression. These results suggest that the cellular expression of EL and LPL in human placenta is different. Nevertheless, the two lipases might have overlapping functions in the mouse placenta. Our data also suggest that the major portions of both proteins are stored in an inactive form in human term placenta.—Lindegaard, M. L. S., G. Olivecrona, C. Christoffersen, D. Kratky, J. Hannibal, B. L. Petersen, R. Zechner, P. Damm, and L. B. Nielsen. Endothelial and lipoprotein lipases in human and mouse placenta. J. Lipid Res. 2005. 46: 2339–2346.

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Placental uptake of fatty acids is essential for the growth and development of both placenta and fetus. FFAs are derived from FFAs bound to albumin or from lipoprotein-associated triglycerides (TGs) and phospholipids. The transport of FFA from maternal blood across the microvillous and basal membranes into the fetal circulation involves fatty acid binding and transfer proteins (fatty acid binding proteins, fatty acid translocase/CD36, and fatty acid transfer protein) (1). In the cytosol of the syncytiotrophoblasts, FFAs may be reesterified, deposited as TGs, and hydrolyzed for later release into the fetal circulation (2).

Although albumin-bound FFAs probably can be transferred directly to the placental membrane-fatty acid binding protein, the release of FFAs from lipoprotein-TGs and phospholipids requires hydrolysis in the maternal circulation. LPL hydrolyzes lipoprotein-TGs (3). Placental microvillous membrane (4), isolated trophoblasts, and macrophages (5) all display LPL TG lipase activity. Human placental tissue also expresses TG lipase activities that are distinct from that of LPL, although the cells and proteins involved have not been identified (4). Like TGs, phospholipids are also hydrolyzed, taken up, and resynthesized in placenta (6). The extracellular phospholipase A_2 type II is responsible for 60–80% of placental phospholipase activity (7) and is widely expressed in placenta (i.e., in vascular smooth muscle cells, endothelial cells, and trophoblasts) (8).

In 1999, endothelial lipase (EL) was discovered as a new member of the TG lipase family, which also includes LPL, pancreatic lipase, and hepatic lipase (9, 10). EL is expressed in vascular endothelial cells and mainly hydrolyzes phospholipids in HDL, but it has also some TG lipase activity (11). In contrast, LPL mainly hydrolyzes TGs in chylomicrons and VLDL. The protein sequence of EL is 44% homologous to that of LPL, and the functional domains, including the catalytic residues, heparin binding sites, and cysteines involved in disulfide bridge formation, are preserved (9, 10).

The role of EL in plasma HDL metabolism has gained widespread attention in recent years. Several in vitro and in vivo studies have demonstrated a role for EL in HDL metabolism, including the regulation of cholesterol efflux from macrophages and the promotion of reverse cholesterol transport.

Abbreviations: DIG, digoxigenin; EL, endothelial lipase; TG, triglyceride.

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vivo studies have contributed to the notion of an inverse relationship between EL activity and plasma HDL concentration (12). This effect is mediated both by lipolysis of phospholipids and by bridging HDL to heparan sulfate proteoglycans to facilitate selective cholesteryl ester uptake and lipoprotein holoparticle internalization (11). Although multiple tissue mRNA analyses have shown that human placenta is among the tissues with the highest expression of EL (9, 10), the biology of EL in placenta has not been studied to date. To improve the understanding of the putative roles of EL and LPL in placenta, we have characterized the expression of EL and LPL mRNA, protein, and lipase activities in human term placenta. Also, by studying genetically modified mice, we tested the idea that placental EL expression is increased when LPL expression is eliminated.

MATERIALS AND METHODS

Tissue samples

Biopsies (0.2–0.6 g) from full-term placentas delivered vaginally or by elective cesarean section were snap-frozen in liquid nitrogen within 15 min after delivery. All participating mothers gave informed written consent, and the study protocol was approved by the local ethics committee (reference number KF 01-048-0/1). Mouse placentas were collected on embryonic day 17 or 18 from NMRI mice (M&B, Ry, Denmark) for in situ hybridization and from L0-MCK mice and wild-type controls for real-time RT-PCR studies. L0-MCK mice lack both mouse LPL alleles but express a human LPL minigene under the control of the mouse creatine kinase promoter (13). All placentas were snap-frozen in liquid nitrogen and stored at −80°C.

mRNA quantification

Total RNA was isolated with TriZol (Invitrogen, Taastrup, Denmark). The RNA integrity was ensured by 1% agarose gel electrophoresis or on a RNA Nano LabChip® (Agilent Technologies, Santa Clara, CA). The absorbance at 260 nm. First-strand cDNA was synthesized from 1 μg of total RNA with Moloney murine leukemia virus reverse transcriptase (40 units; Roche A/S, Avedøre, Denmark) and a FAST START DNAmaster SYBR Green kit (Roche A/S). The RNA concentration was assessed from 9 ml/g tissue) containing 0.025 M NH₄Cl, 0.5 mM EDTA, 1 mg/ml BSA, 1% Triton X-100, 0.1% sodium dodecyl sulfate, and 5 IU heparin/mL, pH 8.2. One tablet of protease inhibitor mix (Complete Mini; Roche, Basel, Switzerland) was added per 50 ml (19). After centrifugation, the combined homogenate (75 ml) was applied to a heparin-Sepharose column (5 ml sedimented gel). The column was washed with 60 ml of 20 mM Tris-HCl (pH 7.4) containing 20% glycerol, 0.1% Triton X-100, and 0.1 M NaCl. Bound proteins were eluted with a linear NaCl gradient from 0.1 to 2.0 M (50 ml + 50 ml) in the same buffer. A flow rate of 1 ml/min was maintained at 4°C, and fractions of 4 ml were collected. All fractions were divided into three parts before storing at −70°C. BSA was added to one part to a final concentration of 1 mg/ml to preserve lipase activity; the other two parts were stored without BSA for electrophoresis. The whole procedure was performed twice with placentas from vaginal deliveries and once with a placenta from a cesarean section.

In situ hybridization

Fragments of the EL (253 bp) and LPL (277 bp) cDNAs were amplified from human placental cDNA with the primers described above and cloned into a pCR®-Blunt II Topo® plasmid with the Zero Blunt Topo PCR cloning kit (Invitrogen). The resulting plasmids with the human EL or LPL cDNA fragment were linearized with appropriate restriction enzymes before the synthesis of sense and antisense riboprobes with SP6 and T7 RNA polymerase, respectively (SP6, number 600152; T7, number 600123; Stratagene, AH Diagnostics A/S, Aarhus, Denmark). The EL sense and antisense riboprobes were labeled with digoxigenin (DIG). However, LPL mRNA could not be visualized with a DIG-labeled probe. Therefore, [32P]UTP-labeled probes were used for visualization of LPL (17).

Frozen human placental biopsies were cut in 12 μm sections with a microtome, mounted on SuperFrost® Plus slides (Menzel GmbH and Co., Braunschweig, Germany), and stored at −80°C until processing. In situ hybridization was performed as described (17, 18) with slight modifications for DIG-labeled probes: before dehydration, the slides were incubated for 12–16 h at room temperature with anti-DIG alkaline phosphatase (Roche A/S), washed three times in phosphate-buffered saline, pH 7.4, with 0.25% (v/v) Triton X-100 for 5 min, once in 0.1 M TBS, pH 8.0, for 30 min, and once in a predetection buffer (0.1 M TBS/0.05 M MgCl₂) for 10 min. The slides were subsequently incubated with nitroblue tetrazolium (75 mg/ml; N 8076; Sigma, Vallensbæk Strand, Denmark) and bromochloroindolyl phosphate (50 mg/ml; B 8503; Sigma) in predetection buffer and finally with 10 mM TBS with 10 mM EDTA for 30 min before they were dehydrated in a series of alcohol dilutions. In situ hybridization studies of EL expression in mouse placentas were performed as described previously for mouse ovaries (16).

Separation of human EL and LPL on heparin-Sepharose

Equal amounts (3.2 g each) of placental biopsy material from the center, from the periphery and from the area in between were washed in NaCl (150 mmol/l) and from the homogenized in buffer (9 ml/g tissue) containing 0.025 M NH₄Cl, 5 mM Na2EDTA, 1 mg/ml BSA, 1% Triton X-100, 0.1% sodium dodecyl sulfate, and 5 IU heparin/ml, pH 8.2. One tablet of protease inhibitor mix (Complete Mini; Roche, Basel, Switzerland) was added per 50 ml (19). After centrifugation, the combined homogenate (75 ml) was applied to a heparin-Sepharose column (5 ml sedimented gel). The column was washed with 60 ml of 20 mM Tris-HCl (pH 7.4) containing 20% glycerol, 0.1% Triton X-100, and 0.1 M NaCl. Bound proteins were eluted with a linear NaCl gradient from 0.1 to 2.0 M (50 ml + 50 ml) in the same buffer. A flow rate of 1 ml/min was maintained at 4°C, and fractions of 4 ml were collected. All fractions were divided into three parts before storing at −70°C. BSA was added to one part to a final concentration of 1 mg/ml to preserve lipase activity; the other two parts were stored without BSA for electrophoresis. The whole procedure was performed twice with placentas from vaginal deliveries and once with a placenta from a cesarean section.

Measurement of human LPL

The substrate used for measurement of human LPL activity was a phospholipid-stabilized TG emulsion with the same composition as the commercial 10% Intralipid (100 mg of soybean TGs and 10 mg of egg yolk phospholipids/ml) but containing trace amounts of triolein labeled with [3H]oleic acid (kindly prepared...
Western blotting

Aliquots (10 μl) of fractions from the heparin-Sepharose columns were run on 12% SDS-PAGE gels. After transfer to a Hybond-P 0.45 μm polyvinylidene difluoride membrane (Amersham Biosciences, Copenhagen, Denmark), the blots were incubated at 4°C with a rabbit antiserum against human EL (P02121; dilution of 1:250) for 14–18 h and with a horseradish peroxidase-conjugated goat anti-rabbit antibody for 1 h. After incubation of the blot with SuperSignal West Pico Chemiluminescent Substrate (Pierce Chemicals, Copenhagen, Denmark), antibody binding was detected with a LAS-1000 Intelligent Dark Box II chemiluminescence reader (Fujifilm, Trørød, Denmark) and an Image Reader LAS-1000 Pro V2.5 and ImageGauge V4.0 program (Fujifilm).

Immunohistochemistry

Human placental biopsies were fixed for 12–16 h in 4% paraformaldehyde, pH 7.0 (Bie and Bernsten, Rødovre, Denmark), embedded in paraffin, cut into 3 μm thick sections, mounted on SuperFrost® Plus slides, and baked at 60°C for 30 min. Paraffin was removed with xylene for 10 min, and the sections were rehydrated through a series of alcohol solutions before treatment with 1 mM citric acid, pH 6.0, at 100°C for 15 min and with 1% H₂O₂ for 10 min. After washing with distilled water, sections were incubated at room temperature with 5% porcine serum (number P0399; DAKO, Glostrup, Denmark) in TBS (0.05 M Tris, 0.15 M NaCl, and 0.01% Triton X-100, pH 7.6) for 20 min and EL antiserum (1:500) or LPL IgG (1:1,000) diluted with 5% porcine serum in TBS for 1 h before being washed three times for 5 min in TBS. Bound antibod-
ies were visualized with the EnVision™ System/HRP, Rabbit (DAB+) (K4011; DAKO Cytomation). Finally, after washing in distilled water, sections were dehydrated through a series of alcohol solutions and mounted in Pertex (00801; HistoLab Products Ab, Göteborg, Sweden). In all experiments, sections without primary antibodies were included as a negative control.

To identify trophoblasts, macrophages, and endothelial cells, immunohistochemistry of frozen tissue sections was carried out with antibodies to cytokeratin AE (M3515; DAKO Cytomation), CD68 (M0876; DAKO Cytomation), and CD34 (347660; Becton Dickinson, Brøndby, Denmark), respectively, and a standard Dako Envision™ System (K5007; DAKO Cytomation) on a Dako Techmate™ 500 Plus routine stainer.

**RESULTS**

**EL and LPL mRNA expression in human placenta**

EL and LPL mRNAs were solidly expressed in human placenta (Fig. 1A). The concentrations of the two transcripts were measured with real-time reverse transcription PCR in transplacental biopsies from the center of placentas close to the insertion of the umbilical cord, the periphery, or from the region in between. There was no difference in the relative expression levels of EL and LPL between the three regions (Fig. 1B).

The cellular expression of EL and LPL mRNA was assessed with in situ hybridization studies (Fig. 2A, B, E, F, G). Endothelial cells and syncytiotrophoblasts were identified with immunohistochemistry using antibodies against CD34 and cytokeratin (Fig. 2C, D, H). EL mRNA was seen in both syncytiotrophoblasts and endothelial cells. LPL mRNA was mainly associated with the trophoblast layer; however, we cannot exclude the possibility that there might also be a low level of LPL expression in endothelial cells (i.e., below the detection limit of the experiment).

**EL and LPL protein expression and lipase activity in human placenta**

To assess EL and LPL protein expression, human placental protein extracts were separated by affinity chromatography on a heparin-Sepharose column. After elution of proteins, EL and LPL proteins as well as phospholipid and TG lipase activities were analyzed in the collected fractions. Western blotting with an EL antiserum showed three bands of ~40, 55, and 75 kDa in fractions 5–8 (Fig. 3B). These bands correspond to three previously described isoforms of EL, although we cannot exclude the possibility that they might represent proteolytic fragments of EL. Neither these fractions nor any other fractions contained measurable phospholipase activity (data not shown).

The major portion of LPL protein eluted slightly after EL in fractions 5–13, with the highest concentration in fraction 10 (Fig. 3A). Catalytically inactive monomeric LPL elutes at this position of the gradient (21, 27), and there was no detectable peak of TG lipase activity around fraction 10 (Fig. 3A). Of note, Western blot analysis with an LPL antibody showed that the major part of the LPL immunoactivity in fractions 5–13 was from intact LPL (~60 kDa) (Fig. 3C, lane 2).

A peak of TG lipase activity eluted in and around fraction 15. Active, dimeric LPL elutes at this position of the gradient (21, 27). The specific activity of LPL in fractions 12–18 was ~200 U/mg, which is comparable to the specific activity of human recombinant LPL in the same assays (270–400 U/mg) (21).

To assess cross-reactivity with LPL of the EL antiserum or with EL of the LPL antibody (both used for immunohistochemistry and Western blotting), fractions 5 and 6, which contain both EL and LPL proteins, were pooled. Western blot analysis of the pool showed three distinct bands with the...
EL antiserum, two bands with the LPL antiserum, and five bands with the mixture of the two antisera (Fig. 3C). Thus, there was no cross-reactivity between the two antisera.

**Immunohistochemistry on human placental tissue**

Immunohistochemistry of human placental sections revealed strong EL staining in endothelial cells and weaker staining in syncytiotrophoblasts (Fig. 4A). In contrast, syncytiotrophoblasts displayed strong LPL immunoreactivity, with a weaker signal associated with endothelial cells (Fig. 4B). In accordance with previous reports (5), LPL was also seen in cells resembling CD68-positive macrophages (Fig. 4D). The expression patterns of EL and LPL mRNA and protein are summarized schematically in Fig. 5.

![Fig. 3. Separation of EL and LPL protein on a heparin-Sepharose column. Placental extracts were bound to a heparin-Sepharose column, eluted with a 0.1–2.0 M NaCl gradient, and collected in fractions 1–24. A: LPL protein (open circles) and LPL triglyceride lipase activity (closed circles). B: Visualization of EL protein with Western blotting using an anti-EL antiserum. C: Cross-reactivity assessment of EL and LPL antisera. Fractions with EL and LPL were combined and analyzed in Western blots with anti-EL antiserum (lane 1), LPL antibodies (lane 2), or a mix of the two antibodies (lane 3). Lane 1 shows three bands of EL of ~40, 55, and 75 kDa. Lane 2 shows two bands of LPL of ~42 and 60 kDa. Lane 3 shows all five bands.](image)

![Fig. 4. Immunohistochemistry of EL and LPL protein in human placenta. Sections were incubated with an anti-EL antiserum ×40 (original magnification) (A), anti-LPL antibodies ×40 (B), porcine serum as a negative control ×40 (C), anti-LPL antibodies ×63 (D), or anti-CD68 (a macrophage marker) antibodies ×63 (E). A shows intense EL staining in endothelial cells and a weaker reaction in syncytiotrophoblasts. B shows a longitudinally sectioned villus with strong LPL staining in syncytiotrophoblasts and a weaker staining of endothelial cells. D shows LPL staining in macrophage-like cells. Closed arrows point at syncytiotrophoblasts, open arrows point at endothelial cells, and broken arrows point at macrophage-like cells.](image)
Increased expression of EL in LPL-deficient mouse placenta

Similar to EL mRNA in human placenta, mouse EL mRNA was strongly expressed in the region of placenta where nutritional exchange occurs (i.e., the labyrinth) (Fig. 6A, B). On real-time RT-PCR analysis, LPL mRNA was also robustly expressed in the mouse placenta (data not shown). Recent results suggest that increased EL expression contributes to fatty acid uptake in adipose tissue when LPL expression is genetically ablated (28). To test whether EL mRNA expression in placenta might also be increased in the setting of LPL deficiency, we examined near-term placentas from L0-MCK mice and controls. EL mRNA expression was increased by ~50% in L0-MCK compared with control placentas ($P = 0.02$) (Fig. 6C). Lack of mouse LPL mRNA confirmed the loss of both mouse LPL alleles in L0-MCK mice, and human LPL mRNA expression was virtually absent in L0-MCK placentas (i.e., <0.1% of that in human placenta) (data not shown).

DISCUSSION

The presence of TG lipase activity in human placenta resembling that of LPL was described in 1965 (29). More recent studies have shown mRNA expression of another member of the TG lipase gene family, the EL gene ($LIPG$), in human placenta (9, 10). In this study, we compared the expression of EL and LPL in placenta and examined whether the absence of LPL results in increased expression of EL.

Although EL and LPL proteins were detected on both the maternal and fetal sides of the human placenta, and therefore both may contribute to lipid uptake from both circulations, the levels of EL and LPL protein expression were different: LPL protein staining was most intense in syncytiotrophoblasts facing the maternal circulation, whereas EL protein staining was most intense in endothelial cells facing the fetal circulation. These findings suggest that EL and LPL are differently regulated in human placenta. In fact, LPL mRNA was predominantly seen in syncytiotrophoblasts, whereas LPL protein was present in both syncytiotropho-
blasts and endothelial cells. In adipose tissue and skeletal muscle, the parenchymal adipocytes and myocytes secrete LPL, which is then transported to the luminal surface of the vascular endothelium. There, LPL binds to heparan sulfate proteoglycans and hydrolyzes TG-rich lipoproteins. The LPL protein associated with placental endothelial cells may be derived in a similar manner either from the syncytiotrophoblast (in analogy with the adipocyte- or myocyte-derived LPL in adipose tissue or skeletal muscle capillaries) or from the fetal blood.

Although the EL protein was present in human placenta in substantial amounts, we could not detect any EL phospholipase activity. Moreover, the results suggest that the major portion of LPL (>95%) in human placenta is catalytically inactive. Dimerization of LPL is necessary for its lipolytic activity (21). Western blotting showed that the inactive LPL was mostly intact (~60 kDa), and it was possible to reconstitute some lipolytic activity in the fractions with inactive LPL by an LPL refolding protocol (Zhang, L. et al., unpublished data). Importantly, the major portion of LPL was inactive even when we analyzed a placenta from a cesarean section, which was snap-frozen immediately after removal. Thus, our studies suggest that the inactive LPL in human term placenta is in fact monomeric LPL. In rat adipose tissue, the fraction of inactive LPL varies with the metabolic state and is ~20–30% in the fed state but 70–80% during fasting (30). It is conceivable that dimerization of LPL serves as a means for the rapid regulation of LPL activity in tissues. Interestingly, a recent study (31) found a 39% increase in LPL activity, without a change in total LPL protein content in placentas from type 1 diabetic compared with normal pregnancies.

It is possible that EL, like LPL, is present predominantly in an inactive monomeric form in the human placenta. Indeed, recent data are compatible with the idea that dimerization is needed for EL’s catalytic phospholipase activity (32). However, there are several other possible explanations for the absence of phospholipase activity in the EL-containing fractions from heparin-Sepharose chromatography. The specific phospholipase activity of EL is unknown, and the concentration of EL in the human placental extracts may simply be too low for detection with our assay. No study has reported EL phospholipase activity in tissue extracts, and EL phospholipase activity is inhibited by serum (10, 33). Thus, placental extracts may contain EL inhibitors, which in a similar manner affect our in vitro studies. It is also possible that cofactors for EL’s lipase activity have been lost during purification.

In addition to their lipolytic function, placental EL and/or LPL could contribute to placental lipoprotein holoparticle uptake from the maternal or fetal circulation. Both EL and LPL bind heparan sulfate proteoglycans and bridge plasma lipoproteins to the cell surface independently of their catalytic activity (11, 34–36). The association of dimeric LPL with lipoproteins enhances the binding of the complex to the LDL receptor-related protein and the LDL and VLDL receptors; all three receptors are expressed in human placenta (37–39). Interestingly, the bridging function is also exerted by monomeric LPL (40). Independent of their catalytic activity, both EL and LPL are also able to stimulate selective cholesteryl ester uptake via the scavenger receptor class B type I (35, 41), which is expressed in both mouse (42) and human (43) placenta. Placenta synthesizes large amounts of progesterone (44) and is dependent on a continuous supply of cholesterol through the entire pregnancy. Thus, yet another possible role for EL and/or LPL in placenta could be to ensure the sufficient uptake of cholesterol for the production of steroid hormones.

It was shown recently that EL can compensate for a lack of LPL in adipose tissue and mediates at least part of the free fatty acid uptake in adipose tissue of mice that lack LPL expression in adipocytes (28). Also, EL-deficient mice have increased LPL and hepatic lipase expression in skeletal muscle and liver (45). These observations suggest that EL and LPL can substitute for each other in adipose and peripheral tissues of adult mice, even though their spatial expression patterns normally differ. The present data suggest that EL may also be capable of substituting for LPL in the mouse placenta. In situ hybridization studies showed that the mouse placenta, albeit having a different structural organization than the human placenta (and with bulk lipid transport occurring in the yolk sac), expresses EL in the labyrinth (which resembles the villous tree in human placenta). Although the EL gene is expressed at very high levels in the placenta of wild-type mice, its expression was further increased in LPL-deficient placentas.

In summary, the present study suggests that even though both EL and LPL are present on the maternal and the fetal sides of the human placenta, the two lipase genes appear to be differentially expressed. Nevertheless, EL mRNA expression is increased when LPL is absent in the mouse placenta. Both lipases appear to be present mainly in their catalytically inactive forms in the human term placenta.

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