The study of progesterone action in human myometrial explants

E. X. Georgiou[1, 2], K. Lei[2], P. F. Lai[1], A. Yulia[1, 2], B. R. Herbert[1], M. Castellanos[3], S.T. May[3], S. R. Sooranna[1, 2], M. R. Johnson[1, 2]

[1] Imperial College Parturition Research Group, Chelsea and Westminster Hospital, 369 Fulham Road, London, SW10 9NH, UK

[2] Imperial College Parturition Research Group, Institute of Reproductive and Developmental Biology, Hammersmith Hospital Campus, Du Cane Road, London W12 0NN, UK

[3] Nottingham Arabidopsis Stock Centre, Plant Science Building, School of Biosciences, Sutton Bonington Campus, University of Nottingham, Loughborough, LE12 5RD

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Corresponding author and person to whom reprint requests should be addressed:

Professor M. R. Johnson, Imperial College Parturition Research Group, Department of Obstetrics and Gynecology, Imperial College School of Medicine, Chelsea and Westminster Hospital, 369 Fulham Road, London, SW10 9NH, UK.

Telephone: + 44 20 88 46 78 78 **Fax:** + 44 20 88 46 77 96

Email: mark.johnson@imperial.ac.uk

Abstract

Study hypothesis: Myometrial explants represent a superior model for the study of human myometrial progesterone (P4) signalling in parturition.

Study finding: The transcriptome of myometrial explants closely resembles the *in vivo* condition and the anti-inflammatory action of P4 is not lost with labour onset.

What is known already: Circulating P4 levels decline before the onset of parturition in most animals, but not in humans. This has led to the suggestion that there is a functional withdrawal of P4 action at the myometrial level prior to labour onset. However, to date, no evidence of a loss of P4 function has been provided, with studies hampered by a lack of a physiologically relevant model.

Study design, samples/materials, methods: Myometrial biopsies obtained at Caesarean section were dissected into explants after a portion was immediately snap-frozen (t=0). Microarray analysis was used to compare the t=0 transcriptome to paired (i) explants, (ii) primary myometrial cell cultures as well as (iii) the hTERT myometrial cell line. Western blotting and chemokine/cytokine assays were used to study P4 signaling in myometrial explants.

Main results and the role of chance: Transcriptomic comparison of t=0 to the three models demonstrated that explants more closely resemble the *in vivo* status. At the protein level, explants maintain both P4 receptor (PR) and glucocorticoid receptor (GR) levels versus t=0 whereas cells only maintain GR levels. Additionally, treatment with 1μM P4 led to a reduction in IL-1β-driven cyclooxygenase-2 in explants but not in cells. P4 signalling in explants was PR-mediated and associated with a repression of p65 and c-Jun phosphorylation. Furthermore, the anti-inflammatory action of P4 was maintained after labour onset.

Limitations/reasons for caution: There is evidence of basal inflammation in the myometrial explant model.

Wider implications of the findings: Myometrial explants constitute a novel model to study P4 signalling in the myometrium and can be used to further elucidate the mechanisms of functional P4 withdrawal in human labour.

Large scale data: Data deposited at http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=gvmpggkurbgxfqf&acc=GSE77830

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Introduction

The seminal work of Csapo and Frydman demonstrated that progesterone (P4) is essential for human pregnancy, the former showing that P4 is responsible for the maintenance of early pregnancy and that its removal results in miscarriage (Csapo *et al.* , 1973) and the latter, that blocking P4 can result in the onset of labour (Frydman *et al.* , 1991). However, while in most animals labour follows a precipitous fall in peripheral P4 levels, no such fall occurs in humans and non-human primates. This has led to the concept of a myometrial functional P4 withdrawal and several theories have been proposed to explain it. The most widely accepted is that there is a change in the balance of expression of the P4 receptor (PR), which is comprised of 2 main isoforms: PR-B, which mediates the effects of P4, and PR-A, which antagonises PR-B-mediated P4 signalling, but is also transcriptionally active in its own right. An increase in the PR-A:PR-B ratio at the time of labour onset has been demonstrated in myometrial samples obtained at the time of labour at the mRNA and protein level (Merlino *et al.* , 2007, Mesiano *et al.* , 2002). Furthermore, in PR-A-dominant myometrial cells, P4 enhances pro-inflammatory gene expression (Tan *et al.* , 2012). Another theory suggests that uterine quiescence is maintained throughout pregnancy by a PR-mediated inhibition of the actions of the pro-inflammatory transcription factor NF-κB (Kalkhoven *et al.* , 1996), possibly via the NF-κB inhibitor IκBα (Hardy *et al.* , 2006), but that with the onset of labour, inflammation-induced NF-κB represses PR action bringing about a uterine switch to a contractile phenotype (Allport *et al.* , 2001). A third theory suggests that changes to PR co-regulator expression may cause labour onset (Condon *et al.* , 2003). All three mechanisms have the common end result of a loss of myometrial sensitivity to P4 action and the onset of labour. Efforts to use a mouse model to study this question have been limited, as PR-A knock-out mice are infertile, while no apparent change in ovarian and uterine function was observed in PR-B knock-out mice (Mulac-Jericevic *et al.* , 2003, Mulac-Jericevic *et al.* , 2000).

P4 supplementation has been shown to reduce the risk of preterm labour in high-risk singleton pregnancies (da Fonseca *et al.* , 2003, Meis *et al.* , 2003). The mechanism involved is

uncertain, but since labour is widely accepted to be an inflammatory event (Bollapragada *et al.* , 2009), it is assumed that P4 acts to maintain pregnancy by repressing inflammation. On that basis, the ability of P4 to repress inflammation-induced cyclooxygenase-2 (COX-2) expression has been widely used as a model of P4 action. COX-2 expression is driven by the inflammatory transcription factors NF-κB and activator protein-1 (AP-1) (Khanjani *et al.* , 2011, Khanjani *et al.* , 2012, Lim and Lappas, 2014, Soloff *et al.* , 2004). Several studies have investigated whether P4 inhibits NF-κB and AP-1 activation to repress COX-2 expression, but these have typically been performed in primary cell cultures or cell lines and have involved the over-expression of PR, NF-κB and/or AP-1 (Bamberger *et al.* , 1996, Hardy *et al.*, 2006, Kalkhoven *et al.*, 1996). Primary cultures of uterine smooth muscle cells (henceforth referred to as primary cells) have been shown to maintain structural and functional characteristics (Lee *et al.* , 2012, Mosher *et al.* , 2013), but are possibly not an optimal model for the study of P4 action as high doses of P4 (10μM) are required to bring about a reduction in IL-1β-driven COX-2 (Lei *et al.* , 2012). Previous work by our group using this model has demonstrated that P4 signals via the glucocorticoid receptor (GR) to reduce COX-2 (Lei *et al.*, 2012) via MAPK phosphatase-1 (MKP-1) (Lei *et al.* , 2015), however, this may be because PR levels are lower in primary cells compared to snap-frozen tissue.

In order to overcome the limitations of myometrial cell culture, we have developed an explant-based model for the *ex vivo* study of myometrial function. We compared the transcriptome of tissue snap frozen at the time of Caesarean section (t=0) to myometrial explants, primary cells and the hTERT myometrial cell line. We subsequently compared PR protein levels in t=0, myometrial explants and primary cells before using this system to study P4 action in myometrial samples before and after the onset of labour to test the hypothesis that a functional withdrawal of P4 action occurs with the onset of labour.

Methods

Ethical approval

The Brompton and Harefield Research Ethics Committee approved this project.

Myometrial biopsies

Myometrial biopsies were obtained from women at term (≥37 weeks) following informed consent at the time of planned or emergency Caesarean section. Women with multiple pregnancy, gestational diabetes mellitus, pre-eclampsia and obstetric cholestasis were excluded. In addition, labouring women were recruited to the study if labouring spontaneously and requiring an emergency Caesarean section due to fetal distress or a breech presentation. Cervical dilatation was used to categorise labour into early (≤3cm) or established (>3cm). Biopsies were collected into sterile universal bottles containing phosphate-buffered saline (PBS) and were processed immediately. Samples used were as follows: term no-labour (TNL): n=35; term early labour (TEaL): n=8 and term established labour (TEsL): n=8.

Explant Culture

Biopsies were dissected into 3x3x3mm^3 pieces (explants) and placed in Dulbecco's Modified Eagle Medium (DMEM) (Sigma-Aldrich Ltd., Dorset, UK) supplemented with penicillin-streptomycin (Sigma-Aldrich Ltd.) or immediately snap-frozen in liquid nitrogen (t=0). Depending on the experimental protocol, explants were either untreated or immediately treated for 6 hours with vehicle control (ethanol ± DMSO), progesterone (100nM, 500nM, 1μM, 5μM or 10μM; Sigma-Aldrich Ltd.), dexamethasone (1μM; Sigma-Aldrich Ltd.), mifepristone (RU486, 1μM; Sigma-Aldrich Ltd.) or onapristone (ZK299, 1μM; Arno Therapeutics, Flemington, NJ, USA). They were subsequently treated for a further 24 hours with IL-1β (1, 10, 20, 50 or 100ng/mL; Sigma-Aldrich Ltd.), at which point all tissues were snap-frozen in liquid nitrogen and stored at -80°C. The media in which explants were cultured were also stored at -80°C.

Cell Culture

Biopsies were digested in a mixture of collagenases as previously described (Sooranna *et al.* , 2004) and passaged by trypsinization in 0.25% trypsin containing 0.02% EDTA (Sigma-Aldrich Ltd.) when confluent. Once confluent at passage 4, cells were serum-starved overnight in 1% charcoal and dextran-stripped fetal calf serum (1% DCC) supplemented with penicillin-streptomycin. They were then cultured and treated using the same protocol as explant cultures and were placed at -80°C once the experiment was completed. Supernatants were stored at -80°C.

The myometrial hTERT cell line was cultured in the same conditions as primary cells with overnight serum-starving once cells reached confluence. Once thawed, cells were not passaged beyond passage 5.

RNA extraction

Total RNA was extracted using a Trizol® Plus RNA Purification kit (Thermo Fisher Scientific, Ambion, Abgene Ltd., West Sussex, UK) with on-column DNase treatment prior to elution, all as per the manufacturer's protocol. Bead homogenization in Precellys® tubes (Stretton Scientific Ltd., Derbyshire, UK) was used for tissue lysis with two 20 second cycles at 5000rpm; cells were lysed directly with Trizol® added to the culture plate. The concentration and purity of RNA was determined by spectrophotometry and integrity was confirmed using an Agilent 2100 Bioanalyzer with an RNA 6000 Nano Kit (Agilent Technologies, Palo Alto, CA, USA).

Microarray Analysis

Whole-genome transcriptome analysis was conducted by hybridizing 6 biological samples of total RNA per condition to Affymetrix Human Gene 2.1 ST Arrays Strips (Affymetrix, Santa Clara, CA, USA). A minimum RIN score of 8 was used as cut off for inclusion in the microarray analysis. All steps were conducted at the Nottingham Arabidopsis Stock Centre. Gene expression data were analysed using Partek Genomics Suite 6.6 software (Partek Incorporated, St. Louis, USA). The raw CEL files were normalised using the RMA

196 background correction with quantile normalization, log base 2 transformation and mean
197 probe-set summarization with adjustment for GC content. Differentially expressed genes
198 (DEG) were identified by a two-way ANOVA, and *p*-values were adjusted using the FDR
199 (false-discovery rate) method to correct for multiple comparisons. DEG were considered
200 significant if *p*-value was ≤ 0.05 at a fold change (FC) of > 2 with FDR <0.5.
201
202 **Quantitative RT-PCR**
203 Following quantification, 1µg RNA was reverse transcribed with oligo dT random primers
204 using MuLV reverse transcriptase (Life Technologies Ltd., Paisley, UK). Primer sets were
205 designed and obtained from Invitrogen (Table I). Quantitative PCR was performed using
206 SYBR Green (Roche Diagnostics Ltd., West Sussex, UK) using the previously described
207 cycling protocol (Lei *et al.*, 2015) and amplicon yield was monitored during cycling in a
208 RotorGene Sequence Detector (Qiagen Ltd., West Sussex, UK). The abundance of mRNA for
209 sequences of interest were expressed relative to the constitutively expressed GAPDH.
210
211 **Protein Extraction and Western Blotting**
212 Protein was extracted from explants using bead homogenization in pre-cooled Precellys®
213 tubes (Stretton Scientific Ltd.) containing lysis buffer (New England Biolabs,
214 Hertfordshire,UK) supplemented with protease (Roche Diagnostics Ltd.) and phosphatase
215 inhibitors (Thermo Fisher Scientific, Abgene Ltd., Epsom, UK). Tissues were immediately
216 homogenised by mechanical disruption by two 20 second cycles at 5000rpm. Protein from
217 cells was extracted via direct lysis using the same lysis buffer mixture as for explants. The
218 supernatant was separated from tissue debris by centrifugation at 13000rpm for 10 minutes at
219 4°C. Protein concentrations were determined by DC protein assay (Bio-Rad Labouratories
220 Ltd., Hertfordshire, UK) and bovine serum albumin (Sigma-Aldrich Ltd.) was used for
221 reference standards.
222 Samples in NuPAGE® LDS Sample Buffer (Life Technologies Ltd.) were denatured at 75°C
223 for 10 minutes and 20µg of total protein for each sample was electrophoresed through a 4-

20% polyacrylamide gel (Bio-Rad Labouratories Ltd.). Transfer was carried out onto a polyvinylidene fluoride membrane (Bio-Rad Labouratories Ltd.) using the Trans-Blot® Turbo Transfer system (Bio-Rad Labouratories Ltd.), followed by blocking in 5% nonfat dried milk powder (AppliChem GmbH, Germany) dissolved in 0.1% Tween-Tris buffered saline (TBS-T) for 1 hour at room temperature. The membrane was incubated overnight at 4°C with primary antibody followed by incubation for 2 hours at room temperature with secondary antibody (Table II). Clarity Western ECL substrate (Bio-Rad Labouratories Ltd.) was used for detection. Protein band size was determined using Precision Plus Protein Standards ladder (Bio-Rad Labouratories Ltd.). All protein abundance data were expressed relative to the amount of constitutively expressed GAPDH after 1 hour incubation at room temperature (Table II).

Chemokine/Cytokine Assays

Human Bio-Plex© Pro™ chemokine/cytokine assays (Bio-Rad Labouratories Ltd.) were used to measure the concentrations of IL-1α, IL-1β, IL-6, IL-8, CCL2, CCL5, CCL11, CCL20, ICAM and LIF in explant culture media. These were performed according to the manufacturer's instructions and were read using a Bio-Plex© 200 reader and Bio-Plex Manager © v6.1 software (Bio-Rad Labouratories Ltd.). Data were normalised to tissue weights.

Statistical analysis

Statistical analysis was performed using Graphpad Prism v5.0 (Graphpad Software Inc., La Jolla, CA, USA). Normality was determined via a Kolmogorov-Smirnov test for up to 6 replicates or a Shapiro Wilks test for more than 6 replicates. Normally distributed data were subsequently analysed using a paired t test for the comparison of 2 groups or an ANOVA followed by Bonferroni's multiple comparison test *post hoc* testing for three groups or more. Data that were not normally distributed were analysed using a Wilcoxon matched pairs test or

a Kruskal-Wallis followed by Dunn's multiple comparisons *post hoc* testing for three groups or more. $p<0.05$ was considered statistically significant.

Results

The myometrial explant transcriptome closely resembles the *in vivo* status

Biopsies obtained from non-labouring women at elective Caesarean section at term, were divided into 3: (i) dissected and immediately snap frozen (t=0), (ii) dissected for myometrial explants and (iii) processed for primary cell culture. Explants, primary cells at passage 4 (the typical passage our group uses for experiments) and the hTERT cell line were cultured for a period of 30 hours without treatment. Transcriptomic analysis via microarray demonstrated that explants most closely resemble t=0 (Fig. 1A). Upon direct comparison between explants and t=0, 1444 genes varied significantly whereas the corresponding number for primary cells was 3840 and for hTERT 4603 (Fig. 1B). 555 genes varied commonly upon comparing all 3 groups to t=0 with gene ontology analysis demonstrating higher enrichment scores for functions including 'immune response', 'inflammatory response' and 'leukocyte migration' (Suppl. 1). Furthermore, explants shared 119 genes exclusively with primary cells and 137 genes with hTERT; the equivalent figure for the primary cells and hTERT overlap was 2111 (Fig. 1B). Of the 633 genes uniquely upregulated in the explant group, the most common gene ontology groups pertained to glucose metabolism including 'glycolysis', 'gluconeogenesis' and 'glucose metabolic process' (Suppl. Table II). Overall, the degree of variability on comparing t=0 to each of the 3 groups was least for explants (Fig. 1C) followed by primary cells (Fig. 1D) and hTERT (Fig.1E).

A second set of biopsies and hTERT cultures were used to validate the microarray results via quantitative RT-PCR. Microarray trends were preserved for a panel of genes of interest including those associated with reproductive function (*PTGS2*, *OXTR*, *PGR*, *GJA1*) and smooth muscle phenotype (*ACTA2*, *MYLK*) (Table III). Overall, 15 genes of interest were chosen for validation of microarray results with 3 comparisons performed per gene (explants

versus t=0, primary cells versus t=0, hTERT versus t=0); microarray and RT-PCR data followed the same trend in 39 of 45 cases (86.7%).

Nuclear receptor levels

Subsequent experiments focused on comparing explants and primary cells to t=0 as the same biopsy could be utilised in matched experiments and these 2 groups most closely resembled t=0 based on the microarray results. A separate set of biopsies obtained from non-labouring women were divided into (i) t=0, (ii) myometrial explants and (iii) primary cells, and utilised to assess key nuclear receptor levels on the protein level. Although the level of both PR isoforms tended to decline, there was no significant difference between t=0 and explants. However, the levels of PR were observed to be significantly lower in primary cells at passage 4 than in either t=0 tissue or explants (Fig. 2A&B). The PR-A:PR-B ratio was similar in explants and cells although this was significantly raised compared to t=0 ($p=0.0327$ and $p=0.0067$ respectively) (Fig. 2C). No difference was observed in GR levels between t=0, explants and cells (Fig. 2A&D).

Progesterone-repression of IL-1β-induced COX-2

Previous group data demonstrated that 10μM was the minimum P4 dose causing a significant reduction in IL-1β-driven COX-2 in primary cells. Based on dose response experiments, the myometrial explant IL-1β EC50 was defined as 10ng/mL and the P4 IC50 P4 was 1μM (Suppl. Fig. 1).

In order to directly compare P4 sensitivity, explants and cells originating from the same biopsy were treated with 10ng/mL IL-1β ± 1 or 10μM P4. Compared t=0, basal COX-2 levels were significantly raised in explants ($p=0.0313$) and not in cells. Although not significant, the addition of IL-1β showed a trend towards a greater increase in COX-2 levels in primary cells than in explants ($p=0.07$, Fig. 3). The IL-1β-induced increase in COX-2 protein levels was reduced by pre-incubation with 1μM P4 in myometrial explants and 10μM P4 in cell cultures (Fig. 3).

306

307 **P4 acts via PR in myometrial explants and represses IL-1β-induced activation of p65**
308 **and AP-1**

309 The mixed PR/GR antagonist RU486 reversed the repressive effect of 1μM P4 on IL-1β-
310 induced COX-2 expression in myometrial explants (Fig. 4). The more PR-selective inhibitor
311 ZK299 at the PR-specific dose of 1μM (Kohmura *et al.* , 2000), also reversed the P4 effect
312 (Fig. 4).

313 IL-1β increased the phosphorylation of ERK (p=0.0156) and p38 (p=0.0313) as well as the
314 transcription factor targets c-Jun (p=0.0078) and p65 (p=0.0078, Fig.4). Although a trend was
315 observed, IL-1β did not significantly increase the phosphorylation of JNK or c-Fos (Fig. 5).
316 Pre-incubation with 1μM P4 reduced the IL-1β induced increase in p65 and c-Jun
317 phosphorylation, but interestingly, there was no reduction in MAPK phosphorylation, nor any
318 change in MKP-1 or IκB levels following P4 treatment alone or in combination with IL-1β
319 (Fig. 5). P4 treatment alone did not drive the MAPKs, c-Jun or p65 phosphorylation, but did
320 lead to an increase in c-Fos phosphorylation (Fig. 5).

321

322 **Lack of a functional withdrawal of myometrial P4 action**

323 In order to determine whether there was any evidence of a functional P4 withdrawal with the
324 onset of labour, we obtained myometrium from women before the onset of labour, in early
325 labour ($\leq$ 3cm, termed the "latent phase" of labour), during which the cervix effaces, begins
326 to dilate and contractions become regular and strong, and in established labour (> 3cms,
327 termed the "active phase" of labour), during which the cervix dilates more rapidly and
328 contractions are regular and strong. The increase in COX-2 levels induced by IL-1β was
329 similar in all 3 groups (Suppl. Fig. 2). Pre-treatment of the explants with 1μM P4, was able to
330 reduce the expression of IL-1β-driven COX-2 in all 3 groups (Fig. 6). Treatment with P4
331 alone was not associated with a significant change in COX-2 levels compared to control (Fig.
332 6).

The role of P4 was further studied by quantification of a panel of pro-inflammatory cytokines in the tissue culture media of explants obtained from non-labouring women as well as women in early and established labour. In the first instance, using non-labouring samples, we sought to identify which cytokines were driven by IL-1β in our model and, if so, whether P4 was able to significantly reduce these levels. We identified a shortlist of cytokines comprised of CXCL2, IL-6 and IL-8 (Fig. 7A-C).

Next we determined whether the effect of IL-1β was altered by labour status by calculating the delta change (Δ) between control and IL-1β treated samples. As with COX-2, we found that the effect of IL-1β was similar in all 3 groups for CXL2, IL-6 and IL-8 (Fig. 7D-F). We then assessed whether P4 treatment alone altered the release of pro-inflammatory cytokines into the medium in either of the labouring groups and found that P4 had no effect on cytokine levels (Fig. 7A-C). Finally, we confirmed that there was no difference in the ability of P4 to repress IL-1β-induced increase in of CXCL2, IL-6 and IL-8 levels in the labouring samples (Fig. 7G-I).

Discussion

In this study, we sought to establish a model that reflects the *in vivo* situation more accurately than the current *in vitro* cell models and to use this model to study P4 signalling in the myometrium.

We demonstrated that the explant transcriptome most closely resembles that of the *in vivo* (t=0) condition as compared to both myometrial cells and the hTERT cell line. Importantly, this pattern was preserved on examining genes relevant to reproductive function and parturition; for example the explant levels of PR (*PGR*), oxytocin receptor (*OXTR*) and connexin-43 (*GJA1*) did not vary significantly compared to t=0 (Table III). In contrast, PR RNA levels were significantly lower in both primary cells and hTERT. Indeed, the same pattern was observed for PR on the protein level with no significant change in explants versus t=0, but a significant reduction in primary cells. In addition, *OXTR* and *GJA1* RNA levels

were significantly lower and higher respectively in primary cells (Table III). COX-2 (*PTGS2*) levels were significantly elevated in explants as well as hTERT, although upon comparing this upregulation in relation to COX-2 levels after IL-1β treatment, the effect was negligible with a signal-to-noise ratio of 17.7 (FDA, 2003). Indeed, it was noted that genes associated with inflammation were elevated in all 3 models as evidenced by gene ontology analysis (Suppl. Table I). We also determined that the expression pattern of smooth muscle markers such as alpha smooth muscle actin (*ACTA2*) and myosin light chain kinase (*MYLK*) remained unaltered in explants whereas both were significantly reduced in hTERT (Table III).

We noted with interest that the uniquely upregulated genes in the explant group comprised glucose metabolism pathways (Suppl. Table II). We hypothesised that this was the result of the explants being cultured in DMEM without any additional nutrient supplementation and hence utilizing alternative biochemical pathways to produce glucose. As these pathways do not relate to reproduction, we feel they are not of great importance to the model. In an attempt to mimic the physiological conditions as closely as possible, we did supplement culture media with P4, estradiol and/or the cAMP agonist forskolin, but found that this had no effect on restoring PR levels even closer to t=0 and hence these conditions were not incorporated into the final experimental model used (data not shown).

One possible limitation of this transcriptome analysis was that it was conducted via microarray and not RNA-Seq, the latter being widely accepted as a superior platform with better concordance with quantitative RT-PCR (Wang *et al.* , 2014). However, the two share high correlation and for the purpose of this study, microarray was deemed appropriate. One notable case of poor concordance between microarray and quantitative RT-PCR data was GR (NR3C1), where the microarray indicated upregulation in all 3 models whereas quantitative RT-PCR showed downregulation (Table III). We sought to clarify our findings by undertaking protein level analysis and confirmed that GR levels do not vary significantly in explants or primary cells compared to t=0.

On the protein level, we demonstrated that the treatment of explants with 1μM P4 significantly reduces inflammation, whereas in myometrial cells it does not. Intriguingly, this

388 occurs despite the fact that the PR-A:PR-B ratio increases in explant culture versus snap
389 frozen tissue. Interestingly, the response to IL-1β is greater in cultured myometrial cells with
390 no evidence of increased sensitivity to P4 treatment, suggesting that explants do not just differ
391 to myometrial cells in their cellular organization, but also in their overall sensitivity to
392 different treatments.
393 Even though other studies have shown that passaging of primary cells does not affect the
394 myometrial phenotype (Mosher *et al.*, 2013), our data suggest that there are marked
395 functional differences between explants and primary cell cultures. Explants contain a
396 heterogeneous collection of cell types, although smooth muscle cells predominate as shown
397 by immunohistochemistry for alpha smooth muscle actin (data not shown); additionally they
398 contract spontaneously and after treatment with oxytocin (data not shown).
399 We have shown that both RU486 and ZK299 are capable of reversing the effect of P4.
400 Although neither drug is a pure PR antagonist (Miner *et al.* , 2003), 1μM ZK299 has
401 previously been shown to reverse the effect of P4 but not that of dexamethasone (Kohmura *et*
402 *al.*, 2000), suggesting that P4, in contrast to our observations from primary cell cultures (Lei
403 *et al.*, 2012), may signal via PR in explants. The reason for this may lie in the difference in
404 nuclear receptor levels in the two models, with the explant model more closely resembling the
405 *in vivo* state. Trial of other 'specific' PR and GR antagonists demonstrated that they have
406 non-specific actions in myometrial explants (data not shown).
407 In support of previous findings (Hardy *et al.*, 2006), we show that P4 is able to repress the IL-
408 1β-induced activation of two major pro-inflammatory transcription factors: NF-κB and AP-1.
409 Both of these transcription factors have previously been shown to be required for IL-1β-
410 mediated upregulation of COX-2 in gestational tissues (Allport *et al.* , 2000). However, the
411 mechanism of P4 action does not appear to be via an increase in IκB as has previously been
412 suggested (Hardy *et al.*, 2006). Further, the reduction in c-Jun phosphorylation does not seem
413 to be mediated via an increase in MKP-1 (Lei *et al.*, 2015). It is possible that total c-Jun levels
414 are reduced by P4 treatment or that other phosphatases are increased. These data further

demonstrate that the *in vivo* mechanism of P4 action may differ markedly compared to the *in vitro* models.

Our data indicate that with the onset of labour, P4 does not become pro-inflammatory as suggested by other groups (Allport *et al.*, 2000, Tan *et al.*, 2012), nor does it lose its anti-inflammatory action. Furthermore, the explant sensitivity to IL-1β does not alter with labour status, nor does the ability of P4 to down-regulate the IL-1β response suggesting that there is no functional withdrawal of P4 action, at least in terms of the ability of P4 to repress inflammation. However, it remains possible that there is a withdrawal of other P4-mediated functions that lead to the onset of labour.

In conclusion, this study has established the validity of using an explant model to study myometrial P4 signalling. We provide evidence that P4 acts via PR to reduce IL-1β-induced COX-2 synthesis in associated with a reduction in NF-κB and AP-1 activation. Further, we show that P4 is able to repress IL-1β-induced gene expression even after the onset of labour, suggesting that, at least in this regard, there is no functional withdrawal of P4 action.

Authors' roles

E.X.G. designed the study, recruited patients, performed the experiments and wrote the manuscript. K.L., P.F.L., S.R.S and M.R.J contributed to the design of the study and data interpretation. A.Y. contributed to the design of the study. B.R.H. assisted with chemokine/cytokine assay data acquisition. M.C. and S.T.M. carried out microarray data acquisition and analysis. All authors assisted with drafting of the article and approved the final version to be published.

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References

Allport VC, Pieber D, Slater DM, Newton R, White JO, Bennett PR (2001) Human labour is associated with nuclear factor-kappaB activity which mediates cyclo-oxygenase-2 expression and is involved with the 'functional progesterone withdrawal'. Molecular human reproduction **7**, 581-6.

Allport VC, Slater DM, Newton R, Bennett PR (2000) NF-kappaB and AP-1 are required for cyclo-oxygenase 2 gene expression in amnion epithelial cell line (WISH). Molecular human reproduction **6**, 561-5.

Bamberger AM, Bamberger CM, Gellersen B, Schulte HM (1996) Modulation of AP-1 activity by the human progesterone receptor in endometrial adenocarcinoma cells. Proceedings of the National Academy of Sciences of the United States of America **93**, 6169-74.

Bollapragada S, Youssef R, Jordan F, Greer I, Norman J, Nelson S (2009) Term labor is associated with a core inflammatory response in human fetal membranes, myometrium, and cervix. American journal of obstetrics and gynecology **200**, 104 e1-11.

Condon JC, Jeyasuria P, Faust JM, Wilson JW, Mendelson CR (2003) A decline in the levels of progesterone receptor coactivators in the pregnant uterus at term may antagonize progesterone receptor function and contribute to the initiation of parturition. Proceedings of the National Academy of Sciences of the United States of America **100**, 9518-23.

Csapo AI, Pulkkinen MO, Kaihola HL (1973) The effect of luteectomy-induced progesterone-withdrawal on the oxytocin and prostaglandin response of the first trimester pregnant human uterus. Prostaglandins **4**, 421-9.

da Fonseca EB, Bittar RE, Carvalho MH, Zugaib M (2003) Prophylactic administration of progesterone by vaginal suppository to reduce the incidence of spontaneous preterm birth in women at increased risk: a randomized placebo-controlled double-blind study. American journal of obstetrics and gynecology **188**, 419-24.

469 FDA (2003) Guidance for Industry: Bioanalytical Method Validation. U.S. Department of
470 Health and Human Sciences.
471 Frydman R, Baton C, Lelaidier C, Vial M, Bourget P, Fernandez H (1991) Mifepristone for
472 induction of labour. Lancet **337**, 488-9.
473 Hardy DB, Janowski BA, Corey DR, Mendelson CR (2006) Progesterone receptor plays a
474 major antiinflammatory role in human myometrial cells by antagonism of nuclear factor-
475 kappaB activation of cyclooxygenase 2 expression. Mol Endocrinol **20**, 2724-33.
476 Kalkhoven E, Wissink S, van der Saag PT, van der Burg B (1996) Negative interaction
477 between the RelA(p65) subunit of NF-kappaB and the progesterone receptor. The Journal of
478 biological chemistry **271**, 6217-24.
479 Khanjani S, Kandola MK, Lindstrom TM, Sooranna SR, Melchionda M, Lee YS, Terzidou V,
480 Johnson MR, Bennett PR (2011) NF-kappaB regulates a cassette of immune/inflammatory
481 genes in human pregnant myometrium at term. Journal of cellular and molecular medicine **15**,
482 809-24.
483 Khanjani S, Terzidou V, Johnson MR, Bennett PR (2012) NFkappaB and AP-1 drive human
484 myometrial IL8 expression. Mediators of inflammation 2012, 504952.
485 Kohmura Y, Kirikae T, Kirikae F, Nakano M, Sato I (2000) Onapristone (ZK299) blocks the
486 suppressive effect of progesterone, but not that of dexamethasone, on inducible nitric oxide
487 synthase gene expression and nitric oxide production in murine macrophages. International
488 journal of immunopharmacology **22**, 765-74.
489 Lee Y, Sooranna SR, Terzidou V, Christian M, Brosens J, Huhtinen K, Poutanen M, Barton
490 G, Johnson MR, Bennett PR (2012) Interactions between inflammatory signals and the
491 progesterone receptor in regulating gene expression in pregnant human uterine myocytes.
492 Journal of cellular and molecular medicine **16**, 2487-503.
493 Lei K, Chen L, Georgiou EX, Sooranna SR, Khanjani S, Brosens JJ, Bennett PR, Johnson
494 MR (2012) Progesterone acts via the nuclear glucocorticoid receptor to suppress IL-1beta-
495 induced COX-2 expression in human term myometrial cells. PloS one **7**, e50167.

Lei K, Georgiou EX, Chen L, Yulia A, Sooranna SR, Brosens JJ, Bennett PR, Johnson MR (2015) Progesterone and the Repression of Myometrial Inflammation: The Roles of MKP-1 and the AP-1 System. Mol Endocrinol **29**, 1454-67.

Lim R, Lappas M (2014) Differential expression of AP-1 proteins in human myometrium after spontaneous term labour onset. European journal of obstetrics, gynecology, and reproductive biology **177**, 100-5.

Meis PJ, Klebanoff M, Thom E, Dombrowski MP, Sibai B, Moawad AH, Spong CY, Hauth JC, Miodovnik M, Varner MW *et al.* (2003) Prevention of recurrent preterm delivery by 17 alpha-hydroxyprogesterone caproate. The New England journal of medicine **348**, 2379-85.

Merlino AA, Welsh TN, Tan H, Yi LJ, Cannon V, Mercer BM, Mesiano S (2007) Nuclear progesterone receptors in the human pregnancy myometrium: evidence that parturition involves functional progesterone withdrawal mediated by increased expression of progesterone receptor-A. The Journal of clinical endocrinology and metabolism **92**, 1927-33.

Mesiano S, Chan EC, Fitter JT, Kwek K, Yeo G, Smith R (2002) Progesterone withdrawal and estrogen activation in human parturition are coordinated by progesterone receptor A expression in the myometrium. The Journal of clinical endocrinology and metabolism **87**, 2924-30.

Miner JN, Tyree C, Hu J, Berger E, Marschke K, Nakane M, Coghlan MJ, Clemm D, Lane B, Rosen J (2003) A nonsteroidal glucocorticoid receptor antagonist. Mol Endocrinol **17**, 117-27.

Mosher AA, Rainey KJ, Bolstad SS, Lye SJ, Mitchell BF, Olson DM, Wood SL, Slater DM (2013) Development and validation of primary human myometrial cell culture models to study pregnancy and labour. BMC pregnancy and childbirth **13** Suppl 1, S7.

Mulac-Jericevic B, Lydon JP, DeMayo FJ, Conneely OM (2003) Defective mammary gland morphogenesis in mice lacking the progesterone receptor B isoform. Proceedings of the National Academy of Sciences of the United States of America **100**, 9744-9.

Mulac-Jericevic B, Mullinax RA, DeMayo FJ, Lydon JP, Conneely OM (2000) Subgroup of reproductive functions of progesterone mediated by progesterone receptor-B isoform. Science **289**, 1751-4.

Soloff MS, Cook DL, Jr., Jeng YJ, Anderson GD (2004) In situ analysis of interleukin-1-induced transcription of cox-2 and il-8 in cultured human myometrial cells. Endocrinology **145**, 1248-54.

Sooranna SR, Lee Y, Kim LU, Mohan AR, Bennett PR, Johnson MR (2004) Mechanical stretch activates type 2 cyclooxygenase via activator protein-1 transcription factor in human myometrial cells. Molecular human reproduction **10**, 109-13.

Tan H, Yi L, Rote NS, Hurd WW, Mesiano S (2012) Progesterone receptor-A and -B have opposite effects on proinflammatory gene expression in human myometrial cells: implications for progesterone actions in human pregnancy and parturition. The Journal of clinical endocrinology and metabolism **97**, E719-30.

Wang C, Gong B, Bushel PR, Thierry-Mieg J, Thierry-Mieg D, Xu J, Fang H, Hong H, Shen J, Su Z *et al.* (2014) The concordance between RNA-seq and microarray data depends on chemical treatment and transcript abundance. Nature biotechnology **32**, 926-32.

Figure 1. The myometrial explant transcriptome most closely resembles the physiological status. Myometrial tissue obtained from term non-labouring women was snap frozen at the time Caesarean section (t=0), finely dissected into 3x3x3mm^3 explants or digested with a collagenase mixture to isolate cells for primary culture. Explants were cultured in DMEM without treatment for 30 hours. After serum-starvation with 1% DCC-FCS overnight, the media of myometrial cells at passage 4 and hTERT cells was refreshed and cells incubated for a further 30 hours without treatment. RNA was extracted as described in *Methods*. Whole-genome transcriptome analysis was conducted by hybridization to Affymetrix Human Gene 2.1 ST array strips and analysed using Partek Genomics Suite 6.6 software. Differentially expressed genes (DEG) were identified by two-way ANOVA, and p-values were adjusted using the FDR (false-discovery rate) method to correct for multiple

comparisons. DEG were considered significant if p-value was $p \leq 0.05$ at a fold change of >2 with FDR <0.5. n=6. **A.** PCA plot. **B.** Venn diagram of genes varying significantly versus t=0 which are common and unique to each model. **C.** Volcano plot of myometrial explants. **D.** Volcano plot of primary cells. **E.** Volcano plot of hTERT.

Figure 2. Myometrial explants maintain nuclear receptor levels in culture. Myometrial tissue obtained from term non-labouring women was snap frozen, finely dissected into 3x3x3mm^3 explants or digested with a collagenase mixture to isolate the cells for primary culture. Explants were treated immediately with ethanol vehicle for 30 hours. After serum-starvation with 1% DCC-FCS overnight, primary cells at passage 4 were treated with ethanol vehicle for 30 hours. Protein was extracted and quantified. Western blotting for the progesterone receptor (PR) isoforms (PR-A and PR-B) and glucocorticoid receptor (GR) was performed as described in *Methods*. **A.** Representative western blot of PR and GR levels **B.** Densitometric analysis of PR levels. **C.** Analysis of PR-A:PR-B ratio **D.** Densitometric analysis of GR levels. The data are expressed as mean + SEM. Normality was tested using a Shapiro-Wilks test followed by Wilcoxon signed rank testing. #, $p<0.05$ cells vs explants for PR-B; ##, $p<0.01$ cells vs t=0 for PR-B; *, $p<0.05$ cells vs explants for PR-A; **, $p<0.01$ cells vs t=0 for PR-A; $, $p<0.05$ t=0 vs explants; $$, $p<0.01$ t=0 vs cells. n=8-9.

Figure 3. Explants respond to 1μM progesterone (P4) in paired biopsies whereas myometrial cells do not. Myometrial tissue obtained from term non-labouring women was snap frozen, finely dissected into 3x3x3mm^3 explants or digested with a collagenase mixture to isolate cells for primary culture. Explants were immediately pre-treated for 6 hours with ethanol vehicle, 1μM P4 or 10μM P4 followed by a 24 hour treatment with IL-1β (10ng/mL). After serum-starvation with 1% DCC-FCS overnight, primary cells at passage 4 were pre-treated for 6 hours with ethanol vehicle, 1μM P4 or 10μM P4 followed by a 24 hour treatment with IL-1β (10ng/mL). Protein was extracted and quantified. Western blotting for cyclooxygenase-2 (COX-2) was performed as described in *Methods*. A representative western

blot is shown at the top of the figure with densitometric analysis below. The data are expressed as mean + SEM. Normality was tested using a Kolmogorov-Smirnov test followed by comparison of control versus IL-1β by Wilcoxon signed rank testing or paired t testing depending on the data distribution; *, $p<0.05$ versus control in that group; $, $p<0.05$ versus t=0. The IL-1β, 1μM P4 & IL-1β and 10μM P4 & IL-1β conditions in each group were compared by ANOVA followed by Bonferroni's Multiple Comparison Test; #, $p<0.05$ versus IL-1β in that group; ##, $p<0.01$ versus IL-1β in that group. n=4-8.

Figure 4. Mifepristone (RU486) and onapristone (ZK299) reverse the progesterone (P4) -mediated reduction in IL-1β-driven cyclooxygenase-2 (COX-2) in myometrial explants. Myometrial tissue obtained from term non-labouring women was finely dissected into 3x3x3mm^3 explants. These were immediately pre-treated for 6 hours with ethanol & DMSO vehicle, 1μM P4 ± 1μM RU486 or 1μM ZK299 followed by a 24 hour treatment with IL-1β (10ng/mL). Protein was extracted and quantified. Western blotting for COX-2 was performed as described in *Methods*. A representative western blot is shown at the top of the figure with densitometric analysis below. The data are expressed as mean + SEM. Normality was tested using a Kolmogorov-Smirnov test. Paired t tests were used to compare control versus IL-1β (*, $p<0.05$) and IL-1β versus P4 & IL-1β (#, $p<0.05$). The shaded groups were compared with a Friedman test followed by Dunn's Multiple Comparison Test; $, $p<0.05$; $$, $p<0.01$. n=6-7.

Figure 5. Progesterone (P4) reduces the phosphorylation of pro-inflammatory transcription factors in myometrial explants. Myometrial tissue obtained from term non-labouring women was finely dissected into 3x3x3mm^3 explants. These were immediately pre-treated for 6 hours with ethanol or 1μM P4 followed by a 30 minute treatment with IL-1β (10ng/mL). Protein was extracted and quantified, and Western blotting for **A.** p-ERK1/2, **B.** p-p38, **C**. p-JNK, **D.** p-c-Jun, **E.** p-c-Fos, **F.** MKP-1, **G.** p-p65 and **H.** IκB was performed as described in *Methods*. A representative Western blot is shown at the top of each figure with densitometric analysis below. The data are normalised to control and expressed as mean +

606 SEM. Normality was tested using a Kolmogorov-Smirnov for 6 replicates or a Shapiro-Wilk
607 test for more than 6 replicates. Wilcoxon signed rank testing was used to compare between
608 pairs; *, $p<0.05$ versus control; **, $p<0.01$ versus control; #, $p<0.05$ versus IL-1β; ##, $p<0.01$
609 versus IL-1β. n=6-8.
610
611 **Figure 6. Progesterone (P4) maintains its anti-inflammatory role throughout labour.**
612 Myometrial tissue obtained from women in term **A.** early labour and **B.** established labour
613 was finely dissected into 3x3x3mm^3 explants. These were immediately pre-treated for 6 hours
614 with ethanol vehicle or 1μM P4 followed by a 24 hour treatment with IL-1β (10ng/mL).
615 Protein was extracted and quantified. Western blotting for cyclooxygenase-2 (COX-2) was
616 performed as described in *Methods*. A representative western blot is shown at the top of each
617 figure with densitometric analysis below. The data are expressed as mean + SEM. Normality
618 was tested using a Shapiro-Wilk test. A Wilcoxon signed rank test was used for non-normally
619 distributed data and a paired t test for normally distributed data. **, $p<0.01$ versus control;
620 ***, $p<0.001$ versus control; #, $p<0.05$ versus IL-1β. n=8.
621
622 **Figure 7. Progesterone does not become pro-inflammatory with labour onset and it**
623 **reduces the expression of proinflammatory cytokines/chemokines irrespective of labour**
624 **status.** The media from explant cultures was used to run human Bio-Plex© Pro™
625 chemokine/cytokine assays for **A, D, G.** CXCL2, **B, E, H.** IL-6 and **C, F, I.** IL-8 as per the
626 manufacturer's protocol. Data were normalised to tissue weight. The data in panels A-C are
627 standardised to control and expressed as mean + SEM. The data in panels D-F are expressed
628 as mean + SEM of the delta (Δ) between IL-1β and control groups. The data in panels E-I are
629 standardised to IL-1β and expressed as mean + SEM. Normality was tested using a
630 Kolmogorov-Smirnov for 6 replicates or a Shapiro-Wilk test for more than 6 replicates. For
631 groups of 3, a Kruskal Wallis test was performed followed by Dunn's Multiple Comparison
632 Test for non-normally distributed data and an ANOVA followed by Bonferroni's Multiple
633 Comparison Test was performed for normally distributed data. Wilcoxon matched pair testing

was used for non-normally distributed paired data and paired t tests for normally distributed data. For comparisons to vehicle control within matched labour status group, statistical significance is indicated as follows: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$. For comparisons to IL-1β within matched labour status group, statistical significance is indicated as follows: #, $p< 0.05$; ##, $p<0.01$; ###, $p<0.001$. n=6-16. Labour status groups are labelled as TNL (term no labour), TEaL (term early labour) and TEsL (term established labour).

Supplementary Figure 1. Myometrial axplant dose response data for IL-1β and progesterone (P4). Myometrial tissue obtained from term non-labouring women was finely dissected into 3x3x3mm^3 explants. These were immediately pre-treated for 6 hours with ethanol followed by a 24 hour treatment with IL-1β (1, 10, 20, 50 or 100ng/mL) (**A**). Alternatively, explants were immediately pre-treated for 6 hours with ethanol or various P4 doses (0.1, 0.5, 1, 5 or 10μM) followed by a 24 hour treatment with IL-1β (10ng/mL) (Panel **B**). Protein was extracted and quantified. Western blotting for cyclooxygenase-2 (COX-2). was performed as described in *Methods*. A representative western blot is shown at the top of each figure with densitometric analysis below. The data are expressed as mean + SEM. Normality was tested a Shapiro-Wilk test. Wilcoxon signed rank test was used for non-normally distributed data and a paired t test for normally distributed data; *, $p<0.05$ versus control;; #, $p<0.05$ versus IL-1β; ##, $p<0.01$ versus IL-1β. n=8.

Supplementary Figure 2. The effect of IL-1β on cyclooxygenase-2 (COX-2) does not alter with labour status in the myometrium. Myometrial tissue obtained from women not in labour at term as well as in term early and established labour was finely dissected into 3x3x3mm^3 explants. These were immediately pre-treated for 6 hours with ethanol vehicle or 1μM P4 followed by a 24 hour treatment with IL-1β (10ng/mL). Protein was extracted and quantified. Western blotting for cyclooxygenase-2 (COX-2) was performed as described in *Methods*. The data are expressed as mean + SEM of the delta (Δ) between IL-1β and control groups. Normality was tested using a Kolmogorov-Smirnov test followed by an ANOVA

662 with Bonferroni's Multiple Comparison Test. n=6-8. Labour status groups are labelled as
663 TNL (term no labour), TEaL (term early labour) and TEsL (term established labour).