

Discrepancy in insulin regulation between GDM-platelets and -placenta

Yicong Li, Anthonya Cooper, Imelda N Odibo, Asli Ahmed, Pamela Murphy, Ruston Koonce, Nafisa K Dajani, Curtis L Lowery, Drucilla J Roberts, Luc Maroteaux, et al.

▶ To cite this version:

Yi
cong Li, Anthonya Cooper, Imelda N Odibo, Asli Ahmed, Pamela Murphy, et al.
. Discrepancy in insulin regulation between GDM-platelets and -placenta. Journal of Biological Chemistry, 2016, 10.1074/jbc. M116.713693 . hal-01280557

HAL Id: hal-01280557 https://hal.science/hal-01280557

Submitted on 2 Mar 2016

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers. L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Discrepancy in insulin regulation between GDM-platelets and -placenta

Yicong Li¹, Anthonya Cooper¹, Imelda N Odibo², Asli Ahmed¹, Pamela Murphy², Ruston Koonce¹, Nafisa K. Dajani², Curtis L. Lowery², Drucilla J. Roberts³, Luc Maroteaux⁴, and Fusun Kilic¹*

¹Departments of Biochemistry and Molecular Biology, and ²Obstetrics and Gynecology, College of Medicine, University of Arkansas for Medical Sciences, Little Rock, Arkansas, USA; ³MGH, Department of Pathology, Boston, Massachusetts, USA; ⁴ UMR-S839 INSERM, Universite ´ Pierre et Marie Curie, Institut du Fer a` Moulin, Paris, France

Running Title: In GDM insulin action is tissue-type dependent

*Corresponding author: kilicfusun@uams.edu

ABSTRACT

Earlier findings have identified the requirement of the insulin signaling on maturation and the translocation of serotonin (5-HT) transporter, SERT to the plasma membrane of the trophoblast in placenta. Due to the defect on insulin receptor (IR) in the trophoblast of the gestational diabetes mellitus (GDM)-associated placenta, SERT is found entrapped in the cytoplasm of the GDM-trophoblast. SERT is encoded by the same gene expressed in trophoblast and platelets. Additionally, alteration in plasma 5-HT levels and the 5-HT uptake rates are associated with the aggregation rates of platelets. Therefore, here, we investigated a novel hypothesis that GDM-associated defect in platelet IR should change their 5-HT uptake rates and this should be a leading factor for thrombosis in GDM maternal blood. The maternal blood and the placentas were obtained at the time of cesarean section from the GDM and non-diabetic subjects (n = 6 for each group) and the platelets and trophoblasts were isolated to determine the IR activity, surface level of SERT and their 5-HT uptake rates.

Interestingly, no significant differences were evident in IR tyrosine phosphorylation or the downstream elements, AKT and S6K in platelets and their aggregation rates in both groups. Furthermore, insulin stimulation upregulated 5-HT uptake rates of GDM-platelets as it does in control group. However, the phosphorylation of IR and the downstream elements were significantly lower in GDM-trophoblast and showed no response to the insulin stimulation while they showed 4-fold increase to insulin stimulation in control group. Similarly, the 5-HT uptake rates of GDM-trophoblast and the SERT expression on their surface were several fold lower compared with control subjects. IR is expressed in all tissues, but it is not known if diabetes effects IR in all tissues equally. Here, for the first time, our findings with clinical samples show that in GDM-associated defect on IR is tissue-type dependent. While IR is impaired in GDM-placenta, it is unaffected in GDMplatelet.

INTRODUCTION

Gestational diabetes mellitus (GDM) is the most common metabolic complication of pregnancy, affecting up to 10-15% of all pregnancies (1-7), depending on the diagnostic criteria. GDM carries risks to both mother and fetus including the development of obesity, diabetes and cardiovascular disease in later life. GDM is defined as carbohydrate intolerance due to the impaired insulin signaling with onset or first recognition during pregnancy (1-5). In GDM, the pancreatic β cells fail to produce enough insulin to overcome insulin resistance which leads to carbohydrate intolerance [5, 6], and a great potential of developing type II diabetes mellitus (1).

Recently, it has been shown that the placenta develops insulin resistance in GDM (8-10), for reasons that are not well understood. The placenta not only transports nutrients to the growing fetus, but importantly, serotonin, (5-HT), 5-hydroxytryptamine, is transported across the placenta and is as an essential molecule for embryogenesis (11, 12). Studies on the age-dependent expression of SERT mRNA demonstrated that during early neurulation, there is direct access of maternal 5-HT to the embryo via placental SERT (13-15). Clinical studies reported the increased rate of congenital malformation, prematurity and defect in fetal forebrain development induced by using selective serotonin uptake inhibitor (SSRI) during pregnancy (16-19). Furthermore, blocking placental SERT with SSRI alters the plasma vs platelet 5-HT ratio.

The action of 5-HT is mediated by various 5-HT receptors but terminated by a single transporter, serotonin transport (SERT) which is expressed on the cell surface of the placenta

and on platelets (20). Proper post-translational modifications are essential regulatory factors in neurotransmitter uptake functions of SERT (21-31) and occur in a host-dependent fashion (26-28). SERT is an oligomeric (22) N-glycan (25-27), following the post-translational modifications SERT adopt more stable, lower energy conformations (32-35) to initiate for correct folding and assembly. The alterations in its structure impact the 5-HT uptake function of SERT, and hence its biological role in neurons and peripheral tissues (21, 36, 37). Consequently, identifying the mechanisms regulating post-translational modifications are critical for understanding SERT conformations and oligomerization in biological processes and diseases.

As reported earlier, we demonstrated that insulin receptor (IR) in the trophoblast of GDMplacentas is impaired. Consequently, SERT which requires insulin signaling to dissociate from the endoplasmic reticulum (ER) proteins is entrapped at ER. Therefore, the 5-HT uptake rates of trophoblast cells in GDM are decreased (38). In different tissues, SERT is encoded by a single copy gene for all tissues (39-42). The SERT mRNA is alternatively spliced, and the splice variants are equally expressed in human placental cells and platelets (43) where it regulates the levels of 5-HT in plasma as well as in platelets. Alterations in the plasma vs platelet 5-HT ratio are associated with thrombosis and vascular resistant at the placental chorionic plate and stem villous vessels (44-49).

The present study was aimed to investigate whether SERT in platelets is differentially regulated in platelet by determining 5-HT system and the platelet function in GDM. First the platelet aggregation rates in GDM and non-diabetic maternal blood were analyzed. Interestingly, GDM-platelets responded to their insulin treatment and the 5-HT uptake rates were upregulated suggesting the differential regulation of SERT trafficking in trophoblast vs platelets. Additional studies on the GDM-associated defect in phosphorylation of IR and the downstream elements was only found in placental trophoblast, but not in the maternal platelets. Here, for the first time we show that in GDM-trophoblast and in GDM-platelets the IR exhibits a differentially response to the impaired insulin level; perhaps due to the cell-type specific binding properties of IR.

MATERIALS AND METHODS

Subjects

Placentas, cord and maternal blood samples from 18 years old or older pregnant women with normal (n=5) or diagnosed with GDM (n=5) were recruited for this study. Our study was carried out after an approval from University of Arkansas for Medical Sciences (UAMS) IRB, which included these procedures, and for which subjects had previously provided written informed consent form. The health conditions of subjects were followed by their physicians (38). Inclusion and Exclusion criteria were evaluated by review of medical history, interviewing the subject, and/or results of routine tests performed for the purpose of clinical care.

Isolation and Purification of Trophoblast

Trophoblast cell isolation and preparation were performed as described previously (38, 66-70). Briefly, each cotyledon of a placenta has been dissected, collected, and rinsed with sterile 0.9% NaCl solution. Placenta tissues were digested with DNase, Dispase and Trypsin containing CMF Hank's. Trophoblast were then purified under 5-70 % gradient solution. Human HLA class I ABC Ab containing Dynabeads were incubated with trophoblast to negatively purify contaminations. Trophoblast cell purify is determined via intracellular cytokeratin-7 (CK-7) Ab and trophoblast membrane protein NDOG1 Ab.

Blood handling and platelet isolation

Maternal blood sample has been obtained from University of Arkansas for Medical Sciences (UAMS) OBGYN department. Blood were maintained in 3.8 % sodium citrate solution tube in order to avoid platelet aggregation and activation. Platelet rich plasma was prepared by adding 1/2 volume of Tyrode's HEPES buffer to maternal blood and centrifuged at 1.0 X10³ rpm for 10mins. In each assay, a dilution of 100,000 /µl of platelet in blood were applied.

Stirred platelet aggregation

For aggregation assays, platelets in plasma were prepared and platelet counts were normalized $(300,000/\mu L)$ using a Hemavet 950 (Drew Scientific, Waterbury, CT). The response to collagen $(3 \ \mu g/ml)$ as a platelet agonist was monitored by light transmittance (Chrono-log Corp., Havertown, PA) (55).

Flow cytometry

The level of platelet activation was assessed using FITC labeled P-selectin Ab (BD Pharmingen, Cat 553744). Platelets ($300,000/\mu$ L) were incubated in Ab and at the end of the incubation, 300 L of 2% formaldehyde in PBS was added to stop the reaction.

The level of SERT proteins on the PM of platelets (300,000 platelets/assay) was determined using a special Ab which is designed by our group and generated by Proteintech Group, Inc. (Chicago, IL) against a synthetic peptide corresponding to the second extracellular loop of SERT (38).

The samples were gated for single platelets based on forward and side scatter profiles and 20,000 events were recorded and read at the UAMS Flow Cytometry Core Facility.

Immuno precipitation (IP) and Western blotting (WB) analysis

Throphoblast (1.5 X 10⁶ cells per IP assay) were lysed in IP buffer (55 mm triethylamine (pH 7.5), 111 mm NaCl, 2.2 mm EDTA, 0.44% SDS, 1% Triton X-100) supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF), and protease inhibitor mixture (PIM) as previously described (38). Initially, cell lysate was incubated with protein A sepharose beads to eliminate none-specific interaction (preclear). Anti-SERT monoclonal (Mab technology,Stone Mountain, GA) Ab, was conjugated to protein A bead for 2 hours prior to incubating together with precleared cell lysate overnight at 4 degree.

WB analysis was done the next day using anti-IR Ab (Santa Cruze Biotech, Santa Cruz, CA) phospho-AKT (T308), and phosphor-S6K (T229)-Abs (Cell Signaling, Danvers, MA) or monoclonal Phospho-tyrosine for primary Ab (eBioscience, S.Diego, CA). Horseradish peroxidase (HRP) conjugated anti-rabbit or anti-mouse was used as the secondary Ab. VersaDoc 1000 gel visualization and analysis system was applied to analysis of densitometry of individual bands.

5-HT uptake assay

Trophoblast $(2.3 \times 10^5 \text{ cells per transport assay})$ and platelet (300,000/uL of platelet in blood)were washed with PBS solution containing 0.1 mM CaCl₂ and 1mM MgCl₂. The intact cells were quickly incubated with 14.6 nM ³H-5-HT at room temperature (RT) for 10 min. Whatman GF/B filters collected the cells after incubation, and excess solution was filtrated through a funnel. The uptake assay was stopped by washing twice with ice-cold PBS solution. The sample containing filters were placed into scintillation vials for counting. 2β -carbomethoxy-3trophane (β -CIT) (Chemical Synthesis Service, NIMH) was used as negative control background (22).

Data analysis

Densitometry analysis was performed with Origin software under nonlinear regression equation. Each experiment was triplicated produced in order for standard deviation (SD) calculation and

means. Data are presented as mean \pm S.D. unless otherwise noted. Statistical significance was considered at P < 0.05.

RESULTS

5-HT uptake rates of trophoblasts and platelets

Trophoblast were isolated and purified from human term non-diabetic and GDM associated placentas as previously described (38). The 5-HT uptake rates of trophoblast were measured in the same number of cells (2.3×10^5) per group (GDM or non-diabetic) and repeated in quintuplicate of 6 different preparations of trophoblast (Fig. 1). Under GDM conditions, the 5-HT uptake rates of GDM trophoblast was determined at 33% lower than the trophoblast of non-diabetic placenta (P<0.01).

Next, we evaluated if platelet 5-HT uptake rates were lower in maternal blood samples of GDM groups than in non-diabetic groups. Platelets were isolated from the maternal and cord blood samples and their 5-HT uptake rates were measured (Fig. 1). No significant difference was found between the uptake rates of platelets of cord blood or of maternal blood of non-diabetic subjects compared to platelets of the blood samples from GDM.

Platelet SERT regulates the plasma vs platelet 5-HT ratio, which plays an important role in 5-HT driven blood pathophysiology and platelet biology. Therefore, platelet aggregation rates were evaluated in maternal and cord blood also between non-diabetic and GDM pregnant blood samples. Platelets ($300,000/\mu$ L) were stimulated with collagen (3μ g/ml) to monitor their behavior in a stirred platelet aggregometer (Fig. 2A). Isolated platelets from GDM-maternal or - cord showed a similar aggregation response to collagen than the platelets from maternal and cord blood of non-diabetic pregnancies (Fig. 2A). Aggregation rates were on average 79% in platelets from the maternal blood samples ($78.6\pm7.0\%$ in normal vs. $77.5\pm5.0\%$ in GDM; n=6 each). Aggregation rates appeared as an average of 85% in platelets from the cord blood samples ($85\pm6\%$ in normal vs. $90\pm4\%$ in GDM; n=6 each).

Platelets ($300,000/\mu$ L) from normal and GDM maternal and cord blood samples were then examined for a marker of platelet activation, P-selectin. Figure 2B shows no significant difference in the expression of surface P-selectin between platelet plasma membranes of normal and GDM maternal (5 ± 2 in normal vs. 6 ± 2 in GDM; n=6 each) and cord blood samples (6.6 ± 1.0 in normal vs. 7.6 ± 2.0 in GDM; n=6 each). While the 5-HT uptake rates of GDM trophoblast were reduced, the GDM platelet uptake rates appeared unaffected by the diabetic condition.

Defective insulin signals in GDM trophoblasts but not in GDM platelets

Given that membrane trafficking of SERT is linked to insulin action in the placenta [38], we investigated the insulin signaling pathways in platelets from non-diabetic and GDM subjects. Insulin binding to its receptor leads to activation of the insulin signaling pathway, by insulin receptor (IR) auto-phosphorylation on cytoplasmic tyrosine residues. To investigate this pathway formally, we determined the basal phosphorylation levels of IR in trophoblast by immunoprecipitation (IP) assays (1.5 X 10⁶ cells per assay). While the expression levels of IR in trophoblast were not altered between non-diabetic and GDM samples (Fig. 3A, left middle panel), the level of basal (non-stimulated) phosphorylated IR was 65.9% lower in GDM- than non-diabetic-trophoblast cells (Fig. 3A, Top, and Fig. 3B). The cell lysate of each sample was blotted for total actin as the loading control (Fig. 3A, bottom panel). On the other hand, neither the expression levels nor the phosphorylation levels of IR in platelets from GDM blood samples

appeared different compared to platelets derived from non-diabetic maternal blood samples (Fig. 3A, right and Fig. 3B). These findings strongly suggest that under basal (non-insulinstimulated) conditions, the IR in GDM trophoblast has a lower auto-phosphorylation state despite a normal content of IR, while platelets in GDM are unaffected and platelets respond differentially to insulin.

IR expression was evaluated between non-diabetic (n=7) and GDM (n=6) trophoblast to illustrate the deviations among the patients (Supplementary data1). In addition to IR autophosphorylation, insulin molecule activates IR substrates (IRS), which initiate the phosphorylation of downstream elements such as AKT, mTOR and S6K (50). Therefore, in verifying our findings on IR in trophoblast and platelets, we investigated the phosphorylation of IR downstream effectors in platelets and trophoblast of non-diabetic and GDM placenta and blood samples.

WB (n =3-4) analysis of the trophoblast $(1.5 \times 10^6 \text{ cells per WB assay})$ from GMD and nondiabetic placenta for AKT and S6K were performed. In GDM-trophoblast, the levels of phosphorylation on AKT (Fig. 4A, B) and S6K (Fig. 4A, C) were lower 28.5% and 71.4%, respectively, than their levels in the trophoblast cells of non-diabetic placentas, consistent with the reported studies (7-9). These data complement our findings of the phosphorylation of IR in GDM-trophoblast (Fig. 3). In contrast to the findings with trophoblast, platelets showed no significant change in the levels of phosphorylation for AKT and S6K between the Normal and GDM blood samples (Fig. 4A-C).

In summary, the 5-HT uptake rates as well as the insulin signaling and downstream elements are specifically down-regulated in the trophoblast of GDM-placentas but not in GDM-associated platelets.

Differential response of IR to insulin between trophoblast and platelet

The impact of insulin on the level of IR phosphorylation was investigated in trophoblast isolated from non-diabetic and GDM placentas and then starved for insulin first and then treated with various concentration (0, 10 or 100 nM) of insulin for 24 hr.

The IR expression and the level of phosphorylation were investigated in these trophoblast with IP assay (Fig. 5). In non-diabetic; but not in GDM-trophoblast, phosphorylation of IR was upregulated by 15% or 23% with 10 or 100 nM insulin treatment, respectively; while IR expression levels were not affected by these treatments (Fig. 5). These findings demonstrate that IR phosphorylation levels show an insulin-concentration dependent pattern. However, the GDM-trophoblast under the same treatment, the level of IR phosphorylation did not show a similar response to the insulin pretreatment. Therefore, the impact of insulin on the level of SERT on plasma membrane of trophoblast and their 5-HT uptake rates were further studied in both non-diabetic trophoblast and platelets.

Next, we tested the 5-HT uptake rates of trophoblast in response to the insulin signaling. Specifically here, we tested if the lower 5-HT uptakes rates of GDM-trophoblast is due to the lack of insulin signaling or defective IR on trophoblast. Equal number of trophoblast $(1.5 \times 10^6 \text{ cells per assay})$ isolated and purified from GDM- or non-diabetic-placentas were treated with various concentration of insulin and the 5-HT uptake rates were determined (n=4). The up-regulatory effect of insulin on the levels of SERT molecules at the plasma membrane of trophoblast was only found in non-diabetic trophoblast (Fig. 6) indicating the defect on IR is independent of the insulin level.

In comparing the 5-HT systems in both tissue, placental trophoblast and platelets by the functional efficiency of IR, next, the impact of insulin treatment on 5-HT uptake rates of platelet isolated from non-diabetic and GDM blood samples was investigated (Fig.7A). The 5-HT uptake rates of both platelets, non-diabetic and GDM, showed a transient increase (45%) peaking at 10

nM and 20 nM of insulin treatment, compared to the uptake rates of the non-treated platelets (Fig. 7A).

In order to validate this transient increase in 5-HT uptake, the levels of SERT at the platelet surface was determined after insulin treatment at different concentrations (Fig. 7B). The peak level of SERT expression at the surface of platelets was also obtained after 10 nM insulin exposure (increased by 37%), compared to the level of SERT on the surface of the non-treated platelets (Fig 7B). Therefore, insulin, specifically at 10 nM and 20 nM concentrations, induces an increase of the 5-HT uptake rates as well as of the surface level of SERT molecules on platelets isolated from non-diabetic and GDM blood samples, respectively.

DISSUSSION

The cell signaling is a cell type-dependent physiological phenomena which occurs by the activation of the receptor but is orchestrated by various, extracellular and intracellular, factors. Errors in the processing of the cellular information cause diseases such as GDM where the processing of the insulin signaling is not transduced due to the defective IR. Although IR is expressed in all tissues, it is still not known if diabetes effects IR equally in all tissues. Here, our findings show that in GDM-trophoblast the IR is defective while in GDM-platelets IR is functionally active, yet patients are identified as diabetic.

Recently, we reported that insulin facilitates the dissociation of SERT from its chaperone ERp44 and its translocation to the plasma membrane (38). However, under GDM-associated defects in insulin signaling, SERT is entrapped at the ER and therefore decreases the 5-HT uptake rates in human placental trophoblast cells. While placental SERT function is affected, the platelet SERT functions normal by the lack of insulin in GDM. Various studies demonstrated the expression of ER proteins in blood plasma (51). Therefore, in platelet the trafficking of SERT to the plasma membrane should not be through ERp44-dependent manner as demonstrated in placental trophoblast.

Platelets are derived from the fragmented cytoplasm of megakaryocytes and enter the circulation in an inactive form. The activation of platelets enlists more platelets at a fibrinstabilized hemostatic area to form a thrombus after associating with the endothelium or each other. As is the case for many membrane proteins, SERT trafficking in platelet is mediated by vesicular packing and interactions with specialized proteins. Upon clearance of 5-HT from plasma to platelet, SERT is translocated from the plasma membrane to be routed elsewhere. The posttranslational modification of SERT regulates transporter function (22, 24, 27-29, 31, 45-49), but given that glycosylation occurs in megakaryocytes, (i.e., the progenitors of platelets); this aspect of SERT regulation may not be altered in platelets. In platelets, the biosynthesis as well as the posttranslational modifications of proteins is minimal.

The clinical and biochemical findings infer a complex process to the role of plasma 5-HT in platelet adhesion, aggregation, and thrombus formation. An elevation in free 5-HT levels in plasma accelerates the exocytosis of dense and -granules (52, 53); in turn, these secrete more 5-HT along with the -granules-located procoagulant molecules that will mediate hemostasis. Supporting these hypotheses is the fact that platelets of 5-HT infused mice, in the absence of cardiovascular problem, show an enhanced aggregation profile; however, when the 5-HT-infused mice were injected with a selective 5-HT reuptake inhibitor (SSRI) (54, 55) or a 5-HT_{2A} antagonist (54, 56) the effect of elevated free 5-HT levels in plasma was reversed and the platelet aggregation profile normalized (52-57). The importance of the plasma 5-HT level and platelet SERT in the platelet aggregation phenomenon is supported by findings in platelets of mice lacking the gene for TPH1 (54, 57) or the gene for SERT (54): where granular secretion rates as well as the risk of thrombosis are significantly reduced (54, 55, 57).

Since the trafficking of SERT to the plasma membrane of trophoblast is regulated by its association with ERp44 in insulin dependent manner (38) and this pathway is not affective in platelet (51), the 5-HT uptake rates as well as the level of SERT on the cell surface in GDM-platelet are at the same level with those in non-diabetic-platelet. More important than this, the IR and the downstream elements such as Akt and S6k are functionally active in GDM-platelet whereas inactive in GDM-trophoblast. We hypothesize that the IR response to insulin signaling may be in a cell-type dependent characteristic. In a separate study (58), we showed the occurrence of the incidence of intervillous thrombi (IVT) is significantly higher in diabetes-associated placentas, particularly in GDM within the absence of the thrombosis in maternal blood. IR in placenta is defected by the GDM but not IR in platelets which result a local placental pathology (IVT) but not systemic pathology (thrombosis) in GDM.

IR response to insulin signaling in trophoblast is important for the role of trophoblast in placenta. Because they function as endothelium also they are metabolically active cells of placenta. So they have transport ability as well as the hormone section. Therefore, defect in insulin signaling have several impact on the integrity of placenta to the growing embryos.

In the current study first, we compared the phosphorylation levels of IR and its downstream elements, AKT and S6K in between trophoblast and platelets prepared from non-diabetic and GDM-placentas and -blood samples, respectively. Initial findings showed that IR and the downstream elements were down-regulated in GDM-trophoblast but not in GDM-platelets. Then, we verified these conclusions by stimulating the normal-trophoblast with insulin if the phosphorylation levels of IR would be altered and if this would be in a concentration-dependent manner. While insulin treatment elevates the level of phospho-IR in trophoblasts it did not change that in platelets. Therefore, all insulin and platelet vs trophoblast related findings fit together well with the surface expression and the 5-HT uptake rates of these tissues. Due to the defect in GDM-trophoblast, but not in GDM-platelets, IR cannot respond to the insulin signaling. The impaired insulin signaling arrests SERT in ER of GDM-trophoblast; however, in GDM-platelets SERT molecules are translocated to the plasma membrane in a good order. Based on our published and current studies we hypothesize that the defective IR on GDM-trophoblast could be a part of the IVT formation in placenta but the functional IR on GDM-platelet prevents the formation of systemic thrombosis in the maternal blood.

ACKNOWLEDGEMENTS

We gratefully acknowledge the UAMS Flow Cytometry Core, and thank Ms. Amber Ward for assistance in obtaining consents from subjects and providing us the samples.

COMPETING FINANCIAL INTERESTS: The authors declare no competing financial interests.

CONTRIBUTIONS

A.C., repeated all the experiments presented in Figure 3 and 4. F.K., L.M. and D.J.R designed and directed the project; Y.L., I.N.O, A.A. and R.K. conducted experiments. P.M., N.K.D. and C.L.L. are the physicians of the project diagnose the subjects, provided the subject parameters and human samples; and F.K. analyzed the data. F.K, L.M. and D.J.R. participated in manuscript writing and scientific discussions, giving detailed feedback in all areas of the project.

REFERENCE

- Catalano PM, Kirwan JP, Haugel-de Mouzon S, King J. (2003).Gestational diabetes and insulin resistance: role in short- and long-term implications for mother and fetus. *J Nutr.* 133, Suppl 2):1674S-1683S.
- 2. Buchanan TA, Xiang A, Kjos SL, Watanabe R. (2007). What is gestational diabetes? *Diabetes Care*. **30**, Suppl 2:S105-11.
- Metzger BE, Buchanan TA, Coustan DR, de Leiva A, Dunger DB, Hadden DR, Hod M, Kitzmiller JL, Kjos SL, Oats JN, Pettitt DJ, Sacks DA, Zoupas C. (2007). Summary and recommendations of the Fifth International Workshop-Conference on Gestational Diabetes Mellitus. *Diabetes Care* **30**, (Suppl. 2):S251–S260.
- 4. Catalano PM, Tyzbir ED, Wolfe RR, Calles J, Roman NM, Amini SB, Sims EA. (1993). Carbohydrate metabolism during pregnancy in control subjects and women with gestational diabetes. *Am J Physiol.* **264**, E60–E67.
- Barbour LA, McCurdy CE, Hernandez TL, Kirwan JP, Catalano PM, Friedman JE. (2007). Cellular mechanisms for insulin resistance in normal pregnancy and gestational diabetes. *Diabetes Care*. **30**, Suppl 2:S112-9.
- Setji, T. Brown, A. Feinglos, M. (2005). Gestational Diabetes Mellitus. *Clinical Diabetes*. 23, 17-24.
- 7. Lager, S. Samulesson, AM. Taylor, PD Poston, L. Powell, TL. Jansson, T. (2014).Dietinduced obesity in mice reduces placental efficiency and inhibits placental mTOR signaling. *Physiol Rep.* **2**, e00242.
- Jansson, N., Rosario, FJ., Gaccioli, F., Lager, S., Jones, HN., Roos, S., Jansson, T., Powell, TL. Activation of placental mTOR signaling and amino acid transporters in obese women giving birth to large babies. *J Clin Endocrinol Metab.* **98**,105-13 (2013).
- 9. Roos, S. Powell, TL. Jansson, T. (2009).Placental mTOR links maternal nutrient availability to fetal growth. *Biochem Soc Trans.* **37**, 295-8.
- Betscher, NA. Wein, P. Sheedy, M. T. Steffer, B. (1996).Identification and treatment of women with hyperglycemia diagnosed during pregnancy can significantly reduce perinatal mortality rates. *Aust. N. Z. J. Obstet. Gynaecol.* 36, 239–247.
- 11. Lauder J. M., Tamir H. and Sadler T. W. (1998).Serotonin and morphogenesis. I. Sites of serotonin uptake and binding protein immunoreactivity in the midgestation mouse embryo. Development 102, 709 720.
- Côté F, Fligny C, Bayard E, Launay JM, Gershon MD, Mallet J, Vodjdani G. (2007)..Maternal serotonin is crucial for murine embryonic development. *Proc Natl Acad Sci U S A*. **104**, 329-34.
- 13. Lauder, J.M., and Krebs, H. (1976). Effects of p-chlorophenylalanine on time of neuronal origin during embryo-genesis in the rat. *BrainRes*. **107**, 638–644.
- Bonnin A, Goeden N, Chen K, Wilson ML, King J, Shih JC, Blakely RD, Deneris ES, Levitt P. (2011). A transient placental source of serotonin for the fetal forebrain. *Nature* 472, 347–350.
- 15. Bonnin, A., and Levitt, P. (2012).Placental source for 5-HT that tunes fetal brain development. *Neuropsychopharmacology* **37**, 299–300.
- 16. FDA Drug Safety Communication (2011) Selective serotonin reuptake inhibitor (SSRI) antidepressant use during pregnancy and reports of a rare heart and lung condition in newborn babies. Available at http://www.fda.gov/Drugs/DrugSafety/ucm283375.htm.
- 17. Cooper, W.O., Willy, M.E., Pont, S. J., Ray, W.A. (2007). Increasing use of antidepressants in pregnancy. *Am.J.Obstet.Gynecol.* **196**, 544.e1–544.e5.

- 18. Greene, M.F. (2007). Teratogenicity of SSRIs—Serious Concern or Much Ado about Little? *N. Engl.J.Med.* **356**, 2732–2733.
- 19. Velasquez JC, Goeden N, Bonnin A. (2013).Placental serotonin: implications for the developmental effects of SSRIs and maternal depression. *Front Cell Neurosci.* **23**, 47.
- 20. Balkovetz DF, Tiruppathi C, Leibach FH, Mahesh VB, Ganapathy V. (1989).Evidence for an imipramine-sensitive serotonin transporter in human placental brush-border membranes. *J Biol Chem.* **264**, 2195-2198.
- 21. Amara S.G. and Arriza J.L. (1993). Neurotransmitter Transporters: three distinct gene families. *Curr. Opin. Neurobiol.* **3**, 3317-3344.
- 22. Kilic, F., and Rudnick, G. (2000).Oligomerization of serotonin transporter and its functional consequences. *Proc. Natl Acad. Sci. USA* **97**, 3106-3111.
- Torres GE, Carneiro A, Seamans K, Fiorentini C, Sweeney A, Yao WD, Caron MG. (2003). Oligomerization and Trafficking of the Human Dopamine Transporter. Mutational Analysis Identifies Critical Domains Important For the Functional Expression of the Transporter. *J Biol Chem* **278**, 2731-2739.
- 24. Kocabas, A., Rudnick, G., and Kilic, F. (2003).Functional consequences of Homo- But Not Hetero-Oligomerization between transporters for the biogenic amine neurotransmitters. *J. Neurochem.* **85**: 1513-1520.
- 25. Nguyen TT, Amara SG. (1996). N-linked oligosaccharides are required for cell surface expression of the norepinephrine transporter but do not influence substrate or inhibitor recognition. *J. Neurochem.* **67**: 645-655.
- 26. Tate C.G. and Blakely R.D. (1994). The effect of N-inked glycosylation on activity of the Na⁺- and Cl⁻-dependent serotonin transporter expressed using recombinant baculovirus in insect cells. *J. Biol. Chem.* **269**, 26303-26310.
- 27. Ozaslan, D., Wang, S., Ahmed, B., Bene, A., Kocabas, A.M., and Kilic, F. (2003).Glycosyl modification facilitates homo- and hetero-oligomerization of serotonin transporter: a specific role for sialic acid residues. *J. Biol. Chem.* 278, 43991-44000.
- 28. Chamba A, Holder MJ, Barnes NM, Gordon J. (2008). Characterisation of the endogenous human peripheral serotonin transporter SLC6A4 reveals surface expression without N-glycosylation. *J Neuroimmunol*. **204**, 75-84.
- 29. Chen, J.G., Lui-Ches, S., Rudnick, G. (1997). External cysteine residues in the serotonin transporter. *Biochemistry*. **36**, 1479-1486.
- 30. Chen R, Wei H, Hill ER, Chen L, Jiang L, Han DD, Gu HH. (2007).Direct evidence that two cysteines in the dopamine transporter form a disulfide bond. *Mol Cell Biochem*. **298**, 41-48.
- S. Freyaldenhoven, T. Li, A. Kocabas, E. Ziu, S. Ucer, Ramanagoudr-Bhojappa, G. Miller, F. Kilic, (2012). The Role of ERp44 in Maturation of Serotonin Transporter Protein. *The Journal of Biological Chemistry.* 18, 17801-11.
- 32. Dobson CM. (2004).Principles of protein folding, misfolding and aggregation. *Semin Cell Dev Biol* **15**, 3–16.
- 33. Anelli, A and Sitia, R. (2008).Protein Quality Control in the Early Secretory Pathway *EMBO* **27**, 315-327.
- 34. Kleizen, B. and Braakman, I. (2004).Protein Folding and Quality Control in the Endoplasimic Reticulum. Current Opinion in Cell Biology **16**, 343-349.
- 35. Sitia, R. and Braakman, I. (2003). Quality control in the endoplasmic reticulum protein factory. *Nature*. **426**, 18-25.
- Rudnick, G., and Clark, J. (1993). From synapse to vesicle: The re-uptake and storage of biogenic amine neurotransmitters. *Biochim. Biophys. Acta Rev. Bioenerg.* 1144, 249– 263.

- Blakely, R. D., Ramamoorthy, S., Qian, Y., Schroeter, S., and Bradley, C. 1997 in Neurotransmitter Transporters: Structure, Function, and Regulation (Reith, M. E. A., ed), pp. 29-72, *Humana Press* Inc., Totowa, NJ.
- 38. Li, Y., Hadden, C., Singh, P., Mercado, CP., Murphy, P., Dajani, NK., Lowery, CL., Roberts, DJ., Maroteaux, L., Kilic, F. (2014).GDM-associated insulin deficiency hinders the dissociation of SERT from ERp44 and down-regulates placental 5-HT uptake. *Proc. Natl Acad. Sci. USA*, **111**, 52.
- 39. DF. Balkovetz, C. Tiruppathi, FH. Leibach, VB. Mahesh, V. Ganapathy. (1989). Evidence for an imipramine-sensitive serotonin transporter in human placental brush-border membranes. *J Biol Chem.* **264**, 2195-2198.
- 40. Blakely, R.D., Berson, H.E., Fremeau, R.T., Jr.Caron, M.C., Peek, M.M. Priace, H.K., Bradley, C.C. (1991). Cloning and expression of a functional the 5HT transporter from rat brain. *Nature* **354**, 66-70.
- 41. S. Ramamoorthy, AL. Baumen, KR. Moore, H. Han, T. Yang-Fen, AS. Chang, V. Ganapathy, RD. Blakely. (1993). Antidepressant- and cocaine-sensitive human the 5HT transporter: molecular cloning, expression, and chromosomal localization. *Proc. Natl. Acad. Sci. U.S.A.* **90**, 2542-2546.
- 42. Lesch, K. P., Wolozin, B. L., Murphy, D. L., and Riderer, P. (1993). Primary structure of the human platelet 5HT uptake site: identity with the brain 5HT transporter. *J. Neurochem.* **60**, 2319-2322.
- 43. Bradley, CC. Blakely, RD. (1997). Alternative Splicing of the Human Serotonin Transporter Gene. *J. Neurochem.* **69**, 1356-1367.
- 44. Brenner, B. JT. Harney, BA. Ahmed, BC. Jeffus, R. Unal, JL. Mehta, Kilic. F. (2007). Plasma serotonin level and the platelet serotonin transporter. *J. Neurochem.* **102**, 206-216.
- 45. Kilic, F. Ziu E. Freyaldenhoven S.. Plasma Serotonin and Platelet Serotonin Transporter: Molecular and Cellular Aspects in Cardiovascular Research. In Brataas, J. and Nanstveit, V. (eds.): Handbook of Cardiovascular Research. 2009. Nova Science Publishers, Inc. Hauppauge, NY. ISBN: 978-1-60741-792-7.
- 46. Mercado, C. Kilic, F. (2010). Molecular mechanisms of SERT in platelets: regulation of plasma serotonin levels. *Molecular Interventions* **10**, 231-241.
- 47. C. Mercado, E. Ziu, F. Kilic. (2011). Communication between 5-HT and small GTPases. *Current Opinion in Pharmacology.* **11**, 23-28.
- 48. Fraer, M. Kilic. F. (2015).Serotonin: A different player in hypertension-induced thrombosis. *AHA Hypertension* **65**, 942-948.
- 49. Hadden, C. D.J. Roberts, F. Kilic. Involvement of serotonin transporter in the retroplacental thrombosis and necrosis. Under Review
- 50. Barbour LA. McCurdy CE, Hernandez TL, Friedman JE. (2011). Chronically increased S6K1 is associated with impaired IRS1 signaling in skeletal muscle of GDM women with impaired glucose tolerance postpartum. *J Clin Endocrinol Metab.* **96**, 1431-41.
- 51. Holbrook LM, Watkins NA, Simmonds AD, Jones CI, Ouwehand WH, Gibbins JM. (2010). Platelets release novel thiol isomerase enzymes which are recruited to the cell surface following activation. *Br J Haematol.* **148**, 627-637.
- 52. Shirakawa R, Yoshioka A, Horiuchi H, Nishioka H, Tabuchi A, Kita T. Small GTPase Rab4 regulates Ca²⁺-induced a-granule secretion in platelets. J Biol Chem. 2000;275:33844–33849.
- 53. Crosby D, Poole AW. Platelet dense-granule secretion: the (3H)-5-HT secretion assay Methods Mol Biol. 2004;272:95-96.

- 54. Mercado CP, Quintero MV, Li Y, Singh P, Byrd AK, Talabnin K, Ishihara M, Azadi P, Rusch NJ, Kuberan B, Maroteaux L, Kilic F. A serotonin-induced N-glycan switch regulates platelet aggregation. Sci Rep. 2013;3:2795
- 55. Ziu, E. C.P. Mercado, Y. Li, P. Singh, B.A. Ahmed, S. Freyaldenhoven, S. Lensing, J. Ware, F. Kilic. (2012).Down-regulation of the Serotonin Transporter in hyperreactive platelets counteracts the pro-thrombotic effect of serotonin. *J Mol Cell Cardiol.* **5**, 1112–1121.
- 56. Przyklenk, K. Targeted inhibition of the serotonin 5HT2A receptor improves coronary patency in an in vivo model of recurrent thrombosis. J Thromb Haemost. 2010;8:331-340.
- 57. Walther, DJ, Peter, JU, Winter, S, Holtje, M, Paulmann, N, Grohmann, M, Vowinckel, J, Alamo-Bethencourt, V, Wilhelm, CS, Ahnert-Hilger, G, Bader, M. Serotonylation of small GTPases is a signal transduction pathway that triggers platelet alpha-granule release. Cell. 2003;115:851-862.
- 58. Basnet K, Bentley-Lewis R, Wexler DJ, Kilic F, Roberts DJ. The prevalence of intervillous thrombi is increased in placentas from pregnancies complicated by diabetes. Pediatr Dev Pathol. 2015 Dec 15. [Epub ahead of print]
- 59. Kliman, H.J. J.E. Nestler, E. Sermasi, J.M. Sanger, J.F. Strauss 3rd. (1986). Purification, characterization, and in vitro differentiation of cytotrophoblasts from human term placentae. *Endocrinology* **118**(4):1567–1582.
- 60. Manoussaka, M.S. D.J. Jackson, R.J. Lock, S.R. Sooranna, B.M. Kumpel. (2005). Flow cytometric characterization of cells of differing densities isolated from human term placentae and enrichment of villous trophoblast cells. *Placenta* **26**(4):308–318.
- 61. Clover, L.M. E. Coghill, C.W. Redman, I.L. Sargent. A three-colour flow cytometry technique for measuring trophoblast intracellular antigens: The relative expression of TAP1 in human cytotrophoblast and decidual cells. *Placenta* **21**(8):743–753 (2000).
- Maldonado-Estrada, J. E. Menu, P. Roques, F. Barré-Sinoussi, G. Chaouat. Evaluation of (2004).Cytokeratin 7 as an accurate intracellular marker with which to assess the purity of human placental villous trophoblast cells by flow cytometry. *J Immunol Methods* 286(1-2):21–34.
- 63. Bulmer, J.N. W.D. Billington, P.M. Johnson. (1984). Immunohistologic identification of trophoblast populations in early human pregnancy with the use of monoclonal antibodies. *Am J Obstet Gynecol* **148**(1):19–26.

SOURCE OF FUNDING:

LM's work has been supported by funds from the Centre National de la Recherche Scientifique, the Institut National de la Santé et de la Recherche Médicale, the Université Pierre et Marie Curie, and by grants from the Fondation pour la Recherche Médicale, the French ministry of research (Agence Nationale pour la Recherche).

This work was supported by the NIH Heart Lung and Blood Institute HL091196, Eunice Kennedy Shriver NICHHD 058697 and 053477; American Heart Association [13GRNT17240014] and the Minnie Merrill Sturgis Diabetes Research Fund, and the Sturgis Charitable Trust to FK.

HOW TO CITE THIS ARTICLE: Li, Y., Cooper, A., Odibo, I.N., Ahmed, A., Murphy, P., Koonce R., Dajani, N.K., Lowery, C.L., Roberts, D.J., Maroteaux, L., and Kilic, F. "Discrepancy in insulin regulation between GDM-platelets and placenta."



Figure 1. **5-HT uptake rates.** The 5-HT uptake rates were measured in trophoblast (2.3 X 10^5 per assay) isolated from non-diabetic and GDM placentas or in platelets ($100K/\mu$ L) isolated from maternal and cord blood (all groups n=6) were measured as described previously (61). Rate of uptake is expressed as the means and STD values of quintuplicate determinations from six independent samples in each group. The (*) represents the results of a two-tailed Student's t-test with p < 0.001, (compared with non-diabetic trophoblast uptake rates).



Figure 2. The functions of platelets in Non-diabetic and GDM-associated maternal and cord blood samples. (A) Representative tracings of stirred platelet aggregation reactions are shown. In response to stimulation with 3 μ g/ml collagen, platelets from maternal or cord blood samples of GDM subjects showed no significant difference in aggregation rates than the platelets from non-diabetic (control) subjects. All assays were performed in triplicate (n=7 group). (B) Surface expression of p-Selectin was determined by flow cytometry as indices of platelet activation. Flow cytometry revealed no significant difference in expression levels of p-Selectin between platelets of non-diabetic or GDM-associated pregnant subjects nor between maternal and cord blood samples. Assays in A and B were performed in triplicate.



Figure 3. IR phosphorylation patterns in GDM-trophoblast and GDM-platelets. (A) Both non-diabetic (lanes 1, 3, 6) and GDM (lanes 2, 4, 5) trophoblast (2.2 X 10^6 cells) or human platelets ($300K/\mu L$) were IP with polyclonal anti-IR Ab, followed by WB of monoclonal phosphotyrosine (pTyr) or monoclonal IR Ab (n=3). Lysates from each group were analyzed with anti-Actin Abs as the loading control. (B) Densitometry quantification of phosphorylation of IR are shown (n=3). Data are normalized with the level of actin which represent as mean ± SD. (*) represents the results of a two-tailed Student's t-test with p < 0.001, compared with the intensity of pTyr bands.



Figure 4. AKT and S6K are de-activated downstream of IR in trophoblast but not in platelets. (A) Both non-diabetic (lanes 1, 2, 3) and GDM (lanes 4, 5, 6) trophoblast (2.2 X 10^6 cells) or human platelets (300K/µL) were analyzed for phosphorylation level of AKT and S6K (n=3). WB analysis of phospho-AKT (Top panel) and phospho-S6K (Middle panel) were shown in the panels (n=3). Actin served as a loading control. The results of WB analysis are the summaries of combined data from three densitometric scans. *represents differences between the phosphorylation levels of AKT (B) and S6K (C) in non-diabetic and GDM-trophoblast (*) represents the results of a two-tailed Student's t-test with p < 0.001, compared with the intensity of the bands at pS6K. Results are representative of 3 individual experiments.



Figure 5. Concentration dependent effect of insulin on IR phosphorylation. Trophoblast trophoblast (2.2 X 10⁶ cells) isolated and purified from non-diabetic and GDM-associated placentas; they were first starved for insulin and then treated with 0, 10, 100 nM insulin for 24 hr. After the incubation time, IP assay was performed with polyclonal IR-Ab and WB with pTyr-Ab to determine the level of phosphorylation in each group of cells. The levels of IR were not changed by insulin treatment or between non-diabetic and GDM samples. However, the levels of phospho-IR were elevated in non-diabetic-, but not in GDM-trophoblast, in parallel to the insulin concentration; the highest level of IR phosphorylation was found in non-diabetic-trophoblast pretreated with 100 nM insulin. The results of WB analysis are the summaries of combined data from four densitometric scans of pTyr.



Figure 6. The impact of insulin treatment on 5-HT uptake rates of trophoblast. Trophoblast (1.2×10^6) isolated and purified from non-diabetic and GDM-placenta were initially incubated with 0, 10, 20 or 100 nM insulin. Next day, their 5-HT uptake rates were measured as described previously (44). Asterisks represent the results of a two-tailed Student's t-test with p < 0.001. (*) indicates statistical difference in 5-HT uptake rates between non-diabetic and GDM-trophoblast; (**) shows that elevation in the 5-HT uptake rates by 10 nM insulin were significantly higher than the control samples.



Figure 7. The impact of insulin treatment on 5-HT uptake rates of platelets. (A) Platelets isolated from the non-diabetic- or GDM-associated maternal blood samples. Platelets were incubated with 0-100 nM insulin overnight, followed with 5-HT uptake assay as described previously (n=3) (44). Asterisks represent the results of a two-tailed Student's t-test with p < 0.001; (*) and (**) show the elevation in the 5-HT uptake rates by insulin treatment of non-diabetic or GDM-platelets, respectively compared to their untreated counterparts. (B) Platelets were prepared from non-diabetic and GDM-maternal blood. Following an overnight treatment with (0-100 nM) insulin, plasma membrane expression of SERT was determined by flow cytometry (55). Mean fluorescence intensity of SERT expression in platelets (300,000 platelets/per assay) isolated from non-diabetic was at similar level but both group show a significant elevation in insulin treatment. Flow cytometry revealed a decrease of 51% in the expression levels of SERT in trophoblast of GDM placentas. Asterisks represent the results of a two-tailed Student's t-test with p < 0.001; (*) and (**) show the elevation in the expression of SERT on platelets, respectively compared to their untreated counterparts.