Low-Density Lipoprotein Inhibits Secretion of Phospholipid Transfer Protein in Human Trophoblastic BeWo Cells

AN-YUE TU,[1] MARIAN C. CHEUNG, XIAODONG ZHU, ROBERT H. KNOPP, AND JOHN J. ALBERS

Northwest Lipid Research Laboratories and Northwest Lipid Research Clinic, Division of Metabolism, Endocrinology, and Nutrition, Department of Medicine, University of Washington, Seattle, Washington 98103

Human plasma phospholipid transfer protein (PLTP) plays an important role in lipoprotein metabolism. In this study, we investigated the effects of lipoproteins on the secretion of PLTP in cultured BeWo choriocarcinoma cells. Low-density lipoproteins (LDLs) decreased PLTP secretion in a dose- and time-dependent manner, whereas very low density lipoproteins and high-density lipoproteins (HDLs) had little effect. LDL suppression of PLTP secretion was not altered by the inhibition of both LDL receptor and LDL receptor–related protein with receptor-associated protein. Mitogen-activated protein kinase (MAPK) kinase (MEK) inhibitor, U0126, could abolish the LDL-mediated inhibition of PLTP secretion. Furthermore, LDL, but not HDL, could stimulate the expression of MAPK phosphatase-1 (MKP-1) in BeWo cells that resulted in the inactivation of p44/p42 extracellular signal-regulated kinase (ERK) 1 and 2, the family members of MAPKs. These results support the conclusion that LDL-mediated suppression of PLTP secretion in BeWo cells is through a LDL receptor-independent MAPK signaling pathway.

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Key words: phospholipid transfer protein; human trophoblastic cells; MAPK signaling pathway

Introduction

Phospholipid transfer protein (PLTP) plays an essential role in reverse cholesterol transport mediated by high-density lipoproteins (HDLs) (1–3). *In vitro*, PLTP facilitates phospholipid transfer from lower-density lipoproteins to HDLs (4–7); modulates HDL particle size and lipid composition (8, 9); generates prebeta HDL, the commonly believed earliest acceptor of cellular cholesterol (10); and enhances HDL interaction with cholesterol-loaded cells and the efflux of excess cholesterol (3, 11).

In cultured cells and in animal models, PLTP can be regulated by physiological and environmental factors such as glucose (12), bile acids (13), and fenofibrate (14, 15). Because plasma PLTP activity is often correlated with various lipid and lipoprotein parameters in both humans and mice (16–18), we decided to test the hypothesis that lipoproteins can also regulate PLTP. Several cell types were screened for PLTP activity level. Of the cells studied, BeWo, the human placental trophoblastic cell line, expressed the highest level of PLTP activity (10 times higher than HepG2 cells, and 50–80 times higher than THP-1, human skin fibroblasts, and human monocyte-derived macrophages). The high level of PLTP activity detected in BeWo cells is consistent with our previous observation that PLTP mRNA is highly expressed in the placenta (9). Thus, BeWo cells were chosen for this study. We found that in these cells, low-density lipoproteins (LDLs), but not very low density lipoproteins (VLDLs) or HDLs, could suppress the secretion of PLTP. This inhibitory effect occurs via a LDL receptor-independent signal transduction pathway. The mechanism involves MKP-1 induction and subsequent inactivation of p44/p42 extracellular signal-regulated kinase (ERK) 1 and 2.

Materials and Methods

Cell Culture. Human choriocarcinoma BeWo cells, originally purchased from the American Type Culture Collection, were maintained in F12 nutrient mixture (Ham's medium; Life Technologies, Inc., Gaithersburg, MD) containing 4 m*M* L-glutamine, 0.05 mg/ml penicillin, and 0.05 mg/ml streptomycin supplemented with 15% FCS, as described (14). The growing medium was changed every other day to supply the cells with sufficient nutrients. These cells were incubated at 37°C in a humidified atmosphere of 5% CO_2/95% air.

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[1] To whom correspondence should be addressed at Department of Medicine, Northwest Lipid Research Laboratories, University of Washington, 2121 N 35th Street, Seattle, WA 98103. E-mail: aytu@u.washington.edu

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Lipoprotein Incubation Studies. On reaching 80% confluence, the BeWo cells were washed two times with phosphate-buffered saline and maintained in serum-free medium containing 0.2% bovine serum albumin for 24 hrs at 37°C before each study. To measure the effect of lipoproteins on PLTP, cultured cells that were serum-starved for 24 hrs were incubated with VLDL, LDL, or apoE-free HDL_3 at 37°C for various time periods. These lipoproteins (VLDL at $d < 1.006$ g/ml, LDL at $d = 1.019–1.063$ g/ml, and HDL_3 at $d = 1.125–1.21$g/ml) were prepared by sequential ultracentrifugation from pooled fresh human plasma (19). Apolipoproteins B and E on the HDL fraction were removed by heparin-agarose column chromatography, as described (20). All lipoproteins were stored in 1 m*M* EDTA under nitrogen to minimize oxidation. At the end of the incubation, conditioned medium was collected for PLTP activity and Western blot studies. Cells were washed twice with ice-cold phosphate-buffered saline for cytoplasm extraction and subsequent analyses. The chemicals used in the lipoprotein incubation studies, including heparin (Sigma, St. Louis), chloroquine (Sigma), and U0126 (Promega, Madison, WI), were prepared according to the manufacturer's protocol. In the experiments in which these chemicals were used, the same amounts of solvent used to dissolve these chemicals were added to the medium as controls.

Measurement of PLTP Activity. PLTP activity (PLTA) in conditioned medium was measured by the transfer of radioactivity from ^{14}C-radiolabeled phosphatidylcholine liposomes to unlabeled HDL particles as described (6) using 20–100 μl conditioned medium per assay.

Cytoplasm Extraction and Western Blot Analysis. Cytoplasm proteins were extracted from washed cells using a NE-PER cytoplasm extraction kit (Pierce Biotech, Rockford, IL) according to the manufacturer's protocols. Protein concentration was measured by the Lowry method (21). To compare the cell contents of proteins of interest, Western blot analyses were performed using either 4%–20% SDS gel (for PLTP) or 10% SDS gel (for MKP-1, phosphorylated ERK1/2, and total ERK1/2), and approximately 30 μg of medium protein or 150 μg of cell protein per sample. Electrophoresis was carried out at 200 volts at room temperature for 45 mins. The molecular weight markers from Invitrogen were applied to each SDS gel as standards. Proteins on the gel were transferred electrophoretically at 4°C to 0.2 μm supported nitrocellulose membrane at 26 volts overnight. The membranes were then blocked with 5% fat-free milk in Tris buffered saline with 0.05% Tween 20 and subjected to immunoblotting procedures. The primary antibodies used were anti-PLTP polyclonal antibody developed in our laboratory (22), anti-MKP-1 and anti-ERK1/2 polyclonal antibody from Santa Cruz Biotech (Santa Cruz, CA) and anti-ACTIVE MAPK polyclonal antibody from Promega Corp. Species-appropriate horseradish peroxidase-conjugated secondary antibodies (Kirkegaard and Perry [Gaithersburg, MD] or Promega) coupled with chemiluminescent substrate (Pierce) were used as the detecting agents.

Results

Inhibition of PLTP Secretion by LDL. When VLDL, LDL, and HDL, at concentrations between 10 and 200 μg/ml of cholesterol were incubated with BeWo cells, only LDL consistently decreased PLTP activity in the conditioned medium in a dose- and time-dependent manner (Fig. 1A, B). In eight experiments performed with different preparations of LDL, near-maximal suppression of PLTP activity was observed with 50 μg/ml of LDL cholesterol. In fact, about 60% of the maximal suppression was obtained with 10 μg/ml of LDL. Maximal LDL-mediated inhibitory effect varied among experiments, ranging from 25% to 75% of the control with the different LDL preparations. This variation is much larger than the 5%–10% variation observed between experiments conducted with the same preparation of LDL, indicating that components other than the cholesterol in LDL may be responsible for the observed inhibitory effect. The suppression of PLTP activity usually approached the maximal level by 8 hrs of incubation (Fig. 1B). Consistent with the reduction in PLTP activity, Western blot analysis also showed that LDL reduced PLTP mass in BeWo-conditioned medium (Fig. 1C). In two experiments, the decrease in PLTP mass (43% and 35%) was similar to the decrease in PLTP activity (38% and 36%). No significant change in PLTP mRNA in BeWo cells was observed within 8 hrs of incubation with 100 μg/ml of LDL (data not shown), indicating that the mechanism responsible for the inhibitory effect of LDL was likely a result of posttranscriptional regulation.

To exclude the possibility that the reduction in PLTP activity detected in BeWo-conditioned medium containing LDL was not caused by the destabilization of PLTP by LDL, or the effect of LDL on the PLTP activity assay, LDL at 25, 50, 100, and 200 μg/ml was added to different aliquots of a BeWo-conditioned medium containing no LDL. The mixtures were incubated at either 4° or 37°C for 24 hrs, and PLTP activity was measured. No reduction in PLTP activity was observed in mixtures containing LDL compared to the control medium with no LDL in samples maintained at 4°C and at 37°C. Thus, LDL at concentration $\leq$200 μg/ml had no effect on the PLTP activity assay and did not affect the stability of the PLTP activity in BeWo-conditioned medium.

Suppression of PLTP Secretion in BeWo Cells Is Independent of LDL Receptor. Placental trophoblast cells contain lipoprotein receptors, including LDL-receptor and LDL-receptor-related protein (LRP) that could bind lipoprotein particles carrying apolipoprotein B and E for further metabolism (23). To investigate the roles of these receptors in the observed LDL inhibition of PLTP secretion, several experiments were performed. Preincubation of 50

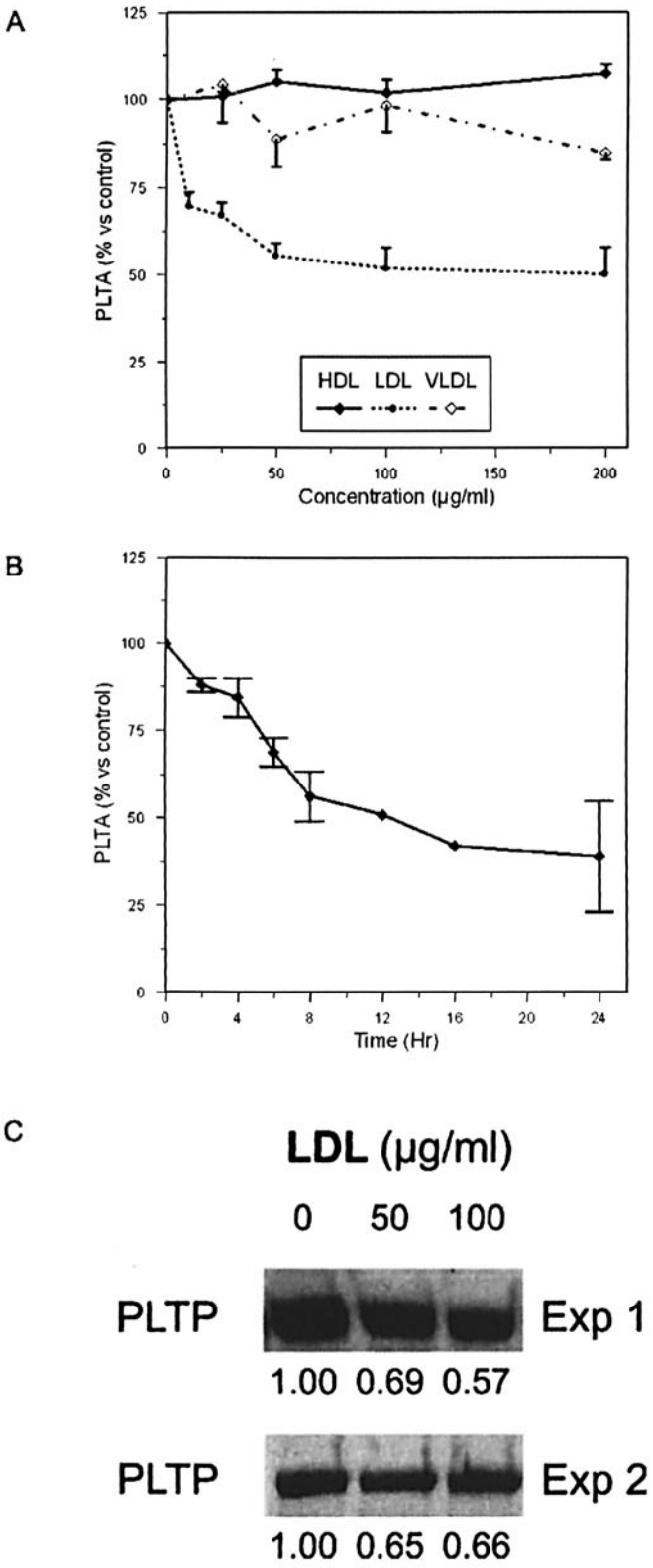

Figure 1. Effects of lipoproteins on phospholipid transfer protein (PLTP) secretion in BeWo cells. BeWo cells were serum-starved for 24 hrs before a study. (A) BeWo cells were incubated with various concentrations of VLDL, LDL, or HDL cholesterol at 37°C for 24 hrs. (B) Cells were incubated with 100 μg/ml LDL cholesterol for various periods of time. (C) Incubation was performed in the presence of 50 or 100 μg LDL cholesterol for 24 hrs. At the end of the incubation, the medium was collected for PLTP activity (PLTA) measurement (A and B) and Western blot analysis for PLTP mass (C). PLTP activity is expressed as a percentage of the activity in control medium; that is, medium without lipoprotein (A) or at zero incubation time (B). PLTP mass is expressed as a ratio to the mass in the control medium without lipoprotein (C). The data in Figure 1A show the mean ± SE from eight experiments with LDL, three experiments with VLDL, and five experiments with HDL. Figure 1B shows the mean ± SE from four experiments, and Figure 1C shows the original data from two experiments.

μg/ml of LDL with heparin (up to 2 mg/ml) that inhibits the binding of LDL to the LDL receptor had no effect on PLTP-mediated phospholipid transfer activity detected in the condition medium, nor did it abolish the inhibitory effect of LDL on PLTP secretion (Fig. 2, top panel). Similar results were obtained with preincubation of BeWo cells with chloroquine (up to 50 μ*M*) which blocks the LDL-receptor-mediated LDL endocytosis process (24) (Fig. 2, middle panel). Likewise, inhibition of both LDL receptor and LRP with receptor-associated protein (1 μ*M*) (25, 26) did not affect PLTP secretion or alter LDL-mediated suppression of PLTP secretion in BeWo cells (Fig. 2, bottom panel). Taken together, these data strongly support the concept that LDL inhibits PLTP secretion in BeWo cells via a LDL-receptor-independent process.

LDL-Suppressed PLTP Secretion via MAPK Pathway. Because the LDL receptor did not play a role in the inhibition of PLTP secretion in BeWo cells, we proceeded to determine whether the inhibition is through a LDL-initiated signal transduction pathway. As LDL has been shown to influence the signaling pathway mediated by mitogen-activated protein kinase (MAPK), we investigated whether the MAPK cascade pathway is also involved in the LDL-mediated suppression of PLTP secretion in BeWo cells. The effect of MAPK kinase (MEK) inhibitor U0126 on PLTP secretion was studied. U0126, which can directly inhibit both activated and inactive MEK (27, 28), completely abolished the inhibition of PLTP secretion by LDL (Fig. 3). These data indicate that the MAPK signal transduction pathway is involved in the LDL-mediated suppression of PLTP secretion in BeWo cells.

Inhibition of PLTP Secretion via LDL-Mediated MKP-1 Induction and ERK1/2 Inactivation. It has been reported that LDL may stimulate the expression of MAPK phosphatase-1 (MKP-1) through a LDL-independent pathway in human vascular smooth muscle cells (29). We therefore tested whether LDL had an effect on MKP-1 in BeWo cells. The addition of LDL into BeWo cells increased MKP-1 after a 10-min exposure time (Fig. 4, top panel). Concomitant with the increase in MKP-1, there was a decrease in the phosphorylated active form of p44/p42 extracellular signal-regulated protein kinases (ERK) 1 and 2, two members of the MAPKs (Fig. 4, middle panel); however, total p44/p42 ERK remained unchanged (Fig. 4, bottom panel). This implies that LDL might induce the

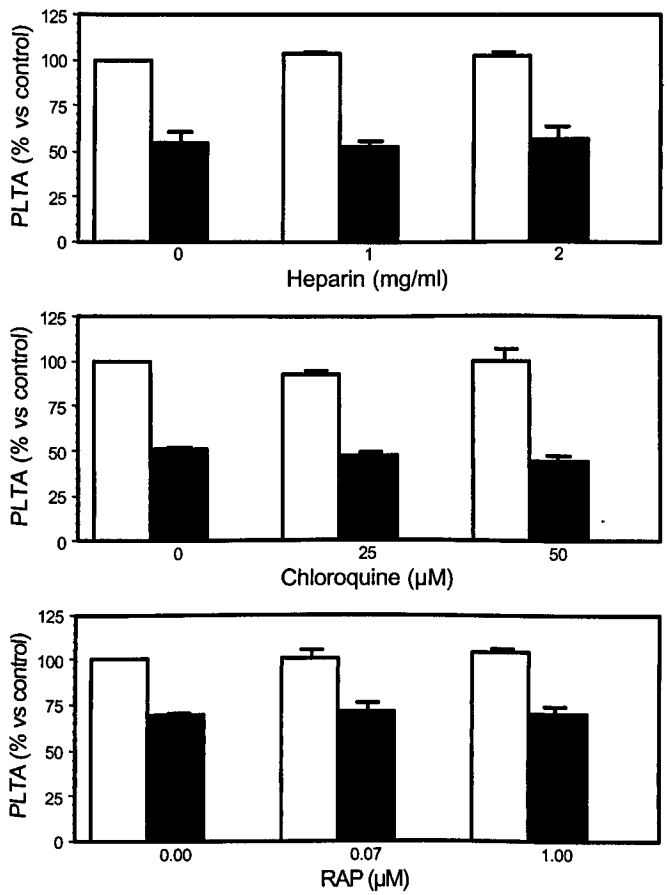

Figure 2. LDL-mediated inhibition of PLTP secretion in BeWo cells is LDL-receptor-independent. Preincubation of LDL with heparin (1 or 2 mg/ml) at 4°C for 1 hr, or preincubation of the cells with chloroquine (25 or 50 μM) at 37°C for 2 hrs, or preincubation of the cells with RAP (0.07 or 1 μM) at 37°C for 1 hr was performed before the addition of LDL (50 μg/ml) into the incubation medium. After a 24-hr (heparin and chloroquine), or 8-hr (RAP) incubation, conditioned medium was collected for the measurement of PLTP activity (PLTA). The data show the mean ± SE from three experiments and are expressed as percentages of the corresponding controls (PLTP in medium without LDL and the study chemicals). Open bars represent incubations without LDL, and closed bars represent incubations with LDL.

expression of MKP-1, which inactivates ERK1/2 and subsequently reduces the PLTP secretion in BeWo cells. As HDL did not show any effect on PLTP secretion, we tested whether HDL could affect MKP-1 and ERKs in BeWo cells. In contrast to LDL, incubation of HDL for 5–60 mins with these cells did not alter MKP-1 or ERK1/2 (data not shown). These data indicate that the LDL-mediated suppression of PLTP secretion in BeWo cells is most likely through a MAPK cascade-signaling pathway.

Discussion

A high concentration of LDL in plasma is a major risk factor for atherosclerosis and coronary heart disease. In addition to cholesterol delivery to peripheral cells, LDL may stimulate proliferation of smooth muscle cells that can lead to the development of atherosclerosis (30, 31). The LDL-initiated signal transduction pathways leading to smooth muscle cell proliferation have recently been studied (29). LDL induces the expression of MKP-1 *via* activation of protein kinase C and tyrosine kinase independent of the LDL receptor. MKP-1 is a member of the dual-specificity tyrosine phosphatase family and has been shown to dephosphorylate some family members of MAPKs, including ERKs, resulting in their inactivation (32, 33). ERKs are very active in smooth muscle cells and macrophages in response to extracellular stimuli, such as LDL (34, 35).

A recent report indicates that human placenta tissues have expressed and activated MAPK molecules, such as ERK1/2 (36), indicating that the MAPK signal transduction pathway could be actively involved in the proliferation, differentiation, and growth of placenta cells. In this study, we reported for the first time that BeWo cells exhibit both active and inactive ERK1/2 molecules, and this signaling pathway could play a role in PLTP secretion. BeWo cells are rapidly growing and differentiated cells and have relatively high amounts of active ERK molecules in cytoplasm, as shown in Figure 4, in contrast to those in the smooth muscle cells, which could be detected only after LDL-mediated activation (29). Our data indicate that ERKs are essential signaling molecules for PLTP secretion. Their abundance may contribute in part to the relatively large quantities of PLTP secreted by BeWo cells. This study also demonstrates that LDL can influence the MAPK signaling pathway in BeWo cells. The LDL-mediated signaling in BeWo cells appears to favor the inactivation of MAPK molecules by inducing MKP-1, while in smooth muscle cells, this signaling leads to the activation of MAPK (29, 34, 35). Because MKP-1 plays an important role in negative feedback regulation of MAPK signaling pathway (37), the balance between MAPK activation and MKP-1 induction appears to be a key determinant of the response of cells to environmental stimuli (38). The effect of LDL on MKP-1 and ERKs appears to result from the alteration in the ratio of MKP-1 and the active and inactive forms of ERKs molecules. This concept would explain why U0126, which can directly inhibit both active and inactive MEKs, could perhaps maintain the balance of these molecules in a cell and could prevent the LDL-mediated suppression of PLTP secretion in BeWo cells (Fig. 3).

Considerable batch-to-batch variability in the suppressive effect of LDL was observed, suggesting the possibility that oxidized lipids could be the active component responsible for the suppression of PLTP secretion in BeWo cells. This does not appear to be the case, as two preparations of oxidized LDL suppressed PLTP secretion in BeWo cells to the same extent as the corresponding amount of native LDL based on LDL protein concentration (50% vs. 55% and 31% vs. 27% for oxidized and native LDL, respectively).

The placenta plays an essential lipid transport role in fetal nutrition (39, 40). Placental PLTP may play a role in this process, based on existing knowledge on the physio-

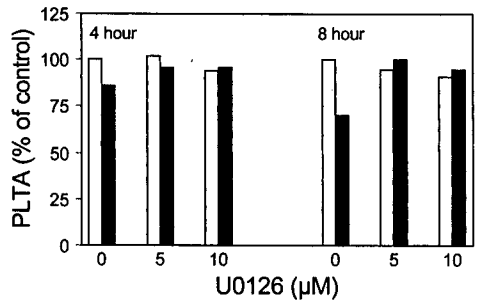

Figure 3. MEK inhibitor, U0126, inhibits the LDL-mediated suppression of PLTP secretion in BeWo cells. BeWo cells were serum starved for 24 hrs, preincubated with U0126 (5 or 10 μM) at 37°C for 1 hr before the addition of 100 μg/ml LDL into the incubation. After a 4- or 8-hr incubation, the medium was measured for PLTP activity (PLTA). The data show the mean value of two experiments and are expressed as percentages of control incubation without LDL and U0126. Open bars represent incubations without LDL, and closed bars represent incubations with LDL

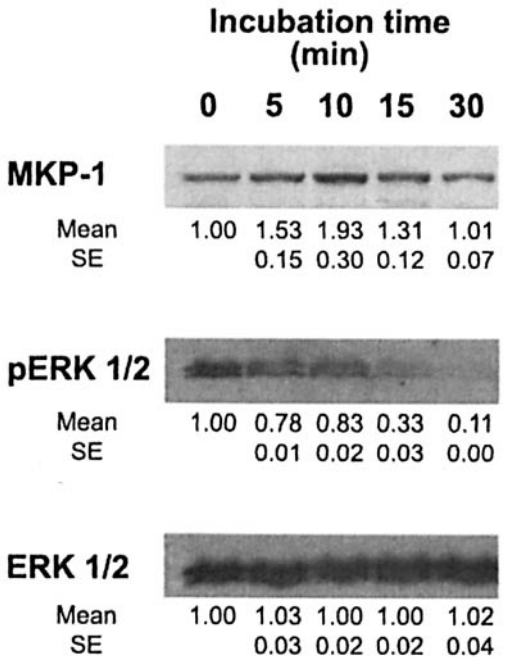

Figure 4. LDL enhances the expression of MKP-1 and inactivation of ERK1/2. BeWo cells were serum starved for 24 hrs and then incubated with 100 μg/ml LDL cholesterol at 37°C for the indicated periods of time. At the end of the incubation, cells were washed twice with ice-cold phosphate-buffered saline. Cytoplasm proteins were extracted from washed cells, and approximately 150 μg of these proteins per sample was separated on a 10% SDS gel and transferred to nitrocellulose membrane for Western blot analyses of MKP-1, phosphorylated (or active) ERK1/2 (pERK1/2), and total ERK1/2. The blots were scanned to calculate the mass of these signaling molecules and are expressed as a ratio to the mass in the same experiment at zero incubation time. The Western blot shown is from a representative experiment, and the mean ± SE are from three experiments.

logical role of PLTP in general. Human placental tissues express high level of PLTP mRNA (9), and among the several cell types we have examined, the BeWo choriocarcinoma cell line secretes the highest level of PLTP. This study shows that LDL suppresses PLTP secretion in BeWo cells but does not address the question of whether these findings are specific for BeWo cells. We speculate that BeWo cells are particularly responsive to LDL suppression of PLTP secretion, as similar concentrations of LDL had a minimal effect on PLTP activity in cultured HepG2 cells. Because LDL levels rise 60% in normal term pregnancy (39–41), placental PLTP expression may be proportionally reduced as gestation proceeds.

The general physiological role of PLTP is to transfer phospholipid from VLDL to HDL, leading to HDL generation and remodeling (9). This process is very important because apo A-I and phospholipid are the building blocks of HDL, which then interacts with various enzymes and tissues to accumulate and transport cholesterol. *In vivo* studies using genetically modified mice support this view. Introduction of the human PLTP transgene into mice alters the ratio of HDL/LDL cholesterol levels (42), and coexpression of PLTP with human apo A-I increases the formation of pre-beta HDL (43), a precursor of mature HDL. In PLTP knockout mice, HDL lipids and apo A-I levels are dramatically reduced, whereas phospholipid and free cholesterol accumulate in VLDL and LDL (44).

If placental PLTP activity declines in the latter half of gestation, one might predict that HDL levels would decrease in the second half of gestation. This prediction is borne out by observations in normal pregnancy. Levels of HDL cholesterol rise from about 55 to 80 mg/dL at 20 weeks' gestation, followed by a decrease to about 65 mg/dL at term (39–41). We have previously speculated that the rise and then partial fall in HDL cholesterol in pregnancy might be a result of the competitive effects of estrogen to increase HDL and apo A-I formation in the first half of gestation, countered in late gestation by fat gain, insulin resistance, and fat-mobilizing hormones such as cortisol, placental growth hormone, and progesterone (39, 40). An increase in placental PLTP secretion in early gestation could contribute to the rise in HDL cholesterol and apo A-I levels. In late gestation, a LDL-driven decline in placental PLTP secretion could plausibly contribute to a decline in HDL generation and HDL cholesterol level. The resulting interplay between rising LDL cholesterol and falling HDL cholesterol levels may balance cholesterol delivery to the placenta and fetus, as both lipoprotein fractions deliver cholesterol to the placenta and stimulate progesterone secretion (45). A gestational PLTP effect on lipoprotein metabolism is further supported by our recent observation that PLTP activity is more strongly associated with lipoprotein lipid levels in women compared to in men (17).

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