Synthesis and release of fatty acids by human trophoblast cells in culture

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Abstract In order to determine whether placental cells can synthesize and release fatty acids, trophoblast cells from term human placentas were established in monolayer culture. The cells continued to secrete placental lactogen and progesterone and maintained specific activities of critical enzymes of triacylglycerol and phosphatidylcholine biosynthesis for 24 to 72 hr in culture. Fatty acid was rapidly synthesized from [^{14}C]acetate and released by the cells. Palmitoleic, palmitic, and oleic acids were the major fatty acids synthesized from [^{14}C]acetate and released. Small amounts of lauric, myristic, and stearic acids were also identified. [^{14}C]Acetate was also incorporated into cellular triacylglycerol, phospholipid, and cholesterol, but radiolabeled free fatty acid did not accumulate intracellularly. In a pulse-chase experiment, cellular glycerolipids were labeled with [1-^{14}C]oleate; trophoblast cells then released ^{14}C-labeled fatty acid into the media as the cellular content of labeled phospholipid and triacylglycerol decreased without intracellular accumulation of free fatty acid. Twenty percent of the ^{14}C-label lost from cellular glycerolipid could not be recovered as a chloroform-extractable product, suggesting that some of the hydrolyzed fatty acid had been oxidized. These data indicate that cultured placenta trophoblast cells can release fatty acids that have either been synthesized de novo or that have been hydrolyzed from cellular glycerolipids. Trophoblast cells in monolayer culture should provide an excellent model for molecular studies of placental fatty acid metabolism and release.—**Coleman, R. A., and E. B. Haynes.** Synthesis and release of fatty acids by human trophoblast cells in culture. *J. Lipid Res.* 1987. **28:** 1335–1341.

Supplementary key words placenta • maternal-fetal exchange • glycerolipid biosynthesis

The developing fetus uses fatty acids to synthesize complex lipids including glycerolipids, sphingolipids, cholesteryl esters, and glycolipids. These complex lipids are essential for the formation of structural components of cellular membranes, intracellular signals such as prostaglandins and diacylglycerol, triacylglycerol stores for postnatal energy production, and secreted products required for postnatal life such as the serum lipoproteins, bile, and pulmonary surfactant. The placenta transports as much as 50% of the daily fatty acid requirement and 100% of the essential fatty acid requirement of the developing fetus (1–5). The major source of these transported fatty acids is unclear, and the relative contributions of maternally derived fatty acids and those synthesized by the placenta have been debated (3, 6). Despite its physiological importance, little is presently known about placental lipid transport on a molecular level.

Studies using human placenta slices (7) and rat placentas (8) showed rapid incorporation of radiolabeled palmitate into placental triacylglycerol and phospholipid. Because normal placenta does not accumulate triacylglycerol, it was suggested that the transport of fatty acid from the maternal to the fetal circulation requires an intermediate esterification step (7, 8). The relatively high activities of the microsomal enzymes of triacylglycerol and phosphatidylcholine biosynthesis and of lysosomal triacylglycerol lipase observed in rat placenta are consistent with this hypothesis (9).

Isolated human placental cells in monolayers and in perifusion systems have been used to study hormone secretion and its regulation (10, 11). We have studied human trophoblast cells in monolayer culture in order to determine whether these cells secrete fatty acids that have been released from cellular glycerolipids or that have been synthesized de novo.

EXPERIMENTAL PROCEDURES

Materials

DNAase I, trypsin type III-S (from bovine pancreas), oleic acid, sodium acetate, bovine serum albumin (essentially fatty acid-free), N-2-hydroxy-ethylpiperazine-N^1,-2-ethanesulfonic acid (HEPES) were from Sigma. Fetal bovine serum and Dulbecco's modified Eagle's medium

Abbreviations: CMFHH, Ca^{2+}-Mg^{2+}-free Hanks, 25 mM HEPES, pH 7.4 DME-G, Dulbecco's modified Eagle's medium with 25 mM glucose; hPL, human placental lactogen; BSA, bovine serum albumin; HPLC, high pressure liquid chromatography.

with 25 mM glucose, trypan blue, penicillin, and streptomycin were from Gibco. Percoll was from Pharmacia. Collagen (Vitrogen) was from Flow Labs. Silica gel G plates were from Analtech. Coat-a-Count Progesterone kit was from Diagnostic Products Corporation. [1,2-^{14}C]Acetate and Aquasol-II were from New England Nuclear. [1-^{14}C]Oleic acid and [2-^{3}H]glycerol were from Amersham. Fatty acid standards were from Serdary.

Trophoblast cell isolation

Trophoblast cells from human placentas were prepared by the method of Kliman et al. (11). Briefly, human placentas were obtained following normal vaginal delivery or uncomplicated Caesarean section of women with uncomplicated pregnancies. Approximately 10-cc pieces of villous tissue were removed from the underlying fibrous tissue and rinsed in a large volume of Ca^{2+}-free Hanks balanced salt solution to remove blood. This tissue was then minced. Large vessels and connective tissue were removed. The tissue was transferred to a flask containing 150 ml of warmed Ca^{2+}-Mg^{2+}-free Hanks, 25 mM HEPES, pH 7.4 (CMFHH) with 0.125% trypsin and 30 mg DNAase I, and incubated for 30 min at 37°C with gentle shaking. After allowing the tissue fragments to settle, six aliquots (13.5 ml each) of the supernatant were layered over 1.5 ml of fetal calf serum in 15-ml polystyrene centrifuge tubes and centrifuged at 1000 *g* for 5 min at room temperature. The pellets were resuspended in Dulbecco's modified Eagle's medium with 25 mM glucose (DME-G). The remaining placenta tissue was twice more digested with 100 ml, then 75 ml of warmed fresh trypsin-DNAase solution. The resuspended cell pellets were pooled and centrifuged at 1000 *g* for 5 min, resuspended in 4 ml of DME-G, and layered over a preformed Percoll gradient in CMFHH. The gradient was formed by centrifuging a 40% Percoll solution at 40,000 *g* for 20 min. Trophoblast cells were isolated at a density of 1.049–1.062 g/ml. Percoll was removed by diluting with CMFHH and centrifuging at 1000 *g* for 5 min. The cells were resuspended in DME-G plus 10% fetal bovine serum, and plated on 60-mm collagen-coated culture dishes at 2–4 × 10^6 cells/dish. An average yield was 1 to 2 × 10^6 cells per gm of placenta. More than 95% of the cells excluded trypan blue.

After 18 to 24 hr in culture, media and unattached cells were removed and 2 ml of fresh DME-G, 1% bovine serum albumin (essentially fatty acid free) was added. Cells were then incubated for various times with 0.1 mM [^{14}C]acetate, 13 μCi/μmol, with 0.1 mM [^{14}C]oleate, 5–30 μCi/μmol, or with 1.3 mM [^{3}H]glycerol, 3 μCi/μmol.

To obtain total particulate preparations, placental villous tissue or cultured cells were homogenized with six up-and-down strokes in a motor-driven Teflon-glass homogenizer in ice-cold medium 1 (0.25 M sucrose, 1 mM EDTA, 10 mM Tris-Cl, pH 7.4). The homogenate was centrifuged at 100,000 *g* for 1 hr. The pellet was resuspended in medium 1 and stored at −70°C for enzyme assay.

Lipid extraction and identification

Cell and media lipids were extracted by the method of Bligh and Dyer (12), dried under N_2, and identified by thin-layer chromatography with appropriate standards. Fatty acids, partial glycerides, triacylglycerol, and cholesteryl esters were chromatographed with heptane–isopropyl ether–acetic acid 60:40:4 (v/v) on silica gel G plates. Cholesterol, which cochromatographs with diacylglycerol in this system, was separated by sequential elution in the Bond-Elut system (13). Extracted media fatty acids were separated by HPLC after conversion to phenacyl esters (14). The mobile phase was 75% (v/v) acetonitrile. Appropriate fatty acid standards were used to quantify mass. Radiolabeled fractions were collected, dried, and counted in Aquasol.

Other methods

Progesterone was measured by ^{125}I radioimmune assay using a commercially obtained kit. Human placental lactogen was measured by ^{125}I radioimmune assay in the laboratory of Dr. Stuart Handwerger. Protein was determined by the method of Lowry et al. (15) using bovine serum albumin as the standard. When cultured cells were used, collagen-protein was removed by sodium hydroxide precipitation (16).

Enzyme assays

Fatty acid CoA ligase activity was measured using 50 μM [^{3}H]palmitate and 5 mM ATP (17). Glycerophosphate acyltransferase activity was assayed using 75 μM palmitoyl-CoA and 300 μM [^{3}H]glycero-3-phosphate (18). Because the total specific activity of glycerophosphate acyltransferase was extremely low, the mitochondrial and microsomal activities were not differentiated. Diacylglycerol acyltransferase activity was assayed using 30 μM [^{3}H]palmitoyl-CoA and 200 μM *sn*-1,2-dioleoylglycerol dispersed in acetone (19). Diacylglycerol cholinephosphotransferase activity was measured using 100 μM CDP-[^{14}C]choline and 100 μM *sn*-1,2-dioleoylglycerol dispersed in acetone (20). All assays were proportional to the time and amount of protein employed. The substrate concentrations gave maximal activities.

RESULTS

Dispersed placental cells isolated at d 1.049-1.062 g/ml of the Percoll gradient attached to collagen-coated plastic dishes within 2 hr of plating. By 18 hr, most cells had flattened and developed long processes, and after 48 hr most cells had aggregated into small groups, contained

cytoplasmic inclusions, and had two or more nuclei (data not shown). These morphological changes are identical to those described by Kliman et al. (11) who presented data supporting the differentiation of human cytotrophoblasts to functional syncytiotrophoblasts during growth in monolayer culture.

The function of trophoblast cells in culture was assessed by examining hormone secretion (**Fig. 1A**). The cells secreted 4.7 and 8.4 ng of progesterone/10^6 cells per hr after 24 and 72 hr in culture, respectively. This secretion rate is higher than previously reported for human cells obtained and cultured similarly without added serum (11). Human placental lactogen secretion did not change during the same time period; values were about 50% of the amount secreted by cells that had been cultured with 10% fetal bovine serum (10). These results indicate that the ability of the cells to synthesize and secrete peptide and steroid hormones was maintained for at least 3 days in culture.

During the 72 hr in culture, the incorporation of [^{3}H]glycerol into triacylglycerol and phospholipids decreased 53 and 59%, respectively, indicating loss of glycerolipid synthetic capability (Fig. 1B). Incorporation of [^{14}C]acetate into cellular glycerolipids declined similarly 72 hr after plating (data not shown). Decreased glycerolipid synthesis did not appear to result from loss of cell viability as measured by trypan blue exclusion; nor was overgrowth of other cell types observed. In order to determine whether the decrease in glycerolipid synthesis from glycerol was related to a decrease in one or more specific enzyme activities, we investigated the putative regulatory microsomal activities of glycerolipid biosynthesis, fatty acid CoA ligase, the activity essential for activating long-chain fatty acids, *sn*-glycerol-3-phosphate acyltransferase, the committed step of the pathway, and diacylglycerol acyltransferase and cholinephosphotransferase activities, the steps unique to triacylglycerol and phosphatidylcholine synthesis, respectively (21). Compared to villous tissue, the specific activities of each of these four glycerolipid synthetic enzymes were the same or greater in cells cultured for 24 hr (**Table 1**). After 72 hr in culture, each activity declined to about 50% of its value at 24 hr, compatible with the decline observed in [^{3}H]glycerol incorporation into glycerolipids. These data do not suggest that regulation occurs at a single step in the pathway.

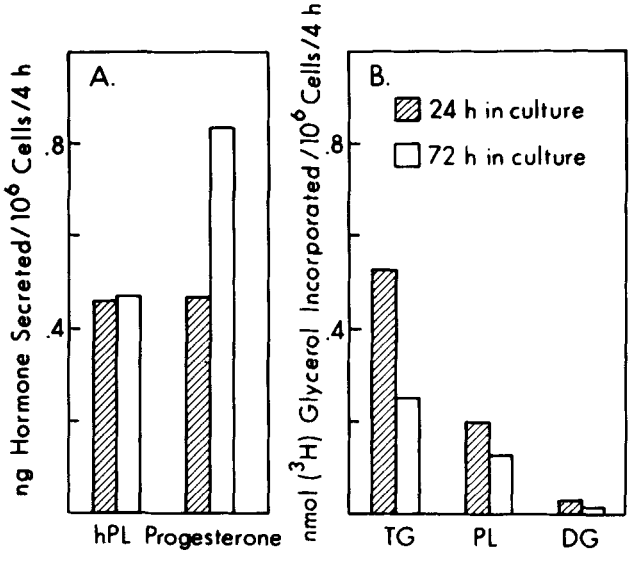

Fig. 1. Hormone secretion and glycerolipid synthesis by cultured trophoblast cells. A). Cells were incubated with DME-G, 10% fetal bovine serum for 72 hr. Media was removed and assayed for human placental lactogen (hPL) and progesterone at 24 and 72 hr in culture. Data represent the average of two independent determinations that varied less than 10%. B). Cells were incubated with DME-G, 10% fetal bovine serum for 24 hr or 72 hr, then the media was replaced with DME-G containing 1.3 mM [^{3}H]glycerol, 3 μCi/μmol, and 0.1 mM oleic acid. Four hours after addition of [^{3}H]glycerol, cells were washed and analyzed for glycerolipid content. hPL, human placental lactogen; TG, triacylglycerol; DG, diacylglycerol; PL, phospholipid. Data represents the average of two independent determinations that varied by less than 10%.

Endogenous fatty acid synthesis and secretion

Because acetyl-CoA carboxylase activity is low in human and rat placenta homogenates, it was suggested that little endogenous synthesis of fatty acid occurs in placenta and that endogenous synthesis would not contribute to placental fatty acid transport (22–24). This hypothesis was tested by incubating cultured trophoblast cells with [^{14}C]acetate and measuring the amount incorporated into cellular and media lipid species (**Fig. 2**). Acetate was rapidly incorporated into cellular triacylglycerol, phospholipid, and cholesterol but little fatty acid accumulated within the cell (Fig. 2A). The cells released [^{14}C]fatty acid into the media at a linear rate during the 4 hr of observation indicating that cultured trophoblast cells can release newly synthesized fatty acid at rates 50- to 200-fold greater than observed for hepatocytes in monolayer culture (Coleman, R. A., E. B. Haynes, T. M. Sand, and R. A. Davis. Developmental co-ordinate expression of triacylglycerol and small molecular weight apoB synthesis and secretion by rat hepatocytes, unpublished results[1]). The fatty acid mass quantified in three media samples was about 8 nmol/10^6 cells per 4 hr. About three times more unlabeled fatty acid than labeled fatty acid was released, indicating that much of the released fatty acid had been derived from preformed fatty acids stored in cell glycerolipids (see below).

Fatty acids synthesized from [^{14}C]acetate and secreted into the media were fractionated by HPLC. The percent

[1]Adult rat hepatocytes in monolayer cultures were incubated with 0.1 mM [^{14}C]acetate and 10% fetal bovine serum for 4 hr. In the media, 0.7 ± 0.09 nmol of acetate/10^6 cells was recovered as glycerolipid and < 0.01 nmol of acetate/10^6 cells was recovered as fatty acid.

TABLE 1. Specific activities of selected enzymes of glycerolipid biosynthesis

Enzyme	Specific Activity (Mean ± SD)		
	0 Hr[a]	24 Hr[b]	72 Hr[b]
	nmol per min per mg protein		
Fatty acid CoA ligase	4.2 ± 2.2	4.6 ± 1.6	2.34 ± 0.7
Glycerophosphate acyltransferase	0.08 ± 0.02	0.19 ± 0.06	0.08 ± 0.02
Diacylglycerol acyltransferase	1.13 ± 0.33	1.18 ± 0.32	0.55 ± 0.15
Diacylglycerol cholinephosphotransferase	0.59 ± 0.38	0.52 ± 0.20	0.22 ± 0.05

[a]Total particulate preparation of placenta villous tissue (n = 5).
[b]Total particulate preparation of isolated, cultured placental cells (n = 4 to 6).

distribution (mean ± SD of 11 samples after 2- or 4- hr incubations) of radiolabel in fatty acid was lauric, 3.4 ± 1.2; myristic, 3.8 ± 2.4; palmitoleic, 18.9 ± 3.3; palmitic, 19.1 ± 8.1; oleic, 42.3 ± 11.2; and stearic, 3.0 ± 1.0. Another 5.7 ± 5.0% of the ^{14}C-label eluted in the 1-4-min fractions and could represent fatty acids shorter than 12 carbons as well as [^{14}C]acetate that had been carried over into the chloroform extract. This variably present fraction was not analyzed further. Incorporation of [^{14}C]acetate into fatty acids longer than 18 carbons was not observed.

Release of fatty acids from cell glycerolipid

In order to determine whether trophoblast cells can release fatty acids from cellular lipids as has been hypothesized (7, 8) and as had been suggested by the fatty acid mass released during the [^{14}C]acetate incubations (see above), cells were incubated with [^{14}C]oleic acid for 24 hr, then chased with unlabeled oleic acid for 24 hr (**Fig. 3**). After 24 hr, 21.6% of the ^{14}C-label in triacylglycerol and phospholipid was recovered in the media as fatty acid. Another 19.6% of the initial label was lost from the chloroform-soluble fraction, probably reflecting cellular oxidation of fatty acid released from cellular glycerolipids. It appeared that 25% of the original label was lost from triacylglycerol and 50% from phospholipid. Intracellular free fatty acid was virtually undetectable at every time point.

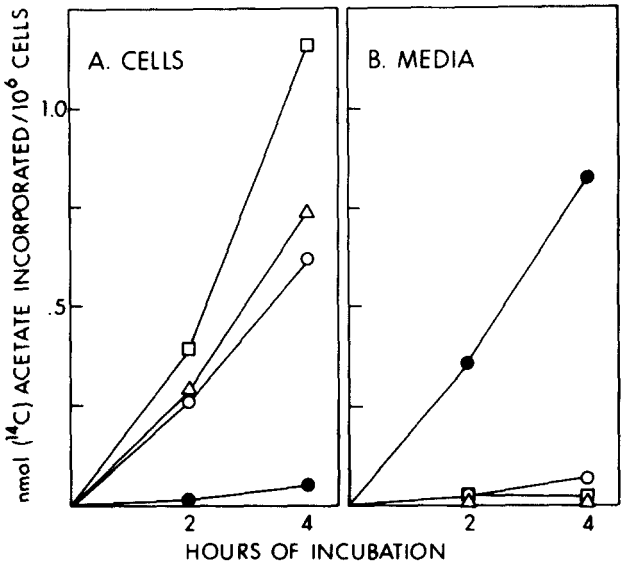

Fig. 2. Incorporation of acetate into (A) cell and (B) media lipids by trophoblast cells. Cells (2.18 × 10^6/dish) were plated with 93% efficiency and preincubated in DME-G and 10% fetal bovine serum as in Experimental Procedures. After 18 hr, the medium was replaced with DME-G, 10 mg/ml BSA, 0.1 mM oleic acid, and 0.1 mM [^{14}C]acetate. Two and 4 hr later the media and cells were extracted and lipids were identified as described: □, phospholipid; △, triacylglycerol; ○, cholesterol; ●, fatty acid. Each point is the average of results from two dishes.

DISCUSSION

Numerous in vitro studies have shown that placentally transported fatty acids provide as much as 50% of the daily fatty acid requirement of the fetus during late gestation (1-6). Placental transport is thus probably crucial for normal fetal development. The fetus derives all of its essential fatty acids from the mother and, indirectly, from the maternal diet. In the fetus, it is likely that both essential and nonessential fatty acids are used almost entirely for synthetic processes, since the activities of carnitine palmitoyl transferase and mitochondrial enzymes of β-oxidation are very low before birth (25); little evidence exists that transported fatty acids are used by the fetus for energy (26, 27). Transported fatty acids, and those synthesized de novo by the fetus, would be used to form complex lipids for the biogenesis of plasma and intracellular membranes, myelin, storage droplets of triacylglycerol and cholesterol esters, and specialized secretory products such as lipoproteins, bile and surfactant.

In vitro studies have demonstrated that fatty acids are transferred from maternal to fetal serum in a variety of animal species (1-6), and that the fatty acids of Intralipid (an emulsion of soybean triacylglycerol that is administered intravenously) can be transferred from the blood of a laboring woman to the cord blood of her infant at birth (28). Studies in guinea pigs show that radiolabeled acyl

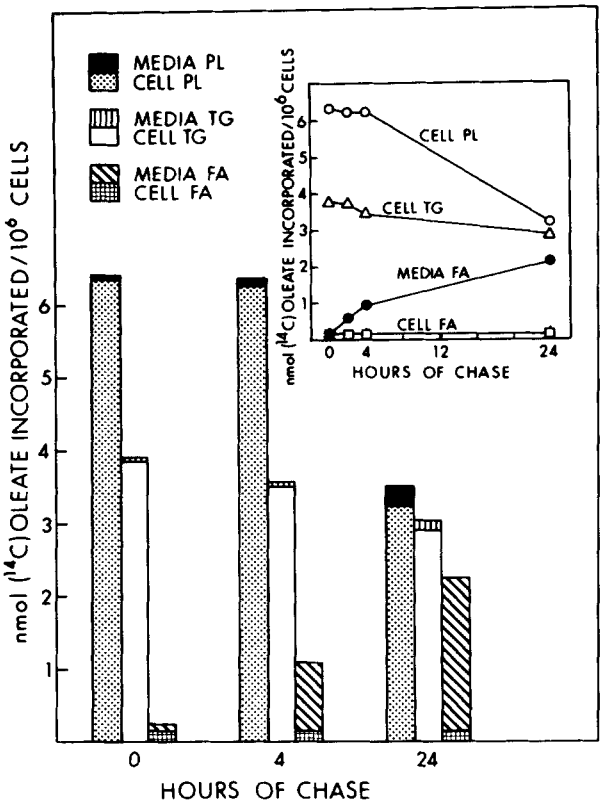

Fig. 3. Incorporation of [^{14}C]oleate into cellular and secreted glycerolipid and fatty acid by trophoblast cells. Cells were plated at 3.5×10^6 cells/dish in DME-G, 10% fetal bovine serum. After 15 hr, fresh medium was added plus 0.1 mM [^{14}C]oleic acid. After incubation for 24 hr the cells were rinsed three times with DME-G, 10 mg/ml BSA. Fresh DME-G, 10 mg/ml BSA, and 0.1 mM unlabeled oleic acid were added. At intervals, cell and media lipid was extracted and identified. Each point is the average of two dishes. A similar experiment, chasing with oleate-free media, showed a similar rate of secretion of fatty acid. PL, phospholipid; TG, triacylglycerol; FA, fatty acid.

groups of very low density lipoprotein-triacylglycerol are rapidly transferred from the serum of pregnant rats or guinea pigs to their fetuses (29, 30). These studies imply that placental lipoprotein lipase hydrolyzes Intralipid or very low density lipoprotein-triacylglycerol before fatty acid is transported, since intact phospholipid and triacylglycerol are not transported across the placenta (30, 31).

It was previously thought that little transported fatty acid was synthesized de novo by the placenta because placental acetyl-CoA carboxylase activity was low (22–24); however, significant fatty acid synthesis by human, pig, and sheep placenta has now been reported (32–34). Our data showing release of radiolabeled fatty acid into the media after trophoblast cells were incubated with [^{14}C]acetate is consistent with the latter studies. Trophoblast cells rapidly incorporated acetate into cellular triacylglycerol, phospholipid, and cholesterol and into media fatty acid. Our studies demonstrate a high rate of fatty acid release despite the fact that the media fatty acid was almost certainly being simultaneously taken up from the media by the cells. It is likely that the actual rate of fatty acid release into the media was greater than observed, since the released fatty acids could also be reincorporated into cellular glycerolipids. The amount of ^{14}C-labeled fatty acid release into the media by placental trophoblast cells was 50- to 200-fold higher than observed for rat hepatocytes incubated under identical conditions. Although a variety of cultured cells can release or exchange small amounts of free fatty acid with the media (35, 36), trophoblast cells appear to be similar to adipocytes and cultured aortic endothelial cells which release relatively large amounts of free fatty acid (35). It is unclear whether newly synthesized fatty acids are released immediately or are first esterified and then hydrolyzed before being released from cells. The fatty acids released by trophoblast cells were primarily 16 and 18 carbons in length, typical of the major fatty acid species synthesized by most mammalian tissues.

The hypothesis that esterified cellular lipids provide a reservoir of fatty acid that can be released from cells was tested by labeling cellular glycerolipids with [^{14}C]oleate and then chasing with unlabeled oleate for 2 to 24 hr (Fig. 3). Four hr after the start of the chase period, as much radiolabeled fatty acid was released into the media as occurred after 4 hr de novo synthesis from acetate (compare Figs. 2 and 3). The rate of release had decreased by 24 hr but in that time 49% of the previously labeled phospholipid and 25% of the triacylglycerol had disappeared from the cell. These data indicate that esterified glycerolipids in trophoblasts can provide a source of releasable fatty acid since, at no time, did unesterified fatty acid accumulate within the cells. Lack of accumulation of triacylglycerol and phospholipid in the media as well as low media lactate dehydrogenase activity indicate that extracellular hydrolysis of glycerolipids had not occurred and that little cellular damage occurred during the chase period.

Of the initial 10.35 nmol of labeled cell lipid/10^6 cells at the start of the chase, 8.25 nmol was recovered 24 hr later as cellular triacylglycerol and phospholipid and media fatty acid. These data imply that acylglycerol lipase(s) and one or more phospholipases acted to release fatty acids from cellular triacylglycerol and phospholpid. It may be that a specific phospholipid species provides a reservoir of acyl-chains for release from the cell. Twenty percent (2.1 nmol) of the original ^{14}C-label in cell glycerolipid was not recovered as chloroform-soluble material. The label was probably lost through β-oxidation and conversion to water-soluble products. Consistent with this hypothesis is a study showing that, in guinea pig placenta, fatty acids that are radiolabeled at carbon-1 appear to be transported less well than those labeled further from carbon-1 (37). It was suggested that these data indicate partial placental oxidation of fatty acid before secretion. Further studies of

the released fatty acids will be required to determine whether secretion of partially oxidized fatty acids is a significant process in cultured trophoblast cells.

The four microsomal activities of glycerolipid biosynthesis measured in total particulate preparations from human placenta and human trophoblast cells differ markedly from our previous studies of rat placenta microsomes (9). In human placenta, fatty acid CoA ligase specific activity is more than 2-fold higher and glycerophosphate acyltransferase is more than 30-fold lower than observed in rat placenta. The relationship between glycerolipid biosynthesis and enzyme specific activities measured under optimum in vitro conditions is not well understood. Despite the relatively low specific activity of the putative committed step of the glycerolipid synthetic pathway, the glycerophosphate acyltransferase, triacylglycerol, and phospholipids were actively synthesized without intracellular accumulation of fatty acid. Clearly, only guarded interpretations can be made concerning the effect of altered in vitro enzyme specific activities and in vivo rates of synthesis.

Human placenta cells in monolayer culture are emerging as a model system in which one can study trophoblast differentiation to syncytiotrophoblast (38), hormone secretion in response to acute stimuli (10, 39, 40), and hormone receptor binding (41, 42). Our data suggest that these cells will also be an ideal model for the study of placental fatty acid metabolism as it relates to transport, since they can be maintained in defined media and selectively stimulated with hormones, substrates, and small molecule effectors.

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