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Lipopolysaccharide and double stranded viral RNA mediate insulin resistance and increase system a amino acid transport in human trophoblast cells *in vitro*

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ABSTRACT

Introduction: Inflammation and underlying low-grade maternal infection can impair insulin signalling and upregulate nutrient transport in the placenta which contribute to fetal overgrowth associated with GDM and/or obese pregnancies. There are, however, no studies on the role of infection on placental nutrient transport in pregnancies complicated by GDM and/or obesity. Thus, the aims of this study were to determine the effect of the bacterial product lipopolysaccharide (LPS) or the viral dsRNA analogue polyinosinic:polycytidylic acid (poly(I:C)) on the insulin signalling pathway and amino acid transport in primary human trophoblast cells.

Methods: Human primary villous trophoblast cells were treated with LPS or poly(I:C). Protein expression of insulin signalling pathway proteins, insulin receptor (IR)-β, insulin receptor substrate (IRS)-1 and protein kinase B (also known as Akt), and phosphatidylinositol-4,5-bisphosphate 3-kinase p85α subunit (PI3K-p85α) protein were assessed by Western blotting. Glucose and amino acid uptake were assessed by radiolabelled assay. Western blotting and qRT-PCR were used to determine amino acid transporter protein and mRNA expression, respectively.

Results: LPS and poly(I:C) significantly decreased phosphorylation of IR-β, IRS-1, Akt, total PI3K-p85α protein expression and glucose uptake. LPS and poly(I:C) also significantly increased expression of System A amino acid transporters SNAT1 and SNAT2, and System A-mediated uptake of amino acids.

Discussion: LPS and poly(I:C) induces insulin resistance and increases amino acid uptake in human primary trophoblast cells. This suggests that the presence of low-grade maternal infection can contribute to excess placental nutrient availability and promote fetal overgrowth in pregnancies complicated by GDM and/or obesity.

1. Introduction

Pre-existing maternal obesity and gestational diabetes mellitus (GDM) are associated with maternal peripheral insulin resistance and the development of fetal macrosomia [1]. In healthy women, the development of insulin resistance is a normal physiological process, particularly in the third trimester of pregnancy, to accommodate the nutrient demands of the fetus for optimal growth and development. Insulin resistance, however, is more pronounced in women with GDM and/or pre-existing obesity which contributes to excess nutrient availability to the fetus, leading to fetal overgrowth and increased fetal adiposity. In conjunction with insulin-mediated glucose uptake by the placenta [2], amino acid transporters [3] are also responsible for the transfer of nutrients across the placenta from the maternal circulation to the growing fetus.

Glucose is the primary source of energy required for fetal and placental development. However, fetal glucogenesis is minimal [4], with the fetus completely dependent on the transport of maternal circulating glucose across the placenta for optimal growth and development. In first trimester of pregnancy, glucose uptake into the placenta is primarily regulated by insulin [2]. In contrast, during third trimester, the process of glucose transportation into term

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placenta becomes altered and is not regulated by insulin [5]. This reduced sensitivity to insulin in term placenta is thought to be caused by the reduced expression of the insulin-sensitive glucose transporter (GLUT)-4 in term syncytiotrophoblast cells when compared to first trimester syncytiotrophoblast cells which highly express GLUT-4 [2].

In normal healthy pregnancies, maternal blood glucose levels are regulated by the insulin signalling pathway. However, in pregnancies complicated by maternal obesity and/or GDM, there are defects in this pathway resulting in increased maternal hyperglycaemia. Pancreatic β-cells secrete insulin, which binds to the β-subunits of the insulin receptor (IR-β). Activation of IR-β results in the autophosphorylation of its tyrosine residues and downstream tyrosine phosphorylation insulin receptor substrates (IRS-1 and 2). Activation of IRS proteins allows for the recruitment of downstream signal transducing molecules such as phosphoinositide 3-kinase (PI3K) p85α [6]. PI3K regulates the Akt/protein kinase B signalling pathway which is involved in activating downstream processes such as glucose and protein metabolism [7]. There are studies that show placental insulin signalling is altered in pregnancies complicated by GDM and/or obesity. Studies have shown insulin activation of the PI3K-p85α/Akt pathway can also regulate protein synthesis by via regulating 4EBP1 [8]. These studies highlight the important relationship between placental insulin signalling and fetal growth and development.

System A transporters are membrane transport proteins that regulate the sodium-dependent uptake of small neutral aliphatic amino acids (e.g. alanine, glutamine, serine, cysteine and proline) [10]. System A transporters can also transport a non-metabolised amino acid analogue known as α-(methylamino)isobutyric acid (MeAIB) [11]. These studies have shown System A activity is upregulated with increasing gestational age and fetal demand for nutrients [12]. Further, women with GDM have increased System A activity on the maternal-facing microvillous membrane of syncytiotrophoblasts [13]. Notably, the same study also found that pregnancies with fetal overgrowth without GDM, had no change in placental System A activity when compared to healthy controls. These studies indicate that changes to the placental amino acid transport system contributes to altered fetal growth and increased fetal adiposity.

In human placenta, three System A transporter subtypes (SNAT1, 2 and 4) are expressed on the maternal-facing microvillus membrane, suggesting their important role in pregnancy and fetal growth and development [14–16]. SNAT1 and SNAT2 share similar functional kinetics and high affinity to neutral amino acids, whereas SNAT4 in comparison has a lower affinity for neutral amino acids and able to transport cationic amino acids in a Na^+-independent manner [17]. Studies have shown placental SNAT4 activity is highest during first trimester of human pregnancy [16], while SNAT1 is the major contributor of placental System A activity at term [18]. Furthermore, amino acid uptake via System A transporters are upregulated in primary human syncytiotrophoblasts cells when stimulated with TNF-α and IL-6 [19,20].

There is growing evidence that show maternal endotoxemia and metabolic inflammation can induce peripheral insulin resistance and are strongly associated with pregnant women who are obese and/or have GDM [21–23]. Activation of toll-like receptors (TLR) signalling pathways by bacterial and viral infection and their products have been implicated to contribute to the pathophysiology of GDM [22–25]. For example, bacterial lipopolysaccharide (LPS) is a TLR4 ligand and induces insulin resistance in metabolic diseases [26]. Notably, activation of the TLR4 signalling pathway is also associated with increased System A amino acid uptake across the placenta [27]. Likewise, dsRNA viral analogue polyinosinic:polycytidylic acid (poly(I:C)), a TLR3 ligand, and has been shown to induce insulin resistance in skeletal muscle and adipose tissue from pregnant women [22–25]. There are no studies, however, that have assessed the effect of LPS (as a model of bacterial infection) or poly(I:C) (as a model of viral infection) on insulin resistance and nutrient transportation in human primary trophoblasts. Therefore, the aims of this study were to assess the effect of bacterial LPS and the viral dsRNA analogue poly(I:C) on the insulin signalling pathway and amino acid transport system in primary human trophoblasts.

2. Materials and methods

2.1. Tissue collection

The Research Ethics Committee of Mercy Hospital for Women approved this study. Written, informed consent was obtained from all participating women. Placenta was obtained from women who delivered healthy, singleton infants at term (>37 weeks gestation). Samples were processed within 15 min of delivery. Indications for Caesarean section were breech presentation and/or previous Caesarean section. Women with any underlying medical conditions such as pre-existing diabetes, asthma, polycystic ovarian syndrome, chorioamnionitis, acute fetal distress, preeclampsia and renal/macrovascular complications were excluded from this study. In addition, smokers and women diagnosed with GDM in this pregnancy were excluded.

For these studies, samples were collected from non-obese (BMI $\leq$ 30 kg/m^2) subjects. The women were classified as non-obese based on their pre-pregnancy BMI. Placental lobules (cotyledons) were obtained from various locations of the placenta; the basal plate and chorionic surface were removed from the cotyledon, and villous tissue was obtained from the middle cross-section. Placental tissue was blunt dissected to remove visible connective tissue and calcium deposits. Tissues were washed extensively with PBS, and immediately used for isolation of trophoblast cells as detailed below.

2.2. Isolation of primary trophoblast cells

Placental villous cytotrophoblasts were isolated as previously described [28]. Briefly, DNase/trypsin digestion and purified by separation on a Percoll gradient. Briefly, placental villous tissue (~25 g) was dissected and washed in saline and then digested three times in DMEM containing 0.25% trypsin, 0.25% grade II dispase, and 0.2 mg/mL DNase. Tissue was shaken at 37 °C for 45 min. The cytotrophoblast cells were separated on a Percoll gradient and resuspended in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics). For the insulin signalling and glucose uptake experiments, cells were plated on 24-well-plates at a density of 5×10^5 cells per well. For the amino acid uptake experiments, cells were plated on 6-well-plates at a density of 2×10^6 cells per well. The cells were cultured for a total of 90 h at 37 °C in 8% O_2, 5% CO_2 atmosphere, and the cell culture media was changed daily. Trophoblast cells purity was confirmed by high protein expression of cytokeratin-7 (epithelial cell marker), absence of vimentin (fibroblast cell marker) expression, and secretion of hCG (measure of biochemical differentiation).

Sixty-six hours after plating, to allow for syncytialisation [28,29], cells were treated in the absence or presence of 10 μg/ml LPS or 5 μg/ml poly(I:C) for 20 h. The concentration of LPS and poly(I:C) were used based on their ability to elicit a pro-inflammatory response in primary trophoblast cells [30,31]. Cell viability was assessed by the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) proliferation assay.