High-glucose levels reduce fatty acid oxidation and increase triglyceride accumulation in human placenta

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25 **Abstract**

26 **Aim/hypothesis:** Placenta of women with gestational diabetes mellitus (GDM) exhibits an
27 altered lipid metabolism. The mechanism by which GDM is linked to alterations in placental lipid
28 metabolism remains obscure. We hypothesized that high-glucose levels reduce mitochondrial fatty
29 acid oxidation (FAO) and increase triglyceride accumulation in human placenta.

30 **Methods:** To test this hypothesis, we measured FAO, fatty acid esterification, *de novo* fatty
31 acid synthesis, triglyceride levels and carnitine palmitoyltransferase activities (CPT) in placental
32 explants of women with GDM or with no pregnancy complication.

33 **Results:** In women with GDM, FAO was reduced by ~30% without change in mitochondrial
34 content, and triglyceride content was 3-fold higher than control group. Likewise, in placental
35 explants of women with no complication high-glucose levels reduced by ~20% FAO and
36 esterification increased linearly with increasing fatty acids concentrations. However, *de novo* fatty
37 acid synthesis remained unchanged between high-and-low glucose levels. In addition, high-glucose
38 levels increased triglycerides content ~2-fold compared to low-glucose levels. Furthermore,
39 etomoxir-mediated inhibition of FAO enhanced by ~40% esterification capacity, and elevated by
40 1.5-fold triglycerides content in placental explants of women with no complications. Finally, high-
41 glucose levels reduced ~70% CPT-I activity, and ~25% phosphorylation levels of acetyl-CoA
42 carboxylase in placental explants of women with no complications.

43 **Conclusion:** We reveal an unrecognized regulatory mechanism on placental fatty acid
44 metabolism by which high-glucose levels reduce mitochondrial FAO through inhibition of CPT-I,
45 shifting flux of fatty acids away from oxidation towards the esterification pathway, leading to
46 accumulation of placental triglycerides.

47 **Keywords:** Carnitine palmitoyltransferase I, *de novo* fatty acid synthesis, esterification of fatty
48 acids, fatty acid oxidation, gestational diabetes mellitus, hyperglycemia, placenta, triglycerides.

Abbreviations: Acetyl-CoA carboxylase, ACC; Carnitine palmitoyltransferase I, CPT-I; Carnitine palmitoyltransferase II, CPT-II; Fatty acid oxidation, FAO; Free fatty acids, NEFA; GDM, Gestational diabetes mellitus.

Introduction

Pregnancies affected by gestational diabetes mellitus (GDM) are characterized by various degrees of maternal glucose intolerance, hyperglycemia and hyperinsulinemia (6). Several epidemiological studies have shown that GDM is independently associated with adverse perinatal outcomes (9, 36, 42). The main adverse outcome of maternal diabetes is fetal macrosomia, which is characterized by fetal fat accretion and overgrowth (27, 42). The HAPO (Hyperglycemia and Adverse Pregnancy Outcome) Study Cooperative Research Group has demonstrated an association between maternal hyperglycemia and fetal macrosomia (1, 26), suggesting that maternal hyperglycemia is a contributing factor to fetal macrosomia by enhancing substrate availability to the fetus, stimulating excessive growth and formation of adipose tissue (13, 34).

The underlying mechanisms by which maternal hyperglycemia translate into fetal adiposity are incompletely understood. In 1954, Pedersen proposed that maternal hyperglycemia results in augmented transplacental glucose transfer leading to hyperglycemia in the fetus, which stimulates the production and secretion of insulin by the fetal pancreatic beta-cells. Hence, glucose surplus and hyperinsulinemia would play a direct role in the accumulation of fat in fetal adipose tissue (30, 31). However, Szabo et al. proposed a different hypothesis to explain fetal macrosomia in diabetic women. The hypothesis postulates that high maternal plasma free fatty acids levels (NEFA), secondary to maternal insulin resistance, lead to increased transplacental transfer of NEFA to the fetus, which are subsequently transported to fetal adipocytes and esterified into triglycerides. In this scenario, maternal hyperglycemia does not contribute directly to fetal fat accretion in the form of energy oversupply, but rather maternal glucose is used as a source of the glycerol, necessary for

NEFA esterification (41). Several clinical studies have reinforced the idea that elevated maternal plasma triglyceride levels may account for fetal fat accretion (15, 22, 23, 28, 38).

Recently, it has been shown that placental lipid metabolism is altered in placentas from diabetic women (16, 25, 34, 35). These findings have prompted the notion that placental lipid metabolism may represent a regulatory step towards fetal macrosomia (14, 34, 39, 41). In this study, we aimed to further understand the role of maternal hyperglycemia on the regulation of placental lipid metabolism. To this end, we tested the hypothesis that high-glucose levels inhibit placental fatty acid oxidation leading to enhanced NEFA esterification and accumulation of placental triglycerides.

Methods

Study subjects

The study was performed on placentas from pregnancies monitored at the Department of Obstetrics and Gynecology, University Hospital “Puerta del Mar” (HUPM). Patient samples were obtained after written informed consent in accordance with the HUPM Ethics Committee requirements and the Declaration of Helsinki. Patients were eligible among consecutive pregnant women attending our antenatal clinic who were planned to deliver by an elective Caesarean section due to clinical reasons other than diabetes, and potentially not affecting placental metabolism (breech presentation or prior Caesarean section). This was so to rule out potential effects of labor on placental energy metabolism. Specific exclusion criteria included women under the age of 18, smokers or those with a history of long-chain 3-hydroxyacyl-CoA deficiency, hemolysis elevated liver function syndrome or acute fatty liver of pregnancy, preeclampsia, chronic hypertension, or other co-morbid disease. The diabetic group was composed of 8 gestational diabetic women. Only cases needing insulin therapy for metabolic control were eligible and offered to participate in the study in order to include only cases with clear metabolic impairment. Maternal diabetes mellitus

was defined as an abnormal glucose tolerance according the criteria defined by the National Diabetes Data Group (18), which have been accepted by the Spanish Group of Diabetes in Pregnancy (11). Screening was performed using a two-steps approach in pregnant women between 24-28 weeks of gestation. The initial screening procedure consisted of a 50-g glucose challenge test, with a 1-h blood glucose cut-off set at ≥ 7.76 mmol/l. Women with a positive screening test underwent a confirmatory 3-hour 100-g oral glucose tolerance test (fasting glucose ≥ 5.82 mmol/l; 1-hour, ≥ 10.54 mmol/l; 2-hour, ≥ 9.15 mmol/l; and 3-hour, ≥ 8.04 mmol/l). Gestational diabetes mellitus was defined when two or more plasma glucose measurements were equal or higher than the cut-off points. Insulin therapy was indicated if more than one-third of capillary peripheral glucose measurements were higher than the targets (> 5.27 mmol/l fasting, > 5.82 mmol/l preprandrial and > 7.76 mmol/l 1-hour postprandrial). In total, 14 women with no pregnancy complication participated in the control group. Randomly chosen subsets of either 6 or 8 controls were used for the experiments as indicated in the legend of Figures. Demographics and baseline data, as well as perinatal variables, are shown in Table 1. All Caesarean sections were performed at term. Placental samples and fasting maternal blood samples from control and GDM group were obtained at the time of the elective Caesarean section. At this time no significant differences were found in lipids, glycemia nor insulinemia levels. Neonatal anthropometric measurements were performed immediately at delivery as usual. Fetuses of women with GDM showed a slight tendency to have higher birthweight and placental weight was significantly higher in this group.

Biochemical parameters

All biochemical parameters were analyzed at the Clinical laboratory, HUPM, using reagents and modular systems from Roche Diagnostics. Plasma insulin was measured by electrochemiluminiscence immunoassay (ECLIA) by E-170 using 20 μ l of sample. Plasma glucose, triglycerides, total cholesterol and high-density lipoprotein cholesterol (HDL-c) were

measured by standard enzymatic methods by C-711 using between 2-3 μ l of sample. Low-density lipoprotein cholesterol (LDL-c) was calculated using the Friedewald-Fredrickson formula.

Placental explants culture

Term placenta obtained from elective Caesarean section was placed on ice and arrived to the laboratory within 10-15 minutes of delivery. Then, decidual tissue and large vessels were removed from villous placenta by blunt dissection on aseptic culture conditions. Afterwards, small fragments of villous tissues (~100 mg wet weight) were rinsed twice in cold-PBS and 6 explants were transferred to each well of a 6-well plate containing 2 ml of culture medium (RPMI-1640 supplemented with 5 mmol/l glucose, 10% FBS (vol/vol), 100 units/ml penicillin G, and 100 μ g/ml streptomycin) and maintained at 37°C in a humidified atmosphere of 5% CO₂/ 95% O₂ for 1h prior to experiments. Villous explant viability and morphological integrity was assessed by XTT (XTT kit, Roche) and haematoxylin-eosin staining respectively.

Materials

Cell culture reagents (RPMI-1640 medium without glucose and fetal bovine serum) were from Invitrogen/Gibco, California, USA. The [9,10-³H]-palmitic acid, [³H]-H₂O, D-[¹⁴C(U)]-glucose and L-[N-methyl-¹⁴C]carnitineHCl were from PerkinElmer, Massachusetts, USA. Etomoxir and essentially fatty acid-free bovine serum albumin were from Sigma, St. Louis, USA.

Fatty acid solution preparation

Stock of fatty acid solution was prepared by conjugating palmitate with essentially fatty acid-free bovine serum albumin (BSA) to generate a stock solution of 25% (wt/vol) BSA, 4 mmol/l palmitate in glucose-free culture medium. Stock solution was filtered-sterilized and diluted into the final culture medium to give concentrations of 1.25% BSA, 0.1 or 0.2 mmol/l palmitate.

143 **Fatty acid oxidation assay in placental explants**

144 Mitochondrial FAO assays were performed ex vivo in placental explants as described
145 previously (2, 32) with the following modifications. Freshly isolated villous explants were
146 incubated in culture media supplemented with low (5 mmol/l) or high (11 mmol/l) glucose
147 concentrations, and in the presence of 1.25% BSA, 0.1 mmol/l cold palmitate, and 18500 Bq/ml
148 [³H]-Palmitate at 37°C for 18h. The glucose concentration in culture medium for the experiments in
149 which glucose was not an experimental factor was 5 mmol/l. Glucose was added to media from a
150 sterile stock solution of 1 mol/l glucose. At the end of the incubation period, the medium was
151 collected, and tritiated water determined by the vapor-phase equilibration method of Hughes et al
152 (21). FAO was defined as nmol of palmitate per mg of tissue per hour.

153 **Esterification into total lipids in placental explants**

154 The esterification rate in placental explants was determined as previously described with some
155 modifications (5). Briefly, after similar incubation conditions to those used for measurements of β-
156 oxidation, with low or high glucose levels in the presence of 1.25% BSA, 0.1 mmol/l cold
157 palmitate, and 18500 Bq/ml [³H]-palmitate for 18h, explants were washed 3 times with 2 ml of ice-
158 cold PBS and homogenized in 500 µl of PBS. An aliquot of 100 µl was used to extract the lipid
159 content from samples according to Bligh and Dyer (3). Afterwards, the radioactive content was
160 determined by liquid scintillation counting. Esterification was defined as nmol of palmitate per mg
161 of tissue per hour.

162 **De novo lipid synthesis in placental explants**

163 *De novo* lipid synthesis was determined using [¹⁴C]-glucose according to the procedure
164 described by Brown et al. with some modifications (5). Villous Placental explants from control
165 group were incubated in RPMI-1640 culture media with low- or high-glucose levels (5 mmol/l and

166 11 mmol/l respectively) and 37000 Bq/ml [¹⁴C]-glucose at 37°C for 18h. At the end of the
167 incubation period, culture media were discarded and explants collected, rinsed 3 times with 2 ml of
168 ice-cold PBS, followed by homogenization in 500 µl of PBS. After a total lipid extraction (as
169 described for measurements of placental esterification rate), the radioactive content was determined.
170 De novo lipid synthesis is expressed as pmol per mg of tissue per hour.

171 **Placental triglyceride determination**

172 Placental triglyceride determination was determined as previously described (33). Frozen
173 placental explants from control and GDM group (~20 mg) were used for experiments showed in
174 Figure 1. For the rest of the experiments, placental explants were preincubated in low or high-
175 glucose in the presence of 0.1 mmol/l palmitate for 18h as described above. Tissues were
176 homogenized in 400 µl HPLC-grade acetone. After incubation with agitation at room temperature
177 overnight, aliquots of 5 µl of acetone-extracted lipid suspension were used to determined
178 triglyceride concentrations using a triglyceride reagent kit (Biosystems, Barcelona, Spain). Proteins
179 were quantified using the bicinchoninic acid method (Thermo Scientific, Madrid, Spain). Placental
180 lipid content was defined as mg of triglyceride per mg of total placental proteins.

181 **Western blot analysis**

182 Placental explants from control group were preincubated in RPMI-1640 culture media
183 containing low- or high-glucose levels for 18h. At the end of the incubation period culture media
184 were discarded, explants were collected and washed with ice-cold PBS, followed by
185 homogenization in lysis buffer (20 mmol/l Tris-HCl pH 7.5, 150 mmol/l NaCl, 1 mmol/l EDTA, 1
186 mmol/l EGTA, 1% (v/v) Triton X-100, 2.5 mmol/l sodium pyrophosphate, 1 mmol/l β-
187 glycerophosphate, 1 mmol/l Na₃VO₄, 1 µg/ml leupeptin, 1 mmol/l phenylmethylsulfonyl fluoride))
188 plus protease inhibitors (Protease Inhibitor Cocktail, Sigma, St. Louis, MO). After 10 min. on ice,

extracts were sonicated and centrifugated at 18,000 X g for 10 minutes at 4°C. Pellets were discarded and solubilized proteins (40-60 µg/sample) were resolved by 5% SDS-PAGE for phospho-acetyl-CoA Carboxylase (p-ACC) and 10% SDS-PAGE for actin, and electrotransferred onto polyvinylidene difluoride filters for immunoblotting by conventional means. After probing with specific p-ACC antibody (1:1000, Cell Signaling, Barcelona, Spain), the membranes were stripped and reprobed with antibody against actin (1:3000, Sigma). Signals were detected by chemiluminescence (Immun-Start western chemiluminescence kit, Bio-Rad, Madrid, Spain), and band densitometry was quantified with the ImageJ software (NIH, USA).

Mitochondrial citrate synthase assay

As an index of mitochondrial content, citrate synthase activity was measured using the Citrate Synthase Assay kit (Sigma, St. Louis, USA) according to manufacturer's instructions, in placenta from control and GDM group. Protein content was determined as above. Citrate synthase activity was defined as nmol/ml/min.

CPT assay

Activities of carnitine palmitoyltransferase I (CPT-I) and carnitine palmitoyltransferase II (CPT-II) were determined in the direction of acyl-carnitine formation, using [¹⁴C]-carnitine as substrate (4). Briefly, placental explants were preincubated in RPMI-1640 culture media containing low- or high-glucose levels at 37°C for 18h. At the end of the incubation period culture media were discarded, explants were collected and washed with ice-cold PBS priory homogenization in lysis buffer (5 mmol/l Tris-HCl, pH 7.2, 150 mmol/l KCl) with a glass homogenizer. For assay of CPT-I, 100 µl of cell homogenate, in which the mitochondria remain largely intact, was incubated in the presence of 50 µmol/l palmitoyl-CoA, 500 µmol/l carnitine and 9250 Bq/ml [¹⁴C]-carnitine, in a 30°C shaking water bath for 10 min. For assay of CPT-II, a portion of the homogenate was adjusted

to 1 % (w/v) of the detergent octylglucoside, which solubilizes the mitochondrial membranes, inactivating CPT-I and releasing CPT-II from the mitochondrial matrix in active form. Afterwards, reactions were stopped by adding 500 µl 1.2 N HCl and palmitoyl-[¹⁴C]-carnitine was extracted by adding 500 µl of 1-butanol. Radioactive content was determined by liquid scintillation counting.

Statistical analysis

Statistical analysis of data was performed using the SPSS software (SPSS, Inc., Chicago, IL). Distributions were checked with a histogram and the Kolmogorov-Smirnov test. When a variable was distributed normally, data were presented as mean ± S.D. In cases of non-normal distribution, data were shown as median and interquartile range. Comparisons were done by using the Mann Whitney's U test or ANOVA. Differences were considered significant at $p < 0.05$.

Results

Reduced fatty acid oxidation and elevated triglyceride levels in placentas from women with gestational diabetes

To reveal the metabolic characteristics of placentas from women with GDM, we determined the FAO capacity in placental explants from control and diabetic women. As shown in Figure 1A, FAO was reduced by ~30% in placentas of women with gestational diabetes compared with the control group. A reduction in FAO capacity could be explained by a lower mitochondrial number in the GDM group. However, as assessed by citrate synthase activity, mitochondrial content was similar between placental explants from control and diabetic women, suggesting that the molecular mechanism underlying reduced FAO capacity in diabetic group may be related to other factors rather than to mitochondrial number (Fig1B). Coinciding with reduced FAO, triglyceride levels in the GDM group were 3-fold higher compared to control group (Fig1C). Taken together, these

results indicate an association between reduced FAO capacity and accumulation of triglycerides in placentas from diabetic women.

Effect of high-glucose levels on fatty acid oxidation and triglyceride levels in explants of human placenta.

Maternal hyperglycemia is a hallmark of women with gestational diabetes. Therefore, it is reasonable to hypothesize that the impaired ability of placentas from women with GDM to oxidize fatty acids is a direct consequence of placental glucose surplus environment, leading to accumulation of placental triglycerides. To test this hypothesis, we measured the effect of low- or high-glucose levels on FAO in placental explants from control group. As shown in Figure 2A, high-glucose levels significantly reduced the FAO rate in placental explants. In parallel, high-glucose levels enhanced fatty acid esterification in the presence of 0.1 and 0.2 mmol/l palmitate (Fig2B). Likewise, esterification augmented at increasing concentrations of palmitate from 0.1 to 0.2 mmol/l, (Fig2B). However, *de novo* fatty acid synthesis using [¹⁴C]-glucose as carbon source remained unchanged (Fig2C). Similar findings were found for *de novo* fatty acid synthesis using [¹⁴C]-acetate as carbon source (data not shown). High-glucose levels significantly increased by ~2-fold the placental triglyceride content (Fig2D), consistent with the expectation that fatty acids are preferentially directed towards esterification under that condition. Taken together, these data indicate that high glucose levels alter the placental triglycerides content through inhibition of FAO.

Etomoxir-mediated inhibition of fatty acid oxidation increases triglyceride accumulation in placental explants

To gain further insight into the molecular mechanism by which high glucose levels alter placental fatty acid partitioning, we used etomoxir, a specific and irreversible inhibitor of the carnitine palmitoyltransferase I (CPT-I), to evaluate the impact of inhibition of mitochondrial fatty

acid entry on FAO, fatty acid esterification and the storage pool of triglycerides in placenta from healthy women. Etomoxir treatment significantly inhibited FAO capacity in placental explants (Fig3A), resulting in augmented esterification (Fig3B), and higher placental triglyceride content (Fig3C).

High-glucose levels decreases carnitine palmitoyltransferase I activity in placental explants

We further investigated the mechanisms by which high-glucose reduced FAO capacity in human placental explants. To this end, we measured the activity of CPT-I and CPT-II in placental explants from control group preincubated in low- or high-glucose levels for 18h. As shown in Figure 4A-B, high-glucose levels reduced by ~70% the activity of CPT-I, whereas CPT-II activity remained unchanged as expected. Because malonyl-CoA is a physiological regulator of CPT-I activity, we quantified the phosphorylation levels of ACC, the enzyme that catalyzes the ATP-dependent carboxylation of acetyl-CoA to form malonyl-CoA. Interestingly, phosphorylation levels of ACC were reduced by ~25% in the presence of high-glucose levels (Figure 4C), suggesting an increased production of malonyl-CoA in placental explants.

Discussion

The availability of maternal nutrients to the fetus is regulated by the placenta involving three main mechanisms: direct transfer of nutrients, placental consumption of nutrients and placental conversion of nutrients into alternative fuel sources (19). Direct transfer has been considered the main mechanism by which placenta regulates the nutrient-exchange between the mother and the fetus (19). However, the placenta exhibits a high metabolic activity, which is severely affected by the intrauterine milieu of diabetic and/or obese women. Specifically, studies performed on placentas from diabetic women have shown major changes in expression levels of genes involved up-regulation of pathways of lipid synthesis and transplacental lipid fluxes (16, 25, 34, 35). These

findings have spurred the notion that alterations in placental lipid pathways perhaps contribute to fetal fat accumulation and adiposity in diabetic women (8, 34).

The FAO pathway has not been evaluated in placenta from GDM women. In this study, we demonstrated that these women exhibited lower FAO oxidation capacity without change in mitochondrial content. To explain these observations, we hypothesized that lower FAO capacity may be related to maternal hyperglycemia, a hallmark of GDM women. However, the metabolic environment of women with GDM is characterized also by the presence of excessive NEFA levels and pro-inflammatory cytokines (6, 35), which makes difficult to tease apart the causing factor involved in reduced placental FAO observed in these women. Thus, we attempted to mimic maternal milieu of women with GDM in our *ex vivo* studies, using low-and-high glucose levels, and low-and-high NEFA levels. Therefore, a limitation of this study is that although our *ex vivo* culture conditions for placental explants clearly allowed mechanistic studies; they may not accurately reflect a GDM milieu and replicate *in vivo* pathology. Thus, our findings in placenta from women with GDM may be explained by other factors related to obesity, such as elevated NEFA and/or pro-inflammatory cytokines, rather than maternal hyperglycemia. However, obesity is not a confounding factor in the phenotype of the GDM women group in our study population (BMI was similar between both groups), which supports the notion that only GDM related factors, such as higher glucose levels, may trigger the observed modifications. Although glycemia and insulinemia levels were only determined in the fasting state, it may be highlighted that the absence of differences between the two groups may be also attributed to the prescription of a strict metabolic control in patients with GDM. Along this line, there were no differences in the levels of glycosylated haemoglobin between the two groups. Nevertheless, further studies are warranted to investigate regulation of FAO pathways using placental explants from women with type I diabetes, type II diabetes, and obese non-diabetic women.

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307 Using placental explants from women with no pregnancy complication, we demonstrated that
308 high-glucose levels inhibited FAO and increased triglyceride accumulation. These results are in
309 agreement with our findings in placentas from GDM women. Because *de novo* fatty acid synthesis
310 remained unchanged, and because etomoxir-mediated inhibition of CPT-I recapitulated the effects
311 of high-glucose on FAO and esterification pathways, we thought that the mechanistic link between
312 high-glucose levels and lower FAO was inhibition of CPT-I activity by its physiological inhibitor
313 malonyl-CoA, which is synthesized from glucose-derived acetyl-CoA by ACC. Following this
314 rationale, we demonstrated that CPT-I activity and phosphorylation of ACC was significantly
315 decreased by high-glucose levels. Because phosphorylation of ACC inhibits its enzymatic activity,
316 our results support the notion that FAO is diminished by high-glucose levels through decreased
317 ACC phosphorylation and enhanced production of malonyl-CoA levels in placental explants, which
318 resulted in lower CPT-I activity. Interestingly, this mechanism results in a shift of fatty acid
319 partitioning away from the β -oxidation pathway towards esterification, allowing the accumulation
320 of triglycerides in human placenta.

321 These alterations in lipid metabolism mediated by high-glucose levels beg for two important
322 questions: 1) What are the consequences of triglycerides accumulation in placenta? 2) Is placental
323 storage of triglycerides a contributing factor to fetal macrosomia? Several studies have
324 demonstrated that maternal serum triglyceride levels are associated with abnormal fetal growth in
325 women with GDM, type 1 and type 2 diabetes (17, 38), spurring the notion that increased maternal
326 lipid availability results in fetal fat accretion. In a hypothetical scenario of maternal triglycerides
327 oversupply and elevated lipolysis rate at the maternal-placental side, esterification of NEFA into

triglycerides in placental cells may indicate a regulatory system to limit maternal fatty acids transfer to the fetus, and serve as a protective mechanism against fetal macrosomia. However, there is no data about the lipolysis rate of very low-density lipoproteins and chylomicron remnants in placentas from women with GDM. Thus, although placental lipid metabolism has been proposed as a regulatory step towards fetal macrosomia (14, 34, 39, 41), it is still missing a direct evidence demonstrating that unbalanced triglycerides storage in placental cells results in augmented transplacental delivery of adipogenic substrates to the fetus. On the other hand, accumulation of triglycerides or its harmful intermediaries, such as ceramide and diacylglycerol, in trophoblast cells may exacerbate the basal pro-inflammatory state of pregnancy. In this hypothetical scenario, accumulation of triglycerides in placental cells would trigger inflammatory pathways in trophoblast cells and deleterious effects on placental and fetal metabolism. Several studies support the idea that GDM and/or obesity induces inflammatory pathways in placenta (7, 12, 24, 35).

Our results on fatty acid partitioning contrast with early studies performed by Pathmapeura et al. in trophoblast isolated from normal term human placentas. They showed that low- or high-glucose (0,5-18 mmol/L) levels had not significant effects on FAO and esterification processes in cultured trophoblast exposed to short (2h) or longer (24h) periods of time (29). The differences between both studies may be explained by the experimental models employed. Firstly, Pathmapeura et al. used cultured trophoblast isolated from human placentas, whereas we used placental explants. The latter technique allows the possibility to investigate trophoblast function in a context that contains other cell types (fibroblasts, macrophages, endothelial cells, etc.) and retains the cellular architecture of the tissue *in vivo*. Secondly, trophoblast cells were maintained in culture media for 16h prior initiation of experimental procedures, whereas placental explants were only maintained in culture media for 1h. Finally, they investigated the effects of glucose levels on fatty acid

partitioning for 24h in the presence of 0.25 mmol/l non-esterified fatty acids (palmitate:oleate ratio 1:1), whereas we used 0.2 mmol/l palmitate as a source of non-esterified fatty acids.

We showed that placenta from healthy women can incorporate [¹⁴C]-glucose into lipids, corroborating previous studies concerning the *de novo* fatty acid synthesis capacity of human placenta (10, 20, 40). Whereas high-glucose did not result in a significant increase in *de novo* lipid synthesis in placental explants, FAO was decreased, suggesting an increase in glucose-derived malonyl-CoA. Under these experimental conditions ACC activity appeared to function primarily as a regulator of the FAO pathway, rather than a regulator of the *de novo* fatty acid synthesis pathway. A similar role for ACC has been described in tissues with low *de novo* fatty acid synthesis capacity, such as skeletal and cardiac muscle (37). Early studies suggested that *de novo* fatty acid synthesis pathway plays a minor role in triglyceride accumulation in diabetic placenta, consistent with our observation on *ex vivo* metabolism (10, 20, 39, 40). Finally, we acknowledge that a limitation of our study is that placental explants were preincubated in the absence of insulin, which is present in the *in vivo* milieu and it is required for *de novo* lipid synthesis. Therefore, taken into consideration our experimental conditions without insulin and given the non-significant trend towards increased [¹⁴C]-glucose incorporation into lipid in the presence of high glucose, we cannot conclude that elevations in glucose do not increase placental *de novo* lipid synthesis *in vivo*.

In conclusion, we demonstrate that high-glucose levels alter the metabolic partitioning of fatty acids in human placenta, shifting flux of fatty acids away from oxidation towards the esterification pathway, leading to accumulation of placental triglycerides. The mechanistic link between high-glucose levels and lower FAO capacity is through reduced activity of the enzyme CTP-I, which regulates the first step of the entry of long-chain acyl-CoA into the mitochondrial matrix for β -oxidation. These findings shed light on the biochemical mechanisms by which maternal hyperglycemia may regulate placental lipid pathways in diabetic mothers.

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in clinical data collection, analyzed data, and revised the manuscript for important intellectual
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Figure legends

Figure 1. Fatty acid oxidation is reduced in placenta from women with gestational diabetes. (a) Mitochondrial fatty acid oxidation. A subset of 8 placentas from women with no pregnancy complication (control group, n=8) and gestational diabetic women (GDM, n=8) were used to obtain villous explants as described in “Methods” section. The explants were preincubated with 0.1 mmol/l (18500 Bq/ml) palmitate for 18 hours, and the production of [^3H]-water was determined as described in the “Methods” section. Values are Mean $\pm$ S.D. for 8 independent experiments in triplicate. Significance is indicated (*p<0.05) relative to control group. (b) Mitochondrial content. Citrate synthase activity, an indicator of mitochondrial content, was assayed in placental explants from control and GDM group. Values are Mean $\pm$ S.D. for 8 independent experiments in duplicate. p=0.845 relative to control group. (c) Placental triglyceride content. Frozen placental tissues (~100 mg) from control (n=8) and GDM group (n=8) were used to quantify placental triglyceride content as described in the “Methods” section. Values are Mean $\pm$ S.D. for 8 independent experiments in triplicate. *p<0.05 relative to control group.

Figure 2. High-glucose levels inhibit fatty acid oxidation in placentas from healthy women. (a) Effect of high-glucose levels on fatty acid oxidation. A subset of 6 placentas from women with no pregnancy complication described in table 1 was used to obtain villous explants. Placental explants from control group were incubated at 5 (5 Gl) or 11 (11 Gl) mmol/l glucose in the presence of 0.1 (0.1 Pa) mmol/l palmitate for 18 hours. Afterwards, [^3H]-water was determined as described in the “Methods” section. Values are Mean $\pm$ S.D. for 6 independent experiments in triplicate. Significance is indicated (*p<0.05) relative to 5 mmol/l glucose. (b) Effect of high-glucose levels on fatty acid esterification. A subset of 6 placentas from women with no pregnancy complication described in table 1 was used to measure the esterification capacity. Placental explants from control group were incubated at 5 (5 Gl) or 11 (11 Gl) mmol/l glucose in the presence of 0.1 (0.1 Pa) or 0.2

(0.2 Pa) mmol/l palmitate for 18 hours. Afterwards, [^3H]-palmitate incorporation into total lipids was determined as described in the “Methods” section. Mean $\pm$ S.D. for 6 independent experiments in triplicate. * $p < 0.05$ relative to 5 mmol/l glucose; $^\dagger p < 0.05$ relative to 0.1 mmol/l palmitate. (c) Effect of high-glucose levels on de novo lipid synthesis. A subset of 4 placentas from women with no pregnancy complication described in table 1 was used to obtain villous explants. Placental explants were incubated at low (5 mmol/l) or high (11mmol/l) glucose levels in the presence of [^{14}C]-glucose for 18 hours. Afterwards, [^{14}C]- glucose incorporation into total lipids was determined as described in the “Methods” section. Mean $\pm$ S.D. for 4 independent experiments in triplicate. * $p < 0.05$ relative to low glucose. (d) Effect of high-glucose levels on placental triglyceride content. The same subset of placentas used for fatty acid oxidation and esterification experiments described above was used to measure triglyceride content. Placental explants were incubated as described above and the triglyceride content was determined as described in the “Methods” section. Mean $\pm$ S.D. for 5 independent experiments in triplicate. * $p < 0.05$ relative to 5 mmol/l glucose.

Figure 3. Etomoxir-dependent inhibition of fatty acid oxidation increases triglyceride accumulation in placentas from healthy women. (a) Fatty acid oxidation in placental explants treated with various concentrations of etomoxir. A subset of 6 placentas from women with no pregnancy complication described in table 1 was used to asses FAO capacity. Placental explants were incubated in the absence or presence of 50 $\mu\text{mol/l}$, 100 $\mu\text{mol/l}$ or 200 $\mu\text{mol/l}$ etomoxir with 0.1 mmol/l (18500 Bq/ml) palmitate for 18 hours, and the production of [^3H]-water was determined as described in the “Methods” section. Mean $\pm$ S.D. for 6 independent experiments in triplicate is shown. * $p < 0.05$ relative to untreated placental explants; $^\dagger p < 0.05$ relative to 200 $\mu\text{mol/l}$ etomoxir-treated placental explants. (b) Fatty acid esterification in placental explants treated with various concentrations of etomoxir. The same subset of placental explants described in panel A were used to assess esterification into total lipids as described in the “Methods” section. Mean $\pm$ S.D. for 6

independent experiments in triplicate. * $p < 0.05$ relative to untreated placental explants. (c) The same subset of placental explants described in panel A were used to assess triglycerides content as described in the “Methods” section. Mean $\pm$ S.D. for 6 independent experiments in triplicate. * $p < 0.05$ relative to untreated placental explants.

Figure 4. High-glucose levels inhibit carnitine palmitoyltransferase I activity and reduce phosphorylation levels of acetyl-CoA carboxylase. A subset of 6 placentas from women with no pregnancy complication described in table 1 was used to obtain villous explants and perform the following experiments. Carnitine palmitoyltransferase I activity (a) and carnitine palmitoyltransferase II activity (b) were determined as described in the “Methods” section in placental explants incubated at low (5 mmol/l) or high (11 mmol/l) glucose concentrations for 18 hours. Mean $\pm$ S.D. for 6 independent experiments in duplicate is shown. * $p < 0.05$ relative to 5 mmol/l glucose. (c) Western blot analysis of phospho-acetyl-CoA carboxylase (p-ACC) in protein extracts from placental explants incubated at low (5 mmol/l) or high (11 mmol/l) glucose concentrations for 18 hours. In the upper panel is shown a representative picture of the western blot. In the lower panel the y-axes represents the ratio of phosphorylated acetyl-CoA carboxylase *versus* β -actin in arbitrary units. Mean $\pm$ S.D. for 4 independent experiments in triplicate. * $p < 0.05$ relative to 5 mmol/l glucose.

Figure 1

Figure 2

Figure 3

Figure 4

Table 1. Anthropometrics and metabolic data of the study population.

	Control group (n=14)	GDM Group (n=8)
Delivery mode	Caesarean section No labour	Caesarean section No labour
Maternal age (yr)	33.4 ± 4.6	36.3 ± 2.0
Gestational age (wk)	37.4 ± 1.81	39 ± 1.0
Maternal pregravid BMI	23.7 ± 4.8	25.8 ± 5.3
Maternal glucose (mg/dL)	77.8 ± 14.6	78,6 ± 10,2
Maternal insulin (pmol/L)	8.8 ± 4.6	8.6 ± 1.4
Maternal triglycerides (mg/dL)	185.8 ± 66.5	195 ± 12.2
Maternal Total Cholesterol (mg/dL)	252.8 ± 64.2	233 ± 47.6
Maternal HDL Cholesterol (mg/dL)	101,2 ± 54.4	62,33 ± 10.2
Maternal LDL Cholesterol (mg/dL)	121.8 ± 48,9	135.6 ± 37.8
Maternal HbA1c (%)	5.3 ± 0.3	5.3 ± 0.1
Placental weight (g)	510 ± 75	612 ± 74*
Birthweight (g)	3048 ± 591	3186 ± 362

When a variable is normally distributed, data are given as mean ± SD. *GDM*, Gestational diabetes mellitus; *BMI*, Body mass index; *HbA1C*, hemoglobin A_{1c}. *HDL*, High-density lipoprotein; *LDL*, Low-density lipoprotein. *p<0.05 vs control group.