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Expression of Glucocorticoid Receptor in Human Myometrium during Pregnancy and Labour

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Abstract

Inflammatory events have been implicated in the process of labour. Glucocorticoids mediate strong anti-inflammatory effects through binding to the glucocorticoid receptor (GR), which on activation translocates to the nucleus and increases or decreases the expression of responsive genes thereby suppressing inflammation. We characterised the expression profile of GR protein and mRNA in human non-pregnant (n=10), term pregnant non-labouring (n=10) and labouring (n=10) myometrium, as well as in first trimester, second trimester and preterm labouring myometrium (n=5-10), by Western blotting, immunofluorescent staining, and RT-PCR. GR-α and GR-β protein were detected in all samples. Compared to non-pregnant myometrium, levels of both isoforms were lower in pregnant myometrium ($p<0.05$ for GR-α, $p<0.01$ for GR-β). No differences were found between labouring vs. non-labouring and upper vs. lower segment myometrium. While immunofluorescent staining for GR was predominantly nuclear in non-pregnant myometrium, staining was more evenly distributed across the nuclei and cytoplasmic compartments in term pregnant samples. Compared to non-pregnant and term pregnant myometrium, levels of GR-α mRNA were lower in term labouring myometrium ($p<0.001$ ad $p<0.01$ respectively). In contrast, GR-β mRNA was only detected in a small number of myometrial samples. It is possible that the observed alterations in GR expression may contribute to the sequence of inflammatory events implicated in parturition.

Keywords: Glucocorticoid receptor; Human; Labour; Myometrium; Pregnancy

Introduction

During pregnancy maternal and fetal tissues are exposed to increasing concentrations of the major physiological glucocorticoid, cortisol produced by the adrenal cortex [1]. In addition to being an important regulator of metabolic, stress and inflammatory pathways, cortisol plays a critical role in the development and maturation of a variety of fetal organs including the lungs [2]. The prelabour increase in cortisol is also thought to prepare uterine tissues for labour by upregulating corticotrophin-releasing hormone and prostaglandins [3,4].

The biological actions of glucocorticoids are typically mediated by the intracellular glucocorticoid receptor (GR), a member of the nuclear hormone receptor superfamily [5]. The human GR gene contains 10 exons and is located on chromosome 5q31-32 [6], the last two exons comprise of exon 9α and 9β which are separated by intron J (Figure 1). Multiple GR isoforms are generated as a result of alternative splicing, the use of alternative promoters and by post-translational modifications [7-9] GR-α and GR-β are two protein isoforms that differ at their carboxy terminal as a result from alternate splicing events involving exon inclusion or exclusion of exon 9 (Figure 1). GR-α and GR-β have molecular weights of about 94 and 91 kDa, respectively [10]. GR-α is the classic GR that functions as a ligand-dependent transcription factor. It is located in the cytoplasm in complex with chaperonic molecules composed of heat shock proteins 90, 70 and 50, immunophilins as well as other proteins [8]. Upon binding to glucocorticoid, GR undergoes conformational change, dissociates from this multi-protein complex and translocates into the nucleus. Within the nucleus the receptor generally binds as a homodimer to glucocorticoid-response elements (GREs) in the promoter region of target genes, and regulates their expression positively or negatively, depending on GRE sequence and promoter context [5]. Four main functional GR-α isoforms exist (GR-A, GR-B, GR-C, and GR-D) alternatively translated from different start codons (placed in the N-terminal region) [11]. Fifteen mRNA spliced variants generated by the actions of multiple promoters linked to untranslated exon 1 regions have additionally been described [12]. It is likely that the different spliced variants mediate different biological effects of glucocorticoids. The β isoform is unable to bind hormone due to a change in the ligand-binding domain. It is located in the nucleus even in the absence of ligand and is thought to act as an inhibitory (dominant-negative) form of the α isofrom [13].

The progesterone receptor (PR) belongs to the same nuclear receptor subfamily as the GR. The receptors are structurally homologous and progesterone is able to bind to the GR [14]. Progesterone is thought to maintain human pregnancy through the activation of genes that promote quiescence and by preventing nuclear factor kappa-B (NFκ-B) activation of contractile genes, such as cyclooxygenase-2 (COX-2) [15,16] Progesterone has also been shown to reduce the risk of recurrent preterm birth [17]. In this context, it is unclear to what extent progesterone's immunomodulatory and anti-inflammatory properties are mediated through binding to the PR and GR [14,18].

Human endometrium, decidua, placenta, membranes and cervix are known to express GR [19-22]. Johnson et al. [23] recently demonstrated that human term placenta expresses a variety of GR mRNA transcripts although GR-α mRNA transcribed from the IC promoter generated the majority of placental GR. In contrast, very little is known about GR

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expression in human myometrium during pregnancy. Giannopoulos et al. [24] demonstrated [3H] dexamethasone binding to cytosolic fractions of human non-pregnant myometrium with a reduced concentration of binding sites in term myometrium. Animal studies have generally confirmed GR expression in non-pregnant and pregnant myometrium [25,26] but it is less clear whether expression is temporally regulated during gestation; Korgun et al. [27] found no change in immunohistochemical expression of GR between days 1-9 of rat gestation while Herman-Gnjidic et al. reported a increase in cytosolic GR levels between 1 and 5 days postpartum in the same species [28].

The purpose of this study was therefore to determine, using RT-PCR, Western blotting and immunofluorescence staining, whether myometrial expression of GR was altered during pregnancy. Specifically we tested the hypotheses that GR-α and GR-β mRNA and protein levels were temporally and/or spatially regulated during pregnancy.

Materials and Methods

Selection of patients and tissue collection

All women were recruited from the Department of Obstetrics and Gynaecology at the Royal Victoria Infirmary, Newcastle-upon-Tyne. The study received approval from the Newcastle and North Tyneside Health Authority Ethics Committee, and all patients gave written, informed consent.

Non-pregnant myometrium

Myometrial biopsies were obtained from non-pregnant (NP) premenopausal women (n=10, aged 32-46 years) undergoing a hysterectomy for a benign gynaecological condition. Patients with uterine leiomyoma were excluded from the study. Non-pregnant uteri were sampled from the lower corpus well clear of the cervix with myometrium being taken from the middle of the uterine wall, allowing generous clearance margins from the serosal and endometrial surfaces. Samples obtained in both the follicular and luteal phase of the cycle were used.

First and second trimester myometrium

Samples of myometrium were obtained from patients undergoing elective first trimester (n=10**,** gestation 10-13 weeks, median 11 weeks) or second trimester termination of pregnancy (n=10**,** gestation 14-20 weeks, median 17 weeks) under general anaesthesia. Samples from the upper corpus of the uterus were taken after evacuation of the uterus using biopsy forceps (Richard Wolf Endoscopes, Wimbledon, UK). Under ultrasound guidance, the forceps were introduced into the myometrium and a biopsy taken, avoiding the site of the placental bed as determined by ultrasound before termination.

Term pregnant myometrium

Upper and lower segment myometrial samples were obtained from healthy women undergoing elective caesarean section at term (P) under either regional or general anaesthesia (n=10**,** age 16-43 years, gestation 37-42 weeks, median 40 weeks). The samples were obtained immediately following delivery of the placenta and membranes before the closure of the uterine cavity. Samples from the upper segment were taken under direct vision using biopsy forceps (Richard Wolf Endoscopes, Wimbledon, UK) introduced into the uterine cavity through the incision as described previously [29]. The forceps were pushed through the decidual layer and into the myometrium. The biopsy was taken from a non-placental bed site, as determined by manual palpation before the delivery of the placenta. Samples from the lower uterine segment were excised using tooth forceps to grasp the myometrium and curved scissors to sample it.

Term and preterm labouring myometrium

Upper and lower segment myometrial samples were obtained from women admitted in spontaneous term labour (L) who required an emergency caesarean section for fetal distress or failure to progress (n=10**,** age 16-41 years**,** gestation 37-42 weeks, median 40 weeks). Spontaneous labour was defined as the onset of regular painful uterine contractions resulting in progressive dilatation of the cervix beyond 3 cm. Augmentation of labour with oxytocin was not a reason for exclusion. Upper and lower segment biopsies were obtained in a similar manner to those from women undergoing elective caesarean section. Upper and lower segment myometrial samples were also obtained from women in spontaneous preterm labour who required emergency caesarean section for fetal distress or failure to progress (n=5, age 21-33 years, gestation 28-34 week, median 31 weeks). Myometrial samples were snap-frozen using liquid nitrogen cooled isopentane and stored at –80°C.

Preparation of myometrial homogenates

All procedures were carried out on ice. Samples were homogenized in sucrose buffer consisting of 25 mmol/L Tris buffer (pH 7.6), 0.25 mol/L sucrose and 1 mmol/L ethylenediamine tetraacetate in the presence of pepstatin, leupeptin, aprotinin, and phenylmethylsulfonylfluoride (Sigma, St. Louis, MO). Homogenates were subsequently centrifuged at 1000 x g to remove tissue debris and the resultant supernatants stored at -80°C until use. The protein concentration was assayed in triplicate using the DC protein assay kit (Bio-Rad Laboratories Inc., Hercules, CA) with BSA as a protein standard.

Western blot immunodetection

SDS-PAGE was performed using 100μg myometrial homogenates solubilised in SDS-sample loading buffer (0.5 mol/l of Tris, 5 mol/l of urea, 2.5% sodium dodecyl sulphate (SDS) and 3.5% β-mercaptoethanol), resolved on 10% polyacrylamide gels at 175 V and electrotransferred onto nitrocellulose at 90 V for 90 min. Protein lysate prepared from cultures of non-pregnant myometrial cells treated with lipopolysaccharide was also included as a positive control. To check for equal loading and transfer efficiency, membranes were stained with Ponceau-S solution (0.5% in 1% acetic acid; Sigma, St. Louis, MO) before being placed in 5% non-fat milk in phosphate-buffered solution (PBS) containing 0.1% Tween 20 for 1 h at room temperature. Membranes were incubated with primary antibody, either a polyclonal anti-GR antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) diluted 1:2000 in PBS containing 5% non-fat milk and 0.1% Tween 20 or, the monoclonal anti-GR antibody (BD Biosciences, Franklin Lakes, NJ) diluted 1:2500 in PBS containing 5% BSA and 0.1% Tween 20 overnight at 4°C on a rocking platform. After three 10 min washes in PBS, the membranes were incubated for 1 h at room temperature with goat anti-rabbit IgG, or goat anti-mouse IgG coupled to horseradish peroxidise (DAKO Corp., Cambridgeshire, UK), as appropriate, at a 1:3000 dilution in PBS. Detection was carried out using an enhanced chemiluminescence (ECL) assay system (Amersham Biosciences, Piscataway, NJ). All membranes were reprobed with an anti-GAPDH antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA), diluted 1:3000 in PBS, for 1 h, as an additional control to confirm equal protein loading. Data were obtained under conditions where a linear relationship existed between the amount of protein loaded and the intensity of the ECL signal from the immunoblots. ECL signals were

quantified by scanning densitometry using a UMAX PS 2400 scanner at 600 dpi coupled to the intelligent quantifier software package from Bio Image Systems Inc. (Jackson, MI).

1st strand cDNA synthesis

Total RNA was extracted from individual myometrial samples (NP=10, 1st Trimester=10, 2nd Trimester=10, P=10 and L=6 samples) using SV total isolation kits (Promega Corp., Madison, WI) according to the manufacturer's protocol. The concentration of RNA was determined by UV spectrophotometry at 260 nm. First strand cDNA was transcribed from 1µg of RNA, using 1 µl oligo(dT) primer (Invitrogen Ltd., Paisley, UK), 2µl of 10 mM dNTPs (Invitrogen Ltd., Paisley, UK), 10µl first-strand reaction buffer (Invitrogen Ltd., Paisley, UK), 1µl of 0.1 M DTT (Invitrogen Ltd., Paisley, UK), 1µl RNase inhibitor (Invitrogen Ltd; Paisley, UK), 1µl Superscript reverse transcriptase (Invitrogen Ltd., Paisley, UK) and nuclease-free water to make up the total volume to 50µl. The reaction mixture was incubated at 50°C for 60 min.

Conventional RT-PCR

To assess the relative endogenous levels of the GR-α and GR-β isoform transcripts in myometrial tissue, RT-PCR was performed. The sense and anti-sense oligonucleotide primers used in this study were derived from published sequences [6,25,27,28]. Further details can be found in Figure 1 and in Table 1. To analyse GR-α and GR-β mRNA levels PCR amplifications were performed using 4µl cDNA, corresponding to 80 ng total RNA. The reactions contained 12.5µl of PCR Master Mix (Promega Corp., Madison, WI), 0.5µM of each primer (forward and backward) and nuclease-free water in a final volume of 25µl. PCR was carried out under standard conditions with an initial denaturation at 94°C for 4 min, followed by 26 cycles of denaturation at 94°C for 1 min; annealing at 55°C for 30 sec (GR-α) or 54°C for 30 sec (GR-β); extension at 72°C for 1 min; with an additional 5 min at 72°C for the final extension. All reactions were terminated in the exponential phase to allow semi-quantitative analyses. PCR reactions using primers for

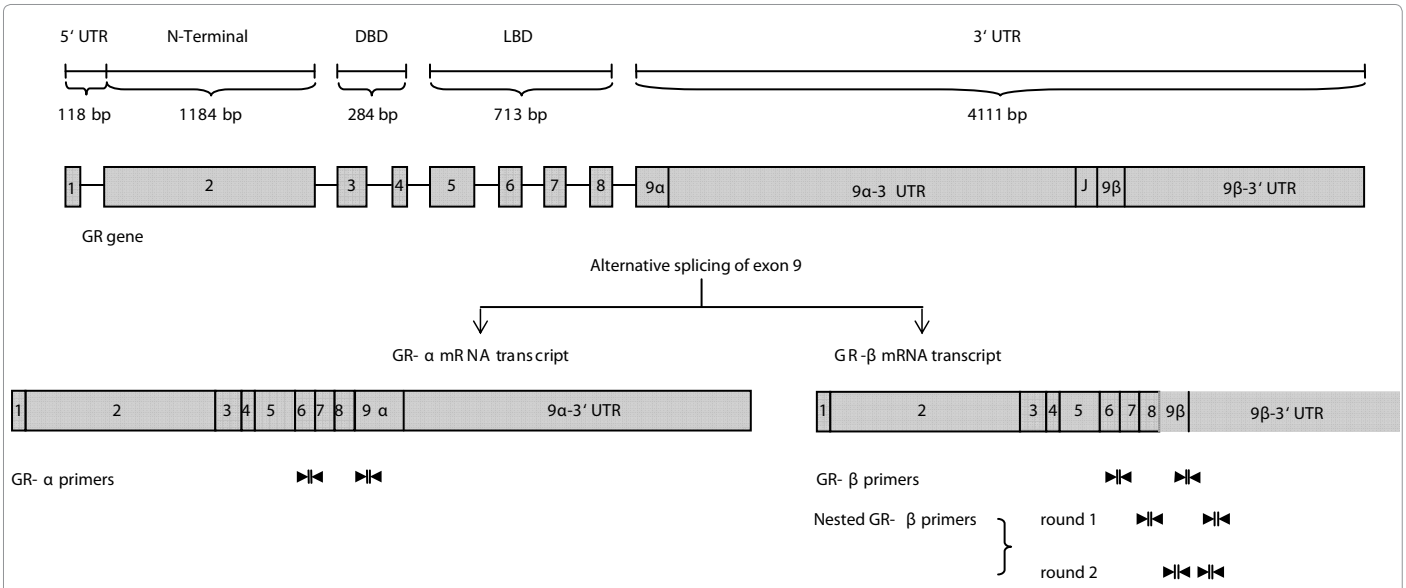

Figure 1: Organisation map of the human glucocorticoid receptor (GR) gene (NR3C1) and gene products (Gen Bank Accession Number NM_001018076). The human GR gene consists of nine exons (designated by the numbered boxes) coding various regions of the receptor. Exon 1 and the first 13 nucleotides of exon 2 encode the 5' untranslated region (5' UTR), with the remainder of exon 2 coding the N-terminal region. Exon 3 and 4 encode the first and second zinc-finger motifs respectively in the DNA binding domain (DBD). The ligand binding domain (LBD) is coded for by the next four exons, with exon 9 containing coding sequences for the 3' untranslated region (3' UTR). Alternative splicing of exon 9 generates two different transcripts: GR-α mRNA contains exon 9α and GR-β mRNA contains exon GR-β. The location of the GR-α and GR-β specific primers used in this study are indicated by the following symbol (▶|◀).

		Sequence	Nucleotide Position	PCR Product Size
Detection Primers				
GR-α	(FW)	5′-TGTTTTGCTCCTGATCTGA-3′	1982 to 2000	~389bp
	(BW)	5′-TCGGGGAATTCAATACTCA-3′	2352 to 2370	
GR-β	(FW)	5′-TGTTTTGCTCCTGATCTGA-3′	1996 to 2014	~383bp
	(BW)	5′-TGAGCCCAAGATTGT-3′	2373 to 2388	
Nested GR-β Primers				
Round 1	(FW)	5′-CCTAAGGACGGTCTGAAGAGC-3′	2144 to 2164	~366bp
	(BW)	5′-CCACGTATCCTAAAAGGGCAC-3′	5119 to 5139	
Round 2	(FW)	5′-AGCACATCTCACACATTAAT-3′	4954 to 4973	~134bp
	(BW)	5′-TATAGTTGTCGATGAGCATC-3′	5071 to 5087	
Housekeeping Gene Primers				
GAPDH	(FW)	5′- CTGCCGTCTAGAAAAACC-3′	840 to 857	~220bp
	(BW)	5′- CCAAATTCGTTGTCATACC-3′	1039 to 1058	

FW, forward; BW, backward

GR primer location according to GenBank accession number NM_001018076 sequence; GAPDH according to GenBank accession number sequence NM_002046

Table 1: Primers used for semi-quantitative RT-PCR.

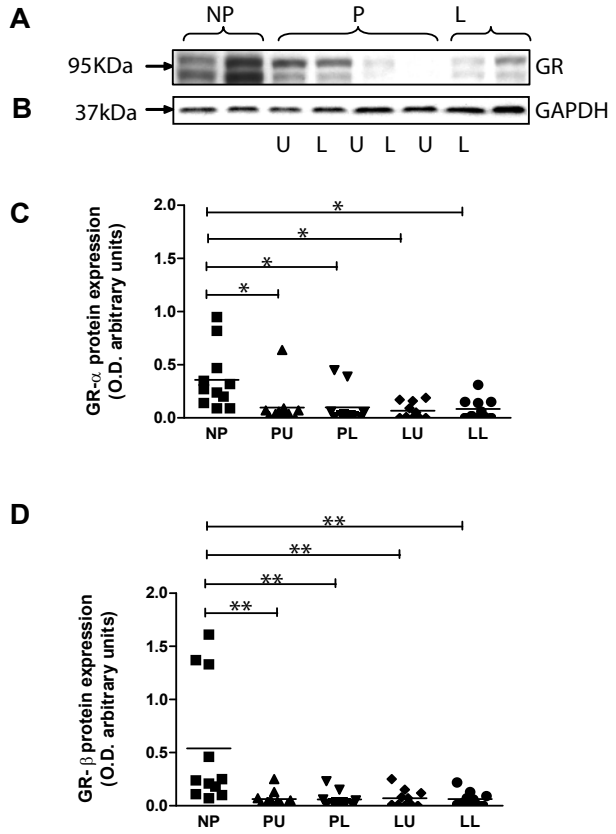

Figure 2: Expression of Glucocorticoid receptor (GR) protein in human myometrium. Immunodetection of GR protein expression in non-pregnant (NP), uterine upper (U) and lower (L) segments from term non-labouring (P) and term spontaneously labouring (L) human myometrium. Tissue homogenates were resolved by SDS-PAGE and the proteins detected using the BD laboratories anti-GR antibody. Two protein bands at 95 KDa (GR-α) and 90KDa (GR-β) were detected. A representative immunoblot is presented in **A**. To ensure equal lane loading and transfer efficiency during Western blotting, the membranes were stained with Ponceau-S solution (not shown) and later re-probed with anti-GAPDH as an additional control. A representative control blot is shown in **B**. Immunodetected bands were quantified by scanning densitometric analysis; densitometry graphs for GR protein are shown in **C**. All samples are plotted within the allotted patient groups. The bars represent means. (GR-α protein: NP vs. PU/PL/LU/LL P<0.05; GR-β protein: NP vs. PU/PL/LU/LL P<0.01). The results are representative of the samples being used in three independent experiments. **D.** Western blot analysis of NP tissue homogenate resolved by SDS-PAGE with (+) and without (–) shrimp alkaline phosphatase (SAP) pre-treatment, with the control shown alongside.

GAPDH or no cDNA template were included as positive and negative controls, respectively. PCR products were resolved by electrophoresis on 2% Tris-acetate-EDTA agarose gels containing ethidium bromide, visualised under ultraviolet light exposure, and photographed. Band intensity was evaluated by densitometric scanning using a UMAX PS 2400 scanner at 600 dpi coupled to the intelligent quantifier software package from Bio Image Systems Inc. (Jackson, MI).The results are expressed as mean ± SEM.

Nested PCR

Nested PCR was additionally used to amplify GR-β because of the reported rare abundance of GR-β mRNA. PCR conditions were adapted from that described by Gagliardo [30]. Briefly, initial denaturation was for 3 min at 95°C; one PCR cycle consisted of denaturation at 95°C for 30 sec; annealing at 55°C for 30 sec; and extension at 72°C for 30 sec; with an additional 10 min at 72°C for the final extension. The first round of PCR amplification consisted of 40 cycles with 0.5μM of each outer primer and 2μl of cDNA from the reverse transcription reaction together with the same reagents as described above. Second round synthesis comprised of 26 cycles with 0.5μM of each inner primer and 4μl of the PCR product generated from the first round amplification as template. The amplified DNA fragments were detected as previously described.

Immunofluorescent staining

Formalin-fixed, paraffin-embedded specimens were cut into 3μm sections, mounted on 3-amino-propytriethoxy-silane (Sigma, St. Louis, MO) pre-treated slides, air dried and stored at room temperature until use. The sections were dewaxed in xylene, and then rehydrated in a graded alcohol series (99%, 95%, 70%, H_2O). Epitope retrieval was by pressure cooking in citrate buffer (pH 6.0) for 1 min. After rinsing in tris-buffered saline (TBS; pH 7.6), all tissue sections were exposed to a non-immune block of 10% goat serum in TBS for 30 min. Sections were then incubated with anti-GR antibody (BD Biosciences, Franklin Lakes, NJ) diluted 1:2000 in TBS containing 1% goat serum overnight at 4°C in a humidified chamber. For the negative controls, sections were incubated with an equivalent volume of TBS without Primary antibody. Primary antisera were then removed and after a further three rinses in TBS, the sections were incubated with FITC-conjugated affinity pure goat anti-mouse secondary antibody (Jackson Immunoresearch Lab. Inc., West Grove, PA) diluted 1:100 in TBS for 30 min at room temperature. Slides were mounted with 4',6-diamidino-2-phenylindole (DAPI) mounting medium and then sealed with coverslips. Fluorescent staining was detected using a Leica TCS-SP2UV confocal laser scanning microscope (Leica Lasertechnik GmbH, Heidelberg, Germany) with Leica TCS-NT software.

Data were compared using one-way analysis of variance (ANOVA) followed by Bonferroni multiple comparisons post test with Prism 4 software (GraphPad Software Inc., San Diego, CA). Statistical significance was determined at $p < 0.05$.

Results

Expression profile for GR protein in human myometrium

Two different GR specific antibodies were initially tested in immunoblotting experiments both of which recognise the alpha (α) and beta (β) isoforms of human GR. Two distinct protein bands of 95kDa and 90kDa, representing GR-α and GR-β were detected using the monoclonal GR antibody therefore this antibody was selected for the study (Figure 2A). GAPDH expression was also included as a protein loading control (Figure 2B). The higher molecular weight GR-α protein band was highly expressed in non-pregnant samples, and decreased in pregnant (NP vs. PU/PL $p<0.05$) and labouring myometrial samples (NP vs. LU/LL $p<0.05$) (Figure 2C). There were no differences in protein expression between the upper and lower uterine segments and between the non-labouring and labouring groups. The level of lower molecular weight GR protein was also lower in the pregnant and labouring samples compared to non-pregnant myometrial samples (NP vs. PU/PL/LU/LL $p<0.01$) (Figure 2D). In an attempt to further corroborate the pattern of expression we also tested a specific GR-α antibody. However, this antibody failed to detect any protein, including a positive protein control (data not shown).

Pregnancy induced changes in GR expression in human myometrium

In light of the observed decrease in GR expression in pregnant

myometrium, we determined the relative expression levels in first trimester, second trimester, and preterm spontaneously labouring myometrium in order to ascertain when in pregnancy down-regulation of GR occurs (Figure 3). Although GR protein expression appeared to be reduced in first and second trimester myometrial samples, relative to non-pregnant control samples, the differences were not statistically significant. However there was a decrease in GR expression in the preterm labour samples (GR-α: NP vs. EPU/EPL $p<0.05$; GR-β: NP vs. EPU/EPL $p<0.05$).

Distribution and tissue subcellular localisation of GR protein within human myometrium by fluorescence confocal microscopy

The presence of GR protein in myometrial tissues was confirmed by immunofluorescent staining visualised by confocal microscopy. Expression of total GR was confined to the nuclei of non-pregnant myometrial cells with negligible cytoplasmic staining evident (Figure 4A). In contrast, in pregnant tissue sections, staining appeared to be more even distributed across the cytoplasmic and nuclear compartments. The expression of GR was similar in the upper (Figure 4B) and lower segment pregnant non-labouring sections (data not shown), providing further verification that GR is not spatially regulated within the myometrium during pregnancy. No staining above background was observed in the control sections when the primary antibody was omitted (data not shown).

GR-α and GR-β mRNA expression in human myometrium

Semi-quantitative RT-PCR was used to examine the expression profile of GR-α and GR-β mRNA in non-pregnant (n=10), first trimester (n=10), second trimester (n=10), term non-labouring (n=10) and term spontaneously labouring (n=10) myometrial samples, using GAPDH as a housekeeping gene (Figure 5). A 389bp product representing GR-α mRNA was detected in all the samples studied. The level of GR-α mRNA was lower in term labouring samples compared to both term pregnant (NP vs. PU/PL $p<0.01$) and non-pregnant myometrial samples (NP vs. LU/LL $p<0.001$) (Figure 5). All samples were equally loaded as determined by uniform expression of GAPDH. No product was obtained after PCR amplification of cDNA negative (water) controls. GR-β mRNA was detected in a small number of the myometrial samples. Nested PCR confirmed the presence of very low levels of GR-β message in some of the samples from each of the groups (data not shown).

Discussion

This is the first study to characterise the expression of GR protein and mRNA levels in myometrium during human pregnancy and labour. The results suggest that levels of GR-α protein fall dramatically during pregnancy, probably from as early as the first trimester. The changes in GR-α mRNA levels were broadly consistent with this pattern, although levels were lower in term labouring compared to non-labouring myometrium. Levels of myometrial GR-β protein also fell during pregnancy while mRNA was only detected in a small number of samples. We found no evidence of spatial regulation of GR within the myometrium.

Western blotting detected GR protein at the predicted molecular weights (95 and 90 KDa) using both a polyclonal and a monoclonal antibody preparation. Additional protein bands of less than 70 kDa were also detected, in keeping with other reports of GR expression in tissue homogenates [31]. It remains to be determined whether these lower molecular weight proteins are related to GR, as a result of post-translational modification or alternative RNA splicing. Both GR-α and GR-β protein expression fell during pregnancy, a finding confirmed by immunofluroescence, with no further change in labour. These findings are consistent with those of Giannopoulos et al. [24] who demonstrated that cytosolic fractions of human myometrium contain high affinity, low capacity binding sites for glucocorticoids and that the number of binding sites was higher in non-pregnant compared to pregnant

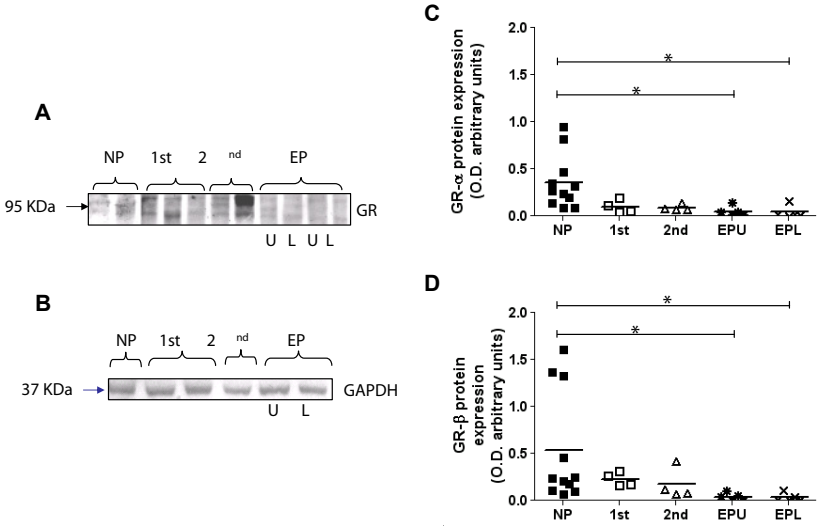

Figure 3: Pregnancy induced changes in GR expression in human myometrium. Immunodetection of GR protein expression in non-pregnant (NP), first trimester (1st), second trimester (2nd), and preterm labouring (EP) human myometrium. Upper (U) and lower (L) uterine segment samples were examined in the preterm labouring group. Tissue homogenates were resolved by SDS-PAGE and the proteins detected using the BD laboratories anti-GR antibody. Two protein bands at 95 KDa (GR-α) and 90KDa (GR-β) were detected. A representative immunoblot is presented in **A.** To ensure equal lane loading and transfer efficiency, membranes were stained with Ponceau-S solution (not shown) and later re-probed with anti-GAPDH as an additional control. A representative control blot is shown in **B.** Immunodetected bands were quantified by scanning densitometric analysis, and densitometry graphs for GR protein shown in **B**. All samples are plotted within the allotted patient groups. The bars represent means. (GR-α protein: NP vs. EPU/EPL $P<0.05$; GR-β protein: NP vs. EPU/EPL $P<0.05$). The results are representative of the samples being used in three independent experiments.

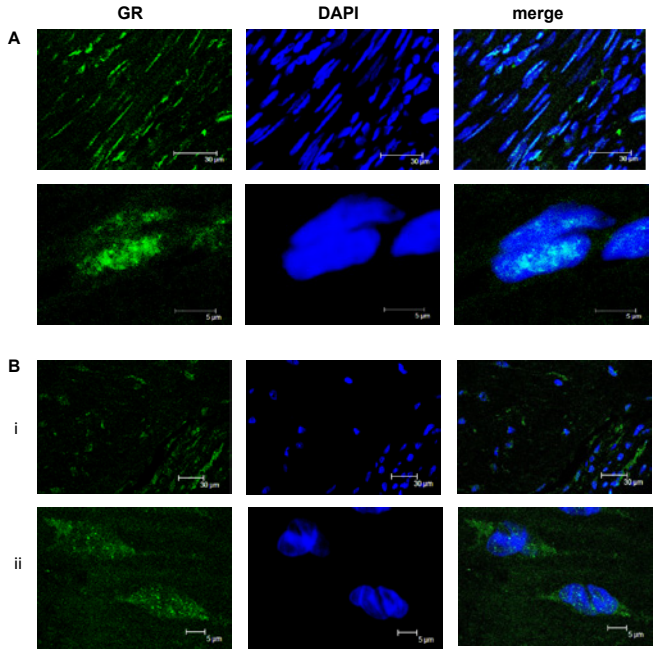

Figure 4: Distribution and tissue subcellular localisation of GR protein within human myometrium by fluorescence confocal microscopy. GR protein localisation and expression in non-pregnant (**A**) and pregnant non-labouring sections of human myometrium (upper uterine sections are shown in **B**; lower segment pregnant non-labouring sections not shown). The left panels show GR protein staining visualised using a FITC conjugated secondary antibody (stains green). Nuclear DNA was defined by counterstaining with DAPI (stains blue) as shown in the middle panels. Colocalisation is illustrated by merging the images as shown in the right panels. Corresponding control reactions for each of the tissue sections were negative as they showed no immunofluorescence staining above background (not shown).

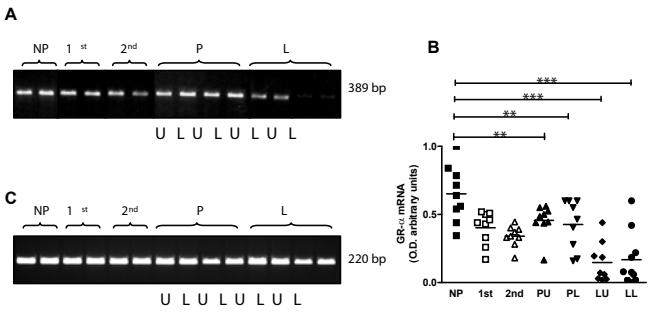

Figure 5: Pregnancy induced changes in GR-α mRNA expression in human myometrium. GR-α expression levels in non-pregnant (NP), first trimester (1st), second trimester (2nd), term pregnant (P) and spontaneously labouring (L) human myometrial samples were assessed by semi-quantitative RT-PCR analysis. Upper (U) and lower (L) uterine segment samples were examined in the P and L groups. Using GR-α specific primers a PCR product of 389bp was observed in all samples. A representative gel is presented in **A** and densitometry graph in **B**. All samples are plotted within the allotted patient groups. The bars represent means. (NP vs. PU/PL $p<0.01$; NP vs. LU/LL $p<0.001$) GAPDH housekeeping RT-PCR was used with each sample to confirm equal loading. No difference in expression was observed between the samples. A representative gel is presented in **C**. No signal was detected when nuclease free water was used as a DNA-free negative control (not shown).

myometrium. Moreover, the number of myometrial binding sites was similar before and during term labour.

Reverse transcription PCR confirmed the presence of GR mRNA in non-pregnant myometrium, as reported previously [32], and showed a predominance of GR-α over GR-β mRNA in all myometrial tissues studied. Previous studies have also found GR-β mRNA to be 200-500 times less abundant than GR-α message in human tissues and cell lines [33,34]. We only demonstrated GR-β mRNA in a minority of samples from each group, even using nested PCR. Stjernholm-Vladic et al. [22] have reported inconsistent detection of GR-β mRNA in non-pregnant, term and post-partum cervical tissues. In contrast GR-α mRNA levels appeared to be regulated during pregnancy although only the difference in expression levels between non-pregnant and pregnant labouring myometrium achieved statistically significance.

Regulation of GR expression is a key determinant for cellular and tissue glucocorticoid sensitivity, with transcriptional responses to both endogenous and exogenous glucocorticoids being directly proportional to the number of intact cellular receptors [35]. Among the factors that alter receptor expression, glucocorticoid itself appears to be the most potent regulator, with homologous down-regulation occurring at transcriptional, post-transcriptional and post-translational levels [36,37]. Circulating maternal cortisol levels are high throughout pregnancy, with levels peaking in labour [1], thus offering one explanation for the down-regulation observed in this study. Changes in sex steroids may also be involved; oestrogen can also down-regulate GR while progesterone is capable of acting as a corticosteroid receptor antagonist [38].

Other mechanisms may contribute to the activity of GR in addition to receptor expression. In this context it is noteworthy that immunofluorescence showed a decrease in nuclear GR staining during pregnancy. Ligand binding classically induces shuttling of the GR from the cytoplasm to the nucleus [5] and yet, despite gestation-related increases in glucocorticoid, most of the GR staining was evenly distributed throughout the myometrial cells inferring reduced transcriptional activity. Phosphorylation of GR is known to influence subcellular localisation [39]. Studies using phospho-specific antibodies have shown that, in the absence of hormone, some receptors are phosphorylated at serine-203 while others remain unphosphorylated. In the presence of hormone, a subpopulation of GR phosphorylated at serine-211 localizes to the nucleus and strongly correlates with GR transcriptional activation. Down-regulation of GR has been shown to occur at the same rate as serine-203 phosphorylation [40]. Whether phosphorylation status of myometrial GR changes during pregnancy remains to be resolved.

It is plausible that the effects of glucocorticoids in pregnancy may be mediated via interactions with other members of the steroid receptor superfamily, including the mineralocorticoid receptor and PR as each of these receptors share various structural and functional characteristics including ligand binding and DNA sequence recognition specificity and PR has been implicated in the mediation of glucocorticoid anti-inflammatory effects.

In conclusion, we provide evidence of the expression profile of GR proteins in the human myometrium and have demonstrated a pregnancy related down-regulation. Further research is needed to understand the physiological significance of this observation and to clarify the role of glucocorticoids and GR in pregnancy maintenance and the onset of labour.

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