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CRediT Statement

- Pei F Lai: conceptualisation, methodology, validation, formal analysis, investigation, data curation, writing – original draft, writing – review & editing, visualisation
- Ektoras X Georgiou: conceptualisation, methodology, validation, formal analysis, investigation, data curation, writing – review & editing, visualisation
- Rachel M Tribe: writing – review & editing, visualisation, supervision
- Mark R Johnson: resources, writing – review & editing, visualisation, supervision, project administration, funding acquisition

The Impact of Progesterone and RU-486 on Classic Pro-Labour Proteins & Contractility in Human Myometrial Tissues during 24-Hour Exposure to Tension & Interleukin-1 β .

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Abstract

Increased expression of pro-labour genes that encode cyclooxygenase-2 (COX-2), oxytocin receptor (OTR) and connexin-43 (Cx43) at parturition is often attributed to P4 functional withdrawal, based on findings from animal models and human primary myometrial cells. However, the cause of reduced myometrial P4 responsiveness that promotes contractions at labour is not fully determined. Uterine stretch occurs with advancing gestation but most *in vitro* experimental models do not take this into consideration. We aimed to examine whether tissue-level myometrial stretch influences the ability of P4 to regulate pro-labour protein abundance by using myometrial biopsies from term gestation pregnant women to assess the impact of 24 h exposure to combinations of (i) stretch-mediated tension, (ii) P4 (100 nM) and (iii) an anti-progestin, RU-486 (1 μ M). Firstly, we observed baseline COX-2 and Cx43 protein levels increased, whereas P4 content along with calponin-1 and progesterone receptor (PR) protein abundance decreased, in vehicle-treated tissues. P4 supplementation subtly reduced COX-2 levels in un-stretched tissues. Spontaneous and oxytocin-augmented contractility were unchanged by tissue culture exposure to P4 and/or RU-486. Interleukin-1 β (IL-1 β ; 1 ng/ml) enhanced COX-2 protein and PGE₂ content in un-stretched tissues. Overall, tissue stretch may, in part, regulate P4-sensitive pro-labour protein levels, but this is likely to be reliant on interaction with other *in utero* factors that were absent in our tissue cultures. More complex culture conditions should be evaluated in future to aid further development of a physiologically relevant model to improve our understanding of *in utero* myometrial P4 responsiveness.

1. Introduction

The ability of ovarian hormones to regulate uterine contractility was reported in 1913 by Stickel, which was soon followed by decades of research, more so with animal models than humans, into the effects of specifically progesterone (P4) on myometrial tissue contractility because of its apparent pro-relaxatory effects (Corner and Csapo, 1953). One central dogma that arose from these early studies is the concept of 'P4 block' (Csapo, 1956), whereby P4 was proposed to repress contractions in the myometrium such that labour is initiated only when P4 concentrations had been reduced. However, in contrast to many animals, circulating P4 levels do not decline before the onset of labour in humans (Mitchell and Taggart, 2009). Instead, it has recently been suggested that human labour is associated with reduced nuclear P4 concentration in myometrial cells caused by increased P4 metabolism from enhanced 20 α -hydroxysteroid dehydrogenase (20 α -HSD) activity (Nadeem *et al.*, 2016).

An alternative and currently more accepted theory for how myometrial P4 action is reduced at labour is that of P4 'functional withdrawal', whereby the myometrium is suppressed in its ability to mediate the pro-quiescent effects of P4 at labour despite no change in its concentrations (Astle *et al.*, 2003, Challis *et al.*, 2000, Konopka *et al.*, 2013, Lofgren and Backstrom, 1997, Rae *et al.*, 2007). This P4 functional withdrawal theory is predominantly associated with the opposing effects of P4 receptor (PR) isoforms A (PR-A) and B (PR-B) demonstrated in myometrial cells, whereby PR-B mediates the pro-gestational effects of P4 and PR-A dimerises with PR-B to inhibit its activity whilst also controlling the expression of pro-inflammatory mediators independently of PR-B (Merlino *et al.*, 2007, Peters *et al.*, 2017, Pieber *et al.*, 2001, Tan *et al.*, 2012). Functional P4 withdrawal at parturition is commonly characterised by an increase in PR-A:PR-B expression ratio (Mesiano *et al.*, 2002, Rezapour *et al.*, 1997, Tan *et al.*, 2012). PR function is also affected by differential histone modification of PR gene promoters (Chai *et al.*, 2014, Chai *et al.*, 2012) and the phosphorylation status of PR-A protein at its Ser345 residue (Amini *et al.*, 2016); both have been examined for their roles in the switch from pro-pregnancy to pro-labour state within myometrial cells in relation to inflammation.

The consequences of a decline in P4 influence on myometrial smooth muscle for animals and human cells in culture have been extensively explored, especially with regards to enhancement of inflammation and contractility at labour (Chwalisz *et al.* , 1995, Mesiano, 2007, Mitchell and Taggart, 2009). Relatively less is understood about what makes the myometrium alter its PR receptor levels and signalling dynamics, especially at tissue level, which has hindered efforts to develop therapies against complications like preterm labour (PTL). Uterine over-distension, inflammation and infection are often cited as major causal factors of PTL (Orsi and Tribe, 2008, Romero *et al.* , 2006), but how they induce labour and how myometrial P4 functional withdrawal contributes to PTL is not clear. Stretch can reduce PR-B abundance in primary human myometrial cells (Lei *et al.* , 2011) and enhance interleukin-1 β (IL-1 β)-driven cyclooxygenase-2 (COX-2) expression, which are not inhibited by medroxyprogesterone acetate (MPA; a synthetic progestin). Stretch can also increase OTR mRNA levels in primary human myometrial cells (Terzidou *et al.* , 2005). However, we have recently identified differences in gene expression profiles between tissue explants and primary cell cultures derived from human myometrial biopsies (Georgiou *et al.* , 2016), which suggests findings from primary cells are not directly translatable to *in vivo* myometrial function and it is better to study the impact of P4 in the myometrium using intact tissue; the same study also demonstrated tissue explants are better at retaining PR expression than primary cells and are P4 responsive in terms of regulating COX-2 expression.

In the present study, we further evaluated the use of myometrial tissue cultures for examining P4 action by incorporating stretch-mediated tension to better model the *in utero* state during advancing pregnancy. This approach was based on a previous study that examined the effect of stretch on the transcriptome profile of cultured human myometrial tissues (Tattersall *et al.* , 2012). Here, we focused on exploring whether tension can impact on the relationship between P4, inflammation and contractility in the myometrium, by measuring P4 concentrations and protein levels of both PR and three ‘classic’ P4-sensitive pro-labour markers that functionally mediate inflammation or contractility, namely COX-2, OTR and connexin-43 (Hadley *et al.* , 2018, Smith, 2007), in *ex situ* stretched tissues. Most importantly, we monitored for changes in protein and P4 concentrations that occurred as a result

of the tissue culture process itself to assess the relevance of our findings to the *in utero* condition. This is an approach that has seldom been applied to previous studies that used primary cells and tissue explants, which makes it difficult to judge their translational value. For our tissue cultures, we hypothesised that stretch alone can change baseline levels of labour-associated proteins. We identified a physiologically relevant P4 concentration to test the hypotheses that increased tension by tissue stretch can (i) repress pro-quiescent actions of P4, by changing PR abundance and P4 accumulation, to increase pro-labour protein levels and contractile output, and (ii) enhance cytokine-driven inflammation, which is commonly associated with labour (Hadley *et al.*, 2018), to increase the abundance of pro-labour proteins.

2. Materials and Methods

2.1. Ethical approval & collection of tissue samples

Myometrial biopsies from non-labouring pregnant women at term (37^{+0} to 40^{+2} weeks) gestation undergoing elective Caesarean section for singleton delivery ($n = 31$, whereby each biopsy was assigned to one of four sets of experiments for the study; Table 1) were collected in accordance with the Declaration of Helsinki guidelines and with approval from the local research ethics committee for Chelsea & Westminster Hospital (London, UK; Research Ethics Committee No. 10/H0801/45). Women were excluded from the study if they had diabetes (type I, type II and gestational), pre-eclampsia or obstetric cholestasis. Informed written consent was obtained from all women who participated. Biopsies excised from the upper margin of incision made to the lower segment of the uterus were promptly immersed into sterile Dulbecco's phosphate buffered saline (DPBS; Sigma-Aldrich, Dorset, UK) for $2-4^{\circ}\text{C}$ storage until dissection to commence tissue culture within the same day.

2.2. Tissue culture

Biopsies were dissected in cold DPBS into tissue strips of longitudinal muscle, which were each tied to polyester thread at both ends to give an assessment region of $\sim 10 \times 2 \times 1$ mm (Supplemental Table S1). Tissues were stretched by attaching one end of each strip to a glass weight of either 0.6-0.7 g or 2.2-2.4 g mass to apply 'low' or 'high' tension, respectively, or not tied to a weight to maintain them under 'no' tension; this approach of applying different amounts of tension to myometrial tissues was adapted from previous studies (Moraitis *et al.*, 2015, Tattersall *et al.*, 2012). Tissues were incubated with different combinations of 100 nM P4 (0.01 % v/v ethanol vehicle), 1 μM RU-486 (0.01 % v/v DMSO vehicle) and 1 ng/ml IL-1 β (0.01 % v/v sterile H₂O vehicle) (all supplied by Sigma-Aldrich), while suspended in serum-free DMEM supplemented with penicillin (20 units/ml) and streptomycin (20 $\mu\text{g/ml}$) (Sigma-Aldrich), for 24 h in a tissue culture incubator maintained at 37°C with 5 % CO₂ and a humidified atmosphere.

After this treatment period, each tissue strip was either (i) flash frozen in liquid nitrogen for -80°C storage after weighing, or (ii) used for isometric tension measurements. For samples stored immediately after 24 h culture, each tissue strip was cut from their tied ends to isolate their region of assessment after measuring their length, and each further cut into two segments before weighing and flash freezing in liquid nitrogen; their tissue dimensions and weights are presented in Supplemental Table S2. All glass beads were weighed after detaching them from their tissue strips for all 4 sets of experiments, and these values were used to calculate muscle stress after correcting their weights by subtracting 40 % to account for buoyancy in DMEM. For all biopsies, segments of DPBS-washed untreated tissue were also flash frozen in liquid nitrogen immediately after initiating 24 h treatment of their matched tissue strips, and these served as 'time zero' (t=0) controls.

2.3. Tissue extraction & protein quantification

Cell lysis buffer (New England Biolabs, Hitchin, UK), supplemented with cOmplete™ Mini Protease Inhibitor Cocktail (Roche, West Sussex, UK) and Halt™ Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific, Hemel Hempstead, UK), was added to frozen tissues and each homogenised in a CK28-R tube using a Precellys 24 bead-based mechanical homogeniser (Stretton Scientific, Stretton, UK), with two cycles of 5000 revolutions per minute mechanical disruption for 20 seconds and a 5-minute cooling interval on ice in-between cycles. Homogenates were centrifuged at 15890 g for 10 minutes at 4°C, and the resulting supernatant was isolated as the final tissue extract to use for immunoblotting (section 2.4) or P4 quantification (section 2.5). Protein quantification was undertaken using a DC (modified Lowry) protein assay kit (Bio-Rad, Hertfordshire, UK) by following the manufacturer's protocol; bovine serum albumin (Sigma-Aldrich) was used as the reference standard.

2.4. Immunoblotting

Samples of tissue extracts for Western blotting were prepared into lithium dodecyl sulphate (LDS)-based sample buffer (Life Technologies, Paisley, UK) with 50 mM dithiothreitol (Sigma-Aldrich). Western blotting, immunodetection and horse radish peroxidase (HRP)-based enhanced chemiluminescence (ECL) imaging were undertaken as previously described (Lai *et al.* , 2016);

Novex® Sharp pre-stained protein standards (Life Technologies, Paisley, UK) were used as molecular weight markers. Additionally, Ponceau-stained PVDF membranes were imaged by placing them between sheets of transparency film and scanning them using a Scanjet 4850 desktop scanner (Hewlett Packard, Reading, UK) to save as Tag Image File Format (TIFF) files at 600 dots per inch (dpi) resolution.

Primary antibodies used are listed in Supplemental Table S3. Protein was electrophoresed at a quantity of 10 µg per sample for immunodetection of COX-2, OTR and Cx43; 25 µg and 5 µg protein per sample was required for immunodetection of PR and calponin-1, respectively. Samples transferred onto polyvinylidene fluoride (PVDF) using the XCell SureLock Mini-Cell and XCell Blot Module System (Life Technologies) were probed with primary antibodies for COX-2 and Cx43 detection. Samples transferred onto PVDF using the Trans-Blot Turbo Transfer System (Bio-Rad) were probed with primary antibodies for OTR, calponin-1 and PR (both PR-A and PR-B isoforms) detection.

Densitometry analysis was undertaken using ImageJ v1.50i software (National Institutes of Health, Bethesda, MD, USA; Research Resource Identifier (RRID) SCR_003070). Histogram-based analysis was used to obtain pixel intensity values from all ECL bands. Pixel intensity values for COX-2, Cx43, calponin-1 and PR were normalised to those of β -tubulin. Pixel intensity values for OTR were normalised to those from Ponceau staining, whereby raw integrated density values were obtained from scanned greyscale images of Ponceau-stained Western blot membranes (~30-110 kDa region of each sample lane) and divided by region of analysis size to give corrected pixel intensity values that represented total protein staining. We had intended to use glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the housekeeping protein for normalisation of OTR data because it is better separated in molecular mass from OTR than β -tubulin, but we found that raw GAPDH abundance values were decreased relative to t=0 controls for tissues maintained under high tension during 24 h tissue culture and hence used Ponceau staining for OTR normalisation instead.

2.5. Progesterone measurements

A MILLIPLEX® MAP Steroid/Thyroid Hormone Magnetic Bead Panel Endocrine Multiplex Assay kit (Millipore, Watford, UK) was used, with guidance from the manufacturer's protocol, to determine the P4 concentrations of tissue extracts. Each sample was used at a quantity of 2 µg total protein per assay well. Immunoassay plates were measured for their fluorescence levels using a Bio-Plex® MAGPIX multiplex reader with Bio-Plex Manager v6.1 software (Bio-Rad; RRID SCR_014330). Plate readings for each sample were converted to P4 concentrations using a calibration curve produced from P4 standards, and subsequently normalised to the weight of tissue from which they were extracted.

2.6. Prostaglandin E2 (PGE₂) ELISA

PGE₂ content in media samples from 24 h tissue cultures (section 2.2) were analysed using a PGE₂ ELISA kit (Enzo Life Sciences, Exeter, UK) following manufacturer's protocol. Samples were diluted with fresh DMEM at 1:5 and 1:30 ratio for experiments without and with IL-1β treatment, respectively; these dilution ratios were determined from optimisation experiments to keep colorimetric readings of samples within the range defined by the PGE₂ standards. All PGE₂ concentration values were normalised to the wet weight of their matched tissue strips.

2.7. Isometric tension measurements and data analysis

Tissue strips were mounted to MLT0201 transducers (ADInstruments, Oxford, UK) attached to an 8-channel organ bath (Panlab; Barcelona, Spain), using the polyester threads tied to them during 24 h tissue culture after glass beads had been detached. Organ bath chambers were filled with physiological saline solution (PSS; same composition as previously used (Lai *et al.*, 2016)), which was maintained at 37°C with 95 % O₂/5 % CO₂ aeration. Tissues were submerged into these PSS-filled chambers, which contained combinations of 100 nM P4, 1 µM RU-486 and vehicle controls that matched their 24 h treatment conditions, immediately after securing them to their transducers. The organ bath was connected to a ML870 PowerLab unit with LabChart 7 software (ADInstruments) for data acquisition.

After 5 minutes from the start of data recording, 29.4 mN tension was applied to all tissues and 1 h 25 minutes of spontaneous activity recorded prior to applying cumulative concentrations of Syntocinon® (synthetic oxytocin; Alliance Pharmaceuticals, Wiltshire, UK), which was added to tissues every 25 minutes (1×10^{-11} to 1×10^{-7} M concentrations to encompass a dynamic range for contractile response (Crankshaw and Morrison, 2011)). After incubation with 1×10^{-7} M oxytocin, PSS at all tissues was exchanged with PSS containing 60 mM KCl (KPSS), to stimulate maximal depolarisation and confirm tissue viability. After 10 minutes in KPSS, this buffer was replaced with PSS for 10 minutes before tissues were cut from their tied ends and each into two segments prior to weighing and flash freezing in liquid nitrogen for -80°C storage.

Absolute mean integral tension (MIT) values (mN/s/mm^2 units) were calculated as described previously (Lai *et al.*, 2016); spontaneous contractile output (the ‘control period’) was determined from the last 15 minutes prior to applying 1×10^{-11} M oxytocin, and oxytocin-induced contractile output was determined from the last 15 minutes of exposure to each oxytocin concentration of interest. Contraction frequency was determined by manual counting of contraction peaks using LabChart 7 chart view of raw data. Summary scores were calculated using a previously described equation (Matthews *et al.*, 1990).

2.8. Statistical analyses

Prism v8.0 software (GraphPad, La Jolla, CA, USA; RRID SCR_002798) was used for statistical analysis. Fit to normal distribution was assessed using either Shapiro-Wilk (where $n = 7$) or D’Agostino-Pearson (where $n = 8$) test. Contractility data was assessed using Kruskal-Wallis with Dunn’s *post-hoc* analysis and 2-way ANOVA with Bonferroni’s *post-hoc* analysis. Western blotting and ELISA data were assessed using 1-way ANOVA with the appropriate *post-hoc* analysis tests as stated in Figure legends; a mixed-effects model was implemented for comparisons between groups that contained different n numbers, for which data not normally distributed were log transformed. Student’s paired t test and Wilcoxon test were also used to assess Western blot data for experiments that involved IL-1 β treatment. Oxytocin $\log\text{EC}_{50}$ values from non-linear regression analysis of ΔMIT

data were compared using an extra sum-of-squares F test. Differences were deemed statistically significant where $p \leq 0.05$ and p values from comparisons between ≥ 3 groups were adjusted for multiplicity of pairings used in the analysis. Differences associated with varying the tension applied to tissues with matched P4, RU-486 and IL-1 β 24 h treatments for all Western blot and ELISA data were assessed using 2-way ANOVA with Bonferroni's *post-hoc* analysis, but no statistical significance was found (all $p > 0.05$).

3. Results

3.1. Change in tissue length and tension after 24 h culture

Based on an experiment with tissue strips from myometrial biopsies not used for the present study (Supplemental Table S1), we determined our 24 h tissue culture protocol results in values for muscle strain ($\Delta L/L$, where 'L' as denominator is length at $t=0$) of approximately -0.1, 0 and 0.5 for tissues maintained under no, low and high tension, respectively. Thus our low tension-cultured tissues had maintained $t=0$ length, high tension-cultured tissues were stretched beyond $t=0$ length by ~50%, and tissues cultured under no tension shortened from $t=0$ length by ~10 %. Tissue lengths after 24 h culture for this $n = 4$ experiment were similar to those associated with the present study (Supplemental Table S2). Muscle stress, which was calculated from the weight of beads attached to tissues (after correcting for buoyancy in DMEM) and normalised to cross-sectional area after 24 h culture, was higher in high tension-cultured tissues compared to those maintained under low tension (Supplemental Table S2).

3.2. Labour-associated and PR proteins in myometrial tissues after 24 h tissue culture with exposure to vehicles and tension

Before we assessed the impact of tissue stretch and PR ligands, we needed to establish how tissue culture itself affected baseline levels of pro-labour and PR proteins. For this, we examined changes in protein content associated with 24 h exposure to vehicles for P4, RU-486 and IL-1 β in serum-free DMEM when compared to $t=0$ controls (Figures 1 & 2). These observations were made from two separate sets of experiments, which were both used to evaluate the effect of PR ligands (P4 and RU-486) on the protein levels of COX-2, OTR, Cx43, calponin-1 and PR. One set of experiments assessed the effect of tension (Figures 4 & 5) and the other set additionally assessed the impact of exogenous IL-1 β (Figures 8 & 9). The 'high tension' condition was only tested for experiments that did not include IL-1 β due to biopsy size restricting the number of conditions that could be included in each experiment.

Tissue culture for 24 h with vehicles increased levels of COX-2 ($p < 0.0001$ for all tension conditions) and Cx43 ($p = 0.02$, no tension; $p = 0.0006$, low tension; $p < 0.0001$, high tension), whereas OTR was unchanged ($p = 0.87$, no tension; $p = 0.08$, low tension; $p = 0.47$, high tension) and calponin-1 decreased ($p = 0.02$, no tension; $p = 0.004$, low tension; $p = 0.0005$, high tension), when compared to biopsy-matched $t=0$ controls (Figure 1). PR protein abundance decreased for both PR-A ($p = 0.004$, no tension; $p = 0.003$, low tension; $p = 0.0008$, high tension) and PR-B ($p = 0.01$, no tension; $p = 0.002$, low tension; $p = 0.004$, high tension) following the same tissue culture conditions; the reduction in both PR-A and PR-B did not result in changes to PR-A:PR-B protein ratio (Figure 2).

3.3. The effect of tension during 24 h culture on P4 accumulation in human myometrial tissues

The ability of myometrial tissues to sustain its P4 content after 24 h culture was assessed in a third set of tissues maintained under different amounts of tension, while treated with P4 or its vehicle (Figure 3). Baseline quantities of P4 in tissues maintained in culture for 24 h were 72.6 ± 7.8 (no tension; $p = 0.01$), 77.1 ± 8.9 (low tension; $p = 0.02$) and 77.7 ± 5.4 (high tension; $p = 0.04$) % less than $t=0$ controls. P4 supplementation at 100 nM concentration resulted in 16.8 ± 1.3 (no tension; $p = 0.007$), 58.2 ± 35.5 (low tension; $p = 0.02$) and 32.5 ± 5.4 (high tension; $p = 0.001$) times higher P4 levels in tissue extracts than tension-matched vehicle controls. Compared to $t=0$, this concentration of exogenous P4 increased its tissue content by 5.0 ± 1.7 (no tension; $p = 0.47$), 5.5 ± 1.7 (low tension; $p = 0.18$) and 7.1 ± 2.0 (high tension; $p = 0.01$) fold; a lack of significant difference, for all except high tension-cultured tissues, indicated 100 nM P4 was a good match to near-physiological levels represented by $t=0$. Differential application of tension to tissues for 24 h demonstrated no impact on their P4 content at the baseline level nor when culture media was supplemented with this steroid.

3.4. Impact of P4 and RU-486 in 24 h culture with tension on labour-associated and PR protein levels in myometrial tissues and their extracellular PGE₂ concentrations

Supplementation of culture media for 24 h with a near-physiological concentration of P4 had a weak suppressive effect on COX-2 protein abundance in myometrial tissues maintained under no tension (26.1 ± 19.5 % reduction; $p = 0.32$) or low tension (39.8 ± 11.9 % reduction; $p = 0.18$) when

compared to vehicle controls (Figure 4a). RU-486, a clinically relevant antiprogesterone (Bygdeman *et al.*, 1993), was used with the intention of examining whether its efficacy in antagonism of PR function can be influenced by tension to result in altered pro-labour protein levels. P4 in combination with RU-486 enhanced COX-2 abundance relative to P4 treatment alone only significantly for tissues maintained under no tension, which demonstrated a mean fold difference of 2.1 ± 0.4 ($p = 0.04$). COX-2 catalyses the rate-determining step of the prostaglandin synthesis pathway and so its expression levels at labour are often correlated with PGE₂ concentrations, which most studies have shown to increase with labour in uterine tissues (Hadley *et al.*, 2018). We measured PGE₂ to assess how well COX-2 protein abundance matched its functional output. From this, we observed changes in COX-2 protein levels in 24 h cultured tissues closely mimicked those in extracellular PGE₂ concentrations but none were statistically significant (Figure 4b). There were no differences in OTR (Figure 4c), Cx43 (Figure 4d) and calponin-1 (Figure 4e) protein content in tissue extracts when all P4 and RU-486 treatment combinations were compared to each other, irrespective of amount of tension applied.

P4 and RU-486 co-treatment resulted in PR-A reduction by 41.0 ± 8.1 (no tension; $p = 0.002$), 46.0 ± 13.7 (low tension; $p = 0.05$) and 56.7 ± 15.0 (high tension; $p = 0.02$) % relative to tension-matched vehicle control tissues (Figure 5). RU-486 treatment for 24 h, in the absence of P4, also reduced PR-A protein content, though only significantly at low tension-incubated tissues, by 29.6 ± 7.7 (no tension; $p = 0.14$), 54.3 ± 6.8 (low tension; $p = 0.01$) and 55.6 ± 5.9 (high tension; $p = 0.14$) % relative to tension-matched vehicle controls. PR-B protein expression was unaffected by all P4 and RU-486 combinations tested, irrespective of amount of tension applied during treatment. Whereas PR-A:PR-B protein ratio was significantly reduced by 30.9 ± 8.2 (no tension; $p = 0.01$), 31.6 ± 5.4 (low tension; $p = 0.005$) and 30.9 ± 11.4 (high tension; $p = 0.04$) % for 24 h treatment with RU-486 alone, when compared to tension-matched vehicle controls. P4 and RU-486 co-treatment for 24 h also decreased PR-A:PR-B ratio by 29.1 ± 9.3 (no tension; $p = 0.01$), 33.0 ± 8.9 (low tension; $p = 0.002$) and 28.2 ± 9.7 (high tension; $p = 0.23$) % relative to tension-matched vehicle controls.

3.5. Contractility of myometrial tissues after 24 h treatment with P4 and RU-486

The functional impact of P4 and RU-486 treatment in 24 h tissue cultures on spontaneous and oxytocin-augmented contractility is presented in Figures 6 & 7. We chose to only measure the contractility of 24 h cultured tissues incubated under low and high tension because these two tension conditions have previously been shown to produce differences in contractile output (Tattersall *et al.*, 2012). Thus we aimed to assess whether an absence of notable difference in abundance of our selection of protein markers, which we observed in tissues incubated for 24 h without subsequent contractility measurements (Figures 4 & 5), translates into no difference in physiological output.

Spontaneous contractility tended to be greater after 24 h tissue culture under high tension, especially for tissues only exposed to vehicles for P4 and RU-486, whereby MIT values were 5.6 ± 2.6 (ethanol & DMSO; $p = 0.02$), 1.9 ± 0.5 (ethanol & RU-486; $p > 1$), 6.0 ± 2.9 (P4 & DMSO; $p = 0.20$) and 3.4 ± 1.0 (P4 & RU-486; $p > 1$) times greater for high tension-incubated tissues than those maintained under low tension prior to isometric tension measurements (Figure 6b). This observation for spontaneous contractions was not associated with differences in their frequency. Basal tensions for low and high tension-cultured tissues during spontaneous contractions, measured from the beginning of the control period represented in Figure 6b, were 0.3 ± 0.03 and 0.5 ± 0.07 mN, respectively, for vehicle (E+D)-treated tissues and were not significantly different ($p = 0.38$); basal tensions prior to oxytocin response were also not significantly different between different P4 and RU-486 combinations (data not shown).

Multiple concentrations of oxytocin were applied to tissues in a cumulative manner to allow observation for changes in response kinetics, such as maximal response and EC_{50} shifts, and mimic the pulsatile nature of *in utero* exposure to this uterotonic (Tribe *et al.*, 2012). Tissues maintained under high tension during 24 h culture demonstrated greater maximal contractile output in response to oxytocin, which contributed to summary score MIT values that were 2.7 ± 1.1 (ethanol & DMSO; $p = 0.33$), 3.5 ± 0.9 (ethanol & RU-486; $p = 0.005$), 3.8 ± 1.5 (P4 & DMSO; $p = 0.01$) and 3.5 ± 0.8 (P4 & RU-486; $p = 0.02$) fold greater than those for tissues maintained under low tension during 24 h culture (Figure 7). Negative log EC_{50} (pEC_{50}) values for oxytocin Δ MIT were not notably different ($p = 0.98$; Table 2).

3.6. Impact of IL-1 β , along with P4 and RU-486, in 24 h culture on labour-associated and PR protein levels in myometrial tissues and their extracellular PGE₂ concentrations

Concentrations of IL-1 β have been shown to increase at labour in the myometrium (Osman *et al.*, 2003) and it has been used to promote myometrial inflammation in primary cells (Lei *et al.*, 2011) and tissue explants (Amini *et al.*, 2016, Georgiou *et al.*, 2016) of human myometrium for the study of P4 signalling. Here, only tissues maintained under no and low tension were used to examine the influence of IL-1 β exposure on the abundance of our protein markers, in order to simulate a pro-inflammatory uterine environment in the absence of any impact from tissue over-distension that may obscure the effects of this cytokine; we were cautious of this due to differences in contractility between low and high tension-incubated tissues (Figures 6 & 7). It was also not possible to include all three tension conditions due to biopsy size restrictions. A concentration of 1 ng/ml IL-1 β was chosen based on our optimisation data obtained from human myometrial tissue explants, which demonstrated this amount of the cytokine to be the minimum required to significantly increase COX-2 protein content (Georgiou *et al.*, 2016).

After 24 h treatment with IL-1 β , tissues contained 3.0 ± 0.8 (no tension; $p = 0.008$) and 2.0 ± 0.5 (low tension; $p = 0.21$) times more COX-2 protein than tension-matched vehicle controls (Figure 8a). All other comparisons between combinations of 24 h treatment with P4, RU-486 and IL-1 β did not show any significant differences for COX-2. Extracellular PGE₂ concentrations (Figure 8b) were increased after IL-1 β stimulation, in the absence of P4 and RU-486, by 3.6 ± 0.8 (no tension; $p = 0.04$) and 2.8 ± 0.8 (low tension; $p = 0.15$) fold. This IL-1 β -driven enhancement of extracellular PGE₂ levels was only significant for tissues maintained under no tension, as observed for COX-2 protein in culture-matched tissues. The protein abundance of OTR (Figure 8c), Cx43 (Figure 8d) and calponin-1 (Figure 8e) were not affected by exogenous IL-1 β , irrespective of amount of tension applied and which combination of P4 and RU-486 was present. IL-1 β exposure itself did not significantly affect PR protein content in tissues, in the absence of P4 and RU-486, for both isoforms after 24 h culture (Figure 9). For all P4 and RU-486 combinations tested in 24 h tissue culture with the application of IL-1 β , the only significant change to PR identified was the 46.1 ± 15.6 % lower levels of PR-A

369 present in tissues treated with RU-486 in the absence of P4 ($p = 0.02$), as observed in experiments
370 without IL-1 β treatment (Figure 5).

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4. Discussion

Most previous work investigating the impact of P4 on human myometrial function used monolayer primary cell cultures. This approach has two limitations: (i) the inability to quantify contractile force output as a measure of function, and (ii) the absence of a 3-dimensional structural arrangement that includes other cell types normally present in the myometrium. These factors that reflect deviations from the *in utero* state may limit the conclusions that can be drawn from their experimental data. We have previously used myometrial explants as a tissue-based model to examine the impact of RU-486 and IL-1 β on inflammation-associated P4 signalling (Georgiou *et al.*, 2016), but did not monitor contractility to assess functional integrity nor examined the influence of tension. Here we incorporated the application of tension by stretch, a notable feature of change at the uterus with advancing gestation, to our tissue culture protocol to assess its impact with exogenous P4, RU-486 and IL-1 β on (i) P4 and PGE₂ concentrations, (ii) myometrial contractility, and (iii) protein content of PR (Georgiou *et al.*, 2016) and P4-regulated contraction-associated proteins (CAPs: COX-2, OTR and Cx43) that act as classic pro-labour markers (Renthal *et al.*, 2010, Williams *et al.*, 2012). Calponin-1, which inhibits actomyosin interactions to promote relaxation in the absence of calmodulin binding and protein kinase C phosphorylation, was used as a smooth muscle marker (Liu and Jin, 2016).

A duration of 24 h for tissue culture, along with the use of 0.6-0.7 and 2.2-2.4 g weights to apply isotonic tension, were chosen based on findings from a previous study that examined the effect of both 24 and 65 h tissue culture, which concluded these durations of tissue culture were not different in their effect on contractile output (Tattersall *et al.*, 2012). Unlike this and other published studies that have used human myometrial tissue cultures to assess contractility (Cordeaux *et al.*, 2010, Moraitis *et al.*, 2015, Young and Zhang, 2004), we did not include serum in our culture media in an attempt to minimise cell proliferation and differentiation that could dissipate the *in utero* state (Ma *et al.*, 1998, Palmberg and Thyberg, 1986), and our previous microarray analysis suggests 30 h culture in serum-free media maintains a transcriptome profile of myometrial tissues that is reasonably similar to freshly isolated biopsies (Georgiou *et al.*, 2016). It has recently been demonstrated that 48 h culture with 5 % v/v charcoal-stripped serum does not alter mRNA levels for COX-2, OTR and PR in human

myometrial tissues, but no corresponding data for protein abundance nor contractility were provided (Ilicic *et al.* , 2017a, Ilicic *et al.* , 2017b). Furthermore, in a separate study, we have measured oxytocin response of tissues cultured for 48 h in serum-free media, which were notably more inconsistent in their contractile output than tissues cultured for 24 h (data not shown). In addition to good physiological stability of 24 h serum-free cultured tissues, we saw this duration of culture to be sufficient for observing changes in protein abundance that would be influenced by P4-regulated gene expression.

4.1. The impact of tension-incorporated tissue culture on baseline conditions

We examined the difference between biopsy-matched t=0 control and 24 h vehicle-treated tissues to determine to what extent stretch-mediated tension itself altered the phenotype of myometrial tissues in the absence of P4 supplementation. For all tension conditions tested, protein levels of COX-2 and Cx43 increased, while those of calponin-1 and PR (both PR-A and PR-B) decreased, after 24 h tissue culture, and there was no truly discernible impact from differential application of tension to these changes. OTR abundance and PR-A:PR-B ratio for protein content were unaffected by both 24 h tissue culture and tension, which suggests increased COX-2 availability had limited impact on these proteins. We have previously shown COX-2 protein abundance can increase following 24 h culture of myometrial explants in the absence of both tissue stretch and exogenous P4 (Georgiou *et al.*, 2016). Similar observations of tissue culture effects on PR have also been reported previously (Georgiou *et al.*, 2016, Peters *et al.*, 2017) using myometrial explants. Overall, factors of 24 h tissue culture other than tension are likely to bear a greater influence on baseline levels of both pro-labour and PR proteins, which remain to be elucidated most likely with the use of more complex tissue culture conditions.

Ilicic *et al* previously reported on the effects of myometrial tissue culture and tension on mRNA levels of COX-2 and OTR (Ilicic *et al.*, 2017a), as well as PR-A and PR-B (Ilicic *et al.*, 2017b). They found that the culture process increased COX-2, but decreased OTR, mRNA levels; their duration of culture was 48 h, which may indicate a time-related explanation for this discrepancy (Ilicic *et al.*,

2017a) if not a difference between the regulation of gene transcription and protein turnover. The same explanation could apply to differences between studies for PR-A abundance and PR-A:PR-B protein ratio (Ilicic *et al.*, 2017b). PR-A:PR-B protein ratio did not increase in un-stretched tissue strips, unlike what we had previously observed from tissue explants, which may have been due to a difference in culture duration (24 *versus* 30 h) (Georgiou *et al.*, 2016). Whereas Ilicic *et al* showed stretch did not change baseline COX-2 and OTR mRNA levels (Ilicic *et al.*, 2017a), which correlates well with protein data from the present study. These comparisons between studies highlight the need to consider time-dependent effects on baseline conditions in future use of this tissue-based experimental model.

Overall, the changes in levels of COX-2, Cx43, calponin-1 and PR protein we observed from the present study together suggest the tissue culture process itself promotes a pro-contractile phenotype that resembles a labour-associated event irrespective of tension, which provides a useful model for parturition research. However, there is limited agreement with regards to changes in myometrial COX-2 and Cx43 protein that occur at human parturition. Some studies suggest myometrial COX-2 protein abundance is unchanged (Slater *et al.* , 1999, Sparey *et al.* , 1999) but others say it increases during labour (Choi *et al.* , 2007, Tattersall *et al.* , 2008). Myometrial Cx43 mRNA levels have been reported to increase at labour (Chow and Lye, 1994, Pierce *et al.* , 2002, Renthall *et al.*, 2010), but those for Cx43 protein have been observed to not change (Chow and Lye, 1994, Pierce *et al.*, 2002, Tattersall *et al.*, 2008) or increase (Sparey *et al.*, 1999). An absence of change in PR-A:PR-B ratio in our present study is in contrast to previous observations from myometrial biopsies of labouring and non-labouring women (Merlino *et al.*, 2007, Pieber *et al.*, 2001) and rhesus macaques (Haluska *et al.* , 2002). However, it is of interest to note that PR-A and PR-B expression has been found to decrease, whilst maintaining a constant PR-A:PR-B ratio, in the myometrium of labouring guinea pigs (Palliser *et al.* , 2010, Welsh *et al.* , 2014), which, like humans, do not demonstrate a decrease in systemic P4 levels at labour.

4.2. Comparison to stretched primary cells

Our previous work with primary myometrial cells, which were also cultured in DMEM, showed 1 and 6 h stretch increased COX-2 mRNA and protein content, respectively (Lei *et al.*, 2011, Sooranna *et al.*, 2004), but COX-2 protein was not different after prolonged periods of stretch (30 h) when compared to non-stretched cells (Lei *et al.*, 2011), consistent with data from the present study. OTR mRNA increased at 1 h but not at 6 h in stretched primary cells (Lei *et al.*, 2011, Terzidou *et al.*, 2005), which suggests prolonged stretch also does not change OTR levels and correlates well with our protein data from 24 h tissue cultures. For PR-A and PR-B, stretch of primary cells for 30 h can decrease both PR isoforms (Lei *et al.*, 2011), which is similar to our observations in the present study except for the decrease in protein levels being more significant for PR-B than PR-A in primary cells. The effect of stretch on Cx43 levels in primary cells has not been assessed. However, myometrial tissue from non-labouring women with singleton and twin pregnancies have been previously compared to show no difference in their Cx43 protein content (Lyall *et al.*, 2002). Together, prolonged *in vitro* stretch at the cell and tissue level using similar culture media leads to similar consequences for their abundance of classic labour-associated proteins except for PR.

4.3. Tissue P4 concentrations

A substantial decrease in P4 concentrations within intact myometrial tissue was observed following 24 h culture in the absence of exogenous P4, alongside changes in the protein abundance of COX-2, Cx43, calponin-1 and PR discussed above. Media supplementation with 100 nM P4 maintained the tissue content of this steroid at levels similar enough to those in t=0 samples to be deemed physiologically relevant. The amount of P4 accumulation achieved was slightly enhanced by tension, and thus the rate of P4 catabolism in myometrial tissue is unlikely to have been increased by stretch. This is consistent with previous studies that have shown myometrial P4 concentrations increase with gestation (Batra and Bengtsson, 1978, Runnebaum and Zander, 1971), implying that a probable increase in uterine wall tension with advancing pregnancy, prior to parturition, does not cause a decline in myometrial P4 content. Instead, it is possible that myometrial tissue stretch caused structural changes, such as reducing rigidity of cellular plasma membranes, to promote P4 retention. On the other hand, other studies have shown no difference in myometrial P4 concentrations between

non-labouring and labouring women at term (Batra *et al.* , 1979, Rezapour *et al.*, 1997, Rezapour *et al.* , 1996).

A more recent study demonstrated labour-associated reduction in P4 concentration specifically located within the nucleus of human myometrial cells (Nadeem *et al.*, 2016). The findings from our present study focus on quantification of total tissue P4 content, which cannot be used to exclude the possibility that organelle-specific changes in P4 concentrations occurred within the myometrial cells of our cultured tissues. The location of a signalling molecule is certainly as important as its quantity at subcellular level in relation to their impact on cellular function. Our understanding of how P4 controls myometrial phenotype will most likely be improved through further examination of the relationship between tissue tension and intracellular P4 localisation, using techniques that can differentiate subcellular compartments of myometrial smooth muscle cells within intact tissue.

4.4. Progesterone responsiveness at protein level

P4 supplementation resulted in a slight tendency to reduce COX-2 abundance in cultured tissues, which was subtly hindered by tension. The latter may have been mediated through ERK1/2 phosphorylation promoted by tension, which has previously been demonstrated in myometrial tissue cultures (Aye *et al.* , 2018) and primary cells (Sooranna *et al.* , 2005). ERK1/2 is capable of phosphorylating PR-A at its Ser345 residue, which can enhance its trans-repressive effect on PR-B activity to reduce suppression of inflammation by P4 (Amini *et al.*, 2016). RU-486 reversed P4-mediated COX-2 reduction significantly in un-stretched tissues only, emphasising the possibility that stretch-mediated tension can repress anti-inflammatory effects of P4. From the perspective of physiological relevance, 24 h culture under low tension preserved t=0 tissue length and, in turn, potentially tension that resembles the *in utero* state more so than no and high tension-cultured tissues. Thus, *ex situ* changes in the absence of stretch-mediated tension could be interpreted as a gain in P4 responsiveness upon complete removal of uterine stretch, which would occur after childbirth. Additionally, an apparent absence of P4 responsiveness in our low tension-cultured tissues could suggest tension-mediated repression of P4 signalling is present at term gestation pregnancy before

labour, but is suppressed by *in utero* factors that were absent in our tissue cultures and lost at parturition.

Exogenous P4 at a near-physiological concentration was not sufficient to completely prevent culture-associated enhancement of COX-2 abundance. Furthermore, protein levels of OTR, Cx43 and calponin-1 for all combinations of P4 and RU-486 treatments were not different, irrespective of the amount of tension applied. The culture process reduced baseline PR protein content, which could have suppressed P4 responsiveness below the threshold levels required to maintain low amounts of these pro-labour proteins. A combination of P4 and stretch, both regarded as important in controlling myometrial phenotype, was not enough to prevent this apparent culture-associated reduction in PR responsiveness, which emphasises the potential importance of additional *in utero* factors for stabilisation of PR protein in human myometrial tissues.

P4 supplementation did not prevent baseline reduction of PR levels by tissue culture, which demonstrated ligand binding does not enhance PR protein content in *ex situ* myometrial tissues. On the contrary, especially in the case of RU-486, it would appear that PR ligand binding in cultured myometrial tissues reduced PR-A abundance, and it is possible that the partial PR agonist characteristics of RU-486 were responsible for such observations from this ‘antiprogestin’ (Khan *et al.* , 2013, Raaijmakers *et al.* , 2009). P4-driven reduction of PR protein has been demonstrated in immortalised human myometrial cells (Peters *et al.*, 2017) and primary human cervical stromal fibroblasts (Ackerman *et al.* , 2016), which has been attributed to enhanced proteasome degradation of PR mediated by its post-translational modification at P4 binding; P4-driven reduction in PR has also been observed in human decidual cells (Lockwood *et al.* , 2010). It is possible that RU-486 activates the same pathways for reducing levels of PR protein when acting as a PR agonist, and it has the potential to do so more effectively than P4 because RU-486 has a higher binding affinity for PR (Pullen *et al.* , 2006). Decreased PR protein abundance in uterine (Fang *et al.* , 1997) and brain (Carrillo-Martinez *et al.* , 2011) tissues following *in vivo* administration of RU-486 in rats has been demonstrated, but without elucidation of the mechanisms responsible. RU-486 is best known for acting as an antiprogestin that mediates P4 functional withdrawal by inhibiting PR activity; RU-486

could achieve this *in utero* by, in part, promoting a decrease PR abundance to suppress P4 responsiveness.

At the same time, mimicry of P4 by RU-486 is partly reliant on low availability of co-repressors, especially those that interact with the C-terminal region of PR (Liu *et al.* , 2002, Xu *et al.* , 1996). The partial agonist effects of RU-486 can also be promoted by a high availability of co-activators (Liu *et al.*, 2002, Wang *et al.* , 2007). Differential impact of RU-486 on PR-A and PR-B is apparent in the present study, which may relate to these PR isoforms having different binding affinities for their co-regulators; PR-A mostly associates with corepressors and PR-B can associate with both coactivators and corepressors (Giangrande *et al.* , 2000). We speculate that selective reduction of PR-A abundance by RU-486 in our cultured myometrial tissues could be indicative of low corepressor activity at P4-responsive genes. Significant reductions in PR-A:PR-B protein ratio in tissues treated with RU-486 did not directly correspond to lower levels of COX-2, OTR and Cx43, which demonstrates PR isoform ratio is not a direct determining factor when it comes to any P4-driven reduction of human myometrial pro-labour proteins. Instead, these observations support the need to examine other factors of PR activity, such as co-regulator interaction and post-translational modification, to enrich our understanding of how P4 signalling promotes suppression of pro-labour protein levels during myometrial quiescence.

4.5. Functional responsiveness to oxytocin

Oxytocin-augmented contractility was enhanced by 24 h tissue culture under high tension, which is in agreement with observations from similar studies (Aye *et al.*, 2018, Moraitis *et al.*, 2015). This increase in maximal contractile output, in the absence of an EC₅₀ shift, occurred following tissue culture under different amounts of stretch-mediated tension that did not change myometrial OTR or Cx43 levels. Instead, it is likely that enhanced efficiency of each contraction occurred *via* changes in structural organisation of the contractile apparatus (Schofield and Wood, 1964), potentially along with alterations to its post-translational modifications and/or protein-protein binding interactions (Aasen *et al.* , 2018, Kim *et al.* , 2017, Wheatley and Hawtin, 1999), during tissue culture. Furthermore, OTR

and Cx43 are by no means the only proteins that contribute to myometrial contractility; others include ion channels (Greenwood and Tribe, 2014) and components of the actomyosin apparatus (Morgan, 2014). Assessment of such alternative contraction-associated proteins were outside the scope of the present study, so we cannot exclude the possibility that changes in their expression and/or activity in our tissue cultures contributed to the tension-associated differences in contractile output we observed. Thus, other proteins that can modulate smooth muscle contractility need to be examined in future to further elucidate the impact of tension on myometrial function in relation to P4 responsiveness. With regards to spontaneous contractility prior to oxytocin stimulation, this was also enhanced by 24 h culture in high tension-cultured tissues, which was suppressed to a minor extent by RU-486 most likely *via* its non-genomic pro-relaxation effects (Perusquia *et al.*, 2009).

4.6. Sensitivity to cytokine stimulation

IL-1 β removed the ability of a near-physiological concentration of P4 to reduce COX-2 abundance in no tension-cultured tissues of the present study. This supports the idea that an increase in pro-inflammatory cytokines suppress the anti-inflammatory effects of P4 in the absence of a change in P4 concentrations (Peters *et al.*, 2017). Our low tension-cultured tissues showed IL-1 β was unable to increase COX-2 protein to levels above those subtly promoted by stretch-mediated tension. IL-1 β had no impact on OTR and Cx43 abundance, irrespective of tension applied and presence of PR ligands, which emphasises the possibility that responsiveness of these pro-labour proteins to P4 regulation may have been hindered by the absence of other *in utero* factors.

IL-1 β lessened RU-486-associated reduction of PR-A levels, especially when P4 was present, which correlates well with a previous study that used myometrial explants to show IL-1 β can enhance PR-A stability (Peters *et al.*, 2017) and our observation in the absence of P4 suggests IL-1 β -mediated stabilisation of PR-A is dependent on its ligand-bound conformational state (Raaijmakers *et al.*, 2009). However, IL-1 β did not increase PR-A:PR-B protein ratio, despite higher amounts of both COX-2 and PGE₂ in the case of no tension-cultured tissues. Similarly, 24 h IL-1 β treatment of human cervical fibroblasts can increase their expression of both PR isoforms without increasing PR-A:PR-B

ratio, despite also increasing COX-2 mRNA levels and PGE₂ concentrations (Pierce *et al.* , 2018). Simultaneous lack of change in myometrial OTR and Cx43 levels supports the concept that their enhancement during cytokine-driven inflammation relies on PR-A dominance. PGE₂ stimulation has been shown to promote PR-A dominance in a myometrial cell line but this was only significant with <1 nM PGE₂, above which PR-B expression also increased to result in no PR-A dominance at the mRNA level (Madsen *et al.* , 2004). Extracellular PGE₂ concentrations in our tissue cultures exceeded 1 nM for both vehicle control treatment and after IL-1 β stimulation, when taking into account an average tissue strip weight of 83.7 ± 9.4 (mean $\pm$ SEM) mg, and this could explain their lack of PR-A dominance if assuming this effect of PGE₂ in a myometrial cell line can be translated to cultured tissues.

5. Conclusion

The key advantage of using a tissue-based model to study myometrial function is that it is physiologically truer in representation of the *in utero* state than traditional primary cell cultures. Here we showed tissue culture in serum-free media increased baseline levels of COX-2, Cx43 and calponin-1, which mimicked what would be expected for a transition from quiescent to contractile state. P4 supplementation, at a near-physiological concentration, did not fully prevent these changes. This apparent culture-associated reduction in P4 response was likely to have been attributed to decreased PR abundance rather than increased PR-A:PR-B protein ratio. PR stabilisation was not achieved by applying tissue stretch and exogenous P4, which demonstrated modulation of myometrial tissue phenotype depends on more than just these two *in utero* factors. Tissue stretch subtly repressed changes in COX-2 protein expected from P4 and IL-1 β exposure, but its impact on contractility was more profound. Tension-related differences in contractile output did not correlate with abundance of classic pro-labour markers; other markers of smooth muscle function need to be measured in future to assess the relationship between stretch and P4 at labour. A lack of PR-A dominance in response to IL-

1 β suggests cytokine-driven inflammation is not the sole cause of enhanced PR-A:PR-B ratio at labour.

This study along with others collectively support efforts to improve the use of human myometrial tissue cultures to model parturition-associated changes. The impact of tissue stretch and IL-1 β on the anti-inflammatory effects of P4 observed in the present study conceivably would have been more profound had less culture-related baseline changes occurred, and it is certainly of interest to identify the factors responsible for the latter so that more can be learnt of how myometrial phenotype is regulated at tissue level. There needs to be further exploration of culture duration and complex media supplementation, along with tissue stretch, to better simulate the *in utero* environment, all to be done prior to pursuing the finer details of physiologically relevant changes in the myometrium upon the onset of human labour.

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855

Figure Legends

Figure 1. Levels of pro-labour and smooth muscle marker proteins in human myometrial tissues from non-labouring women at term gestation after 24 h tissue culture under different amounts

of tension. Tissue strips dissected from myometrial biopsies were treated with vehicle controls for progesterone (0.01 % v/v ethanol; 'E') and RU-486 (0.01 % v/v DMSO; 'D') in serum-free culture media for 24 h while under 'no', 'low' or 'high' tension ('T') for two sets of experiments, one of which also included the vehicle control for IL-1 β (0.01 % v/v sterile H₂O; 'H') and only assessed the effect of no and low T. Time zero ('t=0') samples represent untreated tissues dissected from the same biopsies used for 24 h culture. Western blotting was used to assess their content of (a) cyclooxygenase-2 (COX-2), (b) oxytocin receptor (OTR), (c) connexin-43 (Cx43), and (d) calponin-1; Ponceau staining of total protein or immunodetection of β -tubulin for matched PVDF membranes was used for data normalisation. These samples are identical to those represented in Figures 4 & 8, where representative immunoblot images are presented. All data presented as mean $\pm$ SEM (no and low tension: n = 16 for all except calponin-1, where n = 14 instead; high tension: n = 8 for all except calponin-1, where n = 7 instead). Repeated measures ANOVA (mixed-effects model) with Dunnett's *post-hoc* analysis was used to assess t=0 *versus* 24 h vehicle controls (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$).

Figure 2. Levels of progesterone receptor proteins in human myometrial tissues from non-labouring women at term gestation after 24 h tissue culture under different amounts of tension.

Tissue strips dissected from myometrial biopsies were treated with vehicle controls for progesterone (0.01 % v/v ethanol; 'E') and RU-486 (0.01 % v/v DMSO; 'D') in serum-free culture media for 24 h while under 'no', 'low' or 'high' tension ('T') for two sets of experiments, one of which also included the vehicle control for IL-1 β (0.01 % v/v sterile H₂O; 'H') and only assessed the effect of no and low T. Time zero ('t=0') samples represent untreated tissues dissected from the same biopsies used for 24 h culture. Western blotting was used to assess their content of progesterone receptors, both isoform A

(PR-A) and B (PR-B), along with β -tubulin on matched PVDF membranes for data normalisation. These samples are identical to those represented in Figures 5 & 9, where representative immunoblot images are presented. All data presented as mean $\pm$ SEM (no and low tension: $n = 15$; high tension: $n = 7$). Repeated measures ANOVA (mixed-effects model) with Dunnett's *post-hoc* analysis was used to assess $t=0$ versus 24 h vehicle controls ($^* p \leq 0.05$, $^{**} p \leq 0.01$, $^{***} p \leq 0.001$).

Figure 3. Progesterone levels in term human myometrial tissues after exogenous application for 24 h tissue culture under different amounts of tension. Tissues were incubated with 100 nM progesterone (P4) or ethanol vehicle ('Vh') for 24 h in serum-free culture media while under 'no', 'low' and 'high' tension ('T'). Time zero (' $t=0$ ') samples represent untreated tissues dissected from the same biopsies used for 24 h culture. Tissue extracts from flash frozen tissues were measured for their P4 content using a plate-based immunoassay. Data presented as mean $\pm$ SEM ($n = 7$). Friedman test with Dunn's *post-hoc* analysis was used to assess (i) $t=0$ versus 24 h ethanol vehicle ($^a p \leq 0.05$), (ii) $t=0$ versus 24 h 100 nM P4 ($^b p \leq 0.05$), and (iii) ethanol vehicle versus 100 nM P4 of matched tensions applied to tissues during 24 h incubation ($^* p \leq 0.05$ and $^{**} p \leq 0.01$).

Figure 4. Levels of pro-labour and smooth muscle marker proteins, with extracellular PGE_2 concentrations, after 24 h incubation of term human myometrial tissues with P4 and RU-486. Tissues were incubated with combinations of 100 nM progesterone (P4, 'P'; ethanol 'E' vehicle) and 1 μM mifepristone (RU-486, 'R'; DMSO 'D' vehicle) for 24 h in serum-free culture media while under 'no', 'low' or 'high' tension ('T'). Western blotting was used to assess their content of (a) cyclooxygenase-2 (COX-2), (c) oxytocin receptor (OTR), (d) connexin-43 (Cx43), and (e) calponin-1; immunodetection of β -tubulin or Ponceau staining of total protein for matched PVDF membranes was used for data normalisation. Representative immunoblots are shown, where the first band detected on the left of each image is that of a $t=0$ sample (i.e. untreated tissues dissected from the same biopsies used for 24 h culture; data for which is presented in Figure 1), and bands for the other

12 samples are 24 h incubated tissues in the same order of treatment conditions as shown on their associated histogram. All data presented as mean $\pm$ SEM ($n = 8$ for all except calponin-1, where $n = 7$ instead). **(b)** PGE₂ concentrations in 24 h culture media from the same tissue samples were measured by ELISA; values corrected for tissue weight and presented as mean $\pm$ SEM ($n = 7$). Repeated measures ANOVA with Tukey's *post-hoc* analysis or Friedman test with Dunn's *post-hoc* analysis was used to assess all treatment conditions of matched tensions applied to tissues during 24 h incubation (^b $p \leq 0.05$).

Figure 5. Levels of progesterone receptor proteins in term human myometrial tissues after 24 h incubation with P4 and RU-486. Tissues were incubated with combinations of 100 nM progesterone (P4, 'P'; ethanol 'E' vehicle) and 1 μ M mifepristone (RU-486, 'R'; DMSO 'D' vehicle) for 24 h in serum-free culture media while under 'no', 'low' or 'high' tension ('T'). Western blotting was used to assess their content of P4 receptors, both isoform A (PR-A) and B (PR-B), along with β -tubulin on matched PVDF membranes for data normalisation. Representative immunoblots are shown, where the first lane of bands on the left of each image is that of a $t=0$ sample (i.e. untreated tissues dissected from the same biopsies used for 24 h culture; data for which is presented in Figure 2), and lanes of bands for the other 12 samples are 24 h incubated tissues in the same order of treatment conditions as shown on their associated histograms. All data presented as mean $\pm$ SEM ($n = 7$). Repeated measures ANOVA with Tukey's *post-hoc* analysis or Friedman test with Dunn's *post-hoc* analysis was used to assess all treatment conditions of matched tensions applied to tissues during 24 h incubation (^b $p \leq 0.05$ and ^{bb} $p \leq 0.01$).

Figure 6. Spontaneous contractility (prior to oxytocin stimulation) of term human myometrial tissues after 24 h incubation with P4 and RU-486. Tissues were incubated with combinations of 100 nM progesterone (P4, 'P'; ethanol 'E' vehicle) and 1 μ M mifepristone (RU-486, 'R'; DMSO 'D' vehicle) for 24 h in tissue culture media while under 'low' or 'high' tension ('T'). After 24 h

incubation, tissues were subjected to isometric tension measurements to assess contractile output. **(a)** Representative contractility traces for one patient's biopsy that shows both spontaneous and oxytocin-augmented contractions, where the arrows indicate the times at which oxytocin was applied to tissues. **(b)** Mean integral tension (MIT) and contraction frequency data, all presented as mean $\pm$ SEM ($n = 8$), measured from the 'control period' of spontaneous contractions – i.e. the 15 minutes immediately prior to applying the first concentration of oxytocin (1×10^{-11} M) on the left of each representative trace. Kruskal-Wallis with Dunn's *post-hoc* analysis was used to compare all tissues of matched tensions applied during 24 h incubation prior to contractility measurements (all $p > 0.05$), and 2-way ANOVA with Bonferroni's *post-hoc* analysis was used to assess low *versus* high T conditions between tissues with matched P4 and RU-486 treatments (^a $p \leq 0.05$).

Figure 7. Oxytocin-augmented contractility of term human myometrial tissues after 24 h incubation with P4 and RU-486. Mean integral tension of oxytocin-augmented contractions relative to the 'control period' (Δ MIT), along with frequency of oxytocin-augmented contractions and mean Δ MIT summary scores, calculated from isometric tension measurements of tissues after 24 h incubation with combinations of 100 nM progesterone (P4, 'P'; ethanol ('E') vehicle) and 1 μ M mifepristone (RU-486, 'R'; DMSO 'D' vehicle) in tissue culture media while under 'low' or 'high' tension ('T'). The first concentration of oxytocin (1×10^{-11} M) was applied to tissues after recording spontaneous activity for 1 h and 25 minutes, whereby the last 15 minutes of the latter is referred to as the 'control period' (see Figure 6). Subsequent concentrations of oxytocin were applied in a cumulative manner once every 25 minutes, and data was obtained from the last 15 minutes of exposure for each concentration of oxytocin. All data presented as mean $\pm$ SEM ($n = 8$). For Δ MIT summary score data, Kruskal-Wallis test with Dunn's *post-hoc* analysis was used to assess all tissues of matched tensions applied during 24 h incubation prior to contractility measurements (all $p > 0.05$), and 2-way ANOVA with Bonferroni's *post-hoc* analysis was used to compare low *versus* high T conditions of matched P4 and RU-486 treatments (^a $p \leq 0.05$ and ^{aa} $p \leq 0.01$).

Figure 8. Levels of pro-labour and smooth muscle marker proteins, with extracellular PGE₂ concentrations, after 24 h incubation of term human myometrial tissues with P4, RU-486 and IL-1 β . Tissues were incubated with combinations of 100 nM progesterone (P4, 'P'; ethanol ('E') vehicle), 1 μ M mifepristone (RU-486, 'R'; DMSO ('D') vehicle) and 1 ng/ml interleukin-1 β (IL-1 β , 'I'; sterile H₂O ('H') vehicle) for 24 h in serum-free culture media while under 'no' or 'low' tension ('T'). Western blotting was used to assess their content of (a) cyclooxygenase-2 (COX-2), (c) oxytocin receptor (OTR), (d) connexin-43 (Cx43) and (e) calponin-1; Ponceau staining of total protein or immunodetection of β -tubulin for matched PVDF membranes was used for data normalisation. Representative immunoblots are shown, where the first band detected on the left of each image is that of a t=0 sample (i.e. untreated tissues dissected from the same biopsies used for 24 h culture; data for which is presented in Figure 1), and bands for the other 10 samples are 24 h incubated tissues in the same order of treatment conditions as shown on their associated histograms. All data presented as mean $\pm$ SEM (n = 8 for all except calponin-1, where n = 7 instead). (b) PGE₂ concentrations in 24 h culture media from the same tissue samples were measured by ELISA; values corrected for tissue weight and presented as mean $\pm$ SEM (n = 7). Repeated measures ANOVA with Tukey's *post-hoc* analysis or Friedman test with Dunn's *post-hoc* analysis was used to assess all IL-1 β -included treatment conditions (i.e. E+D+I, P+D+I, E+R+I, P+R+I) of matched tensions applied to tissues during 24 h incubation (all $p > 0.05$). Wilcoxon test or Student's paired t test was used for 'all vehicle' (E+D+H) *versus* 'IL-1 β only' (E+D+I) treatment of matched tensions applied during 24 h incubation (* $p \leq 0.05$, ** $p \leq 0.01$).

Figure 9. Levels of progesterone receptor proteins in term human myometrial tissues after 24 h incubation with P4, RU-486 and IL-1 β . Tissues were incubated with combinations of 100 nM progesterone (P4, 'P'; ethanol ('E') vehicle), 1 μ M mifepristone (RU-486, 'R'; DMSO ('D') vehicle) and 1 ng/ml interleukin-1 β (IL-1 β , 'I'; sterile H₂O ('H') vehicle) for 24 h in serum-free culture media

986 while under ‘no’ or ‘low’ tension (‘T’). Western blotting was used to assess their content of P4
987 receptor proteins, both isoform A (PR-A) and B (PR-B), along with β -tubulin on matched PVDF
988 membranes for data normalisation. Representative immunoblots are shown, where the first lane of
989 bands on the left of each image is that of a $t=0$ sample (i.e. untreated tissues dissected from the same
990 biopsies used for 24 h culture; data for which is presented in Figure 2), and lanes of bands for the
991 other 10 samples are 24 h incubated tissues in the same order of treatment conditions as shown on
992 their associated histograms. All data presented as mean $\pm$ SEM ($n = 8$). Repeated measures ANOVA
993 with Tukey’s *post-hoc* test or Friedman test with Dunn’s *post-hoc* test was used to assess all IL-1 β -
994 included treatment conditions (i.e. E+D+I, P+D+I, E+R+I, P+R+I) of matched tensions applied to
995 tissues during 24 h incubation (^b $p \leq 0.05$). Wilcoxon test or Student’s paired t test was used for ‘all
996 vehicle’ (E+D+H) *versus* ‘IL-1 β only’ (E+D+I) treatment of matched tensions applied during 24 h
997 incubation (all $p > 0.05$).

(a) **(b)** **(c)** **(d)** **(e)**

(a)

(b)

(c)

(d)

(e)

Table 1:**Patient****Demographics****for Myometrial****Tissue Samples**

	24 h 100 nM P4 treatment only (n = 7); Figure 3	24 h 100 nM P4 ± 1 µM RU-486 treatment only (n = 8); Figures 1-2 & 4-5	24 h 100 nM P4 ± 1 µM RU-486 treatment, followed by organ bath experiments (n = 8); Figures 6-7	24 100 nM P4 ± 1 µM RU-486 ± 1 ng/ml IL-1β treatment only (n = 8); Figures 1-2 & 8-9
<i>Maternal age (years; median with range)</i>	37 (26 to 43)	38 (32 to 50)	35 (26 to 40)	34 (31 to 41)
<i>Gestational age (weeks^{+days}; median with range)</i>	39 ⁺⁰ (38 ⁺² to 40 ⁺²)	39 ⁺⁰ (38 ⁺⁵ to 39 ⁺⁴)	39 ⁺¹ (37 ⁺⁰ to 40 ⁺²)	39 ⁺⁰ (38 ⁺⁴ to 40 ⁺⁰)
<i>Gravida (median with range)</i>	2 (1 to 6)	2 (2 to 3)	3 (1 to 7)	2 (1 to 7)
<i>Parity (median with range)</i>	Viable = 1 (0 to 3) Non-viable & abortus = 0 (0 to 2)	Viable = 1 (0 to 1) Non-viable & abortus = 0 (0 to 2)	Viable = 1 (0 to 4) Non-viable and abortus = 1 (0 to 5)	Viable = 1 (0 to 2) Non-viable and abortus = 0 (0 to 4)
<i>Booking BMI (median with range)</i>	24.0 (19 to 33)	21.5 (19 to 29)	24.0 (22 to 25)	22.6 (18 to 32)
<i>Ethnicity</i>	White European = 3 Stated as 'other' = 2 Not stated = 2	White European = 7 Stated as 'other' = 1	White European = 5 Stated as 'other' = 1 Black Caribbean = 1 Asian = 1	White European = 4 White other = 1 Black African = 1 Asian = 2

<i>Reason for Caesarean</i>	Previous Caesarean = 4 Breech = 2 Maternal request = 1	Previous Caesarean = 4 Other indications = 2 Maternal request = 2	Previous Caesarean = 6 Breech = 1 Maternal request = 1	Previous Caesarean = 5 Other indications = 2 Breech = 1
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Table 2: EC_{50} for Oxytocin-Augmented Myometrial Contractility (ΔMIT) after 24 h Tissue Culture with P4 and RU-486

24 h Isotonic Tension in Tissue Culture	24 h Treatment in Tissue Culture	Oxytocin EC_{50} (M)	Lower 95 % CI for Oxytocin EC_{50} (M)	Upper 95 % CI for Oxytocin EC_{50} (M)	Oxytocin pEC_{50}
Low Tension	Ethanol + DMSO	2.3×10^{-10}	5.2×10^{-11}	9.3×10^{-10}	-9.6
	Ethanol + 1 μ M RU-486	4.0×10^{-10}	1.3×10^{-10}	1.1×10^{-9}	-9.4
	100 nM P4 + DMSO	5.2×10^{-10}	1.7×10^{-10}	1.3×10^{-9}	-9.3
	100 nM P4 + 1 μ M RU-486	6.2×10^{-10}	1.5×10^{-10}	2.2×10^{-9}	-9.2
High Tension	Ethanol + DMSO	2.0×10^{-10}	4.6×10^{-11}	8.2×10^{-10}	-9.7
	Ethanol + 1 μ M RU-486	3.8×10^{-10}	9.6×10^{-11}	1.2×10^{-9}	-9.4
	100 nM P4 + DMSO	2.8×10^{-10}	6.6×10^{-11}	1.1×10^{-9}	-9.6
	100 nM P4 + 1 μ M RU-486	3.0×10^{-10}	1.5×10^{-10}	5.9×10^{-10}	-9.5

1 Highlights:

- 2 • Baseline content of myometrial pro-labour proteins can be altered by tissue culture
- 3 • Myometrial tissues in culture should be supplemented with progesterone (100 nM)
- 4 • Myometrial tension alone is unlikely able to control *in utero* progesterone response
- 5 • Stretch-mediated tension impacts on function more than pro-labour protein levels
- 6 • Interleukin-1 β does not promote PR-A dominance in cultured myometrial tissues