Dynamic regulation of lipid metabolism in the placenta of in vitro and in vivo models of gestational diabetes mellitus†

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Abstract

The purpose of this study was to investigate lipid metabolism in the placenta of gestational diabetes mellitus individuals and to evaluate its effect on the fetus. We examined the expression of lipogenesis- and lipolysis-related proteins in the in vitro and in vivo gestational diabetes mellitus placenta models. The levels of sterol regulatory element binding protein-1c were increased, and fat accumulated more during early hyperglycemia, indicating that lipogenesis was stimulated. When hyperglycemia was further extended, lipolysis was activated due to the phosphorylation of hormone-sensitive lipase and expression of adipose triglyceride lipase. In the animal model of gestational diabetes mellitus and in the placenta of gestational diabetes mellitus patients during the extended stage of gestational diabetes mellitus, the expression of sterol regulatory element binding protein-1c decreased and the deposition of fat increased. Similar to the results obtained in the in vitro study, lipolysis was enhanced in the animal and human placenta of extended gestational diabetes mellitus. These results suggest that fat synthesis may be stimulated by lipogenesis in the placenta when the blood glucose level is high. Subsequently, the accumulated fat can be degraded by lipolysis and more fat and its metabolites can be delivered to the fetus when the gestational diabetes mellitus condition is extended at the late stage of gestation. Imbalanced fat metabolism in the placenta and fetus of gestational diabetes mellitus patients can cause metabolic complications in the fetus, including fetal macrosomia, obesity, and type 2 diabetes mellitus.

Summary Sentence

In this study, we suggest that lipogenesis and lipolysis are stimulated in the circumstance of gestational diabetes mellitus.

Graphical Abstract

Keywords: pregnancy, diabetes, placenta, lipid, metabolism
Introduction

Gestational diabetes mellitus (GDM) is a condition wherein a woman without diabetes experiences hyperglycemia during pregnancy [1]. With the incidence of GDM increasing in correspondence with the global rise of metabolic diseases [2], 9–15% of the pregnant women develop GDM [3]. During pregnancy, several metabolic changes occur in the mothers to enable meeting the energy demands of the fetus [4]. For example, maternal insulin resistance increases resulting in enhanced supply of glucose to the fetus. Although the exact mechanism is unclear, decreased insulin sensitivity and resistance are probably induced in response to hormonal alterations. Indeed endogenous hormones, such as human chorionic somatomammotropin (hCS), progesterone, cortisol, and prolactin, induce changes in the responsiveness of pancreatic beta cell with respect to insulin synthesis [5]. However, if insulin resistance is maintained continuously, the women enter a hyperglycemic state and the balance between insulin resistance and its supply is lost, which may result in the development of GDM [6]. The pathology of GDM is not entirely understood, but some characteristics of type 2 diabetes mellitus (T2DM) and several metabolic syndromes, like hyperglycemia, reduced maternal insulin sensitivity, and hyperlipidemia, have been described for GDM [7, 8]. Women diagnosed with GDM have high risk of developing T2DM in the future, cesarean delivery, and hypertension. Moreover, offspring born to pregnant women with GDM could later develop obesity, T2DM, hypertension, and metabolic syndrome [9].

The placenta is formed at the interface between maternal and fetal tissues [10, 11], and it plays a significant role during pregnancy. It can react to maternal and fetal signals and control placental nutrient transport and metabolic function [12, 13]. The placenta uses various substrates to meet its energy needs [14], and nutrients like glucose, amino acids, and lipids are transported to the fetus during pregnancy [15]. Glucose is an important energy substrate of the placenta [16], and is primarily derived from the maternal blood. However, the transfer of fatty acids in the placenta also influences fetal growth and development [17]. Free fatty acids can accumulate in the body organs including the placenta if their levels remain continuously elevated, and they might cause lipotoxic damage and metabolic dysfunction [18]. Diabetic conditions alter placental development and function at the onset of GDM [19]. In GDM, nutrient transport in the placenta may be altered [15]. One of the major effects of maternal hyperglycemia on the fetus is the development of high plasma glucose levels [20]. In addition, glucose has been identified to result in the accumulation of lipids and fats in human placenta [21] as well as fetuses [22]. Pedersen group hypothesized that maternal high glucose levels result in increased fetal weight and length during pregnancy. Maternal blood sugar affects the weight and length of the infants directly because the fetus consumes more glucose and the fetal insulin levels rise due to the increasing supply of glucose from maternal blood [23].

Lipids are synthesized and degraded in cells via a process called lipid metabolism. Many studies have primarily focused on lipid metabolism in adipose tissue, while studies on placental fat metabolism and its effect on the fetus are insufficient. Therefore, we hypothesized that hyperglycemia in GDM patients induces metabolic and functional changes in the placenta. We investigate lipid metabolism in the placenta of GDM patients and to evaluate its effect on the fetus.

Methods

Cell culture and GDM-like in vitro model

The human choriocarcinoma-derived cell line JEG3 was used as a trophoblast model. The JEG3 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Sigma D2902, Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% charcoal-stripped fetal bovine serum (cPBS, Gibco, Invitrogen Co., Carlsbad, CA, USA), 100 IU/mL penicillin, and 100 μg/mL streptomycin, and incubated at 37°C in an atmosphere containing 5% CO2. To establish a GDM-like in vitro model, high glucose conditions were generated by culturing the cells in the presence of 30 mM D-glucose (G6152; Sigma-Aldrich) in a time-dependent manner (24, 48, and 72 h).

Experimental animals

This study was reviewed and approved by the Pusan National University-Institutional Animal Care and Use Committee (PNU-IACUC; Approval Number PNU-2019-2409) (Busan, Republic of Korea). Also, the study followed the ARRIVE guidelines for reporting results. The 12-week old female Sprague-Dawley rats were purchased from Samtako (Osan, Korea), housed in standard cages, and fed with basal feed. Rats were maintained on a 12-h light/dark cycle. All rats were handled in the Pusan National University-Laboratory Animal Resources Center, accredited by the Korea Food and Drug Administration. After 1 week of acclimatization before the experiment, rats were mated overnight, and the presence of sperm in the vagina was checked the following morning (day 0 of pregnancy). Pregnant rats were randomly assigned to the following two groups: control pregnancy (n = 5) and GDM group (n = 5). Diabetes was induced using a single intraperitoneal injection of streptozotocin (STZ, Sigma-Aldrich) in 0.1 M citrate buffer (pH 4.0) at a dose of 45 mg/kg body weight on day 1 of pregnancy. Control animals received an equal volume of citrate buffer. On gestation day (GD) 6, diabetes was confirmed by measuring fasting (8 h) blood glucose concentrations obtained using a tail prick. The animals with fasting plasma glucose above 300 mg/dL were categorized as having GDM. On GD 18, final fasting blood glucose levels were obtained and euthanasia was performed using CO2 gas to prepare tissue samples. Blood samples were collected in SST tubes (Becton Dickinson company, NJ, USA), and the retro-orbital plexus was collected in EDTA-coated micro tubes (BD Biosciences, Franklin Lakes, NJ, USA). To collect amniotic fluid, a 23-gage needle was inserted to each gestational sac and the fluid was aspirated. The collected tissue samples were fixed in 10% formalin until analysis or stored at −70°C for further experiments.

Hematology

Blood analysis and serum biochemistry were performed for all collected samples. The levels of white blood cells (WBC), lymphocytes (LYM), monocytes (MON), and neutrophils (NEU) were measured using a VetScan hematology system (Abaxis, Sunnyvale, CA, USA). Serum biochemical components, including total cholesterol (TC), triglyceride (TG), glucose (GLU), high-density lipoprotein (HDL), and low-density lipoprotein (LDL), were assayed using an automatic serum analyzer (Hitachi 747; Hitachi, Tokyo, Japan). All assays were performed in duplicate using fresh serum.
**Human placental tissue collection and processing**

The biospecimens and data used for this study were provided by the Biobank of Pusan National University Hospital (PNUH), a member of the Korea Biobank Network. This study was approved by the Institutional Review Board (IRB) of PNUH (H-1703-004-006) and complied with the Declaration of Helsinki. The need for signed informed consent from patients was not necessary. Human placental tissues were collected and immediately stored at −80°C; samples were divided into normal (n = 20) and GDM (n = 18). Gestational diabetes mellitus was defined as a condition wherein glycosylated hemoglobin (HbA1c) > 6.23 for glyceamic values. Clinical characteristics of sample groups are shown in Table 1.

**Quantitative real-time PCR and western blotting**

Total RNA was extracted using TRIzol reagent (Invitrogen Co.) according to the manufacturer’s protocol. Real-time quantitative PCR (Q-PCR) was performed in a single reaction to determine gene expression using SYBR Green Realtime PCR Master Mix (TOYOBO, Osaka, Japan) following the manufacturer’s protocol. The absorbance was directly read at 540 nm in the wells using a microplate spectrophotometer (Epoch Bioteck, VT, USA).

**Glycerol release assay**

JEG3 cell and amniotic fluid were used to analyze glycerol release. Each media and cell lysate sample (10 μL) were mixed with 200 μL of Free Glycerol Reagent (F6428, Sigma-Aldrich) following the manufacturer’s protocol. The absorbance was directly read at 540 nm in the wells using a microplate spectrophotometer (Epoch Bioteck, VT, USA).

**Statistical analysis**

The results are presented as the mean ± standard error of the mean. The data were analyzed using one-way analysis of variance (ANOVA) (SPSS for Windows, 10.10, standard version; SPSS Inc., Chicago, IL, USA) and GraphPad Prism® version 8.0.2 (San Diego, CA). P < 0.05 indicates significant differences.

**Results**

**Lipogenesis in GDM placenta model in vitro**

To generate the in vitro GDM placenta model, JEG3 cells were cultured under hyperglycemic conditions induced in response to 30 mM glucose (24, 48, and 72 h), and the expression of human placental lactogen (hPL) and glucose transporter 1 (GLUT1) mRNA were examined using qPCR. The hPL and GLUT1 mRNA were considered as a GDM biomarker in this study [24, 25]. The hPL mRNA levels were increased at 24 and elevated significantly at 72 h after glucose treatment (Figure 1A). The GLUT1 mRNA levels were increased significantly at 24, 48, and 72 h (Figure 1B). Several genes, including SREBP-1, FAS, and PPARγ, are known to be associated with lipogenesis [26]. The levels of lipogenesis-related proteins were examined under hyperglycemic conditions (Figure 1C), and SREBP-1c levels were found to be significantly increased at 24 and 48 h. Although FAS and PPARγ did not show a significant alteration in their levels, their expressions were
Fat metabolism in the placenta in GDM

Figure 1. Lipogenesis in the cell model of GDM placenta. (A, B) mRNA expression of the GDM marker hPL and GLUT1 under in vitro GDM conditions. (C) Levels of lipogenic proteins in the in vitro GDM placenta model. JEG3 placenta cells were exposed to hyperglycemic conditions, i.e., in vitro GDM conditions for 24, 48, and 72 h. The protein levels of SREBP-1c (125 kDa), fatty acid synthase (FAS; 270 kDa), and peroxisome proliferator-activated receptor-γ (PPARγ; 43–70 kDa) were analyzed by western blot. Data normalized to β-actin are expressed as the mean ± standard deviation. \( \ast P < 0.05 \) compared to the control group. The blots were cropped to improve the clarity and conciseness of the presentation. (D) Lipid uptake was analyzed in the JEG3 cells after staining with Oil Red O. Magnification: 100× (scale bar: 100 μm).

Figure 2. Lipolysis in the cell model of GDM placenta. (A) Levels of lipolysis proteins in the in vitro GDM placenta model. The protein levels of hormone-sensitive lipase (HSL; 81, 83 kDa), phosphorylated HSL Ser 660 (p-HSL (Ser 660); 81, 83 kDa), and adipose triglyceride lipase (ATGL; 54 kDa) were analyzed by western blot. Data normalized to β-actin are expressed as the mean ± standard deviation. \( \ast P < 0.05 \) compared to the control group. The blots were cropped to improve the clarity and conciseness of the presentation. In addition, lipolytic activity was measured in the in vitro GDM placenta model. After JEG3 cells were exposed to hyperglycemic conditions, the media was harvested and cell lysates were prepared. (B, C) Glycerol release was measured in hyperglycemic placenta cell media and lysate. Data were expressed as the mean ± standard deviation. \( \ast P < 0.05 \) compared to the control group.

Increased at an early stage (24 and 48 h) of hyperglycemia. The total protein level was normalized to those of β-actin.

To measure lipid accumulation, the result of lipogenesis, Oil Red O was used to stain hyperglycemic placenta cells; staining was found to be intensified at 24 h. These results indicate that lipid generation and accumulation were elevated in early hyperglycemia (Figure 1D).

GDM placenta lipolysis cell model

To analyze lipolysis in the JEG3 placenta cells under GDM conditions, the levels of HSL, p-HSL (Ser 660), and ATGL were assessed by western blotting (Figure 2A). ATGL expression and HSL phosphorylation at Ser 660 were significantly enhanced upon extended hyperglycemia exposure (48 and 72 h), but not in 24 h. The activation of adenyl cyclase is induced in response to the stimulation of lipolytic activity, which again increases cAMP levels. The cAMP activates protein kinase A (PKA) and the sequential steps involved in the hydrolysis of TGs, which are mediated by several lipases [27]. The release of glycerol, a lipolysis indicator, in cell media, was examined under hyperglycemic conditions to measure lipolytic activity in placental cells. During lipolysis, the TG in the cell is metabolized into glycerol and three fatty acids.
Figure 3. Induction of GDM in animal models by STZ treatment. Measurement of (A) blood glucose levels, (B) insulin concentration, (C) placenta weights, (D) fetus weights, (E) amniotic fluid glucose levels, and (F) amniotic fluid glycerol levels in rats with STZ-induced GDM. Blood glucose was measured after fasting for 8 h on GD6 and GD18. Data are expressed as the mean ± standard deviation. *P < 0.05 compared to the control group.

acids, which are released from the cell [28]. The amount of glycerol in the cell media was significantly increased at 48 and 72 h after glucose treatment (Figure 2B). Also the glycerol amount in the cell lysate was increased at 72 h significantly (Figure 2C).

GDM induction in an animal model using STZ treatment

To establish in vivo GDM models, pregnant rats were treated with a single intraperitoneal injection of STZ on GD1. On GD6 and GD18, blood glucose levels were measured using a tail prick after fasting for 8 h from the control and STZ groups. The STZ treated rats showed increased blood glucose compared to the control group during the gestation period (Figure 3A). Also, the insulin concentration was significantly decreased in STZ groups (Figure 3B). Interestingly, the glucose (Figure 3E) and glycerol (Figure 3F) concentrations in the amniotic fluid were significantly increased in STZ animals, while the change in glycerol concentration was not significant.

Parameters related to lipid metabolism such as TC, TG, HDL, and LDL were also investigated. The levels of TG and LDL were increased in STZ rats, while those of HDL were decreased (Table 3). Maternal body weight did not significantly differ between the control and STZ groups. There was no significant change in the hematological values in STZ treated animal (Table 4). However, the placental weight markedly increased in the STZ group compared to that in the control group (Figure 3C). Furthermore, the fetal weight (Figure 3D) in the STZ group was higher than that in the control group, but this was not significant. These results suggest that the diabetic conditions are well induced in STZ-injected rats, and that placental function was dysregulated and more glucose may be transferred to the fetus and amniotic fluid because of the hyperglycemic state.

| Blood parameter | CON | STZ |
|-----------------|-----|-----|
| TC (g/mL)       | 77.67 ± 8.66 | 79.5 ± 41.65 |
| TG (mg/dL)      | 348.22 ± 79.75 | 568.17 ± 242.19 (*) |
| HDL (mg/dL)     | 41.19 ± 9.37 | 27.7 ± 8.08 (*) |
| LDL (mg/dL)     | 19.33 ± 4.21 | 27.19 ± 18.41 |

Table 3. Lipid metabolism-related parameters in STZ rat

Data were expressed the mean ± standard deviation. *P < 0.05 compared to the control group.

| Blood parameter | CON | STZ |
|-----------------|-----|-----|
| WBC (10^3cells/μL) | 8.92 ± 1.48 | 9.92 ± 0.85 |
| LYM (10^3cells/μL)  | 6.02 ± 0.87 | 6.6 ± 1.14 |
| MON (10^3cells/μL)   | 0.40 ± 0.25 | 0.39 ± 0.09 |
| NEU (10^3cells/μL)   | 2.48 ± 0.92 | 2.93 ± 0.19 |

Table 4. Blood hematological values in STZ groups

Lipid metabolism in the placenta of GDM animals

The expression of genes related to lipogenesis and lipolysis was analyzed to study lipid metabolism in the placenta of GDM animals. In the placenta of STZ rats, the expression of lipogenesis-associated proteins, including SREBP-1c, FAS, and PPARγ, was examined by western blotting (Figure 4A). The expression of SREBP-1c was significantly decreased in the STZ group compared to that in the control group, while that of FAS and PPARγ showed no significant change. We also analyzed the levels of lipolytic proteins, including HSL, p-HSL (Ser 660), and ATGL, in the placenta of GDM animals (Figure 4B). Similar to the results obtained in the in vitro GDM placenta model, the protein levels of p-HSL (Ser 660), HSL, and ATGL were increased significantly in the STZ group. Lipid droplet accumulation was examined using Oil Red O staining of the placenta from GDM animals, and the accumulation was enhanced in the STZ-treated animals in both maternal and fetal sides, while it was more drastic in maternal side (Figure 4C).
Lipid metabolism in placenta from GDM patients

Placental tissues were collected from normal pregnant women (n = 20) and GDM women (n = 18) as mentioned in the materials and methods to compare the lipid metabolism. The expression of lipogenesis-related proteins, including SREBP-1c, FAS, and PPAR γ, was examined using western blotting (Figure 5A). SREBP-1c expression decreased in the GDM group compared to that in the normal group, while that of FAS and PPAR γ showed no significant changes. These results were similar to animal data, indicating that lipogenesis is inhibited in the placenta of GDM individuals. The levels of lipolytic proteins, including HSL, p-HSL (Ser 660), and ATGL, were analyzed. Although it was not significant, the levels of p-HSL (Ser 660) were increased, and the expression of total HSL was unchanged. The expression of ATGL significantly reduced in placenta from GDM patients (Figure 5B). Placenta from GDM patients was also stained with Oil Red O (Figure 5C); the results showed higher lipid content in the GDM group than that in the normal group.

Discussion

The incidence of GDM has been gradually increasing, reflecting the prevalence of obesity and T2DM in the population [29, 30]. However, the mechanisms underlying GDM are unknown [31]. Due to the presence of placental hormones and some other factors, the effect of insulin is altered and fat accumulation is increased in GDM patients during pregnancy. Many studies have focused primarily on lipid metabolism in adipose tissue, but studies on fat metabolism in the placenta of GDM individuals and its effect on the fetus are insufficient. Maternal lipids and fatty acids contribute to elevated fetal fat levels, and are also related to increased birth weight. However, the physiological function of the placenta with respect to maternal and fetal fat metabolism has not yet been studied. In one previous study, the activity of lipoprotein lipase (LPL) was examined in the placenta of GDM individuals. Activity of LPL in the placentas of individuals with insulin-dependent diabetes mellitus was increased, but it was unchanged in individuals with GDM. In addition, the expression of LPL protein was not altered in either case [32]. In other study, the placental expression of LPL was increased significantly in the GDM group and the increased placental LPL is associated with fetal size [33]. However, other lipases and indicators of lipid metabolism were not investigated in this study. Therefore, we assessed lipid metabolism in the placenta of GDM individuals and tried to understand how it affects the fetus.

We developed a GDM-like in vitro model by exposing JEG3 cells to hyperglycemic media. We examined the mRNA
expression of hPL and GLUT1, which is known to be related to GDM development in placenta cells. The hPL modulates the mother's metabolic state during pregnancy and helps supply energy to the fetus. Furthermore, it decreases maternal insulin sensitivity [34]. In addition, GLUT1 changes the levels of plasma glucose or insulin [35]. The level of hPL mRNA was increased in JEG3 cells at 24 and 72 h, and the GLUT1 mRNA was increased significantly at 24, 48, and 72 h after exposure to hyperglycemic conditions. These results indicate that our in vitro cell model is relevant to GDM conditions. To test lipid metabolism, fat accumulation in the placental cells was tested in under hyperglycemic conditions. Fat was more accumulated than normal at 24 h after exposure to hyperglycemic conditions (early GDM stage). Additionally, the levels of lipogenesis-related protein SREBP-1c were increased at this hyperglycemia time point. When the cells were incubated under glycemic conditions for 48 and 72 h (extended or late stage GDM), lipolysis was stimulated via HSL activation and ATGL expression at high levels. In addition, the glycerol concentration increased in the culture media after exposure to hyperglycemic conditions for 48 and 72 h, indicating that lipolysis was enhanced in placenta cells. Based on these results, we assume that the fat is accumulated in the placenta during the early GDM stage because of higher glucose levels, and that the deposited fat can be hydrolyzed and delivered to the fetus at the extended GDM stage via lipolysis.

To support our hypothesis, we employed an animal model of GDM by treating pregnant rats with STZ. In the placenta of GDM animals that was collected at the late pregnancy stage, the levels of lipogenesis-related protein SREBP-1c were decreased, while those of lipolysis-related proteins HSL, p-HSL (Ser 660), and ATGL were increased. The increased lipolysis at the late GDM stage was similar to that observed in the in vitro model. Interestingly, the weight of the placenta in GDM animal and the levels of glucose in the amniotic fluid were increased. These results suggest that the increased glucose in the blood of GDM animals leads to fat accumulation in the placenta, elevated placenta weight, and enhanced nutrient transfer to the amniotic fluid and the fetus. Also in our results, TG and HDL were altered in GDM animal model, while LDL slightly increased without significance. In previous research, women with GDM have increasing TG and LDL levels and lower HDL than normal in general. The regulation of TG, LDL, and HDL are considered as a risk element of T2DM and possible marker in the diagnosis of GDM [36]. To investigate the placenta’s function depending on the region, we examined TG accumulation in maternal and fetal placenta. In general, TG accumulation in both the maternal and fetal side of the placenta in GDM animal was higher than that in normal individuals, and this was more drastic on the maternal side. These results suggest that the nutrients accumulated in the placenta during the early and mid-stages of pregnancy probably more in maternal side, while the accumulated TG might be delivered to the fetus via lipolysis at the late GDM stage.

Lipid metabolism was also tested in placenta collected from GDM patients at the late GDM stage. Similar to the results in the animal study, the lipogenesis was inhibited at the late stage. Lipolysis indicators exhibited a more complicated pattern in placenta from GDM patients. The expression of the lipolytic protein ATGL was significantly decreased, while the levels of p-HSL were increased. These results suggest that the lipolysis process in human GDM placenta is not as strong as the GDM animal model. These distinct results may be attributed to the fact that GDM symptoms in humans are not as severe as those in the animal models.

In summary, we found that lipogenesis in the placenta during the early GDM stage is enhanced, which stimulates fat accumulation in the placenta until the late pregnancy stage. At the late GDM stage, the accumulated fat in the placenta is hydrolyzed, and the excessive TG moieties and glucose units can be transferred to the fetus. The dysregulated nutrient metabolism in the placenta and fetus may in turn affect incidence of metabolic complications in the fetus, including fetal macrosomia, obesity, and T2DM.

**Conflict of interest**

The authors have declared that no conflict of interest exists.

**Data availability**

The data underlying this article will be shared on reasonable request to the corresponding author.
Declarations
Ethics approval and consent to participate
The biospecimens and data used for this study were provided by the Biobank of Pusan National University Hospital, a member of the Korea Biobank Network.

Authors’ contributions
B.S.A. and S.C.K. designed research equally; S.Y.K. and Y.J.L. performed research; S.Y.K., Y.J.L., S.M.A., M.J.K., J.S.J., D.S.K., Y.L., and E.M.J. analyzed data; S.Y.K. and Y.J.L. wrote the paper.

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