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Real-Time Microscopic Assessment of Fatty Acid Uptake Kinetics in the Human Term Placenta

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Abstract

INTRODUCTION: The placenta employs an efficient and selective fatty acid transport system to supply lipids for fetal development. Disruptions in placental fatty acid transport lead to restricted fetal growth along with cardiovascular and neurologic deficits. Nevertheless, little is known about the molecular mechanisms involved in human placental fatty acid trafficking during the initial steps of uptake, or the importance of fatty acid chain length in determining uptake rates.

METHODS: We employed BODIPY fluorophore conjugated fatty acid analogues of three chain lengths, medium (BODIPY-C5), long (BODIPY-C12), and very-long (BODIPY-C16), to study fatty acid uptake in isolated human trophoblast and explants using confocal microscopy. The three BODIPY-labeled fatty acids were added to freshly isolated explants and tracked for up to 30 minutes. Fatty acid uptake kinetics were quantified in trophoblast (cytotrophoblast and syncytiotrophoblast together) and the fetal capillary lumen.

RESULTS: Long- (BODIPY-C12) and Very long-chain (BODIPY-C16) fatty acids accumulated more rapidly in the trophoblast layer than did medium-chain (BODIPY-C5) whereas BODIPY-C5

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Contributors

KSK and KLT conceived the experiments, KSK conducted the experiments, all authors analyzed the results. All authors reviewed the manuscript. KLT supervised the experiments.

Competing Interests

The authors have no competing interests to declare.

accumulated more rapidly in the fetal capillary than did the longer chain length fatty acids. The long-chain fatty acids, BODIPY-C12 and BODIPY-C16, are esterified and stored in lipid droplets in the cytotrophoblast layer, but medium-chain fatty acid, BODIPY-C5, is not.

DISCUSSION: Fatty acids accumulate in trophoblast and fetal capillaries inversely according to their chain length. BODIPY-C5 accumulates in the fetal capillary in concentrations far greater than in the trophoblast, suggesting that medium-chain length BODIPY-labeled fatty acids are capable of being transported against a concentration gradient.

Introduction

The placenta delivers oxygen and nutrients to the fetus and produces pregnancy-sustaining hormones. Of the major nutrient classes transported by the placenta to support human development, the daily caloric requirement for fatty acids is the greatest[1]. Long-chain polyunsaturated fatty acids (LCPUFA) in particular, are crucial for developing embryonic and fetal organs including the lipid rich brain and cardiovascular systems[2]. Thus, their transport must increase with gestational age to match increasing fetal requirements[2–6]. The human placenta selectively takes up LCPUFA in their non-esterified form via several transport proteins and translocases[7,8] (FATP1, FATP4, FATP2, and FAT/CD36[9–12]) but when these systems are compromised, detrimental developmental consequences such as cognitive impairment and delayed brain maturation occur[13–15].

Placental transport of fatty acids is selective by fatty acid species. This selectivity in transport ensures the appropriate quantities of various fatty acid types are delivered to meet fetal lipid requirements for organ development. The mechanisms regulating the maternofetal transport of fatty acids, including sorting by chain length is less well understood compared than those that regulate glucose and amino acids transport[16]. Studies have proposed that subcellular processes such as fatty acid metabolism and esterification underlie the selective nature of fatty acid sorting and export of fatty acids from placenta[17–19], but these subcellular processes have yet to be discovered.

Unexpectedly, long-chain fatty acid BODIPY-C12 applied to the maternal side of the placenta demonstrates preferential shuttling into the cytotrophoblast where it is stored and metabolized, suggesting a preferential uptake and metabolism of fatty acids by one particular placental cell types[20,21]. While this process in the cytotrophoblast may underlie the intermediate esterification step involved in long-chain fatty acid transport proposed by others [18,19,22], it is unknown if the cytotrophoblast, or any placental cell type, exhibits specificity by fatty acid chain length.

The objective of this study was to quantify the uptake kinetics of different chain length fatty acids. We hypothesized that fatty acid uptake rates by trophoblast and transport into the fetal capillary would be dependent on chain length, with the shorter chain lengths being taken up much faster than the longer lengths. We employed BODIPY-labeled fatty acid analogues to track and quantify fatty acid uptake in human placental explants and isolated trophoblast.

Materials and Methods

Studies were approved by the Oregon Health & Science University (OHSU) Institutional Review Board (IRB# 5684), and all study subjects were provided written, informed consent.

Subject Details

Women (>37 wks gestation, Table 1) scheduled for cesarean section at OHSU were screened for enrollment. Exclusion criteria included multiple gestations, fetuses with chromosomal or structural anomalies including cardiac defects, preeclampsia, diabetes, maternal hypertension, and any other significant co-morbidity.

Tissue overview

We used twenty-eight placentas for different aspects of the study. In brief, placentas were collected at the time of delivery by cesarean section. The fetal membranes were removed prior to obtaining placental weight and dimensions. All placentas were processed within 30min of delivery. Placental tissue samples were used for (i) explants (<1mm³) isolated as previously described[10,20] for live imaging studies, and (ii) isolated cytotrophoblast cell studies for *in vitro* cell culture and Western Blotting.

Primary villous cytotrophoblast isolation and culture

Cytotrophoblast cells were isolated using trypsin-DNAse I digestion followed by Percoll enrichment as previously described[20,23]. Using Cytokeratin-7 and Vimentin immunohistochemical markers, the average CTB isolation purity used for these studies is 90.8% ± 0.3%. A representative image of the purity of our 8hr CTB is shown in Figure S1. Cytotrophoblasts were plated at a density of 3.0 × 10⁵ cells cm⁻² on tissue culture dishes or glass coverslips. Cells were cultured in Iscoves Modified Dulbecco's Medium (IMDM, ATCC) supplemented with 10% Fetal Bovine Serum (FBS)(Gibco) and 100U/ml penicillin and 100µg/ml streptomycin and incubated in a 5% CO₂/95% air incubator at 37°C. Cell viability was assessed by Trypan blue exclusion. After 8h in culture, nonadherent cells were removed by washing with pre-warmed culture media, and the media was subsequently replaced every 24h. Cells cultured on glass coverslips were used for fatty acid uptake studies, and Western Blot analyses were performed from plated tissue cultured cells. Cells were studied at 8h(cytotrophoblasts) or 72h(syncytiotrophoblast) when the majority of cytotrophoblasts have aggregated, fused and differentiated into multinucleated, syncytial giant cells[24].

Fluorescent fatty acid tracking and microscopy (explants, cells).

The BODIPY fluorophore is an intensely fluorescent, photostable, and intrinsically lipophilic molecule, unlike most other long-wavelength dyes. BODIPY conjugated fatty acids analogues undergo native-like cellular transport and metabolism, making them effective as a tracer for lipid trafficking[25]. The BODIPY-moiety effectively adds 4-6 carbons to the conjugated fatty acid (e.g. BODIPY-C12 is most similar to C-16 and C-18 natural fatty acids and BODIPY-C16 is similar to very long-chain fatty acids C-20 and C-22) [26]. BODIPY-C12, C16, C5 are well established as tools to study fatty acid uptake and esterification in multiple models[25].

We prepared 10 μ M solutions of BODIPY-C12 (Molecular Probes) by diluting a 2.5mM DMSO stock solution (1:250) in M199-HEPES containing 0.1% fatty acid-free bovine serum albumin (FAF-BSA) (Fisher Scientific)[20]. Explants and cells were exposed to 2 μ M BODIPY-fatty acids for 30min for most experiments because the uptake consistently reached a plateau by 15min.

Explants: Explants were maintained in IMDM media. To assess explant viability, 2 μ L ethidium homodimer (Molecular Probes) was added to each well containing explants to distinguish dead regions of tissue. Imaging was restricted to regions with no evidence of ethidium homodimer uptake. For lipid droplet (LD) labeling, 1:500 of HCS LipidTOX Deep Red (Molecular Probes) was mixed into each well containing placental explants. After 30min, 2 μ M BODIPY-fatty acid solution was added to each well and mixed by trituration. Explants were immobilized for confocal microscopy using 8 \times 8mm pieces of stainless steel mesh (TWP, Inc) and an incubation chamber (37°C; Okolab) was used.

Quantification of total BODIPY-C12 uptake in cells

Total BODIPY-C12 uptake was determined in cytotrophoblast cultures using a plate-reader assay as described[27]. Cytotrophoblast were plated at 3.0×10^5 cells cm^{-2} in 96-well plates (BD Falcon) and cultured as described. Cells were studied at 8h (cytotrophoblasts) and 72h of cell culture. Cells were pre-incubated in 80 μ L serum free IMDM for 1h with or without inhibitors. Inhibitors used included 10% FBS, Triacsin C to inhibit Long-chain Acyl-CoA Synthetase (Santa Cruz Biotech), and the FATP2 inhibitor, CB-2 (EMD Millipore). After 1h, 20 μ L of assay solution (25 μ M BODIPY-fatty acid, 25 μ M FAF-BSA, 10mg/ml Trypan Blue) was added to each well, mixed, and incubated for 20min at 37°C with 5% CO₂/95% air. Fluorescence (485nm excitation, 525nm emission) was measured using a BioTek Synergy H1 hybrid plate reader (BioTek). After measurement, the medium was removed, and plates were carefully washed with Hank's Buffer for 10min at 37°C. Cells were then lysed using RIPA buffer (EMD Millipore) and protein was measured using a BCA method (Thermo Pierce).

Immunofluorescence

Cells cultured for 8h and 72h on glass coverslips were incubated with BODIPY-C12 for 30min as described above and fixed in 4% paraformaldehyde for 20min at room temperature. Samples were blocked and permeabilized in Block-aid (Life Technologies)/ 0.1% saponin (Sigma) for 1h before overnight incubation with primary antibody against desmoplakin (1:200, ab16434, Abcam) at 4°C. Following primary antibody incubation, samples were washed with PBS and labeled with secondary antibodies (donkey F(ab')₂ anti-mouse Alexa Fluor 647 (1:500, ab150103), for 1h at room temperature. The samples were washed and counterstained with 1 μ g/ml Hoechst 33342 for 10min, mounted in Slowfade Diamond (Molecular Probes), and imaged using a Zeiss 880 LSM Confocal with Airyscan.

Image Analysis

For live-imaging of explants, 2 separate explants were imaged. All images were analyzed using Fiji software[28]. The mean fluorescence intensity was measured in the trophoblast layer, representing a combination of the syncytiotrophoblast and cytotrophoblast, and the

fetal capillary lumen using a consistently sized region of interest after automatic video stabilization. Because the BODIPY fluorophore is relatively insensitive to environmental conditions, quantification of fluorescence intensity is a reliable measure of tracer concentration[29].

Co-localization analyses were performed with Fiji using the automatic method of Costes et al.[30]. The Pearson co-localization coefficient indicates the degree to which BODIPY-C12 is being incorporated into the LD organelles identified by LipidTOX[31]. To segment and quantify particles, we used the analyze particles process on the thresholded images (Otsu method) in Fiji. This method distinguishes particles by their brightness relative to their surroundings.

Western Blotting

Protein was isolated after 8h or 72h of cell culture using RIPA lysis buffer (EMD Millipore) and concentrations were measured using a BCA protein assay kit (Thermo Pierce). SDS-PAGE, loaded with 15 μ g, and blotting was performed onto nitrocellulose using a Biorad Minicell (Biorad). Blots were probed with mouse anti-human FATP2 (1:500, ab175373, Abcam) and incubated overnight at 4°C. The blots were washed and probed with an anti-mouse HRP conjugated secondary antibody (1:1000, sc-2005, Santa Cruz Biotech) and developed for 5min using a chemiluminescent substrate (SuperSignal, Thermo Pierce). Gels were imaged using a UVP gel doc and all images were quantified using Fiji.

Statistical Methods

The linear portion(0-15min) of the uptake curve in explant studies was analyzed using linear regression. Two-way ANOVA with Sidak's multiple comparisons test was used for all *in vitro* uptake plate reader experiments. Paired Student t-test was used for western blot quantification. P-values less than 0.05 were considered significant.

Results

To continuously quantify the uptake of various chain length fatty acids in live placenta tissue at two locations simultaneously, we used live-confocal microscopy and three BODIPY-labeled fatty acids differing only by fatty acid chain length incubated with human term placental explants. In all experiments, the BODIPY-fatty acids are too dilute to be detectable in the extra-villous space, but become visible as they accumulate in tissue. BODIPY-fatty acid uptake was first visible at the microvillous membrane and proceeded gradually inward toward the fetal capillaries. Within 15min of incubation, fatty acids of different chain lengths accumulated to differing degrees throughout the placental layers and in fetal capillaries(Figure 1A–C, Supplemental Movie S1,S2,S3).

The uptake rates into trophoblast layers were different according to fatty acid chain length (BODIPY-C5: 42 ± 4.4 Fluorescence Units (FU) per min, $\text{FU} \cdot \text{min}^{-1}$, $r^2=0.29$, BODIPY-C12: 46 ± 2.9 $\text{FU} \cdot \text{min}^{-1}$, $r^2=0.54$ and BODIPY-C16: 61 ± 3.3 $\text{FU} \cdot \text{min}^{-1}$, $r^2=0.59$ $p<0.001$) (Figure 1E). By 15min, fluorescence in the trophoblast layer equilibrated and was not different between fatty acids, $p>0.05$ (Figure 1E). BODIPY-C5, the shortest chain fatty acid, was rapidly transported into fetal capillaries where it accumulated(Figure 1A). For BODIPY-

C5, the uptake rate was $110 \pm 13 \text{ FU} \cdot \text{min}^{-1}$, $r^2=0.26$ compared to $50 \pm 2.5 \text{ \% min}^{-1}$, $r^2=0.64$ for BODIPY-C12 and $6.1 \pm 0.46 \text{ \% min}^{-1}$, $r^2=0.44$ for BODIPY-C16 $p<0.0001$ (Figure 1F). Thus, as the fatty acid chain length increased, accumulation rates into fetal capillaries became slower. The mean fluorescence in the capillaries at 15min was greater for BODIPY-C5 ($1610 \pm 623 \text{ FU}$) than BODIPY-C12 ($705 \pm 57.4 \text{ FU}$ $p<0.05$) or BODIPY-C16 ($131 \pm 28.0 \text{ FU}$ $p<0.0001$). BODIPY-C12 and BODIPY-C16 mean fluorescence in capillaries at 15min was not different. Uptake of all fatty acid analogues plateaued by 30 minutes (not shown).

In other cell types, esterification of Long- (BODIPY-C12) and Very-long (BODIPY-C16), but not short-chain fatty acids mediates uptake and accumulation of fatty acids through incorporation into lipid droplets (LD)[32]. To determine whether esterification might be occurring in placental tissue and whether these processes are specific to long- and very long-chain fatty acids, we repeated the live explant studies using a LD counterstain, LipidTOX, to identify pre-existing LD and measure the extent of newly incorporated BODIPY-fatty acids. BODIPY-C5 highlighted punctate structures in the trophoblast layer (Figure 2A), but these punctate structures did not co-localize with LD (Figure 2B,C). In contrast, within min of addition of BODIPY-C12, numerous LD emerged and predominantly located in the cytotrophoblast (Figure 2D, Movie S4). BODIPY-C12 also incorporated into pre-existing LD (Figure 2E, F), signifying esterification of BODIPY-C12.

We quantified the total number of BODIPY-containing particles, the bright punctate structures highlighted by BODIPY-fatty acids, as an estimate of LD accumulation after 15min. The number of particles (irrespective of cell layer) accumulated by 15min was higher with BODIPY-C12 ($2 \times 10^7 \pm 7 \times 10^6 \text{ particles mm}^{-3}$) than BODIPY-C5 ($4 \times 10^6 \pm 3 \times 10^6 \text{ particles mm}^{-3}$; $p<0.05$, Figure 2G), but was not different with BODIPY-C16 ($1 \times 10^7 \pm 4 \times 10^6 \text{ particles mm}^{-3}$; $p<0.05$, Figure 2G). There was no difference between the volumes of the BODIPY-labeled particles incorporating BODIPY-C12 ($0.21 \pm 0.12 \mu\text{m}^3$) vs BODIPY-C5 ($0.10 \pm 0.046 \mu\text{m}^3$) vs BODIPY-C16 ($0.19 \pm 0.13 \mu\text{m}^3$) at 15min (Figure 2H). There was, however, greater co-localization (Pearson correlation) of LipidTOX and BODIPY-C12 ($r=0.24 \pm 0.053$), LipidTOX and BODIPY-C16 ($r=0.29 \pm 0.11$) than BODIPY-C5 and LipidTOX ($r=-0.067 \pm 0.063$ $p<0.05$, Figure 2I).

Fatty acid esterification to Acyl-CoAs occurs concertedly with uptake [33]. We have previously shown that long-chain fatty acid esterification is significantly higher in the cytotrophoblast (CTB) compared to syncytiotrophoblast (SCT) [20]. To explore how different transport systems contribute to fatty acid uptake in each cell type, we tested a panel of inhibitors. We compared uptake of BODIPY-C5, BODIPY-C12, BODIPY-C16 in CTB at 8h compared to SCT at 72h to test in the presence of natural fatty acids present in fetal bovine serum, inhibition of FATP2 (CB-2), or ACSL (1,3,4,5) inhibition (Triacsin C).

Total uptake of BODIPY-fatty acid was quantified after 20min of incubation using a plate reader [27]. Consistent with explant experiments, minimal BODIPY-C5 accumulated within cells (Figure 3A). CTB uptake of BODIPY-C5 at 15min was not significantly different from the SCT (29 ± 8.9 vs $15 \pm 7.4 \text{ Fluorescence Units (FU)} \times \mu\text{g protein}^{-1}$, $p>0.05$), and was not affected by any of the inhibitors (CTB: serum: $15 \pm 8.2 \text{ FU} \times \mu\text{g protein}^{-1}$, Triacsin C: 23 ± 13

FU \times μ g protein⁻¹, CB-2: 59 \pm 18 FU \times μ g protein⁻¹, $p > 0.05$ vs control; SCT: serum: 5.1 \pm 2.2 FU \times μ g protein⁻¹, Triacsin C: 23 \pm 7.8 FU \times μ g protein⁻¹, CB-2: 28 \pm 7.5 FU \times μ g protein⁻¹, $p > 0.05$ vs control) (Figure 3A).

Total accumulation of BODIPY-C12 and BODIPY-C16 was substantially greater than BODIPY-C5. The accumulation of BODIPY-C12 by CTB was significantly higher than for SCT (556 \pm 128 vs. 131 \pm 10.6 FU \times μ g protein⁻¹, $p < 0.0001$). The uptake of BODIPY-C12 into CTB was inhibited by FBS (165 \pm 42.3 FU \times μ g protein⁻¹, $p < 0.0001$), Triacsin C (394 \pm 81.2 FU \times μ g protein⁻¹, $p < 0.05$), and CB-2 (339 \pm 91.2 FU \times μ g protein⁻¹, $p < 0.01$) (Figure 3B). In contrast, the uptake of BODIPY-C12 into SCT was not inhibited by FBS (57.5 \pm 3.62 FU \times μ g protein⁻¹, $p = n.s.$), Triacsin C (142 \pm 39.5 FU \times μ g protein⁻¹, $p = n.s.$), or CB-2 (114 \pm 10.8 FU \times μ g protein⁻¹, $p = n.s.$) (Figure 3B).

Of the fatty acid analogues tested, BODIPY-C16 accumulated most in CTB. Uptake of BODIPY-C16 by CTB at 20min was greater than SCT (1000 \pm 285 vs. 277 \pm 23.4 FU \times μ g protein⁻¹, $p < 0.001$). The CTB uptake was decreased in the presence of FBS (225 \pm 68.8 FU \times μ g protein⁻¹, $p < 0.0001$) and CB-2 (445 \pm 84.0 FU \times μ g protein⁻¹, $p < 0.01$), but not triacsin C (915 \pm 227 FU \times μ g protein⁻¹, $p = n.s.$) (Figure 3C). The SCT uptake was not different with FBS (66.7 \pm 11.6 FU \times μ g protein⁻¹, $p = n.s.$), triacsin C (383 \pm 30.1 FU \times μ g protein⁻¹, $p = n.s.$), or CB-2 (170 \pm 23.6 FU \times μ g protein⁻¹, $p = n.s.$) (Figure 3C).

Differentiation of CTB to SCT is associated with suppression in long and very long-chain fatty acid uptake, but it is possible this effect is a byproduct of *in vitro* culture. Thus, we tested if blocking differentiation could preclude the suppression in long-chain and very long-chain fatty acid uptake. Similar to previous reports, we found CTB cultured for 72h predominantly fused and lost intercellular junctional divisions as evidenced by desmoplakin staining (Figure 4A), and SB203580 prevented fusion as discrete CTB demarcated by desmoplakin remained by 72h (Figure 4B). We measured total uptake of BODIPY-C12 in trophoblast cultured for 8h, 24h, and 72h with and without SB203580, p38 MAPK inhibitor to block differentiation. At 8h and 24h the uptake of BODIPY-C12 at 20min was not different between treated and untreated CTB: (8h: control 521 \pm 31.4 FU \times μ g protein⁻¹ vs SB203580 495 \pm 42.8 FU \times μ g protein⁻¹, $p = n.s.$), (24h: control 316 \pm 35.7 FU \times μ g protein⁻¹ vs SB203580 357 \pm 43.5 FU \times μ g protein⁻¹, $p = n.s.$) (Figure 4C). However, by 72h, when the majority of CTB have differentiated to SCT in the control group, uptake of BODIPY-C12 at 20min was greater in the SB203580 treated trophoblast (72h: control 248 \pm 41.8 FU \times μ g protein⁻¹ vs SB203580 351 \pm 52.1 FU \times μ g protein⁻¹, $p < 0.001$) (Figure 4C).

We hypothesized that our observations of the greater CTB uptake of long and very long-chain fatty acids could be explained by higher levels of FATP2 protein levels, which is a LCPUFA transporter [34,35]. We tested this hypothesis by comparing CTB and SCT FATP2 levels via western blot. FATP2 protein levels were higher in CTB (2.3 \pm 0.25) than in SCT (1.5 \pm 0.083, $p < 0.01$) (Figure 5).

Discussion

We used live confocal microscopy to track fluorescently labeled fatty acid analogues in the human term placenta. The analogues represented medium- (BODIPY-C5), long- (BODIPY-C12) and very-long- (BODIPY-C16) fatty acids. We hypothesized that fatty acid uptake rate into trophoblast would be inversely proportional to chain length, consistent with passive diffusion. However, we were surprised to find such large differences in the uptake kinetics between the fatty acids tested. The rates of incorporation into the various cell layers of placenta were more different than expected by molecular weight (MW: BODIPY-C5: 320 Da; BODIPY-C12: 418 Da; BODIPY-C16: 474 Da)[36,37]. One possible explanation for our observations are differential transport rates by the action of selective transporters or co-transporters. The higher metabolic rate observed in CTB relative to SCT may support its role in transport and esterification, as these are metabolically demanding[21].

The shorter chain fatty acid, BODIPY-C5, accumulated to a lesser degree than the longer chain length fatty acids in the trophoblast layers and rapidly appeared within fetal capillaries. BODIPY-C5 appears to be highly permeable to trophoblast and accumulates in fetal capillaries, potentially reflecting a specific transport system. In contrast, BODIPY-C12 and BODIPY-C16, representing long- and very long-chain fatty acids, respectively, accumulated to high degrees in trophoblast. Within the 30min durations and concentrations tested, BODIPY-C12 accumulated in the capillaries, but little of BODIPY-C16 was found in the capillaries by 30min.

We have previously shown that BODIPY-C12 is stored in LD in the CTB and is FATP2 dependent[20]. Consistent with this, BODIPY-C12 and BODIPY-C16, known substrates of FATP2, were esterified and incorporated into LD in CTB but not the SCT. BODIPY-C5 esterification into LD was not detectable in explants or cells in culture. These results suggest chain length dependent uptake and metabolism of fatty acids in the human placenta.

Fatty acid esterification occurs concertedly during cellular uptake, but how esterification might be related placental fatty acid transport remains untested[19,22,38,39]. We investigated this question and others using a panel of transporter and esterification inhibitors *in vitro*. Trophoblast LD formation and BODIPY-C12 uptake were inhibited by phloretin, triacsin C, and CB-2. Phloretin is a non-specific inhibitor of protein-mediated transport, triacsin C inhibits Long-chain Acyl-CoA Synthetase (ACSL1,3,4 and 5)[40], and CB-2 inhibits FATP2[27].

Similar to our results observed in explants, minimal BODIPY-C5 accumulated within either CTB or SCT. Uptake of BODIPY-C5 appeared to be insensitive to competition with natural fatty acids in FBS. This may be because there is no natural lipid in FBS that competes with BODIPY-C5 transporters, or that its uptake is not transporter dependent. As expected, we observed no inhibition of uptake with Triacsin C, or CB-2, because these systems are specific for long- and very long-chain fatty acids[27,40]. BODIPY-C5 differs from the other two tracers we studied in that it is a medium-chain fatty acid with an odd number of carbons. The uptake and metabolism of odd chain and medium-chain fatty acids may be specialized to serve a crucial role in the placenta for the maintenance of mitochondrial Krebs cycle

intermediates[41,42]. We found that the total uptake of BODIPY-C12 and BODIPY-C16 was greater than for BODIPY-C5, even by as much as an order of magnitude.

Similar to our previous report[20], we found CTB uptake of BODIPY-C12 was greater than SCT. Uptake of long- and very long-chain fatty acids into CTB were sensitive to competition from FBS fatty acids, ACSL inhibitor Triacsin C (BODIPY-C12 only), and FATP2 inhibition with CB-2; but the SCT uptake was not affected by these inhibitors. The ACSL family effects long-chain fatty acids, but does not behave as a plasma membrane transporter. Because Triacsin C inhibits CTB BODIPY-C12 uptake, it appears that long- and very long-chain fatty acid uptake into CTB is facilitated by esterification.

FATP2 is especially relevant in the human placenta because it is the most highly expressed fatty acid transporter[43], and FATP2 is selective for the uptake and esterification of LCPUFA like Docosahexaenoate[34]. In the human placenta, FATP2 is appears to be localized to the CTB[11,20,43], but others have argued FATP2 is localized to the SCT as well. Consistent with the model that FATP2 is localized to the CTB, the greatest long- and very long-chain fatty acid uptake and esterification was observed in the CTB. Taken together, these data may suggest that CTB plays a unique role in the uptake of fatty acids similar to Docosahexaenoate. Other FATP isoforms (e.g. 1 and 4) expressed in placenta may be relevant to the trafficking phenomenon observed in this study, but these mechanisms were not specifically tested.

It is not immediately clear whether the lower uptake of BODIPY-C12 and BODIPY-C16 after trophoblast differentiation was associated with the reduction in the expression of FATP2[20]. We used a CTB differentiation inhibitor, SB203580, that directly blocks p38-MAPK activation, and CTB syncytialization *in vitro*[44,45]. With SB203580 treatment we observed a decrease in BODIPY-C12 uptake over the differentiation 72hr time course, but the decrease was not as large as found in normal differentiation. This effect was seen in spite of the fact that the syncytialization process appeared to be blocked by SB203580. Blocking differentiation of CTB was sufficient to preserve the ability of CTB to maintain higher levels of BODIPY-C12 uptake than would be expected if differentiation had occurred.

One unexpected result of our study was the degree of fatty acid tracer accumulation in fetal capillary spaces and the rapid rate at which the tracer intensity increased. Our experimental model differs from studies using the dually perfused placenta in that we allow tracer to accumulate in the capillaries in which there is no flow. It may possible that a portion of this fluorescence in the capillary represents the export of long-chain fatty acids incorporated into esterified lipids. While the concentration of unesterified fatty acids is less in umbilical than maternal blood, the absolute concentration of LCPUFA in esterified lipids is much higher in umbilical than maternal blood[46]. It is possible that the export of esterified BODIPY-fatty acids, presumably integrated within lipoproteins, is significant[47]. We cannot optically distinguish the form of lipid exported to fetal capillaries in our study, and future studies should address this. This study therefore highlights the significance of placental lipid processing in maternal-fetal lipid partitioning.

Other studies have suggested placental fatty acid transport is inadequately described by passive or facilitative mechanisms. La Fond et al. found arachidonic acid uptake, most similar in chain length to BODIPY-C16, is ATP and Na dependent in SCT basal plasma membrane[48]. BODIPY-C16 is similar in chain length to arachidonic acid, which accumulates in placental tissue greater than oleic acid[17]. Thomas et al. observed cord blood enrichment of medium- and long-chain fatty acids of partially oxidized long-chain fatty acids and suggested peroxisomal beta-oxidation may facilitate fatty acid transport[49]. These reports have proposed placental fatty acid transport appears to be regulated and is metabolically expensive.

All of the fatty acids we tested were taken up in trophoblast and fetal capillaries against a concentration gradient, which cannot be explained by diffusion alone. Similar to prior studies[10,50,51] the uptake of long and very long-chain fats are transporter dependent. The mechanism of fatty acid uptake by FATP and transporters complexed with ACSL involves an esterification step that utilizes 2-ATP equivalents per fatty acid molecule[52,53]. Unlike other placental nutrient transport systems, placental fatty acid transport appears to be regulated and metabolically demanding.

In conclusion, our experiments suggest fatty acid uptake in the human term placenta differs according to chain length. Selective esterification may account for differences in uptake in trophoblast cells. Differentiation of CTB to SCT results in a down regulation in long- and very long-chain fatty acid analogue uptake, suggesting these cells play differing roles in lipid transport and processing in the human term placenta. Our observations showing that BODIPY-fatty acids enter the SCT, the CTB and the fetal capillary against a concentration gradient are compatible only with the conclusion that these molecules are actively transported. Future investigations delving into the regulation of these kinetics and the pathological conditions caused by alterations in fatty acid transport and metabolism are needed.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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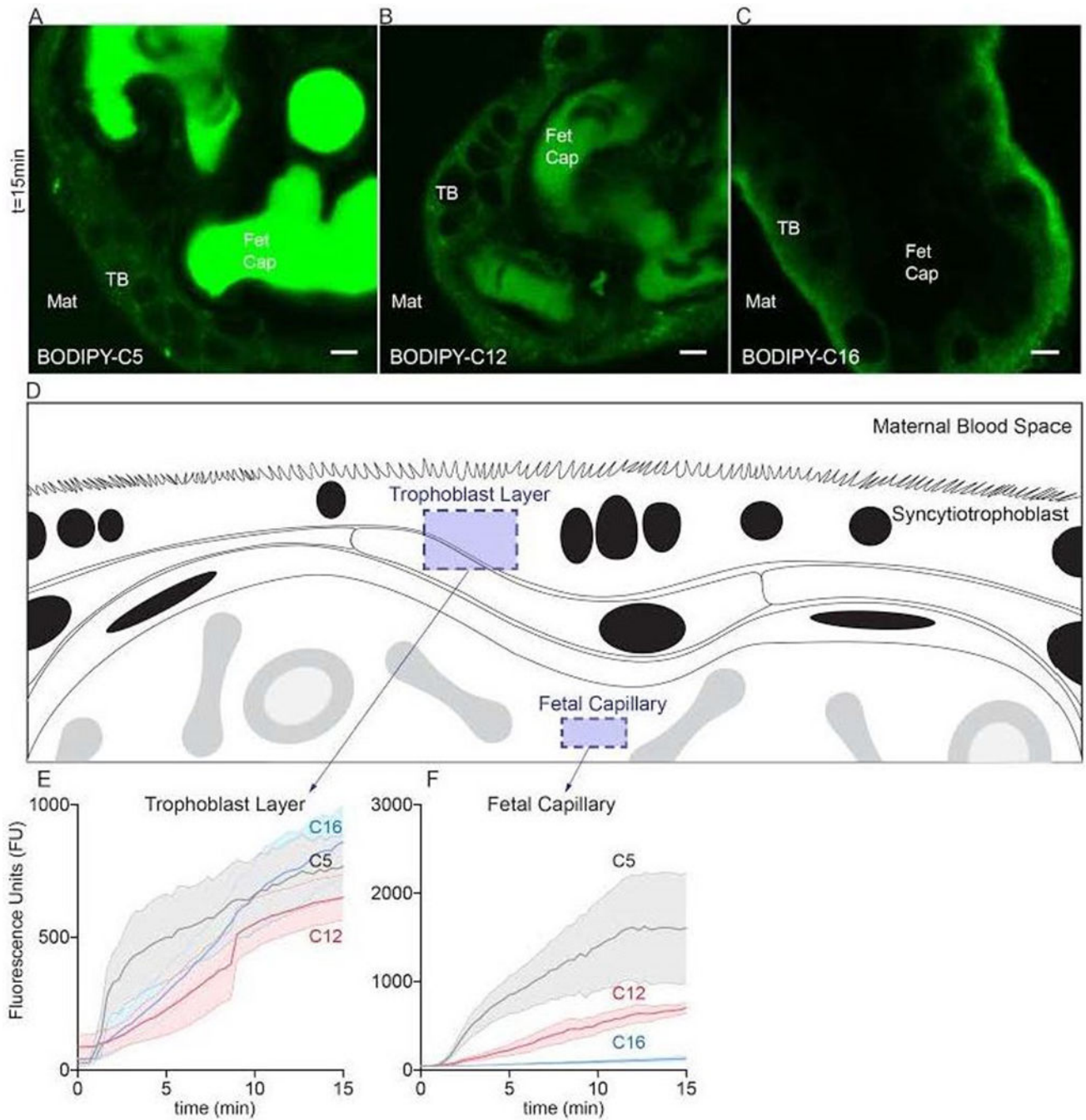


Figure 1. Kinetic distribution of various fatty acid analogues during uptake in living human term placental explants imaged via confocal microscopy.

Explants isolated immediately after cesarean section (< 2h) were placed into HBSS on a stage top incubator (37°C/ambient air) of a confocal microscope. 2µM fluorescent fatty acids were added to explant medium, representing the maternal blood space (Mat), after initialization the image acquisition. The tracer concentration in the medium was not sufficient to register a signal at these settings. Three fluorescent fatty acids were compared, BODIPY-C5 (medium-chain fatty acid), BODIPY-C12 (long-chain fatty acid), and BODIPY-C16 (very long-chain fatty acid). Uptake distributions varied according to fatty

acid chain length over 20min of time. (A) BODIPY-C5 accumulated to the greatest degree within the fetal capillaries (Fet Cap) against an apparent concentration gradient; very little tracer was present in trophoblast (TB) or other placental cells at 20min. (B) BODIPY-C12 appeared to distribute equally between all layers and compartments of placental explants by 20min. (C) BODIPY-C16 largely incorporated into the trophoblast layer; little was present in capillaries at 20min. (D) Schematic representation of regions quantified in explants to produce panels E and F. (E,F) Quantification of total fluorescence, of BODIPY-C5, BODIPY-C12, and BODIPY-C16 at two different locations in explants over time. Data are Mean±SEM. Scale Bar: 5µm. n=5 placentas.

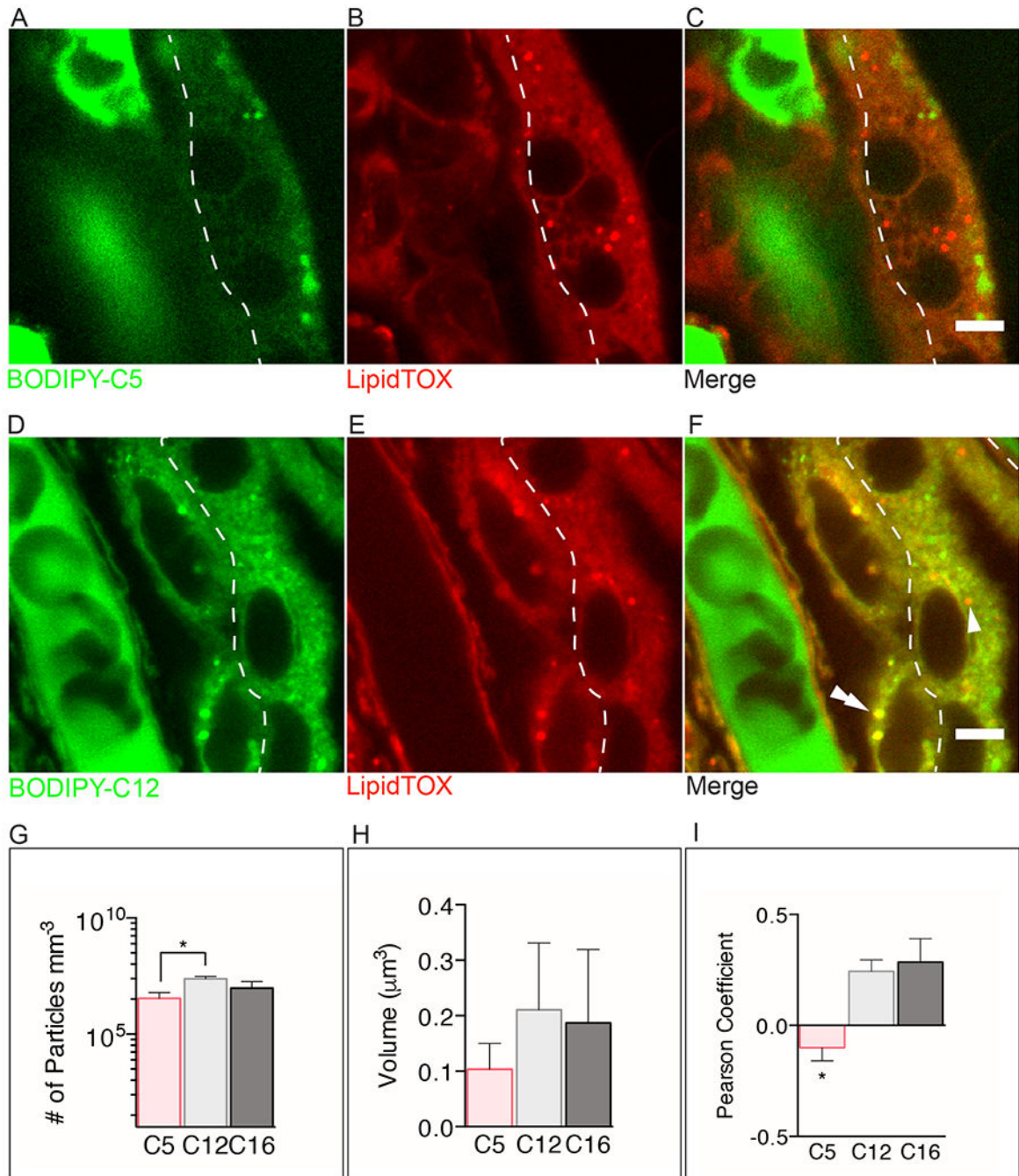


Figure 2. Comparison of short- and long-chain fatty acid uptake in human term placental explants after 15min of incubation with BODIPY-C5 or BODIPY- C12.

The dashed line separates the syncytial and cytotrophoblast layers. (A-C) BODIPY-C5 (green) (D-F) BODIPY-C12 (green) (A) The BODIPY-C5 fatty acid is found diffusely throughout all tissues and within immobile, unidentified punctate structures. (B) The green punctate structures shown in A are not stained with LipidTOX suggesting that they are not lipid droplets (LD). (C) Merge image shows that BODIPY-C5 is not esterified nor incorporated into LD. (D-F) In contrast, BODIPY-C12 is esterified and incorporated into LD (double arrowhead) in the cytotrophoblast but not the syncytiotrophoblast (arrowhead). (G)

There are significantly more punctate structures in explants incubated with BODIPY-C12 than with BODIPY-C5 (log scale). (H) Volumes of BODIPY-C12, BODIPY-C16, and BODIPY-C5 particles are not different. (I) The long- and very long-chain fatty acids BODIPY-C12 and BODIPY-C16, respectively, co-localize with a greater frequency with LipidTOX stained droplets than does BODIPY-C5. This suggests that BODIPY-C12 and BODIPY-C16 are being incorporated into LD to a greater extent than BODIPY-C5. (A-F): Dashed line represents the syncytiotrophoblast-cytotrophoblast interface. Scale Bar: 5 μ m. (g-i): Data are mean \pm SEM; n=5 BODIPY-C5, n=7 BODIPY-C12, n=5 BODIPY-C16. Unpaired t-test, *= p<0.05, **=p<0.01 vs BODIPY-C12.

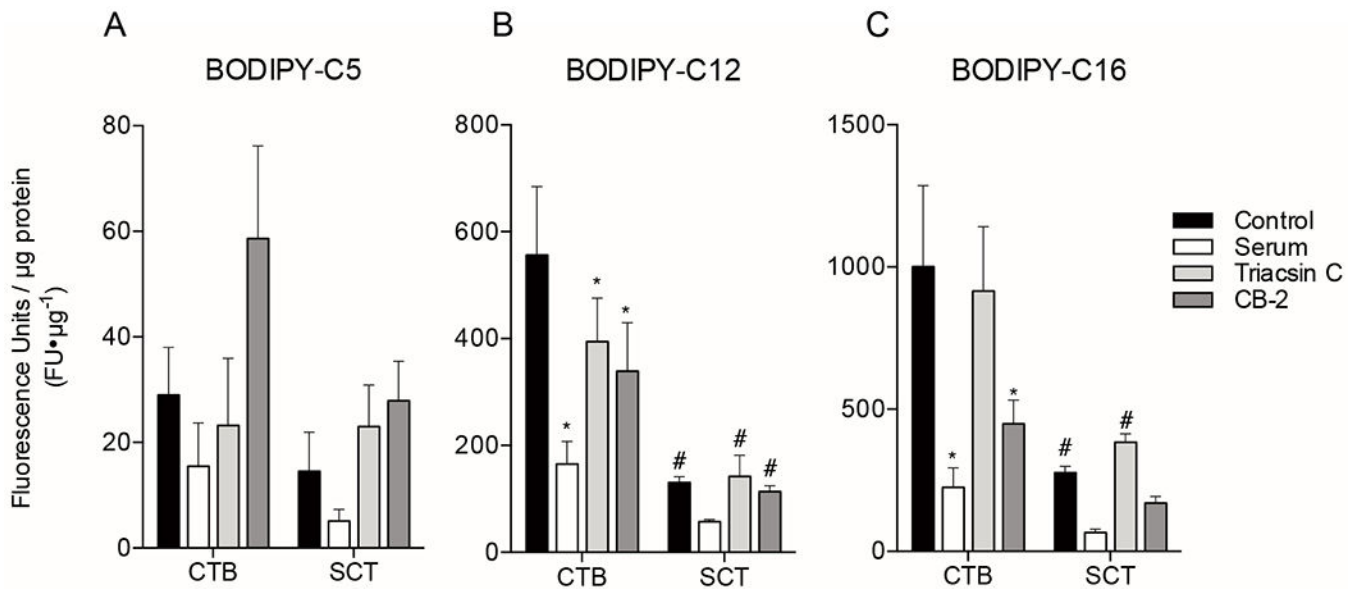


Figure 3. Quantification of total BODIPY-fatty acid uptake in primary isolated human term trophoblast cells with and without inhibitors.

Primary human trophoblast were isolated and plated onto 96 well plates. Cells were pre-treated with inhibitors (fetal bovine serum [FBS], Triacsin C [Acyl-CoA inhibitor], CB-2 [FATP2 inhibitor]) 1h before addition of 2 μ M BODIPY-fatty acid, and total uptake was measured after 20min of incubation with BODIPY-fatty acid using plate reader fluorescence as a marker. Trophoblast tissues were assayed at two time points after initial plating, at 8h to measure cytotrophoblast uptake, or at 72h allow for differentiation to syncytiotrophoblast (SCT) before measuring uptake. (A) BODIPY-C5, (B) BODIPY-C12, and (C) BODIPY-C16 uptake was measured. These data indicate uptake of long and very long-chain fatty acids are transporter dependent. Data are Mean \pm SEM, n=5 placentas. *= p<0.05, **=p<0.01 vs corresponding cell type control. #=p<0.05 CTB vs SCT.

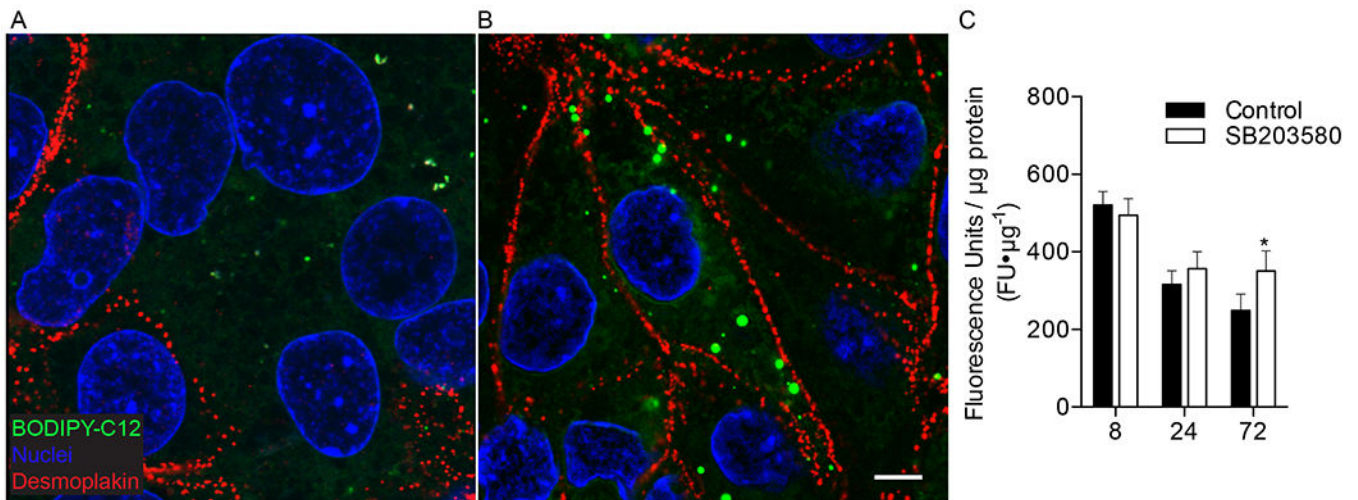


Figure 4. The suppression in long-chain fatty acid uptake during differentiation of cytotrophoblasts to syncytiotrophoblast can be prevented with p38 MAPK inhibition. (A-B) Trophoblast were cultured for 72h with and without 10μM SB203580 (p38 MAPK inhibitor), incubated with 2μM BODIPY-C12 (green) for 20min, fixed and immunolabeled with desmoplakin (red) to visualize intercellular junctions and fatty acid uptake using confocal microscopy. Nuclei are labeled blue (Hoechst) (A) Desmoplakin shows large syncytial aggregates in control 72h cultures, representing syncytiotrophoblast (SCT). (B) 72h cultures treated with SB203580 show cytotrophoblast (CTB) have aggregated, but remain as discrete cells, as evidenced by clearly demarcated desmoplakin labeled intercellular junctions, and suggests SB203580 maintains the CTB phenotype *in vitro* and prevents CTB differentiation to SCT (C) Total uptake of BODIPY-C12 long-chain fatty acid in isolated primary human term trophoblast was measured after 20min of incubation using a plate reader. Uptake was measured at 8, 24, or 72h after initial plating with and without a differentiation blocker SB203580. Treatment with SB203580 had no detectable effect on total uptake of BODIPY-C12 at 8 or 24h time points in culture. Trophoblast at 8 and 24h represent CTB because the majority of cells have not syncytialized at these time points. However, by 72h when CTB have largely fused to become SCT, uptake of BODIPY-C12 is greater in cultures treated with SB203580. These data indicate blocking differentiation of CTB to SCT is sufficient to preserve greater levels of BODIPY-C12 long-chain fatty acid uptake. Data are Mean±SEM, n=8 placentas.

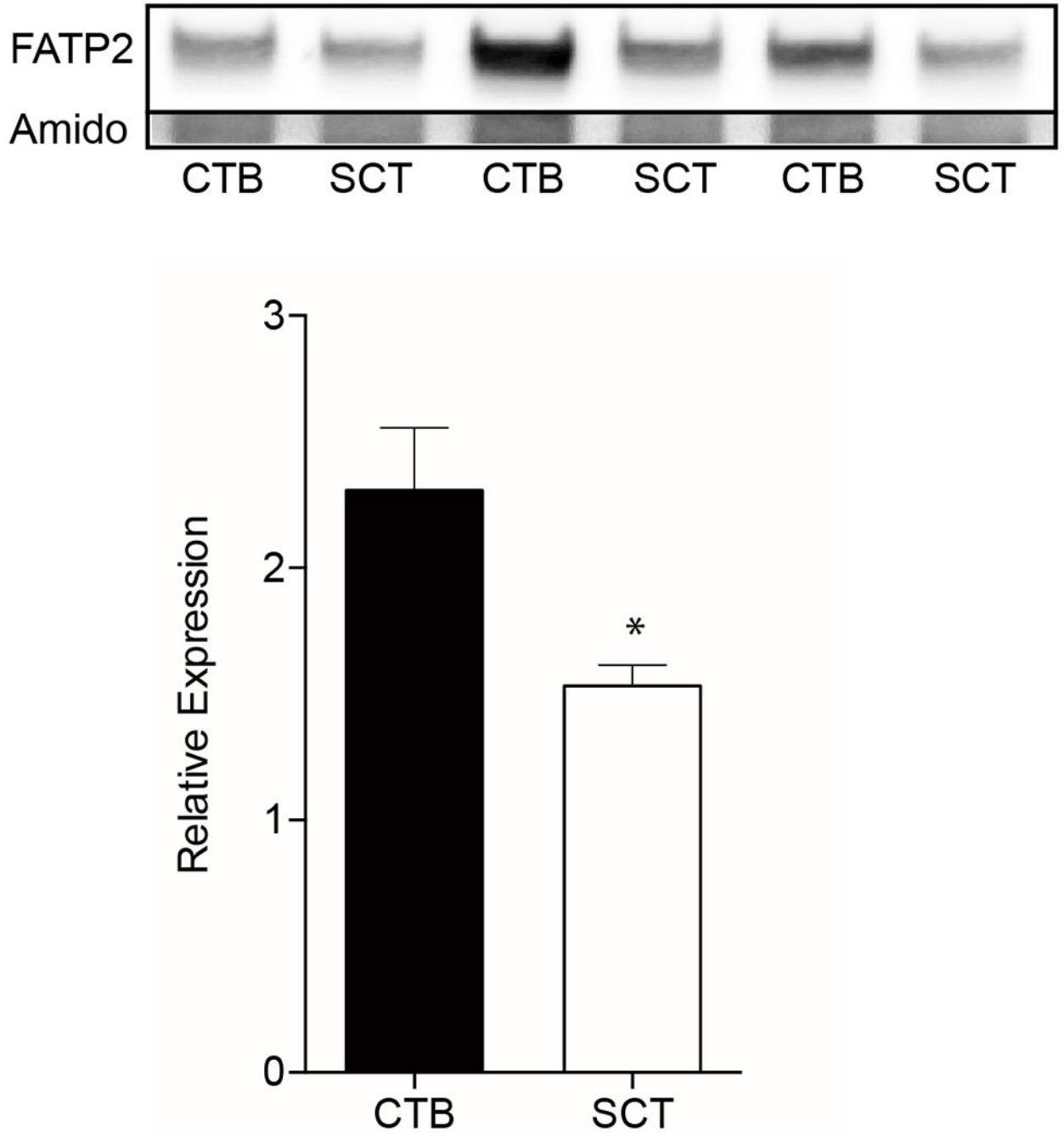


Figure 5. FATP2 western blots of cytotrophoblast and syncytiotrophoblast *in vitro*.

Primary human cytotrophoblast (CTB) were isolated and cultured for 8h and 72h to allow for differentiation to syncytiotrophoblast (SCT) before protein collection. Lysates were analyzed by western blots and probed for Fatty acid transporter type 2 (FATP2). These data suggest the decrease in BODIPY-C12 and/or BODIPY-C16 fatty acid uptake after syncytialization may be due to the reduction in FATP2 protein expression. FATP2 levels

were normalized to total protein visualized by amido-black. Data are Mean±SEM, n=6 placentas

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Table 1:

Maternal Characteristics

	Mean±S.D. (n=28)	95% Confidence Interval
Age (yr)	31±5	[29, 33]
Body Mass Index (BMI: kg/m ²)	27±8	[24, 30]
Parity	2±1	[1, 3]
Gestational Age (weeks)	39±1	[38, 39]
Birth Weight (g)	3400±500	[3200, 3600]
Placenta Weight (g)	546±120	[500, 590]

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