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Placental Pim-1 expression is increased in obesity and regulates cytokine- and toll-like receptor-mediated inflammation

Stella Liong [a, b], Gillian Barker [a, b], Martha Lappas [a, b, *]

[a] Obstetrics, Nutrition and Endocrinology Group, Department of Obstetrics and Gynaecology, University of Melbourne, Victoria, Australia
[b] Mercy Perinatal Research Centre, Mercy Hospital for Women, Victoria, Australia

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ABSTRACT

Introduction: Obesity is a growing epidemic, and as a consequence the number of obese pregnancies has also increased. Pregnancy is characterised by maternal and placental inflammation which is intensified with maternal obesity. The proviral integration site for Moloney murine leukemic virus (Pim)-1 protein is a serine/threonine kinase involved in a wide range of inflammatory diseases. In relation to obesity, however, its role has not been elucidated in human placenta. The aims were to determine the placental expression of Pim-1 with pre-existing maternal obesity and its role in regulating placental inflammation associated with obesity.

Methods: Human placenta was obtained at the time of term Caesarean section from lean and pre-existing obese pregnant women to determine the effect of maternal obesity on Pim-1 expression. To determine the effect of Pim-1 on the inflammatory response induced by bacterial endotoxin LPS and pro-inflammatory cytokines TNF-α or IL-1β, the chemical inhibitor SMI-4a and siRNA were used.

Results: Pim-1 protein and mRNA expression was significantly increased in placenta of obese women. SMI-4a significantly suppressed the expression and release of pro-inflammatory cytokine IL-6 and chemokines GRO-α and MCP-1 when stimulated with LPS or TNF-α in placenta. Primary trophoblast cells transfected with Pim-1 siRNA had decreased expression and release of pro-inflammatory cytokines IL-1β, IL-6, chemokines GRO-α and MCP-1, when stimulated with LPS, TNF-α or IL-1β.

Discussion: The findings from this study implicate Pim-1 may contribute to placental inflammation in pregnancies complicated by maternal obesity. Thus, therapeutic targets for Pim-1 may improve fetal outcomes complicated by obese pregnancies.

1. Introduction

The global incidence of obesity is rapidly increasing [1]. In Australia, it has been predicted that by 2025, 75% of females of reproductive-age will be either overweight or obese [2]. There is strong evidence that maternal nutritional and metabolic status are important for fetal development and the long-term health of offspring [3–5]. Excessive fetal overgrowth and increased fetal adiposity is closely associated with maternal obesity [6]. These infants from obese pregnancies also have increased risk of metabolic disease, including obesity, diabetes, cardiovascular disease, and certain cancers [7–12].

Pregnancy is associated with low-grade inflammation. In pregnancies complicated by obesity however, this inflammation is significantly heightened. Obese pregnant women are associated with increased levels of pro-inflammatory cytokines IL-1β, IL-6 and TNF-α in the placenta and in maternal circulation [13–17]. Recent studies have also shown low-grade maternal endotoxemia can activate systemic and adipose tissue inflammation in obese pregnant women [13]. Pro-inflammatory cytokines and endotoxemia have been shown to promote intrauterine inflammation and placental dysfunction, such as reduced mitochondrial respiration and ATP generation, insulin resistance and excessive amino acid and free fatty acid delivery to the fetus [18–20]. It is hypothesised that this altered placental state is responsible for the poor pregnancy outcomes and fetal morbidity associated with maternal obesity. Therefore, understanding the effect of maternal obesity on placental function is important for improving neonatal outcomes.

* Corresponding author. Department of Obstetrics and Gynaecology, University of Melbourne, Mercy Hospital for Women, Level 4/163 Studley Road, Heidelberg 3084, Victoria, Australia.
E-mail address: mlappas@unimelb.edu.au (M. Lappas).

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The proviral integration site for Moloney murine leukemic virus (Pim) proteins are a family of serine/threonine kinases that have been demonstrated to regulate inflammation [21,22]. There are three Pim kinases (Pim-1-3) which are structurally and functionally similar, however each are encoded by distinct genes. The Pim-1 gene has different translation initiation sites resulting in two isoforms, Pim-1L (44 kD) and Pim-1S (33 kD). Both Pim-1 isoforms are reported to have similar kinase activities *in vitro* [23]. Unlike many protein kinases, human Pim-1 kinase does not require phosphorylation for activation; as such, Pim-1 kinase are constitutively activated [24]. Studies have shown regulation of Pim-1 kinase activity occurs at the expression level including transcription, translation, and proteosomal degradation [25,26].

Pim-1 expression is upregulated in response to pro-inflammatory cytokines, growth factors, mitogenic stimuli and infection [27–30]. For example, Pim-1 expression is increased during Epstein-Barr virus infection [30] and in response to LPS exposure in RAW264.7 cells [28]. There are also studies which have shown Pim-1 can regulate inflammation and the immune response. Pim-1 blockade suppresses allergen-induced airway inflammation and cytokine production in mice [22] and reduces the development of dextran sodium sulfate-induced colitis in mice by suppressing macrophage activation and Th1- and Th17-type immune responses [28]. In addition, Pim-1 overexpression studies using animal models have shown Pim-1 increases inflammation and pyelonephritis in mice [31].

The role of Pim-1 in regulating placental inflammatory pathways associated with pre-existing maternal obesity remains unknown. Therefore, the aim of this study was to characterise the expression of Pim-1 in placenta from non-obese and obese pregnant women. The role of Pim-1 on inflammation in placenta was also assessed using a Pim-1 chemical inhibitor on placental tissue or Pim-1 siRNA knockdown on primary trophoblast cells. Tissues and cells were stimulated with bacterial product LPS (used as a model of metabolic endotoxemia) or pro-inflammatory cytokines TNF-α and IL-1β to mimic the inflammatory environment associated with obesity. The Pim-1 inhibitor used in this study was SMI-4a, a benzylidene-thiazolidene-2,4-dione that has a strong affinity against Pim-1 and a modest affinity to Pim-2.

2. Materials and methods

2.1. Tissue collection

Approval for this study was obtained from the Mercy Hospital for Women's Research and Ethics Committee and written informed consent was obtained from all participating subjects. Women were invited to provide samples on the day of admission for surgery. All tissues were obtained at the time of term Caesarean section in the absence of labour to ensure there were no effects of labour on Pim-1 expression. Indications for Caesarean section included repeat Caesarean section or breech presentation. Women fulfilling any of the following criteria were excluded; vascular/renal complication, gestational diabetes mellitus, multiple gestations, asthma, smokers, preeclampsia, chorioamnionitis, placental abruption, acute fetal distress and women with any other adverse underlying medical conditions.

Placenta was obtained within 15 min of delivery. 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 (i) immediately snap frozen in liquid nitrogen and stored at −80 °C for analysis of protein expression by Western blotting as detailed below; (ii) fixed, and paraffin embedded for immunohistochemical analysis; or (iii) immediately used for explant studies or siRNA studies as detailed below.

For Pim-1 expression and immunohistochemistry (IHC) studies, placenta was obtained from normal glucose tolerant women who entered pregnancy lean (BMI between 18 and <25 kg/m^2; n = 10 patients) or obese (BMI ≥30 kg/m^2; n = 10 patients). The relevant clinical details of the subjects in this cohort are detailed in Table 1.

2.2. Placental explants

Placental tissue explants were performed to determine the effect of the Pim-1 kinase inhibitor SMI-4a on inflammation. For these experiments, fresh placenta was obtained from non-obese normal glucose tolerant (n = 6 patients), and tissue explants were performed immediately as previously described [32]. Briefly, placenta was finely diced and placed in DMEM at 37 °C in a humidified atmosphere of 8% O_2 and 5% CO_2 for 1 h. Tissues were blotted dry on sterile filter paper and transferred to 24-well tissue culture plates (100 mg/well). The explants were incubated in 1 ml DMEM containing 100 U/ml penicillin G and 100 μg/ml streptomycin. Tissues were incubated in the absence or presence of 5 μM SMI-4a (AdooQ BioScience; Irvine, CA, USA) for 60 min before the addition of 10 μg/ml LPS (derived from *Escherichia coli* 026:B6; Sigma-Aldrich; St. Louis, MO, USA), or 10 ng/ml TNF-α (PeproTech; Rocky Hill, NJ, USA) for 20 h. After final incubation, tissue and media were collected separately and stored at −80 °C for further analysis as detailed below. The concentration of SMI-4A was based on previous studies [33].

2.3. Gene silencing of Pim-1 in primary villous trophoblast cells

Isolation and purification of primary villous trophoblast cells was performed using fresh placenta from non-obese pregnant women. Placental villous cytotrophoblasts were isolated as previously described [34] by 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 a HEPES-buffered salt solution containing 0.25% trypsin and 0.2 mg/ml DNAse. Tissue was shaken at 37 °C for 30 min. The cytotrophoblast cells were separated on a Percoll gradient and resuspended in standard cell culture medium (5.5 mM glucose, 44.5% DMEM, 44.5% Ham's-F12, and 10% fetal calf serum supplemented with antibiotics). The cells were plated on 24-well plates at a density of 5×10^5 cells per well. The cells were cultured for a total of 90 h at 37 °C in 8% air, 5% CO_2 atmosphere and the cell culture media was changed daily.

Transfection of trophoblast cells was performed as previously described [34] using Pim-1 siRNA (siPIM-1) and negative control siRNA (siCONT) obtained from Ambion (Thermo Fisher Scientific; Scoresby, Vic, Australia). Briefly, cells were transfected with 100 nM siCONT or siPIM-1 and 0.3% Lipofectamine 2000 (Life Technologies, Mulgrave, Victoria, Australia), incubated for 24 h, and removed, and fresh medium was added to wells. After 66 h (total culture time), cells were treated with or without 1 μg/ml LPS, 10 ng/ml TNF-α or 1 ng/ml IL-1β, and the cells were incubated at 37 °C for an additional 24 h. Cells were collected and stored at −80 °C until assayed for mRNA expression by qRT-PCR as detailed below. Media was collected and stored at −80 °C until assayed for cytokine release as detailed below. The response to LPS, TNF-α and IL-1β between patients varied greatly, as we have previously reported [35]. Thus, data is presented as fold change in relative to LPS-, TNF-α- and IL-1β-stimulated siCONT transfected cells, which was set at 1. MTT assay was performed on all treatments to determine any significant effects on cell viability. Experiments were performed on placenta