The human placenta expresses multiple glucocorticoid receptor isoforms that are altered by fetal sex, growth restriction and maternal asthma

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The human placenta expresses multiple glucocorticoid receptor isoforms that are altered by fetal sex, growth restriction and maternal asthma


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Abstract

Introduction: We have previously identified sex-specific differences in the fetal-placental response to cortisol. Our recent studies suggest that this differential response to cortisol is driven by differences in glucocorticoid receptor (GR) protein function rather than through changes in gene transcription or protein expression.

Methods: This study was designed to define whether the human placenta expresses different isoforms of the GR and whether expression was altered by fetal sex and maternal asthma. Asthma and non-asthma pregnant women were prospectively recruited at their first antenatal visit and placenta collected at delivery. Placental GR expression was examined in relation to maternal asthma, fetal sex and birthweight.

Results: Twelve specific bands for the GR were identified at molecular weights of 94, 91, 81, 74, 69, 68, 65, 60, 55, 50, 48 and 38 kDa. The 12 isoforms were localised to the placental trophoblast and expression varied in relation to cellular location in either the cytoplasm or nucleus, fetal sex, fetal size and the presence and absence of maternal asthma.

Conclusion: This is the first study to identify the presence of several protein isoforms of the GR in the human placenta. The data suggest glucocorticoid resistance observed in male placenta may be mediated through increased GRβ, GR A and GR P localisation to the nucleus. While female placentae may be more sensitive to cortisol in the presence of maternal asthma through a decrease in GRβ and an enhancement of GRα activity via an interaction with GRα D3 and GRα C.

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1. Introduction

Excess exposure to maternal glucocorticoids during pregnancy can significantly impact lifelong health [1]. There is some evidence to suggest that programming effects on fetal development are mediated by the placenta and induced in a sex-specific manner via changes in placental function [2]. We have previously identified sex-specific differences in the fetal-placental response to cortisol [3–5]. From this body of work, male fetuses appear to induce a state of glucocorticoid resistance in an environment of excess glucocorticoids while females appear hypersensitive to changes in glucocorticoid concentration [6]. Our recent studies suggest that this differential response to cortisol is driven by differences in glucocorticoid receptor (GR) protein function [4] rather than changes in GR gene transcription or GR protein expression [6].

GR is a ubiquitously expressed member of the nuclear receptor transcription factor superfamily. There is one human GR gene which is comprised of 8 translated exons and 9 untranslated first exons (exon 1) spanning 80 kB. The untranslated exon 1 of the GR gene can be spliced into 9 different promoter variants [7] that function in a tissue specific manner to regulate GR protein expression. Exons 2–9 can generate various isoforms of GR through alternative splicing [8–10] or through alternative initiation of translation [10,11] resulting in the expression of GRα, GRβ, GRγ, GR-A and GR-P proteins. GRα is the functional isoform involved in transcriptional activation and transcriptional repression of multiple targets. Moreover, eight different GRα translational isoforms can originate from GRα mRNA. GRα-A and GRα-B encode for proteins of 94 and 91 kDa respectively. GRα C1–C3 (81–83 kDa) and GRα D1–D3 (50–55 kDa) proteins are also GR translational variants. It has been suggested that the translation of these isoforms function

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in a tissue specific manner [10]. All translational Grz variants have the ability to translocate into the nucleus to regulate transcriptional activities. GRβ inhibits activation of Grz through a dominant negative mechanism. Splice variants GRγ (95 KDa), GR-A (65 KDa) and GR-P (75 KDa) have low transactivation activities [12,13]. We have previously shown that splice variants of Grz including GRβ, GR-P and GRγ mRNA are all detectable in the human placenta with Grz and promoter 1C showing the highest expression [14]. In pregnancies complicated by asthma we have additionally shown Gr gene activity was positively correlated with cord blood cortisol compared to healthy controls [6]. Based on the evidence we have collected so far, we hypothesise that sex-specific differences in cortisol sensitivity in fetal-placental tissues are controlled by the interaction of the functional Grz with other GR isoforms. In the current study we aim to define the sex-specific expression, phosphorylation status and intracellular locality of the human GR isoforms in the term placentae of normal pregnancies and pregnancies complicated by maternal asthma.

2. Methods

2.1. Maternal and neonatal characteristics

Maternal characteristics including maternal height, weight, age, parity and gravidity were recorded at the first antenatal visit. Women with any complications other than asthma were excluded including those who subsequently developed pre-eclampsia, gestational diabetes, infection and preterm delivery. Women who smoked or were obese were included in the study. Neonatal data collected at delivery includes gestational age and birthweight. Birthweight centile (BWC) was calculated using www.gestation.net. “Small for gestational age” (SGA) birthweights were babies less that the 10th BWC at delivery and “appropriate for gestational age” birthweights were > 10th BWC. Gestational age was determined by date of the last menstrual period and confirmed by 18 week ultrasound.

2.2. Asthma assessment

Asthmatic subjects were clinically assessed at 18, 30 and 36 weeks gestation as previously outlined [15]. Asthma control was assessed from the Juniper asthma control questionnaire [16], estimated inhaled glucocorticoid dose for pregnancy, reliever use, oral steroid use, asthma symptoms and a record of extra unplanned doctor visits or hospitalisations for asthma. Lung function, asthma severity, inhaled glucocorticoid dose for each trimester and before pregnancy, bronchodilator use, medication compliance, knowledge of asthma education, usage of asthma action plan, asthma symptoms at each trimester, number of exacerbations were noted [17]. Following delivery case notes were reviewed for birth outcomes of birth weight, placental weight, mode of delivery, complications and fetal sex.

2.3. Placenta and cord blood collection

Placental tissue (n = 134) and cord blood (n = 72) were collected within 1 h of delivery. The tissue was snap frozen in liquid nitrogen and stored at -80°C until analysis. Cord plasma was collected and stored at -20°C.

2.4. Cord blood cortisol ELISA

Cortisol was measured in umbilical vein plasma (n = 72) using a commercial ELISA kit (IBL International Germany). Samples were tested in duplicate and the intra-assay co-efficient of variation was less than 4%. Inter-assay variation was 13.6%. Cross-reactivity with other glucocorticoids was less than 0.01%. Limit of detection was 0.005 ng/dL.

2.5. Primary cells and cell lines used in the study

Human trophoblast cell line BeWo was maintained in DMEM-F12 medium supplemented with 10% FBS and penicillin, streptomycin and l-glutamine (PSG) system at 37°C containing 5% CO2. PSG medium was used. Primary trophoblast from placental tissue was isolated according to the previously published protocol [18]. Umbilical endothelial cells (HUVECS) were isolated using a previously published technique [19]. Mono-nuclear peripheral blood cells were isolated from cord blood at the time of delivery using Lymphoprep density gradient (Axis Shield, Norway) as per company instructions.

2.6. Western blot

2.6.1. Cellular fraction preparation from placental tissue

Placental tissue was homogenised in complete cytosolic fractionation buffer [20] containing complete protease inhibitor cocktail. Lysates were spun at 8000 rpm for 5 min. Supernatants were kept for the cytosolic fraction. Nuclear fraction buffer with complete protease inhibitor cocktail was added to cell pellets. Nuclear fractions were rotated at 4°C for 30 min, sonicated twice for 10 s at 30% amplitude using ultrasonic processor VX2 130 (Sonics, USA) and spun at full speed for 8 min to remove debris. Supernatants were stored at -80°C. Protein concentrations for each fraction were measured using Bradford assay.

2.6.2. Visualisation of target proteins

Cytosolic and nuclear protein fractions (60 µg) were electrophoresed on 3–8% Tris-acetate precast gels (Invitrogen, Life technologies, Carlsbad, California, USA) as previously described [6]. Blots were incubated with either anti-GRα total (1:10,000), anti-GRβ (1:1000), GR phospho specific antibodies (anti-GR S211 and anti-GR S226 (1:750), Abcam, Cambridge, MA, USA) and anti-GR total antibody (1:1500) (Bethyl Laboratories, Montgomery, TX, USA) antibodies. The appropriate secondary antibody (goat anti-rabbit, goat anti-mouse or donkey anti-goat 1:2500) was applied for 1 h. Membranes were subsequently probed with anti-β actin (1:4000, Bethyl Laboratories, USA) and anti-lamin A/C (1:1500, Santa Cruz Biotechnology, Santa Cruz, California, USA) antibodies as loading controls for cytoplasmic and nuclear fractions, respectively. The densitometric analysis was carried out using G:BOX ChemGel Imaging Systems (SYNGENE) to quantify the expression levels of different GR isoforms relative to β actin. Peptide competition with anti GR total antibody (1 µg 1.5 ml) incubated with 1 × (1:1) and 2 × (2/2 µg) concentration of the control peptide (Bethyl Laboratories, USA) was performed as a specificity control.

2.7. GR knockdown in BeWo cell line

BeWo cells were plated in 6 well plates and grown to 85–90% confluence at 37°C, 95% air, 5% CO2. Control siRNA (siRNA control-1, 4390843s), siRNA for an unrelated gene (GRIN-1, s516,8) and two GR-specific siRNA (GR-siRNA-2, s168 and GR-siRNA-1, s228,90, Invitrogen, Australia) each siRNA (90 pmol/L) were transfected into BeWo cells using Lipofectamine RNAMax reagent. Cells were kept at 37°C for 48 h post transfection then harvested and protein extracted for GR protein expression was analysed by Western blot analysis.

2.8. Immunohistochemistry

For immunohistochemistry, paraffin embedded placental tissues (n = 91) were used from women with or without asthma. Immunohistochemistry was performed on 2 µm tissues sections fixed in formalin and embedded in paraffin mounted on glass Superfrost Ultra Plus microscope slides (HDS Scientific Supplies, Wetherill Park, NSW, Australia). Immunohistochemical detection was performed using the streptavidin–biotin complex method. Antigen retrieval was performed using a water bath at 100°C in 10 mM citrate buffer (pH 6.5). Sections were treated with primary antibodies to either GR total (1:2000), GRα (1:2000) or GRβ (1:6000) (abcam, Cambridge, MA, USA) overnight at 4°C. Negative control consisted of omission of the primary antibody.

2.9. Analysis of immunostaining

Each tissue sample was scored according to the intensity of nuclear or cytoplasmic staining (0 = negative staining; score 1 = weak staining; score 2 = moderate staining; score 3 = strong staining). A random selection of 10 fields per tissue section was captured at 400× magnification for scoring assessment. The mode nuclear and/ or cytoplasmic scores for 10 fields was calculated for each tissue section and used for statistical analysis.

2.10. Statistical analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS v 19). An a-priori decision was made to analyse the data separately by sex when comparing the effects of asthma severity and treatment, as past research indicates that placental cortisol metabolism differs between males and females in the presence of asthma [21,22]. CR data was not normally distributed so non-parametric tests were used which included Mann–Whitney tests. Frequency data was analysed using Chi Squared. Spearman’s correlations were performed to assess associations. Univariate and multivariate analyses were conducted to assess impact of multiple variables on GR expression. The alpha level was set at 0.05. GR data is expressed as median and interquartile range.

3. Results

3.1. Subjects

There were significant differences in maternal or neonatal characteristics between women with asthma (n = 27) when
compared to healthy controls \((n = 53)\) (Table 1) in relation to inhaled corticosteroid use (ICS) and BMI. Cord blood cortisol concentrations \((n = 72)\) were significantly lower in males whose mothers had asthma relative to control males (Table 1).

3.2. GR isoforms expressed in the placenta

Total GR antibody (Bethyl Biosciences) identified 12 specific bands in protein extracts of whole placenta \((n = 134)\) at a molecular weight (MW) of 94, 91, 81, 74, 68, 65, 60, 55, 50, 48 and 38 kDa (Fig. 1). Some of these MWs are equivalent to known isoforms including GR\(\alpha\) (94 kDa), GR\(\beta\) (91 kDa), GR\(\gamma\) (81 kDa) GR\(\delta\) (74 kDa) GR\(\alpha\) (65 kDa), GR\(\beta\)-D 1-3 (50–55 kDa). Some isoforms have not been previously reported including the 69, 68, 60, 48 and 38 kDa proteins. Not all isoforms were expressed in every individual and there were some observed differences in relation to maternal asthma and fetal sex (Table 2). Following pre-absorption of the GR antibody with the control peptide all MW forms were removed (data not shown).

We also performed knockdown experiments of the GR gene in BeWo \((n = 3)\) and HEK293T \((n = 3)\) cell lines to determine whether all known and unknown GR protein bands were specific to the GR gene translation. A decrease in protein expression of different GR isoform particularly GR\(\alpha\), GR\(\beta\), GR-P, GR-D and 38 kDa size isoforms was observed in protein lysates from BeWo cells \((n = 3)\) 48 h post siRNA treatment (Figs. 2A and B). GR\(\alpha\)-C (range: 6–98% repression), 68–69 kDa (range: 36–85% repression, GR-A (range: 4–77% repression) were not significantly repressed in this experimental series but there was varying repression between individual experiments due to transfection efficiency differences. As a control, GR was knocked down in HEK293T cell line \((n = 3)\) and downstream pathways measured to confirm the siRNA was specific for GR and affected glucocorticoid-regulated pathways. The cytoplasmic anchoring protein for GR, FKBP51 was reduced and a pro-inflammatory cytokine inhibited by GR DNA binding, Tumour Necrosis Factor (TNF) \(\alpha\) was increased at the protein level in the presence of GR knockdown (Fig. 2C and D).

Interestingly, when examining the expression of all GR isoforms as a percentage of the total GR expression, it can be observed that GR\(\alpha\) A expression is quite low relative to the other GR isoforms in both the nucleus and the cytoplasm and regardless of sex (Fig. 4). GR\(\beta\) D isoforms and the 48 and 38 kDa unknown isoform account for over 60% of the total GR expression in the placenta (Fig. 4).

3.3. Localisation of GR isoforms using immunohistochemistry and western blot

3.3.1. GR immunostaining

Placental sections were stained with GR\(\alpha\) (Fig. 3A) GR\(\beta\) (Fig. 3C) and total GR (Fig. 3A) specific antibodies. Immunostaining identified that glucocorticoid receptors were localised to syncytiotrophoblast \((S)\) (pointed by arrow in (Fig. 3), cytotrophoblast, macrophage, vascular smooth muscle, cord blood immune cells \((C)\) and endothelial cells \((E)\) of placenta \((n = 91)\). GR\(\beta\) predominated in the syncytiotrophoblast. While GR\(\alpha\) and total GR antibody staining was observed in most cell types. There were no significant differences between the sexes or in relation to maternal asthma using this technique (Fig. 3).

3.3.2. Western blot localisation of GR isoforms

All 12 isoforms of GR were expressed in either the nucleus or the cytoplasm of whole placental extracts, isolated primary trophoblast cells \((n = 3)\) and the BeWo cell line \((n = 3)\). In human umbilical vein endothelial cells (HUVECS) \((n = 3)\) there was low expression of the unknown isoform at 68 kDa, GR\(\alpha\) A and GR\(\alpha\) D1 in the cytoplasm and in the nucleus, there was low expression of GR\(\alpha\), GR\(\beta\) and GR\(\alpha\) D1 isoforms. Only GR\(\alpha\) D1 can be observed in the nucleus of HUVECS in Fig. 5. In cord blood polymorphonuclear cells (CBMNC) \((n = 3)\) there were 4 GR isoforms; GR\(\alpha\), unknown unknown isoform 68 kDa, GR\(\alpha\) A and GR\(\alpha\) D1 localised to the nucleus. No isoforms were detected in the cytoplasm of CBMNC (Fig. 5).

3.4. Effect of sex on GR isoform expression in the nucleus or cytoplasm

Both males \((n = 65)\) and females \((n = 70)\) had greater nuclear expression of GR\(\alpha\) (94 kDa) than cytoplasmic expression (Female: nuclear: median: 0.09 interquartile range: \(0–0.31\) vs cytoplasmic: 0.0 \((0.0–0.14)\); Male: nuclear: 0.08 \((0–0.22)\) 4 vs cytoplasmic: 0.0 \((0.0–0.13)\); non-\(P = 0.002\)). In female placenta, there was greater nuclear expression of 68 kDa protein (nuclear: 0.0 \((0–0.36)\) vs cytoplasmic: 0.0 \((0–0.21)\); \(P = 0.03, n = 70\) and GR\(\alpha\) D1 (55 kDa) (nuclear: 0.24 \((0–1.25)\) vs cytoplasmic: 0.1 \((0–0.42)\); \(P = 0.03, n = 70\)) relative to cytoplasmic expression of the other GR isoforms. In male placentae, there was greater nuclear expression of GR\(\alpha\) (nuclear: 0.0 \((0–0.12)\) vs cytoplasmic: 0.0 \((0–0.1)\); \(P = 0.006, n = 65\)) and GR\(\alpha\) A (65 kDa) (nuclear: 0.06 \((0–0.73)\) vs cytoplasmic: 0.0 \((0–0.31)\); \(P = 0.01, n = 65\)) relative to cytoplasmic expression of these GR isoforms.

3.5. Effect of maternal asthma and fetal sex on GR isoform localisation

In relation to the presence of maternal asthma and sex; control placentae of male pregnancies had greater nuclear expression of

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Table 1

<table>
<thead>
<tr>
<th>Maternal and neonatal characteristics.</th>
<th>Female</th>
<th>Male</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Asthma</td>
</tr>
<tr>
<td>(n)</td>
<td>29</td>
<td>41</td>
</tr>
<tr>
<td>Age (yrs)</td>
<td>26.1</td>
<td>25.6</td>
</tr>
<tr>
<td>SEM</td>
<td>0.6</td>
<td>0.75</td>
</tr>
<tr>
<td>BMI</td>
<td>25.7</td>
<td>29.3^a</td>
</tr>
<tr>
<td>SEM</td>
<td>0.5</td>
<td>1.1</td>
</tr>
<tr>
<td>Gravidity</td>
<td>2.3</td>
<td>2.03</td>
</tr>
<tr>
<td>SEM</td>
<td>0.29</td>
<td>0.21</td>
</tr>
<tr>
<td>Parity</td>
<td>0.76</td>
<td>0.58</td>
</tr>
<tr>
<td>SEM</td>
<td>0.16</td>
<td>0.11</td>
</tr>
<tr>
<td>1CS dose (ug/mL/day)</td>
<td>180.3^a</td>
<td>0</td>
</tr>
<tr>
<td>SEM</td>
<td>7.12</td>
<td>0</td>
</tr>
<tr>
<td>Smokers (%)</td>
<td>14</td>
<td>46</td>
</tr>
<tr>
<td>Gestational age (days)</td>
<td>277.3</td>
<td>276.9</td>
</tr>
<tr>
<td>SEM</td>
<td>1.8</td>
<td>1.7</td>
</tr>
<tr>
<td>Birthweight (g)</td>
<td>3405.4</td>
<td>3400.4</td>
</tr>
<tr>
<td>SEM</td>
<td>90.9</td>
<td>85.8</td>
</tr>
<tr>
<td>Birthweight centile</td>
<td>45.8</td>
<td>43.5</td>
</tr>
<tr>
<td>SEM</td>
<td>5.27</td>
<td>4.61</td>
</tr>
<tr>
<td>SGA (&gt;10th BWC, %)</td>
<td>10.3</td>
<td>12.5</td>
</tr>
<tr>
<td>SEM</td>
<td>50.6</td>
<td>600.4</td>
</tr>
<tr>
<td>Placenta weight (g)</td>
<td>12.3</td>
<td>25.7</td>
</tr>
<tr>
<td>SEM</td>
<td>1.8</td>
<td>1.9</td>
</tr>
<tr>
<td>Cord cortisol (nmol/L)</td>
<td>0.19</td>
<td>0.09</td>
</tr>
<tr>
<td>SEM</td>
<td>18</td>
<td>25</td>
</tr>
</tbody>
</table>

Ages expressed as medians (interquartile range). Values are means SEM.

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^a P < 0.05; SEM — standard error of the mean; BMI — body mass index; ICS — inhaled corticosteroid; FEVI: FVC = forced expiratory volume at 1 s relative to forced vital capacity; SGA — small for gestational age.
68 kDa \( (P = 0.03, n = 24, \text{Table 2}) \) and GR-A (65 kDa) \( (P = 0.04, n = 24, \text{Table 2}) \) relative to cytoplasmic expression of these GR isoforms. In the presence of maternal asthma, placentae of male pregnancies had greater nuclear expression of GRz (94 kDa) relative to cytoplasmic expression of the GRz \( (P = 0.003, n = 24, \text{Table 2}) \).

In placentae of control females nuclear expression of GRz (94 kDa) \( (P = 0.03, n = 29, \text{Table 2}) \) and unknown 60 kDa \( (P = 0.03, n = 29, \text{Table 2}) \) were greater than cytoplasmic expression. In placentae of females whose mothers had asthma, nuclear expression of GRz (94 kDa) \( (P = 0.02, n = 41, \text{Table 2}) \) and GRz D1 (55 kDa) \( (P = 0.04, n = 41, \text{Table 2}) \) was greater than cytoplasmic expression.

### Table 2

<table>
<thead>
<tr>
<th>Using GR( \beta ) Ab</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>% Detected</td>
<td>Asthma</td>
</tr>
<tr>
<td>GR( \beta )</td>
<td>0.24 (0.16–0.63)</td>
<td>87.5</td>
</tr>
<tr>
<td>Nucleus</td>
<td>0.33 (0.15–1.4)</td>
<td>87.5</td>
</tr>
<tr>
<td>Using GR total Ab</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GRz</td>
<td>0.05 (0–0.165)</td>
<td>58.3</td>
</tr>
<tr>
<td>Nucleus</td>
<td>0.1 (0–0.365)*</td>
<td>70.8</td>
</tr>
<tr>
<td>GR( \beta )</td>
<td>0 (0–0.08)</td>
<td>37.5</td>
</tr>
<tr>
<td>Nucleus</td>
<td>0 (0–0)</td>
<td>16.7</td>
</tr>
<tr>
<td>GRz-C</td>
<td>0 (0–0.04)</td>
<td>29.2</td>
</tr>
<tr>
<td>Nucleus</td>
<td>0 (0–0)</td>
<td>20.8</td>
</tr>
<tr>
<td>GR P</td>
<td>0 (0–0.03)</td>
<td>25.0</td>
</tr>
<tr>
<td>Nucleus</td>
<td>0 (0–0.12)</td>
<td>41.7</td>
</tr>
<tr>
<td>69</td>
<td>0 (0–0)</td>
<td>4.2</td>
</tr>
<tr>
<td>Nucleus</td>
<td>0 (0–0)</td>
<td>12.5</td>
</tr>
<tr>
<td>68</td>
<td>0 (0–0.4)</td>
<td>41.7</td>
</tr>
<tr>
<td>Nucleus</td>
<td>0 (0–0.00)*</td>
<td>33.3</td>
</tr>
<tr>
<td>GR-A</td>
<td>0 (0–0.28)</td>
<td>33.3</td>
</tr>
<tr>
<td>Nucleus</td>
<td>0.33 (0–1.415)*</td>
<td>50.0</td>
</tr>
<tr>
<td>60</td>
<td>0.03 (0–0.335)</td>
<td>50.0</td>
</tr>
<tr>
<td>Nucleus</td>
<td>0.00 (0–0.295)</td>
<td>37.5</td>
</tr>
<tr>
<td>GRz-D1</td>
<td>0.12 (0–0.62)</td>
<td>70.8</td>
</tr>
<tr>
<td>Nucleus</td>
<td>0.19 (0–0.89)</td>
<td>70.8</td>
</tr>
<tr>
<td>GRz-D2</td>
<td>0.79 (0.22–1.46)</td>
<td>79.20</td>
</tr>
<tr>
<td>Nucleus</td>
<td>1.14 (0–2.835)</td>
<td>70.80</td>
</tr>
<tr>
<td>GRz-D3</td>
<td>0 (0–0)</td>
<td>20.80</td>
</tr>
<tr>
<td>Nucleus</td>
<td>0.22 (0–0.145)</td>
<td>33.30</td>
</tr>
<tr>
<td>38</td>
<td>0.74 (0–3.29)</td>
<td>66.70</td>
</tr>
<tr>
<td>Nucleus</td>
<td>0.89 (0–3.085)</td>
<td>62.50</td>
</tr>
</tbody>
</table>

*Post-hoc analysis showing significantly altered factor \( P < 0.05\); GR = glucocorticoid receptor; % detected = number of subjects with GR isoform present; values are expressed as median GR protein relative to \( \beta \)-actin and interquartile range.
3.6. Effect of maternal asthma severity and fetal sex on GR isoform expression and localization

In the presence of mild asthma there was increased expression of cytoplasmic GRαD3 (50 kDa) in male (0 (0.4–1.75), n = 8) and female placentae (0.8 (0.4–1.6), n = 13) relative to the other groups (Control male: 0 (0–0), n = 24; Remission 0.1 (0–0.3) n = 3; Intermittent: 0 (0–0), n = 21; Moderate-severe male: 0.0 (0–0) n = 8; Control female 0 (0–0) n = 29; Remission 0 (0–0) n = 4; Intermittent: 0 (0–0); n = 15 Moderate-severe female 0 (0–0) n = 9). Nuclear expression of GRαD3 (50 kDa) was significantly increased in female placentae in the presence of mild asthma (Control female 0 (0–0.2) n = 29; Remission 0 (0–0); n = 4 Intermittent: 0 (0–0.02); n = 15 Mild: 1.1 (0.5–1.8), n = 13; Moderate-severe female 0 (0–0) n = 9). In male placentae of pregnancies complicated by mild asthma, cytoplasmic GRβ was significantly increased (Control male: 0.2 (0.16–0.6), n = 24; Remission 0.15 (0.05–0.2) n = 3; Intermittent: 0.3 (0.12–1.7), n = 21; Mild 0.5 (0.2–0.8) n = 8; Moderate-severe male: 0.3 (0.1–0.5) n = 8). Asthmatic women pregnant with a female who had well controlled asthma (n = 25) had higher cytoplasmic expression of GRαC (81 kDa) than asthmatic women who had uncontrolled asthma (n = 23) during pregnancy (Controlled 0.09 (0–0.9) vs Uncontrolled 0.05 (0–0.13)).

3.7. Effect of maternal asthma and its treatment and fetal sex on GR isoform expression and localization

The use of inhaled corticosteroids for the treatment of asthma was associated with decreased placental nuclear expression of GRβ relative to cytoplasmic expression in females (nuclear: 0.33 (0–1.1) vs cytoplasmic 0.2 (0–0.61) P = 0.03, n = 15). No other GR isoforms were altered by asthma treatments.

3.8. Cord blood cortisol and its relationship to GR isoform expression

There were no relationships between cord blood cortisol concentrations and GR protein expression in female placentae (n = 43).
In male placentae nuclear expression of GRβ was positively correlated with cord blood cortisol ($R^2 = 0.43$, $P = 0.02$, $n = 29$).

3.9. Effect of birthweight, placental weight and labour on GR isoform expression

There were no associations of placental GR expression in relation to birthweight centile (BWC) overall. When placentae were split by appropriate for gestational age (AGA) (>10th BWC, $n = 118$) and small for gestational age (SGA) (<10th BWC, $n = 15$); cytoplasmic expression of GRβ was higher (SGA: 1.5 (0.3–2.2) vs AGA: 0.3 (0.13–0.6) $P = 0.005$) and nuclear expression of GRβ was higher (SGA: 0.8 (0.2–1.7) vs AGA 0.42 (0.2–0.9), $P = 0.04$) in SGA pregnancies relative to AGA pregnancies. Nuclear 48 kDa protein expression was significantly lower in SGA placentae (SGA: 0 (0–0) vs AGA 0 (0–2.6), $P = 0.02$). Nuclear expression of GRα-D1 was significantly higher (SGA: 0.9 (0.04–12.9) vs AGA 0.2 (0–0.6) $P = 0.001$) in SGA placentae. Nuclear and cytoplasmic expression of unknown 38 kDa isoform was significantly increased (nuclear: SGA: 1.9 (0.01–3.8) vs AGA 0.4 (0–1.5), $P = 0.02$ and cytoplasmic: SGA: 0.6 (0–1.8) vs AGA 0.3 (0–1.9) $P = 0.0001$) in SGA placentae.

There was no relationship between placental weight ($n = 114$) or mode of delivery (vaginal delivery ($n = 89$), elective Caesarean ($n = 18$), emergency Caesarean ($n = 25$) on placental GR isoform expression.

3.10. Phosphorylation of GRα at serine 211 and 226

Placental extracts from pregnancies complicated with asthma and normal pregnancies were analysed for the expression of GRα serine 211 (Fig. 6 right panel) and serine 226 (Fig. 6 left panel). GRα was phosphorylated at serine 211 and 226 in both males ($n = 64$) and females ($n = 70$). When GRα was phosphorylated at serine 211 we observed localisation to the nucleus ($n = 134$), and when phosphorylated at serine 226 ($n = 134$) localisation was predominantly restricted to the cytoplasm of whole human placental extracts (Fig. 6). There was no effect of sex on phosphorylation status of GRα. There was greater phosphorylation of cytoplasmic GRα at serine 226 ($P = 0.027$) in the presence of maternal asthma (0.16 (0.08–0.3), $n = 82$) relative to the non-asthmatic population (0.1 (0.06–0.2) $n = 53$) regardless of sex. There was greater phosphorylation of cytoplasmic GRα at serine 226 in placentae from SGA pregnancies relative to AGA pregnancies (SGA: 0.2 (0.1–1.0) vs AGA 0.15 (0.08–0.25) $P = 0.0001$, respectively).

4. Discussion

This is the first study to identify the presence of several protein isoforms of the GR in the human placenta. These isoforms are cell specific with 12 isoforms present in the trophoblast, 5 isoforms present in the endothelium and 4 isoforms identified in the cord blood immune cells. The data shows that GR isoform expression varies with cell type, cellular location, and can be altered by the presence of maternal asthma, growth restriction or fetal sex. We have consistently observed a sex difference in cortisol-regulated pathways of the placenta and a difference in birthweight outcomes in association cord blood cortisol concentrations suggesting that males are glucocorticoid resistant and females are hypersensitive to glucocorticoids in the presence of high concentrations of cortisol. Based on these current findings, we propose that males are glucocorticoid resistant in a high cortisol environment due to the
dominant negative effects of GRβ, and possibly through GR A and GR P localisation to the nucleus. Females may be more sensitive to glucocorticoids through an interaction of GRα A with GRα C and GRα-D3.

The different isoforms of the GR are derived from a single gene and produced by alternate splicing or alternate translation initiation. The GR gene has multiple start codons present in its N terminal region at residues 1, 27, 86, 90, 98, 316, 330 and 336 which allows ribosomes to initiate translation at different sites along the mRNA expression in the human placenta [24]; however, in the current study, positive staining for GRβ protein was localised to the syncytiotrophoblast, endothelium and villous macrophages using immunohistochemistry, and confirmed expression in the trophoblast and endothelium. GRβ does not bind cortisol but exerts its antagonistic effect by binding to GRE sites on the target genes and blocking the action of GRα [23]. Many studies have reported that high levels of GRβ confer

Western are GR proteins. However these unknown proteins will need to be sequenced before they can be confirmed as GR proteins and more gene silencing experiments are required. Given the placenta has a diverse range of activities during pregnancy, it is not surprising there may be many different isoforms of the GR, including some that are yet to be characterised.

GRβ predominates in the nucleus and is expressed abundantly in specific cell types including epithelial cells of the lung, thymus and liver [23]. The presence of GRβ in the placenta and reproductive tissues has been controversial with numerous studies reporting low expression. We have previously reported low GRβ mRNA expression in the human placenta [24]; however, in the current study, positive staining for GRβ protein was localised to the syncytiotrophoblast, endothelium and villous macrophages using immunohistochemistry, and confirmed expression in the trophoblast and endothelium. GRβ does not bind cortisol but exerts its antagonistic effect by binding to GRE sites on the promoter of target genes and blocking the action of GRα [23]. Many studies have reported that high levels of GRβ confer

Fig. 4. Percentage expression of GR isoforms. Percentage was calculated as the individual GR isoform expression divided by total expression which was calculated by the sum of each densitometric measure of each GR isoform relative to β-actin. Values are a mean of all individuals (n = 134) separated by fetal sex and asthma. Panel A represents % expression in the cytoplasm and Panel B represents % expression in the nucleus.

Fig. 5. Identification of GR isoforms in different cell types of the human placenta. Cytoplasmic and nuclear extracts of whole placental tissue (n = 3), isolated placental trophoblast (n = 3), BeWo trophoblast cells (n = 3), umbilical vein endothelial cells (HUVECS) (n = 3) and cord blood mononuclear cells (CBMNC) (n = 3) were exposed to total GR antibody on Western blot to determine which GR isoforms are expressed in each cell type. Whole placenta, isolated trophoblast and BeWo cells had 12 GR isoforms detectable in cytoplasmic and nuclear extracts. HUVECs had 4 isoforms and CBMNCs had 5 GR isoforms.
glucocorticoid insensitivity or resistance [25–29] and low levels are associated with glucocorticoid sensitivity [25,29]. In particular, increased expression of GRβ in the airway epithelium, airway T cells and circulating monocytes are associated with glucocorticoid resistant asthma [28]. We have identified that the localisation of GRβ to the nucleus increases in male placentae in association with a rise in cord blood cortisol. GRβ protein expression is increased in the cytoplasm of male placentae from pregnancies complicated by asthma and in the cytoplasm and nucleus of placentae of SGA pregnancies. These data suggest GRβ has a role in modulating the response to cortisol in the human placenta, especially in the presence of a male fetus or an SGA fetus.

Our previous work had identified that female placentae of pregnancies complicated by asthma were more sensitive to cortisol induced inhibition of cytokine production in vitro [4]. This increased sensitivity was associated with no change in GR protein expression [4] suggesting sensitivity was not conferred by increased concentrations of receptor. Our current data suggests a number of GR isoforms may play a role in conferring greater placental sensitivity to cortisol in the presence of a female fetus and maternal asthma. These include decreased nuclear expression of GRβ, increased expression of GRα D3 and increased cytoplasmic expression of GRα C. The exact interaction between these isoforms will need to be clarified in future studies; however, previous work has shown GRα C has greater transcriptional activity than GRα A, B and D isoforms and can enhance the activity of GRα when co-expressed in COS-1 cells in vitro [10]. GRα D3 can activate glucocorticoid regulated pathways in the absence of agonist but its transactivation activity is much lower than the other GRα isoforms in vitro [10]. The co-expression of GRα C3 and GRα D3 may be a mechanism to enhance the immunosuppressive effects of glucocorticoids and modulate glucocorticoid-induced apoptosis in female placentae.

Fundamental to GRα A activity is its phosphorylation status. GRα A can still function in the absence of phosphorylation but its activity is not as potent. In order for GRα A to translocate to the nucleus it must be phosphorylated at serine 211. We identified there was no effect of sex or asthma on this fundamental process. However, the movement of GRα A out of the nucleus requires phosphorylation at serine 226 and our data suggests that maternal asthma is associated with an increase of serine 226 phosphorylation. This may be another mechanism involved in altering the response to cortisol in pregnancies complicated by asthma through translocation of GRα A out of the nucleus.

The data generated from this work provides important insight into understanding the sex specific differences in placental glucocorticoid sensitivity. Based on this data we propose in the presence of increased exposure to cortisol males induce a state of glucocorticoid resistance through increased expression and localisation of GRβ in the nucleus which then exerts a dominant negative effect on GRα A activity. This state of resistance may also be modulated by the co-localisation of GR A and GR P to the nucleus of male placental trophoblast. Female placentae become sensitive to cortisol in the presence of maternal asthma through a decrease in GRβ and an enhancement GRα A activity through an interaction with GRα D3 and GRα C. Further studies are planned to examine this model. The current data implies there is a significant level of complexity in the placental response to cortisol which may be modified by an individual’s GR proteomic profile and in response to the maternal environment.

Disclosure statement

The authors have nothing to disclose.

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References


