Discrepancy in Insulin Regulation between Gestational Diabetes Mellitus (GDM) Platelets and Placenta*

Received for publication, January 1, 2016, and in revised form, February 20, 2016 Published, JBC Papers in Press, February 26, 2016, DOI 10.1074/jbc.M116.713693

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Earlier findings have identified the requirement of insulin signaling on maturation and the translocation of serotonin (5-HT) transporter, SERT to the plasma membrane of the trophoblast in placenta. Because of 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 in trophoblasts. 5-HT levels were severalfold lower compared with control group. Similarly, the response to the insulin stimulation while they showed 4-fold significantly lower in GDM-trophoblast and showed no phosphorylation of IR and the downstream elements were disrupted. Furthermore, insulin stimulation up-regulated 5-HT uptake and S6K in platelets and their aggregation rates in both groups.

5-HT uptake rates of GDM-trophoblast and the SERT expression on their surface were severalfold lower compared with control subjects. IR is expressed in all tissues, but it is not known if diabetes affects IR in all tissues equally. Here, for the first time, our findings with clinical samples show that in GDM-associated placenta, it is unaffected in GDM-platelet.

Gestational diabetes mellitus (GDM) is the most common metabolic complication of pregnancy, affecting up to 10–15% of pregnancies globally. Type II diabetes mellitus (21) presents with onset or first recognition later in life. GDM is defined as carbohydrate intolerance due to impaired insulin signaling with onset or first recognition during pregnancy (16–19). Furthermore, blocking placental SERT with SSRI alters the plasma versus 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), fol-

This article has been withdrawn by Yicong Li, Anthonya Cooper, Imelda N. Odibo, Nafisa K. Dajani, Curtis L. Lowery, Drucilla J. Roberts, Luc Maroteaux, and Fusun Kilic. Pamela Murphy, Ruston Koonce, and Asli Ahmed could not be reached. Dr. Kilic contacted the editorial office to report a concern raised in some figures of this article. An investigation by the Journal determined that a portion of the pAkt immunoblot from platelets in Fig. 4B was reused as actin in their earlier published articles. The authors state that the immunoblots were not reused; however, due to the dated material, the authors could not provide the actin immunoblots from the previously published articles.

*This work was supported by National Heart, Lung, and Blood Institute, National Institutes of Health HL091196; Eunice Kennedy Shriver NICHD 058697 and 053477; American Heart Association (13GRNT17240014); and the Minnie Merrill Sturgis Diabetes Research Fund; and the Sturgis Charitable Trust (to F. K.) This work was also 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) (to L. M.). The authors declare that they have no conflicts of interest with the contents of this article. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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WITHDRAWN
May 21, 2019
In GDM Insulin Action Is Tissue Type Dependent

subsequently, 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 GDM-placentas 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 (20,40–42). 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 versus platelet 5-HT ratio are associated with thrombosis and vascular resistant at the placental chorionic plate and stem villous vessels (44–48).3

The present study was aimed to investigate whether SERT in platelets is differentially regulated in platelet by determining the 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 up-regulated suggesting the differential regulation of SERT trafficking in trophoblast versus platelets. Additional studies on the GDM-associated defect in phosphorylation of IR and the down-regulated 5-HT system and the platelet function in GDM. First the platelets is differentially regulated in platelet by determining the 5-HT uptake rates of trophoblast cells in GDM-placentas is impaired. Consequently, a differentially response to the impaired insulin level; perhaps that in GDM-trophoblast and in GDM-platelets the IR exhibits a differentially regulation of SERT trafficking.

Materials and Methods

Isolation and Purification of Trophoblast—Trophoblast cell isolation and preparation were performed as described previously (38, 39, 49, 59–61). 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. Trophoblasts 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 membrane protein NDOG1 Ab.

Blood Handling and Platelet Isolation—Maternal blood sample has been obtained from University of Arkansas for Medical Sciences (UAMS) OB/GYN department. Blood were maintained in 3.8% sodium citrate solution tube 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 × 10^3 rpm for 10 min. In each assay, a dilution of 100,000/μl of platelet in blood was applied.

Stirred Platelet Aggregation—For aggregation assays, platelets in plasma were prepared, and platelet counts were normalized (300,000/μl) using a Hemavet 950 (Drew Scientific, Waterbury, CT). The response to collagen (3 μg/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/μl) were incubated in Ab and at the end of the incubation, 300 μl of 2% formaldehyde in PBS was added to stop the reaction.

The level of SERT proteins on the PM of platelets (300,000 platelets/assays) was determined using a special Ab, which is designed and generated by Proteintech Group, Inc. (Chicago, IL) against a synthetic peptide corresponding to the amino acid sequence (38). The samples were stained on the side and side scatter parameters were recorded and read at the UAMS Flow Cytometry Core Facility.

WB analysis was done the next day using anti-IR Ab (Santa Cruz Biotech, Santa Cruz, CA) phospho-AKT (Thr-308), and phospho-S6K (Thr-229)-Abs (Cell Signaling, Danvers, MA) or monoclonal phospho-tyrosine for primary Ab (eBioscience, San 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—Trophoblasts (2.3 × 10^5 cells per transport assay) and platelets (300,000/μl of platelet in blood) were washed with PBS solution containing 0.1 mM CaCl2 and 1 mM MgCl2. The intact cells were quickly incubated with 14.6 nM [3H]5-HT at room temperature 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-3-tropane (β-CIT) (Chemical Synthesis Service, NIMH) was used as a negative control background (22).

3 C. Hadden, D. Roberts, and F. Kilic, manuscript in review.
In GDM Insulin Action Is Tissue Type Dependent

Platelet SERT regulates the plasma versus 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/μl) were stimulated with collagen (3 μg/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 ± 7.0% in normal versus 77.5 ± 5.0% in GDM; n = 6 each). Aggregation rates appeared as an average of 85% in platelets from the cord blood samples (85 ± 6% in normal versus 90 ± 4% in GDM; n = 6 each).

Platelets (300,000/μl) isolated from normal and GDM maternal and cord blood samples were examined for a marker of platelet activation, P-selectin. No significant difference in the expression levels of P-selectin between platelet plasma membranes of normal and maternal (5 ± 2 in normal versus 5 ± 2 in GDM; each) were measured as described. While the expression of surface P-selectin between platelet plasma membranes of normal and GDM maternal (5 ± 2 in normal versus 5 ± 2 in GDM; each) were measured as described. While the expression of surface P-selectin between platelet plasma membranes of normal and maternal (5 ± 2 in normal versus 5 ± 2 in GDM; each) were measured as described.

Next, we evaluated whether platelet 5-HT uptake rates were affected by the diabetic 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) trophoblasts to illustrate the deviations among non-diabetic subjects compared with platelets of the blood samples from GDM.

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 × 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 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 with platelets derived from non-diabetic maternal blood samples (Fig. 3A, right and 3B). These findings strongly suggest that under basal (non-insulin-stimulated) 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) trophoblasts to illustrate the deviations among the patients.

In addition to IR auto-phosphorylation, the 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 placental and blood samples.

WB (n/H11005 3–4) analysis of the trophoblast (1.5/H11003 10^6 cells per WB assay) from GMD and non-diabetic placenta for AKT and S6K was performed. In GDM-trophoblast, the levels of phosphorylation on AKT (Fig. 4, A and B) and S6K (Fig. 4, A and 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. 4, A–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 concentrations (0, 10, or 100 nM) of insulin for 24 h. 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 up-regulated 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 × 10⁶ cells per assay) isolated and purified from GDM- or non-diabetic-placentas were treated with various concentrations 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%) peak at 10 nM and 20 nM of insulin treatment, compared with the uptake rates of the non-treated platelets (Fig. 7A).

To validate this transient increase in 5-HT uptake rates of SERT at the platelet surface was treatment at different concentrations, induces an increase of SERT expression at the surface by insulin treatment of non-diabetic or GDM-platelets (Fig. 7B). Therefore, insulin treatment at different concentrations, induces an increase of SERT expression as well as of the surface level of SERT on platelet membranes, respectively.

Discussion

The cell signaling is a cell type-dependent physiological phenomenon 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 affects 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 function is 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 fibrin stabilized 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 post-translational modification of SERT regulates transporter function (22, 24, 27–29, 31, 45–48), 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 post-translational 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 α-granules (52, 53); in turn, these secrete more 5-HT along with the α-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-HT2A 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 an 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 as those in non-diabetic-platelets. More important than this, the IR and the downstream elements such as AKT and S6K are functionally active in GDM-platelet whereas they are inactive in GDM-trophoblast. We hypothesize that insulin signaling appears impaired differently in placenta then in platelet as the insulin signaling appears impaired in GDM-trophoblast. In a separate study, we showed the 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 versus trophoblast-related findings fit together well with the surface expression and the 5-HT uptake rates of these tissues. Because of 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-trophoblasts; 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.

Acknowledgments—A. C. repeated all the experiments presented in Figs. 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.

In GDM Insulin Action Is Tissue Type Dependent

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**Acknowledgments**—We gratefully acknowledge the UAMS Flow Cytometry Core and the November Ward for assistance in obtaining and providing us the samples.

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