Uptake of long chain fatty acids by human placental choriocarcinoma (BeWo) cells: role of plasma membrane fatty acid-binding protein

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Abstract In order to understand the mechanisms by which fatty acids are taken up by the placenta, the uptake of oleic, linoleic, arachidonic, and docosahexaenoic acids by cultured human placental choriocarcinoma (BeWo) cells was examined. Fatty acid uptake by BeWo cells was temperature-dependent and exhibited saturable kinetics. Oleic acid was taken up least and docosahexaenoic acid most by these cells. Moreover, competitive studies of fatty acid uptake by BeWo cells also indicated preferential uptake compared with oleic acid in the order of docosahexaenoic acid, arachidonic acid, and linoleic acid. Western blot analysis demonstrated that BeWo cells express a protein immunoreactive with antibodies to the human placental plasma membrane fatty acid-binding protein (p-$FABP_{pm}$). Furthermore, pre-treatment of BeWo cells with these antibodies inhibited most of the uptake of docosahexaenoic (64%) and arachidonic acids (68%) whereas oleic acid uptake was inhibited only 32% compared with the controls treated with preimmune serum. These results clearly demonstrate that the p-$FABP_{pm}$ may be involved in the preferential uptake of essential fatty acids (EFA) and their long chain polyunsaturated fatty acids (LCPUFA) by these cells. Studies on the distribution of radiolabeled fatty acids in the cellular lipids of BeWo cells showed that docosahexaenoic acid was incorporated mainly in the triacylglycerol fraction, followed by the phospholipid fraction, whereas for arachidonic acid the reverse was true. The preferential incorporation of docosahexaenoic acid into triacylglycerol suggests that triacylglycerol may play an important role in the placental transport of docosahexaenoic acid to the fetal circulation. Together these results demonstrate the preferential uptake of EFA/LCPUFA by BeWo cells that is most probably mediated via the p-$FABP_{pm}$. We thus propose that the p-$FABP_{pm}$ may be involved in the sequestration of maternal plasma LCPUFA by the placenta.—**Campbell, F. M., A. M. Clohessy, M. J. Gordon, K. R. Page, and A. K. Dutta-Roy.** Uptake of long chain fatty acids by human placental choriocarcinoma (BeWo) cells: role of plasma membrane fatty acid-binding protein. *J. Lipid Res.* 1997. **38:** 2558–2568.

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During the past decade, increasing attention has been focused on the role of n–3 and n–6 essential fatty acids (EFA) and their long chain polyunsaturated fatty acid (LCPUFA) derivatives in fetal growth and development (1–5). During the intrauterine life, the LCPUFA, docosahexaenoic acid, 22:6n–3 and arachidonic acid, 20:4n–6 rapidly accumulate in fetal tissue phospholipids and play a critical role in membrane structure and function, and as eicosanoid precursors (2, 5–8). Dietary deficiency in these fatty acids is associated with visual abnormalities in rats (9), in non-human primates (10), in preterm infants (11), and in formula-fed term infants (12). In humans the vulnerable period of brain development and the maximal rate of LCPUFA deposition occurs in the third trimester and the first 6 to 9 months postnatally (2, 13). These LCPUFA are transported from the maternal circulation across the placenta into fetus venous blood, from which they subsequently cross the blood–brain barrier into the fetal nervous system (14). Placental transport of maternal plasma LCPUFA is in practice crucial for fetal growth and development because fetal synthesis is considered to be very low (1, 4, 15–18). As human placental tissue lacks both the delta 6 and delta 5 desaturase activities (2, 19–21), any LCPUFA in the fetal circulation are most probably the desaturated metabolites of EFA produced by the mother. However, the biochemical mechanisms responsible for higher levels of LCPUFA in the fetal circulation compared with the maternal plasma (2, 15) are still not clear.

In the materno-fetal unit, free fatty acids (FFA) are the main class of naturally occurring lipids transferred across the placenta, irrespective of species or of

Abbreviations: EFA, essential fatty acid; LCPUFA, long chain polyunsaturated fatty acids; $FABP_{pm}$, plasma membrane fatty acid-binding protein; FFA, unesterified free fatty acids; BSA, bovine serum albumin.

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the maternal source from which they originate (1, 4, 19, 22, 23). During the last trimester, FFA concentrations increase rapidly in order to support fetal demand for LCPUFA (21, 23). Recently, we have suggested that the placental membrane fatty acid-binding protein (p-$FABP_{pm}$) which is exclusively located on the membrane-facing the maternal circulation of the human placenta may play an important role in the preferential uptake of LCPUFA from maternal plasma FFA pool by the placenta (23–27). All these studies were, however, performed using prepared human placental membranes and no information is available on intact trophoblast cells with metabolic activity. To understand the uptake and metabolism of EFA/LCPUFA and role of p-$FABP_{pm}$ in placental cells, we have therefore utilized the human placental choriocarcinoma (BeWo) cell line as a model of the human trophoblast. The BeWo cell line displays some placental differentiation markers including the production of placental specific proteins (28) and has been used extensively to study lipoprotein metabolism (29). These cells have, however, not been characterized with regard to fatty acid uptake and metabolism.

In the present study, we examined the uptake and metabolism of linoleic acid (18:2n–6), arachidonic (20:4n–6), docosahexaenoic acid (22:6n–3) and a nonessential fatty acid, oleic acid (18:1n–9) by BeWo cell and also the role of the p-$FABP_{pm}$ in the uptake processes. These studies demonstrate for the first time that the p-$FABP_{pm}$ may be responsible for preferential uptake of maternal LCPUFA by the placenta.

MATERIALS AND METHODS

Materials

[1-^{14}C]linoleic acid (sp act 55 mCi/mmol), [9,10-^{3}H]oleic acid (sp act 10 Ci/mmol) and [1-^{14}C]arachidonic acid (sp act 58 mCi/mmol) were obtained from Amersham, UK, while [4,5-^{3}H]docosahexaenoic acid (sp act 60 Ci/mmol) was purchased from New England Nuclear, U.K. Unlabeled fatty acids, trypsin-EDTA solution, penicillin–streptomycin solution, molecular weight markers, glutamic–oxaloacetic transaminase assay kit (procedure 58-UV), and lipid standards were obtained from Sigma, Poole, U.K. Nutrient Ham's Mix-F-12 with glutamax-I and William's medium E with glutamax-I were obtained from Gibco Life Technologies, Scotland. Horseradish peroxidase (HRP)-linked anti-rabbit IgG was from Scottish Antibody Production Unit, Scotland. Silica-gel LK5 plates were obtained from Whatman, U.K. All other chemicals and solvents were high purity commercial materials obtained from either Sigma, Aldrich Chemical Co., U.K. or BDH, U.K.

Cell culture

BeWo cells were obtained from European Collection of Animal Cell Cultures and were grown in Ham's F12 medium containing 10% fetal bovine serum, 6 mM glutamine, and 100 IU/ml penicillin–streptomycin. Cells were maintained as monolayers in 25 cm^2 tissue culture flask at 37°C with a 5% CO_2-balanced air, and 100% humidity atmosphere. At confluence they were subcultured using a trypsin-EDTA solution to suspend the cells. Media was renewed three times weekly. Cell viability was routinely tested through the exclusion of trypan blue. For experiments, cells from early confluent monolayers were dispersed and replated in 58 mm × 115 mm culture dishes. Prior to the start of the incubation experiments, media were removed and cultures were washed twice with phosphate-buffered saline (PBS), pH 7.4. In some experiments the human hepatoma cell line, HepG2 grown in William's medium, was used as a control.

Preparation of radiolabeled fatty acids

The long chain fatty acids were complexed with bovine serum albumin (BSA) for introduction to cells as described earlier (30). Typically, the radiolabeled fatty acids ([^{3}H]oleic, [^{14}C]linoleic, [^{14}C]arachidonic, and [^{3}H]docosahexaenoic acids) were dissolved in 50 μl of 0.1 M NaOH at 37°C to which appropriate quantities of the corresponding unlabeled fatty acids were added in order to achieve the desired final concentrations (200 μM) (30). Defatted bovine serum albumin (BSA) dissolved in PBS was added to obtain the desired fatty acid: BSA molar ratios (0.25:1, 0.5:1, 1:1, 2:1, 4:1, and 5:1), the pH was adjusted to 7.4 and the fatty acid/BSA solution was diluted to its final concentration with PBS (24–26). The free fatty acid concentration at equilibrium with BSA at 37°C was calculated using the stepwise equilibrium method and the affinity constants previously published for these fatty acids (31). The free fatty acid concentrations were calculated using the EQUAL computer program (Biosoft, U.K.).

Fatty acid uptake studies

Radiolabeled fatty acid uptake studies were performed essentially following the method of Schurer et al. (32). After the confluent growth, the medium was removed from the cell culture plates and the cells were washed with 1 ml of PBS. Both the cells and the radiolabeled ligand were brought to 37°C by incubation in a water bath. The cultures containing approximately 8.25×10^6 cells per dish were then incubated with 500 μl of radiolabeled fatty acid by gentle agitation in a water

bath at 37°C. The cells were incubated at various time intervals to determine the time-course of fatty acid uptake. The fatty acid uptake was stopped by the addition of 3 ml of ice-cold 0.5% BSA. The plates were washed three times with 0.5% BSA and twice with 0.9% NaCl to remove any surface-bound fatty acid. The cells were dissolved by the addition of 1 ml of 1 M NaOH and left overnight at 4°C. Aliquots (500 μl) were added to 5 ml scintillation cocktail (Opti-Fluor) and the radioactivity was determined using a scintillation counter. Additional 20-μl aliquots were processed for determination of the protein content of the cells by the Bradford method using BSA as the protein standard (33).

The effect of temperature on fatty acid uptake by BeWo cells was investigated by incubating these cells at 4°, 26°, and 37° with [^{3}H]oleic acid, [^{14}C]linoleic acid, [^{14}C]arachidonic acid, and [^{3}H]docosahexaenoic acid for 30 min. In order to determine the effects of increasing concentrations of free fatty acid on the uptake activity, cells were incubated for 30 min with 200 μM of the radiolabeled ligands bound to varying quantities of albumin (i.e., fatty acid/BSA molar ratios ranging from 0.25:1, 0.5:1, 2:1, 4:1, and 5:1). Parallel uptake experiments were carried out to examine whether fatty acid uptake was a function of unbound fatty acids or albumin concentration in the medium by keeping a fixed concentration of defatted BSA (200 μM) and varying concentrations of radiolabeled fatty acids (50 to 400 μM) (34).

Competition for fatty acid uptake was investigated by incubating these cells with radiolabeled fatty acid in the presence of a 10-fold excess of unlabeled competitive fatty acids, as described (27, 32).

Purification of human placental membrane fatty acid-binding protein (p-FABP$_{pm}$)

p-FABP$_{pm}$ was purified to electrophoretic homogeneity from the human term placenta following the method as described previously (25, 26). Briefly, human placental membranes were solubilized with 1% Triton X-100, and thereafter the solubilized membrane proteins were subjected to oleate–agarose affinity column. The affinity column was washed with PBS until no further protein appeared in the washes as monitored by absorbance at 280 nm. The affinity column was then washed with 7 M urea and the eluate was dialyzed and concentrated. Purity and activity of the protein preparation were examined by SDS-polyacrylamide gel electrophoresis (PAGE), PAGE autoradiobinding, and radioligand binding, as described earlier (26).

Determination of glutamic-oxaloacetic transaminase activity of human placental FABP$_{pm}$

We examined whether the purified p-FABP$_{pm}$ has glutamic–oxaloacetic transaminase (GOT:L-asparatate:2-oxaloglutarate aminotransferase, E.C. 2.6.1.1) activity like that of the ubiquitous plasma membrane fatty acid-binding protein (FABP$_{pm}$, 40 kDa) (35). This FABP$_{pm}$ has been shown to be present in many tissues such as liver, gut, and adipose (30,35) and it is therefore termed FABP$_{pm}$ in this paper without prefix. The GOT activity of the purified p-FABP$_{pm}$ and human placental membranes was measured using a commercially available GOT kit, as described previously for FABP$_{pm}$ (35).

Preparation of polyclonal antisera to p-FABP$_{pm}$

Rabbit polyclonal antisera against the purified p-FABP$_{pm}$ were prepared, as described (25). Polyclonal antisera against p-FABP$_{pm}$ were raised in New Zealand white rabbits by an initial immunization with 100 μg of the purified p-FABP$_{pm}$ dissolved in PBS together with an equal volume of Freund's complete adjuvant and one subsequent immunization of 100 μg of the protein in 250 μl of PBS and 250 μl of Freund's incomplete adjuvant. Preimmune serum was collected from the rabbits prior to the first immunization. On Western blot analysis the antiserum showed a clear immunopositive band ~40 kDa with purified p-FABP$_{pm}$ and with human placental membranes. It showed no cross-reactivity with other human placental membrane proteins.

Electrophoretic separation and Western blot analysis of membrane proteins from BeWo cells

Western blot analysis of membrane proteins from BeWo cells, HepG2 cells, and human placental membranes was carried out as described previously (25,26). HepG2 cells which contain FABP$_{pm}$ (36) were used here as control. Cell membranes were prepared as described before (26, 37). Polyacrylamide gel electrophoresis of these membranes in the presence of sodium dodecyl sulfate (SDS) was carried out under reducing conditions on SDS-PAGE homogeneous 20 gels (Phast System, Pharmacia, U.K.) as described (26). After electrophoresis, proteins were transferred onto a PVDF membrane by diffusion at 70°C for 1 h. The membrane was probed for the presence of p-FABP$_{pm}$ by incubating with rabbit polyclonal antiserum to human p-FABP$_{pm}$. Antibody–antigen complexes were then detected with HRP–anti-rabbit IgG fraction of donkey polyclonal antiserum (Scottish Antibody Production Unit).

Antibody inhibition of fatty acid uptake by BeWo cells

In order to investigate the role of p-FABP$_{pm}$ in the preferential uptake of LCPUFA by these cells, fatty acid uptake experiments were carried out using BeWo cells treated with anti-p-FABP$_{pm}$ antibody or as control with rabbit preimmune serum. Typically, monolayer cultures were pre-incubated with 1 ml of a 1:40 dilution in PBS

of a polyclonal antiserum raised in rabbit against human p-$FABP_{pm}$ or as a control with 1 ml of 1:40 dilution of the rabbit pre-immune serum in 1 ml PBS, pH 7.4, as described before (25, 26). After pretreatment of cells with the antisera for 30 min at 37°C, cells were washed three times with PBS and incubated with radiolabeled fatty acid as described above. At the end of this preincubation, trypan blue exclusion remained greater than 95% in both antiserum- and pre-immune serum-pretreated cell preparation.

Distribution of radiolabeled fatty acids into cellular lipids

To investigate the incorporation of radiolabeled fatty acid into cellular lipids, cells were incubated for 30 min with radiolabeled fatty acids as described above. After stopping the fatty acid uptake by the addition of 0.5% BSA, the cells were washed three times with 0.5% BSA, followed by two washes with 0.9% NaCl. The attached cells were then washed with ice-cold PBS solution (1 ml) and harvested by scraping with a rubber policeman. The cells were sonicated using an ultrasonicator. The volume was brought to 1 ml. An aliquot of the cell suspension was removed for protein estimation. The remainder was adjusted to pH 3.5 by the addition of 1 M HCl and total lipids were extracted according to the method of Bligh and Dyer (38), dried by rotary evaporation, and redissolved in 100 μl chloroform–methanol 2:1 (v/v). An aliquot was taken for scintillation counting and the remainder was applied to thin-layer chromatography (TLC) silica gel plates (Silica gel LK5). Separation was achieved using a hexane–ether–formic acid 80:20:2 (v/v) solvent system. Lipid fractions were identified by co-chromatography with suitable standards and visualized under UV light after spraying with 0.02% dichlorofluorocene in ethanol. Appropriate bands were scaped directly into vials containing scintillation fluid (Opti-Fluor) and counted. Percentage incorporation into each lipid fraction was calculated by dividing the radioactivity found in that lipid fraction by the total radioactivity incorporated into cellular lipids. In this study the extraction and TLC recovery efficiency using [^{14}C]linoleic acid for [^{3}H]oleic acid, [^{14}C]arachidonic acid for [^{3}H]docosahexaenoic acid, and vice versa, as internal standard were more than 80%.

Statistical analysis

Results are given as mean ± standard error of the mean (SEM). Each experimental condition was carried out in triplicate and all experiments were performed at least three times. The statistical significance was determined using a two-tailed Student's *t* test; *P* values equal to or less than 0.05 were considered significant.

RESULTS

Fatty acid uptake by BeWo cells

Fatty acid uptake by BeWo cells was examined using radiolabeled fatty acids (oleic acid, linoleic acid, arachidonic acid, and docosahexaenoic acid). **Figure 1** shows the time-course of fatty acid uptake by BeWo cells up to 90 min. The fatty acids were present (200 μM) in the incubation mixture with BSA at 1:1 molar ratio. BeWo cells took up these fatty acids in a time-dependent manner. After 30 min of incubation, oleic acid uptake leveled off, whereas uptake of docosahexaenoic, arachidonic, and linoleic acids continued to increase beyond 30 min. The increase was, however, very small. The uptake of docosahexaenoic acid was always the greatest of the four fatty acids throughout the incubation period. After 30 min of incubation, the uptake of oleic, linoleic, arachidonic, and docosahexaenoic acids was 5.36 ± 0.14, 6.52 ± 0.54, 5.94 ± 0.34, and 15.75 ± 2.58 nmol/mg of protein, respectively.

For the study of fatty acid uptake as a function of unbound free fatty acid concentration in the medium, a series of fatty acid–BSA preparations with various concentrations of BSA resulting in fatty acid/BSA molar ratios ranging between (0.25:1 and 5:1) were used. Fatty acid uptake was measured after 30 min incubation at 37°C. Uptake increased with fatty acid/BSA molar ratios up to 1:1; however, it was not a linear function of free unbound fatty acid in equilibrium with BSA beyond a 1:1 molar ratio (**Fig. 2**), and the rate of increase in fatty acid uptake by BeWo cells with increasing concentrations of unbound FFA did not change significantly, indicating saturation of the uptake mechanism. No difference in the fatty acid uptake was observed at particular incubated unbound fatty acid concentration achieved by varying either the fatty acid or BSA. **Table 1** shows only oleic acid uptake data; however, similar results were observed with linoleic and arachidonic acids. Uptake of DHA was not performed. This indicates that changes in albumin concentrations in the medium have no effect on the uptake of fatty acids by these cells under the experimental conditions.

Uptake of various fatty acids by BeWo cells at different temperatures (4°, 26°, and 37°C) was determined. The amount of fatty acid taken up by BeWo cells was greater for all of these fatty acids at 37°C compared with both 4° and 26°C. At 37°C, the uptakes of docosahexaenoic acid (15.75 ± 2.58 nmol/mg protein) and oleic acid (5.36 ± 0.14 nmol/mg protein) were 3-fold greater than those at 26°C (5.09 ± 0.88 nmol/mg protein for docosahexaenoic acid, and 1.68 ± 0.46 nmol/mg protein for oleic acid) ($P < 0.02$); and the corresponding increase in uptake for linoleic acid was 6-fold

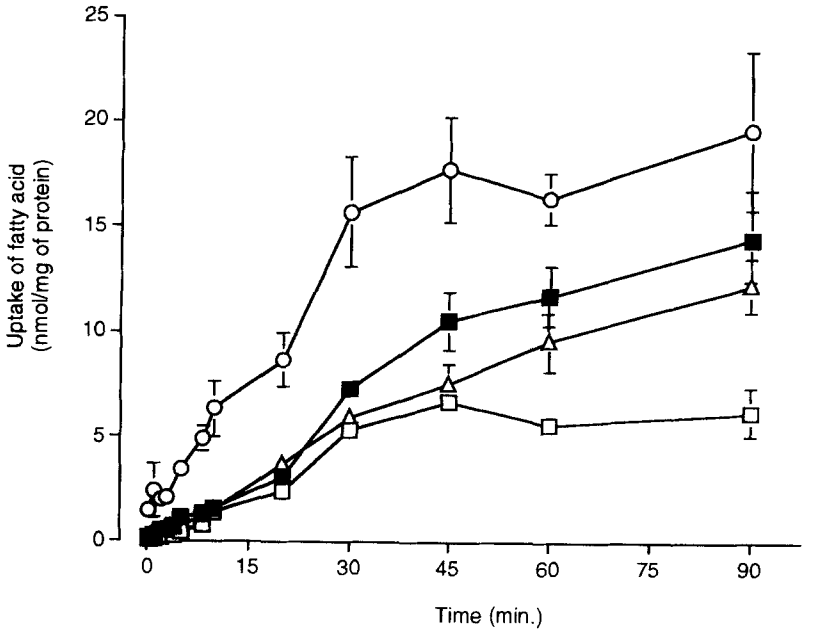
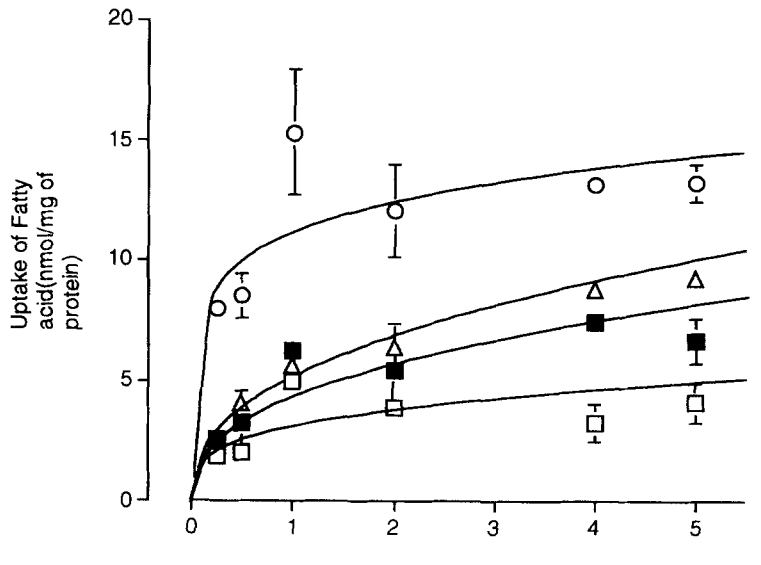


Fig. 1. Time course of fatty acid uptake in cultured BeWo cells. Cells were incubated with oleic acid (□), linoleic acid (■), arachidonic acid (△), or docosahexaenoic acid (○) (200 μM: FA/BSA, 1:1) at 37°C, and uptake was stopped at intervals at different time points. Data represent the mean ± SEM obtained from three separate experiments.

(1.08 nmol/mg protein at 4°C vs. 6.49 ± 0.44 nmol/mg protein at 26°C) ($P < 0.05$). These data indicate temperature dependency of fatty acid uptake which supports the link of fatty acid uptake with cell metabolism.

Studies were then conducted to determine whether uptake of fatty acids was competitive. **Table 2** shows the results of experiments conducted in the presence of a 10-fold excess of unlabeled fatty acid. Unlabeled oleic



Unbound Fatty acid (μM)						
	OA	0	0.0068	0.0156	0.091	0.359
	LA	0	0.0124	0.0295	0.548	1.748
	AA	0	0.0162	0.3430	0.731	2.208
	DHA	0	ND	ND	ND	ND

Fig. 2. Uptake of fatty acids as a function of the unbound fatty acid concentration. Cultured BeWo cells were incubated at 37°C with fixed concentration of fatty acid (200 μM) bound to various concentrations of BSA to achieve fatty acid/BSA ratios 0.25:1, 0.5:1, 1:1, 2:1, 4:1, and 5:1 from which unbound fatty acid concentrations for each fatty acid were calculated. Data represent the mean ± SEM obtained from three separate experiments. Oleic acid (□), linoleic acid (■), arachidonic acid (△), or docosahexaenoic acid (○).

TABLE 1. Uptake of oleic acid by BeWo cells by varying oleic acid or albumin concentrations in the incubation medium

Oleate:BSA Ratio	Uncomplexed Oleate	Fatty Acid Uptake: Constant Oleate (200 μM)	Fatty Acid Uptake: Constant BSA (200 μM)
	nM	*nmol/mg of protein*	
0.25:1	1.79	1.93 ± 0.37	1.95 ± 0.30
0.5:1	3.43	2.02 ± 0.26	1.90 ± 0.12
2:1	15.43	3.62 ± 0.47	4.28 ± 0.24

Fatty acid uptake by BeWo cells was determined keeping a fixed concentration of 200 μM of oleate bound to various concentrations of BSA or with various concentrations of oleate bound to a fixed concentration of BSA. Data represent the mean ± SEM of three separate experiments in which triplicate determinations were performed. Student's *t* tests were performed as described in Methods. No significant difference was observed.

TABLE 2. Competition of fatty acid uptake by BeWo cells

Fatty Acids	BeWo Cells Fatty Acid Uptake
	nmol/mg of protein
[^{3}H]oleic acid	5.36 ± 0.14
+Linoleic acid (10-fold)	1.41 ± 0.15[a]
+Docosahexaenoic acid (10-fold)	0.69 ± 0.17[a]
[^{14}C]linoleic acid	6.72 ± 0.50
+Oleic acid (10-fold)	5.40 ± 0.30
+Docosahexaenoic acid (10-fold)	0.40 ± 0.07[a]
[^{14}C]arachidonic acid	5.94 ± 0.34
+Oleic acid (10-fold)	4.79 ± 0.54
+Linoleic acid (10-fold)	3.31 ± 0.56[b]
+Docosahexaenoic acid (10-fold)	3.47 ± 0.20[c]
[^{3}H]docosahexaenoic acid	15.75 ± 2.58
+Oleic acid (10-fold)	12.20 ± 0.90
+Linoleic acid (10-fold)	6.15 ± 0.18[b]

Competition between fatty acids for uptake by BeWo cells was carried out as described in Materials and Methods. The cells were incubated with or without a 10-fold excess of unlabeled fatty acid. Data represent the mean ± SEM of three separate experiments in which triplicate determinations were performed.

[a] $P < 0.0001$, [b] $P < 0.02$, [c] $P < 0.005$; between uptake by controls and in the presence of 10-fold of excess unlabeled fatty acid.

acid had no significant inhibitory effect on the uptake of any of the other three fatty acids ($P < 0.5$), whereas uptake of oleic acid (5.56 ± 0.16 nmol/mg protein) was inhibited 3.8-fold (1.41 ± 0.15 nmol/mg protein) and 7.8-fold (0.69 ± 0.17) by the presence of excess unlabeled linoleic acid ($P < 0.0001$) and docosahexaenoic acid ($P < 0.0001$), respectively. Docosahexaenoic acid inhibited linoleic acid uptake by 16.8-fold, ($P < 0.0001$). Arachidonic acid uptake was inhibited by linoleic acid and docosahexaenoic acid to almost similar extent (by 1.7-fold). Unlabeled linoleic acid inhibited the uptake of docosahexaenoic acid uptake only by 2.5-fold ($P < 0.02$). These competition studies indicate that the fatty acid uptake system in BeWo cells has a greater affinity for docosahexaenoic acid and the preference order was docosahexaenoic acid>arachidnoic acid>linoleic acid>>>>>oleic acid.

Further characterization of human p-FABP$_{pm}$ and its presence and role in BeWo cells

We have shown earlier that p-FABP$_{pm}$ has an amino acid composition and pI value different from the FABP$_{pm}$ isolated from hepatic and other tissues (26). As FABP$_{pm}$ and mGOT have been shown to be an identical protein (35), we investigated whether the purified p-FABP$_{pm}$ and the placental membranes from which this protein was purified have GOT activity. The p-FABP$_{pm}$ and the placental membranes did not show any appreciable GOT enzymic activity. This clearly indicates that this p-FABP$_{pm}$ is different from the FABP$_{pm}$ present in various tissues. Western blot analysis of membrane proteins of BeWo cells was performed to determine whether BeWo cells have a protein that is immunoreactive to polyclonal anti-human p-FABP$_{pm}$ antiserum. **Figure 3** shows that this antiserum reacts with a protein of 40 kDa mass in BeWo cells but not with any of the membrane proteins of HepG2 cells.

As it has been suggested that this placental membrane protein may be involved in the preferential uptake of long chain fatty acids by placenta (21, 23, 27), fatty acid uptake in BeWo cells was examined by pretreating these cells with the antiserum raised against human p-FABP$_{pm}$ or with pre-immune serum. Pretreatment of these cells with anti-p-FABP$_{pm}$ antiserum inhibited the uptake of oleic, linoleic, arachidonic, and docosahexaenoic acids to different degrees compared with that in pre-immune serum treated cells. With the same amount of antisera, oleic acid uptake was inhibited least (32%, $P < 0.05$), whereas inhibition of the uptake of arachidonic acid (68%, $P < 0.001$) and docosahexaenoic acid (64%, $P < 0.0001$) was greatest, followed by linoleic acid uptake (50%, $P < 0.001$) (**Table 3**). There were significant differences observed in the degree of inhibition of uptake by the antibody between oleic acid and EFA/LCPUFA ($P < 0.005$). These results clearly indicate that fatty acid uptake is at least partly mediated via the p-FABP$_{pm}$ and that this protein may be responsible for preferential uptake of EFA/LCPUFA by these cells.

Distribution of radiolabeled fatty acid into cellular lipids

To determine whether the apparent differences in the uptake between these fatty acids reflect their rates of esterification, the distribution of radiolabeled fatty acids (oleic acid, linoleic acid, arachidonic acid, and docosahexaenoic acid) into different lipid fractions in BeWo cells after 30 min of incubation was examined

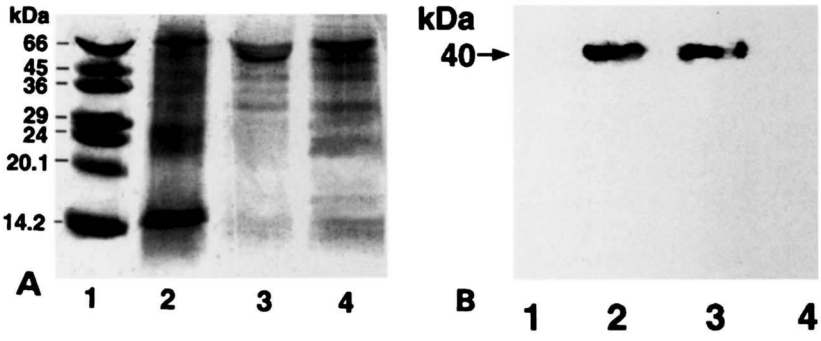


Fig. 3. Western blot analysis of membrane proteins. Panel A: Representative SDS-PAGE gel of membrane proteins as described in Methods section. Lane 1: Molecular weight protein standards, bovine serum albumin (66 kDa), egg albumin (45 kDa), glyceraldehyde 3 dehydrogenase (36 kDa), carbonic anhydrase (20 kDa), and alpha lactalbumin (14.2 kDa); lane 2: human placental membrane proteins (15 μg); lane 3: BeWo cell membrane proteins (15 μg), lane 4: HepG2 membrane proteins (15 μg). Panel B: Representative Western blot of the membrane proteins showing immunoreactivity with human placental anti-$FABP_{pm}$ antisera. The Western blots were carried out as described in Methods. The lanes were as for panel A.

(**Fig. 4**). The percentage values of unesterified free fatty acids over total incorporation of radioactivity reveal that esterification of docosahexaenoic acid is the highest of four fatty acids after 30 min of incubation. This conclusion follows as the unesterified form for docosahexaenoic acid was the least of all the essential fatty acids (for docosahexaenoic acid, 7% vs. 21% for arachidonic acid ($P < 0.005$); 16% for linoleic acid ($P < 0.05$), 20% for oleic acid ($P < 0.05$).

The esterified forms of all these fatty acids (linoleic acid, oleic acid, arachidonic acid, and docosahexaenoic acid) were found mainly in the triacylglycerols and phospholipid fractions, but a small portion was present in the cholesteryl ester and diacylglycerol fractions, the latter being preferred compared to cholesteryl ester. Nevertheless, comparison of the respective labeling after 30 min incubation demonstrated differences between these fatty acids and docosahexaenoic acid. The incorporation of oleic acid (43%) and docosahexaenoic acid (40%) in triacylglycerol fraction was similar. Among EFA/LCPUFA, incorporation of docosahexaenoic acid in triacylglycerol was highest ($P < 0.005$) while its incorporation was lowest in phospholipid fractions ($P < 0.05$) (Fig. 4). The incorporation of docosahexaenoic acid into triacylglycerol was approximately 2-fold higher than that of the other EFA/LCPUFA (41% for docosahexaenoic acid vs. 20% for arachidonic acid, and 24% for linoleic acid). Docosahexaenoic acid incorporation in the cholesteryl ester fraction was also the highest of these fatty acids ($P < 0.001$), but incorporation into diacylglycerol was similar. When incorporation of radiolabeled arachidonic acid and linoleic acid into BeWo cell lipids was compared, significant differences were observed in the phospholipids and cholestyl ester fractions but there were no differences between the incorporation into FFA, triacylglycerol, or diacylglycerol fractions. Incorporation of arachidonic acid in phospholipids was less than that of linoleic acid (55% vs. 44%, $P < 0.05$) whereas the reverse was true in the case of the cholesteryl ester fraction (4.27% for arachidonic acid vs. 2.2% for linoleic acid, $P < 0.005$).

DISCUSSION

During the last trimester, major differences in the quantitative and qualitative spectrum of the plasma

TABLE 3. Effect of anti-human p-$FABP_{pm}$ antibody on fatty acid uptake by BeWo cells

	Uptake of Fatty Acid			
Treatment	Oleic Acid	Linoleic Acid	Arachidonic Acid	Docosahexaenoic Acid
	nmol/mg protein			
Pre-immune serum	4.89 ± 0.10	6.27 ± 0.36	4.60 ± 0.25	13.47 ± 0.33
Anti-p-$FABP_{pm}$ antibody	3.31 ± 0.14[a]	3.12 ± 0.23[b]	1.45 ± 0.17[b]	4.82 ± 0.23[c]

Effect of rabbit anti-human placental $FABP_{pm}$ versus control preimmune serum on uptake of fatty acids by BeWo cells. Cells were pre-treated with either the antiserum or the control preimmune serum for 30 min at 37°C and then incubated with radiolabeled fatty acid (200 μM, fatty acid/BSA 1:1) for 30 min. Uptake of fatty acid was determined as described in Methods. Data represent the mean ± SEM of three separate experiments.
[a] $P < 0.05$, [b] $P < 0.001$, [c] $P < 0.0001$: between antibody and preimmune serum treatment.

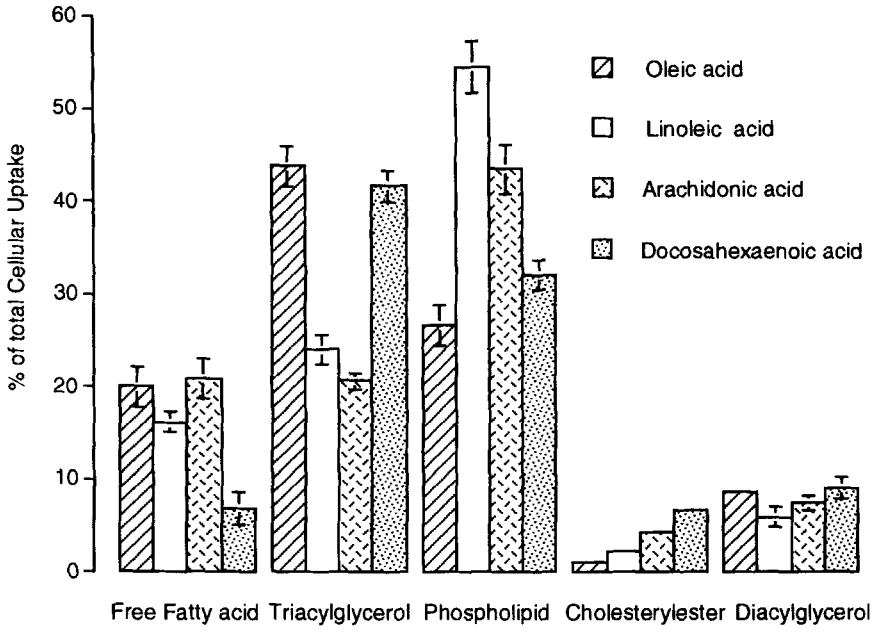


Fig. 4. Distribution of radiolabeled fatty acids into cellular lipids. BeWo cells were incubated with radiolabeled fatty acids (oleic acid, linoleic acid, arachidonic acid, and docosahexaenoic acid) (200 μM each) after 30 min of incubation. Lipids were then extracted and fractionated by TLC as described in Methods. The fractions were placed in scintillation fluid and the radioactivity was counted. Data are expressed as the percentage of total lipid recovered in a lipid fraction ± SEM of three different experiments.

FFA pool in the materno-feto-placental unit are reported especially in the levels of LCPUFA (23). Therefore, LCPUFA present in the maternal FFA plasma pool have to be transported preferentially by the placenta in order to support increasing fetal demand for its rapid growth during the near term. As there is increasing evidence that the transfer of the FFA from the plasma to target tissues occurs by the dissociation of the albumin-bound FFA to the cell surface via a process of facilitated membrane translocation involving plasma membrane fatty acid binding/transporter proteins (21, 34), we have therefore examined the uptake of four different fatty acids of both the n–6 and n–3 series (linoleic acid, arachidonic acid, docosahexaenoic acid) and the non-essential fatty acid (oleic acid) by human placental choriocarcinoma (BeWo) cells and the role of p-FABP$_{pm}$ in these processes. This paper describes, for the first time, the preferential uptake of EFA/LCPUFA by these cells which is most probably mediated via the p-FABP$_{pm}$.

The kinetic data and temperature-dependency of long chain fatty acid uptake by BeWo cells indicate a saturable process. Because the saturation of fatty acid uptake by BeWo cells was attained at 1:1 molar ratio of fatty acid/BSA this ratio was used to examine the uptake and metabolism of these fatty acids. The involvement of cell metabolism with the uptake process is shown by the marked enhancement of the uptake with temperature, which takes place when the temperature exceeds 26°C. The concentrations of unbound free fatty acid at 1:1 BSA/FA molar ratio were within 0.0068 to 0.0162 μM for all three fatty acids except docosahexaenoic acid (whose association constant with albumin is not known). Fatty acid uptake by BeWo cells was similar at a given ratio whether the ratio was obtained by keeping albumin or oleate concentration constant, suggesting that fatty acid uptake is a function of unbound fatty acid concentration in the medium, as observed in many cell types (34).

The presence of competitive inhibition by fatty acids demonstrates the presence of a carrier-mediated uptake mechanism with a higher affinity for LCPUFA, docosahexaenoic acid and arachidonic acid. The demonstration of competitive inhibition of fatty acid uptake further supports the saturable nature of this process with a preferential EFA/LCPUFA uptake system in BeWo cells. A similar preferential uptake system for linoleic acid was also observed in keratinocytes which are known to have specific requirements for EFA in the maintenance of a competent permeability barrier (32).

FFA uptake has been studied extensively in several tissues, and a variety of mechanisms of FFA uptake have been proposed, including passive diffusion and specific binding to a plasma membrane fatty acid-binding protein (34). There are now several reports providing evi-

dence of the involvement of the several membrane-associated fatty acid-binding proteins ($FABP_{pm}$, FATP, FAT) in the uptake of FFA into various mammalian tissues, including the placenta (21, 25–27, 32, 34, 39–44). These proteins are different in structure and postulated function. Attempts were, therefore, made to demonstrate more directly the presence and involvement of the p-$FABP_{pm}$ in fatty acid uptake in the placental cell line, BeWo. Using a polyclonal antibody to the human p-$FABP_{pm}$, Western blot analyses demonstrated the presence of the p-$FABP_{pm}$ in BeWo cells which is absent in HepG2 cells. The $FABP_{pm}$ has previously been shown to be present in HepG2 cells which also do not show any preference for particular fatty acids (32, 36). The placental protein is different from the hepatic protein in terms of amino acid composition, pI values, and GOT enzymic activity (26, 27). Furthermore, the lack of inhibition of linoleic acid uptake by keratinocytes by anti-$FABP_{pm}$ also indicates that the ubiquitous $FABP_{pm}$ may not be involved in the preferential uptake of EFA by these cells. Anti-p-$FABP_{pm}$ antibody blocks the uptake of LCPUFA followed by EFA, indicating that the p-$FABP_{pm}$ may be involved in preferential uptake of LCPUFA. Similar results were also obtained with fatty acid uptake by human placental membranes using this antibody (27). Although a fatty acid-binding protein with preference for EFA has been postulated previously (32), for the first time we provide evidence of such a protein. Recently we have observed that the purified p-$FABP_{pm}$ preferentially binds LCPUFA over non-essential fatty acid (unpublished data). p-$FABP_{pm}$ appears to be distinctly different from other membrane-associated fatty acid-binding proteins ($FABP_{pm}$, FAT, and FATP). Both p-$FABP_{pm}$ and ubiquitous $FABP_{pm}$ are peripherally membrane-bound proteins of similar size (~40 kDa), but they differ in amino acid composition, pI value, and GOT activity. However, definitive evidence about the structure and function of p-$FABP_{pm}$ must await analysis of its complete amino acid and/or cDNA sequence.

We also compared the metabolism of the four radiolabeled fatty acids by BeWo cells after 30 min of incubation. Marked differences were observed in the metabolism of the four fatty acids. First, the proportion of unesterified docosahexaenoic acid was the lowest of the three essential fatty acids. Both the uptake and subsequent esterification of this fatty acid seem to be the greatest among all these fatty acids. Second, the esterification of docosahexaenoic acid into triacylglycerol was more efficient than that of the other three fatty acids. Given the absence of delta 5 and delta 6 desaturation and elongation activities in the human placenta (2, 19–21, 23), the radioactivity incorporated into the cellular lipids cannot reflect desaturated and/or elongated metabolites of the exogeneously added fatty acids. Several studies on the comparative incorporation of arachidonic acid and docosahexaenoic acid into retinal cells (45), rat brain (46), and pineal cells (47) have also pointed out the peculiarity of docosahexaenoic acid which is preferentially used for the synthesis of neutral lipids. As one of the main functions of the placenta is to deliver docosahexaenoic acid into the fetal circulation, it is possible that the triacylglycerol form may favor the transport of docosahexaenoic acid to the fetal circulation. However, it is not known how and in which form docosahexaenoic acid is released from the placenta into the fetal circulation.

In conclusion, the results on fatty acid uptake and metabolism by BeWo cells presented in this paper are consistent with a saturable uptake mechanism with preference for LCPUFA similar to that observed with human placental membranes (21, 25–28, 48). In addition, antibody studies for the first time clearly demonstrate that the p-$FABP_{pm}$ is most probably involved in the preferential uptake of EFA/LCPUFA. However, it remains to be determined how the fatty acids, after binding to p-$FABP_{pm}$, are delivered for placental transport to the fetus. Further studies are also required on the fatty acid-binding domain of the p-$FABP_{pm}$ which allows the preferential binding of LCPUFA over non-essential fatty acids. Increased knowledge about p-$FABP_{pm}$ and the possible environmental modifiers of this protein is required to understand feto-placental lipid metabolism.

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