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The NR4A receptors Nurr1 and Nur77 are increased in human placenta from women with gestational diabetes

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ABSTRACT

Introduction: Members of the NR4A subfamily are involved in a wide range of diseases including obesity and diabetes. The aim of this study was to determine the effect of maternal obesity and gestational diabetes mellitus (GDM) on the expression of the NR4A receptors Nurr1, Nur77 and NOR1.

Methods: Human placenta was obtained at the time of term Caesarean section from (i) lean and obese and normal glucose tolerant (NGT) pregnant women; and (ii) women with GDM and BMI-matched NGT women ($n = 16$ patients). Primary trophoblast cells, isolated from human term placenta, were used to determine the effect of pro-inflammatory cytokines on NR4A protein expression. Primary trophoblast cells were also used to assess the effect of Nurr1, Nur77 and NOR1 siRNA knockdown on pro-inflammatory cytokines.

Results: There was no effect of pre-existing maternal obesity on Nurr1, Nur77 or NOR1 expression. Nurr1 and Nur77 expression were significantly higher in GDM placenta compared to NGT placenta, and in the presence of the pro-inflammatory cytokines TNF-α and IL-β in primary trophoblast cells. Knockdown of Nurr1 and Nur77 in human primary trophoblast cells significantly decreased TNF-α induced expression and secretion of IL-6 and IL-8.

Discussion: Nurr1 and Nur77, which were increased in human placenta from women with GDM, are involved in TNF-α induced-expression of pro-inflammatory cytokines. Pro-inflammatory cytokines are known to play a role in placental nutrient transport. Thus, the regulation of pro-inflammatory cytokines by Nurr1 and Nur77 suggest that these proteins may play a role in placental function and transport mechanisms.

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1. Introduction

The NR4A subfamily includes nuclear receptor subfamily 4 group A member 1 (NR4A1; also known as Nur77); nuclear receptor subfamily 4 group A member 2 (NR4A2; also known as nuclear receptor related 1 protein or Nurr1); and nuclear receptor subfamily 4 group A member 3 (NR4A3; also known as neuron derived orphan receptor 1 or NOR1) [1]. Akin to other nuclear receptors, their structure includes an *N*-terminal transactivation domain (TAD), a central DNA binding domain (DBD), and a C-terminal ligand-binding domain (LBD). However, NR4As are true orphan nuclear receptors in that they do not require ligand-binding to be activated; as a consequence protein expression is closely correlated with their activity [2].

Members of the NR4A subfamily are classified as early response genes, which are induced by a diverse range of stimuli, including fatty acids, stress, growth factors and inflammatory signals [1,3–7]. Consistent with these findings, NR4A receptors regulate a number of key cellular functions, including inflammation, proliferation, adhesion, metabolism and cell survival [1,4,5,8–12]. As such, they have been implicated in a number of diseases including atherosclerosis [5,9], diabetes [10,13], arthritis [6,14,15] and cancer [16].

Notably, NR4A receptors have recently been implicated in the response to energy excess (over-eating), diet induced obesity and diabetes [11–13,17–22]. These studies have, however, focused mainly in liver, skeletal muscle and adipose tissue. Of note, there is only one reported study of NR4A receptors in placenta; in this study, the authors studied the expression of Nur77 only in human placenta [23]. Nur77 was localised to trophoblast cells, including

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villous cytotrophoblast cells and extravillous trophoblast cells, where its expression was significantly lower in women with preeclampsia [23].

There is, however, no data available on the expression of Nurr1, Nur77 and NOR1 in human placenta from obese women or women with gestational diabetes mellitus (GDM). Thus, one of the aims of this study was to determine the effect of pre-existing maternal obesity and GDM on Nurr1, Nur77 and NOR1 in human placenta. Low-grade inflammation is a central feature of pregnancies complicated by maternal obesity and GDM [24–32]. There is increased maternal systemic inflammation which is associated with increased pro-inflammatory cytokines and accumulation of activated macrophages in the interstitial stroma of the placenta of women with GDM or maternal obesity [24,27,29,33–35]. Cross-sectional studies have shown that circulating concentrations of TNF-α are higher in pregnant women with GDM when compared to the normal pregnant women [36,37]. Notably, TNF-α is a significant predictor of insulin resistance during pregnancy [38]. An overproduction of placental TNF-α have been demonstrated in patients with GDM [39] and is associated with increased fetal adiposity [29]. Other circulating pro-inflammatory cytokines like monocyte chemoattractant protein-1 (MCP-1) [35], IL-1β [40] and IL-6 [41] are also positively correlated with GDM. Notably, the upstream factors involved in the regulation of these pro-inflammatory cytokines in human placenta are not known. Thus, a further aim of this study was to elucidate the effect of Nurr1, Nur77 and NOR1 knockdown on pro-inflammatory cytokine expression in human primary placental trophoblast cells.

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 NR4A receptor expression. Indications for Caesarean section included repeat Caesarean section or breech presentation. Women fulfilling any of following criteria were excluded; vascular/renal complication, multiple gestations, asthma, smokers, preeclampsia, chorioamnionitis, placental abruption, acute fetal distress and women with any other adverse underlying medical conditions.

Placenta was collected for two studies; expression studies using placenta villous tissue and cell culture studies using freshly isolated trophoblast cells. 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 immediately stored at −80 °C for analysis of gene and protein expression by qRT-PCR and Western blotting as detailed below; or (ii) used for isolation of trophoblast cells as detailed below.

For the expression study, placenta was obtained from 2 cohorts of women. In cohort 1, placenta was collected from normal glucose tolerant (NGT) women ($n = 16$ patients) who entered pregnancy lean (BMI between 18 and <25 kg/m^2; $n = 8$ patients) or obese (BMI ≥30 kg/m^2; $n = 8$ patients). The relevant clinical details of the subjects are detailed in Table 1A.

In cohort 2, placenta was obtained from NGT women ($n = 16$ patients) or BMI-matched women with GDM ($n = 16$ patients). Women with GDM were diagnosed according to the criteria of the Australasian Diabetes in Pregnancy Society (ADIPS) by either a fasting venous plasma glucose level of ≥5.5 mmol/l glucose, and/or ≥8.0 mmol/l glucose 2 h after a 75 g oral glucose load at approximately 26–28 weeks gestation. Women with GDM were managed by diet alone ($n = 8$ patients) or insulin in addition to diet ($n = 8$ patients). Women diagnosed with GDM required where prescribed insulin according to hospital guidelines for insulin therapy in GDM. Women were controlled by diet if their fasting glucose readings were maintained <5.5 mmol/l over a 2 week period post diagnosis. Women with fasting glucose readings >5.5 mmol/l over a 2 week period post diagnosis were placed on insulin for optimal glucose control. The relevant clinical details of the subjects in this cohort are detailed in Table 1B.

Table 1A
Characteristics of the NGT study group.

	NGT Lean ($n = 8$)	NGT Obese ($n = 8$)
Maternal age (years)	34.0 ± 1.5	31.6 ± 2.0
Pre-pregnancy BMI (kg/m^2)	22.9 ± 0.5	38.9 ± 1.2#
Maternal BMI at delivery (kg/m^2)	26.6 ± 0.6	42.6 ± 1.0#
Gestational weight gain (kg)	9.3 ± 0.5	9.6 ± 0.5
Gestational age at birth (weeks)	38.8 ± 0.2	39.0 ± 0.2
Fetal birth weight (g)	3241 ± 112	3766 ± 141*
Fetal gender	4 Female 4 Male	4 Female 4 Male
Apgar score at 5 min	9.3 ± 1.1	8.9 ± 1.1

Values represent mean ± SEM.
*$P < 0.05$ vs. NGT lean (unpaired Student's *t*-test).
#$P < 0.05$ vs. NGT (Mann–Whitney *U* test).

2.2. Isolation and purification of primary trophoblast cells from term placentas for culture

Isolation and purification of primary trophoblast cells was performed from placenta from NGT non-obese pregnant women. These cells were used for the cell culture experiments and siRNA studies detailed below. Placental villous cytotrophoblasts were isolated as previously described [42] 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. 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).

2.3. Trophoblast cell culture treatments

Sixty-six hours after plating, to allow for syncytialisation, cells were treated in the absence or presence of 10 ng/ml TNF-α and 1 ng/ml IL-1β for 24 h. The concentration of TNF-α and IL-1β used in this study were based on previous studies [43,44]. Cells were collected and stored at −80 °C until assayed for Nurr1, Nur77 and NOR1 mRNA expression by qRT-PCR as detailed below. MTT assay was performed on all treatments to determine any significant effects on cell viability. Experiments were performed on placenta obtained from 6 patients.

2.4. Gene silencing of Nurr1, Nur77 and NOR1 with siRNA

Primary trophoblast cells were also used to investigate the effect of siRNA-mediated gene silencing of Nurr1, Nur77 or NOR1 on inflammation. Twenty hours after plating, the trophoblast cells were transfected with cell culture media containing 200 nM non-specific (NS) siRNA, 200 nM Nurr1 siRNA (HSC.RNAI.N006186.12.1; IDT), 200 nM Nur77 siRNA (HSC.RNAI.N002135.12.1; IDT)

Table 1B
Characteristics of the GDM study group.

	NGT ($n = 16$)	GDM ($n = 16$)
Maternal age (years)	32.8 ± 1.3	33.3 ± 1.2
Pre-pregnancy BMI (kg/m^2)	30.9 ± 2.1	31.1 ± 2.1
Maternal BMI at delivery (kg/m^2)	34.6 ± 2.1	34.0 ± 2.0
Gestational weight gain (kg)	9.4 ± 0.3	7.3 ± 0.5*
Gestational age at birth (weeks)	38.9 ± 0.1	38.3 ± 0.2
Fetal birth weight (g)	3503 ± 110	3410 ± 137
Fetal Gender	8 Female 8 Male	8 Female 8 Male
Apgar score at 5 min	9.1 ± 1.0	9.0 ± 1.1
OGTT at ~28 weeks gestation		
Fasting plasma OGTT (mmol/l)	4.6 ± 0.1	5.4 ± 0.3#
1 h plasma OGTT (mmol/l)	7.4 ± 0.4	10.6 ± 0.6*
2 h plasma OGTT (mmol/l)	5.9 ± 0.3	9.2 ± 0.3*

Values represent mean ± SEM OGTT, oral glucose tolerance test.
*$P < 0.05$ vs. NGT (unpaired Student's *t*-test).
#$P < 0.05$ vs. NGT (Mann–Whitney *U* test).

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