Pro-labour myometrial gene expression: are preterm labour and term labour the same?

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M Tattersall and N Engineer contributed equally to this work

Abstract

Preterm labour (PTL) is the most important cause of neonatal morbidity and mortality. While some causes have been identified, the mechanisms involved remain elusive. This study investigates whether term labour (TL) is an appropriate model for PTL by examining pro-labour gene expression, using quantitative rtPCR, and protein synthesis, using Western analysis, in preterm and term myometrial samples obtained from the upper and lower uterine segments before and after the onset of labour. In the lower segment, the levels of prostaglandin H synthase type-2 (PGHS-2), interleukin-1β (IL-1β), IL-6 and IL-8 mRNA expression were significantly higher in TL compared with PTL samples. Compared with non-labour controls, the expression of IL-1β and IL-8 mRNA was increased in both PTL and TL samples and the expression of PGHS-2 and IL-6 mRNA was increased in TL samples only. In the upper segment, there were no differences between PTL and TL samples and the mRNA expression of PGHS-2 and IL-1β was increased in TL compared with term no labour samples. No effect of PTL or TL was seen on either oxytocin receptor or connexin-43 mRNA expression or protein levels. The multiple regression analysis and studies in primary cultures of uterine myocytes suggest that the inflammatory cytokines, IL-1β and tumour necrosis factor-α, are the most important regulators of PGHS-2 and IL-8. Our data show that preterm and term labouring myometrium are significantly different and that the most marked labour-induced changes in gene expression are in the lower segment. These changes may occur in response to the release of inflammatory cytokines by the labour-associated inflammatory infiltration.

Introduction

Preterm labour (PTL), particularly before 32 weeks, is associated with a high fetal morbidity and mortality and consequently is the subject of intense research with the aim of devising preventative or therapeutic strategies. One approach has been to define what triggers term labour (TL) and key roles have been ascribed to the oxytocin receptor (OXTR) and connexin-43 (CX-43). However, more recently, the importance of prostaglandins (PG) and inflammatory cytokines in the process has been realised.

Early studies reported that myometrial OXTR levels are low in early pregnancy, increase with advancing gestation, peak in early labour and decline in advanced labour (Fuchs et al. 1984). Other studies suggested that OXTR expression increases with gestational age rather than with the onset of labour (Kimura et al. 1996, Wrathes et al. 1999), but more recently OXTR mRNA expression was reported to increase with the onset of TL (Mesiano et al. 2002, Terzidou et al. 2005). CX-43 is also thought to play a critical role in the onset of labour by increasing myometrial cell coupling. In the rat, a small increase in CX-43 mRNA and protein levels occurs prior to the onset of labour and a marked increase during labour itself (Risek et al. 1990, Lye et al. 1993, Ou et al. 1997). In the human, Sparey et al. (1999) found that CX-43 protein was significantly increased in labouring samples and that this was most marked in the upper segment (US) of the uterus.

There is a massive influx of macrophages, neutrophils and T-lymphocytes into the myometrium and gestational tissues with labour (Thomson et al. 1999, Keski-Nisula et al. 2000). The activated leucocytes express pro-inflammatory cytokines increasing myometrial levels of interleukin (IL)-1β and IL-8 (Osmers et al. 1995, Winkler et al. 1999, Osman et al. 2003). The latter promotes...
further neutrophil infiltration and activation, and the former stimulates PG production. PG production is increased in fetal membranes and myometrium with labour (Turnbull et al. 1977, Skinner & Challis 1985) resulting in increased circulating and amniotic fluid PG levels (Sellers et al. 1981, Brennecke et al. 1985, Romero et al. 1994). Consistent with these observations, labour is associated with increased myometrial levels of PGHS type-2 (PGHS-2) mRNA and protein (Mesiano et al. 2002, Sooranna et al. 2004). Increased PG levels act on the myometrium to increase uterine contractility and so promote the onset and progression of labour.

In this study, we have tested the hypothesis that there is no difference in myometrial pro-labour gene expression in PTL and TL samples. To achieve this, we have examined the mRNA expression of a group of pro-labour genes, OXTR, CX-43, IL-8 and PGHS-2 and related the pro-labour gene expression to those of the pro-inflammatory cytokines IL-1β, IL-6 and tumour necrosis factor-α (TNF-α) in preterm and term myometrial samples obtained from the upper and lower uterine segments before and after the onset of labour.

Results

Gene expression

PTL versus TL

In the lower segment, the expression of IL-8, PGHS-2, IL-1β and IL-6 mRNA was greater in TL samples than in PTL samples (P<0.01 for IL-8 and PGHS-2, and P<0.05 for IL-1β and IL-6; Fig. 1A, B, E and F). There were no significant differences in the US samples for OXTR, IL-8, PGHS-2, CX-43, IL-1β, IL-6 and TNF-α mRNA expression (Fig. 1A–G).

Non-labour versus labour

In the lower segment PTL samples, IL-8 and IL-1β mRNA expression was increased compared with preterm no labour (PTNL) lower segment samples (both P<0.05; Fig. 1A and E). There were no significant differences in the US samples. In TL lower segment samples, the expression of IL-8, PGHS-2, IL-1β and IL-6 mRNA was increased compared with term no labour (TNL) samples (P<0.01 for IL-8, IL-6 and PGHS-2 and P<0.05 for

Figure 1 (A) Interleukin-8 (IL-8), (B) prostaglandin H synthase-2 (PGHS-2), (C) oxytocin receptor (OXTR), (D) connexin-43 (CX-43), (E) IL-1β, (F) IL-6 and (G) tumour necrosis factor-α (TNF-α): β-actin mRNA ratio (median, interquartile range and range) in paired upper and lower human myometrial samples obtained from the following four groups of women: at the time of caesarean section (LSCS) under the conditions of preterm with labour (PTL; 33.9±1.5 weeks, n=9), preterm no labour (PTNL; 30.4±1.5 weeks, n=9), term with labour (TL; 39.2±0.5 weeks, n=10) and term no labour (TNL; 38.4±0.4 weeks, n=8). *A difference of P<0.05 and **a difference of P<0.01; a difference of P<0.05 and §§a difference of P<0.01 in term labour compared with preterm labour samples and a difference of P<0.05 and §§§a difference of P<0.01 in the upper segment compared with the lower segment samples.
IL-1β; Fig. 1A, B, E and F); and in the US samples, the expression of PGHS-2 and IL-1β mRNA was increased in TL compared with TNL samples (both \( P<0.01 \); Fig. 1B and E).

**Upper versus lower segment**

In paired US and lower segment samples (preterm and term, non-labour and labour), the expression of IL-8, PGHS-2, IL-1β and CX-43 mRNA was greater in the lower segment samples than in the US samples (\( P<0.0001 \) for PGHS-2 and IL-1β and \( P<0.01 \) for IL-8 and CX-43; Fig. 1A, B, D and E). OXTR mRNA expression was greater in the US than in the lower segment samples (\( P=0.025 \), Fig. 1C).

**Western blotting for protein concentration**

Western analysis for PGHS-2, OXTR (both antibodies) and CX-43 confirmed the mRNA data (Fig. 2A–D). The similarity in the phospho-CX-43 suggests that there is no change in activity (Fig. 2E). The expression of PGHS-2 protein was greater in TL lower segment samples compared with TNL lower segment samples (\( P<0.05 \), Fig. 2A). The levels of CX-43 tended to be greater in the lower segment and OXTR (detected with the C-terminus antibody) tended to be greater in the US. No differences in PGHS-2 were apparent.

**Gestational age**

In order to identify changes in gene expression that may occur with advancing gestation and prior to the onset of labour and which may therefore have a role in the onset of labour, we performed a Spearman test for non-parametric data looking for associations between gestational age and mRNA expression in the non-labour samples (\( n=17 \)). In the lower segment, CX-43 mRNA expression was associated with gestational age (\( r=0.58 \), \( P=0.014 \)) and IL-8 mRNA expression was also weakly associated (\( r=0.47 \), \( P=0.055 \)). In the US, PGHS-2 mRNA expression was negatively associated with gestational age (\( r=-0.55 \), \( P=0.023 \)). There was no association between gestational age and OXTR mRNA expression in either lower or US samples.

**Multiple regression analysis**

Multiple regression analysis for OXTR, CX-43, PGHS-2 and IL-8 was performed including OXTR, IL-8, CX-43, PGHS-2, IL-1β, TNF-α and IL-6 mRNA levels, gestational age, sample site and labour status. For PGHS-2, the resulting equation could explain 60% (\( r^2, P<0.0001 \)) of the variance in its levels, for IL-8, 77% (\( r^2, P<0.0001 \)), for OXTR, 19% (\( r^2 \), \( P=0.13 \)) and for CX-43, 70% (\( r^2 \), \( P<0.0001 \), Table 1).

**Figure 2** (A) Western analysis of prostaglandin H synthase-2 (PGHS-2), (B) oxytocin receptor (OXTR, antibody directed to the N-terminus), (C) OXTR (antibody directed to the C-terminus), (D) connexin-43 (CX-43) and (E) phospho-CX-43. *A difference of \( P<0.05 \) in labour compared with non-labour samples.
We studied the time course of the effects of TNF-α, IL-1β, IL-6 and IL-8 on IL-8, PGHS-2, OXTR and CX-43 mRNA expression (Fig. 3A–D). The mRNA expression of IL-8 was increased by IL-1β (at 1, 6 and 24 h, \( P < 0.05 \)) and IL-8 (at 24 h, \( P < 0.05 \)); of PGHS-2 was increased by IL-1β (at 1, 6 and 24 h, \( P < 0.05 \), TNF-α (at 1, 6 and 24 h, \( P < 0.05 \)), IL-6 (at 24 h, \( P < 0.05 \)) and IL-8 (at 24 h, \( P < 0.05 \)); of OXTR was reduced by IL-1β (at 6 and 24 h, \( P < 0.05 \)) and of CX-43 was increased by TNF-α (at 6 and 24 h, \( P < 0.05 \); Fig. 3A–D).

We then studied the time course of the effects of the pro-labour factors PGE2 (10 nM), PGF2α (10 nM) and OXT (100 nM) on IL-8, PGHS-2, OXTR and CX-43 mRNA expression (Fig. 4A–D). The mRNA expression of IL-8 was increased by PGE2 at 1 h (\( P = 0.05 \)) and OXT at 1 h (\( P < 0.05 \)) and then reduced by OXT at 24 h (\( P < 0.05 \)); of PGHS-2 was increased by PGE2 (at 1 h, \( P < 0.05 \)) and OXT (at 1 and 6 h, \( P < 0.05 \)); of OXTR was reduced by PGE2 (at 6 and 24 h, \( P < 0.05 \)) and OXT (at 6 and 24 h, \( P < 0.05 \)) and of CX-43 unaffected (Fig. 4A–D).

### Table 1

The multiple regression analysis for oxytocin receptor (OXTR), connexin-43 (CX-43), prostaglandin H synthase type-2 (PGHS-2) and interleukin-8 (IL-8). The analysis was performed including OXTR, IL-8, CX-43, PGHS-2 IL-1β, tumour necrosis factor-α (TNF-α) and IL-6 mRNA levels, gestational age, sample site and labour status.

<table>
<thead>
<tr>
<th>Pro-labour gene (( r^2 ) and ( P ) value)</th>
<th>Related factor</th>
<th>( t ) value (( P ) value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGHS-2 (60%, ( P &lt; 0.0001 ))</td>
<td>IL-8</td>
<td>3.54 (( P = 0.0008 ))</td>
</tr>
<tr>
<td></td>
<td>IL-6</td>
<td>4.26 (( P &lt; 0.0001 ))</td>
</tr>
<tr>
<td></td>
<td>CX-43</td>
<td>2.63 (( P = 0.01 ))</td>
</tr>
<tr>
<td></td>
<td>IL-1β</td>
<td>2.09 (( P = 0.04 ))</td>
</tr>
</tbody>
</table>
| OXTR (19%, \( P = 0.13 \))                    | Upper or lower segment | 2.24 (\( P = 0.03 \))      
|                                               | IL-6           | 2.18 (\( P = 0.03 \))       |
| CX-43 (70%, \( P < 0.0001 \))                 | PGHS-2         | 2.68 (\( P = 0.01 \))       |
|                                               | IL-1β          | 3.13 (\( P = 0.003 \))      |
|                                               | TNF-α          | 2.59 (\( P = 0.01 \))       |
|                                               | Upper or lower segment | 3.71 (\( P = 0.0004 \))    |
| IL-8 (77%, \( P < 0.0001 \))                  | PGHS-2         | 3.54 (\( P = 0.0008 \))     |
|                                               | IL-1β          | 7.68 (\( P < 0.0001 \))     |
|                                               | IL-6           | 2.03 (\( P = 0.047 \))      |

**Figure 3** The response of (A) interleukin-8 (IL-8), (B) prostaglandin H synthase-2 (PGHS-2), (C) oxytocin receptor (OXTR), (D) connexin-43 (CX-43):GAPDH mRNA ratio (median, interquartile range and range) in primary cultures of human uterine smooth muscle cells to incubation for 0, 1, 6 and 24 h with IL-1β (1 ng/ml), TNF-α (1 ng/ml), IL-6 (1 ng/ml), IL-8 (1 ng/ml) as described in Materials and Methods (\( n = 6–12 \)). *A difference of \( P < 0.05 \).
Discussion

This study shows that pro-labour gene expression is different in term and preterm myometrial samples and that of the pro-labour genes studied only PGHS-2 and IL-8 were significantly elevated with the onset of labour and that these changes were most marked in the lower segment. The multiple regression analysis suggested that the pro-labour gene mRNA expression was associated with that of the cytokines and of PGHS-2, while the cell culture data suggested that the most important modulators of pro-labour gene expression were the inflammatory cytokines IL-1β and TNF-α. Physiologically, these data imply that the myometrial inflammatory infiltration associated with both preterm and TL is important in the onset and progression of labour.

OXTR mRNA expression is a consistent marker of labour in animal models of parturition (Soloff et al. 1979), has been reported to be increased in human labour by several groups including our own (Mesiano et al. 2002, Terzidou et al. 2005) and used by others to differentiate labouring samples (Condon et al. 2003). However, it is also reported that OXTR mRNA expression does not increase with the onset of labour but with advancing gestation, prior to the onset of labour (Kimura et al. 1996, Wathes et al. 1999). Our data show no increase in OXTR mRNA either with the onset of labour (whether at or before term) or with advancing gestation. Furthermore, our mRNA data are supported by Western analysis of OXTR levels that also show no change. Overall, the evidence suggests that OXTR mRNA expression is increased in some but not all women with the onset of labour, and that perhaps the well-recognised increase in OXTR sensitivity to OXT (Fuchs et al. 1983) is more important than an increase in OXTR mRNA expression. Our data are consistent with several recent gene array studies that failed to find an increase in OXTR with the onset of labour (Aguan et al. 2000, Charpigny et al. 2003, Esplin et al. 2005, Havelock et al. 2005, Bukowski et al. 2006). Interestingly, the factors we included in the multiple regression analysis accounted for relatively little of the variation in OXTR mRNA expression, suggesting that other factors regulate OXTR mRNA expression during pregnancy. Our in vitro studies found that OXTR mRNA expression was either unchanged or reduced (as reported previously for IL-1β (Schmid et al. 2001, Helmer et al. 2002, Soloff et al. 2006) and OXT (Phaneuf et al. 2000)) in response to cytokines and pro-labour factors supporting our myometrial data.

CX-43 is held to have an important role in TL. Animal data consistently show that both mRNA and protein levels of CX-43 increase with the onset of labour (Lye...
et al. 1993, McNutt et al. 1994, Ou et al. 1997, Cook et al. 2000) and that ablation of CX-43 delays the onset of parturition (Doring et al. 2006). Human data are less robust, with some studies showing increases in protein alone (Sparey et al. 1999), some in mRNA but not protein (Chow & Lyte 1994, Pierce et al. 2002) and some no change in mRNA (Rezapour et al. 1997). The spatial expression of CX-43 protein was assessed in one study and found to be greater in the US in both non-labour and labour samples (Sparey et al. 1999). Two studies suggested that failure to progress in labour was associated with lower levels of CX-43 mRNA expression (Pierce et al. 2002, Cluff et al. 2006). We found that CX-43 mRNA expression, protein levels and phosphorylation status were unchanged with either term or PTL. Furthermore, several studies have been performed using differential display or gene arrays and none have mentioned CX-43, either because it was unchanged or because it was not included in the array (Aguan et al. 2000, Chan et al. 2002, Charpigny et al. 2003, Esplin et al. 2005, Havelock et al. 2005, Bukowski et al. 2006). Our multiple regression studies found associations between the mRNA levels of CX-43 and those of IL-1β, TNF-α and PGHS-2, but the in vitro data found that only TNF-α affected CX-43 mRNA expression. Overall, these data suggest that in the human at least CX-43 is not consistently up-regulated with the onset of labour.

The expression of PGHS-2 mRNA was increased in both the upper and lower uterine segments with TL, but not PTL, and there was no increase with gestational age in either segment. The term data are consistent with most recent reports (Mesiano et al. 2002, Sooranna et al. 2004), but contrast with our previous observations and the trend reported by Sooranna et al. (2006) and Astle et al. (2007). The mechanism involved in the up-regulation of PGHS-2 activity with the onset of labour is uncertain, but several stimuli including IL-1β are known to increase PGHS-2 expression via the transcription factor nuclear factor κ B (NFκB) (Allport et al. 2001). Indeed, most data suggest that inflammatory cytokines increase myometrial contractility via increased PG synthesis (Khatun et al. 1999). The multiple regression data revealed associations between the levels of PGHS-2 mRNA and those of IL-8, IL-6 and IL-1β and our in vitro studies confirmed the important role played by IL-1β; in contrast both IL-6 and IL-8 had a small effect. Interestingly, TNF-α had a marked stimulatory effect on PGHS-2 mRNA expression, but we found no association between their levels in myometrial samples. OXT is known to activate ERK1/2 and to increase PGHS-2 in an ERK-dependent manner (Strakova et al. 1998). OXT-induced ERK1/2 activation may also explain the increase in IL-8 mRNA expression observed in response to OXT. These inconsistencies may reflect the difference between the behaviour of uterine myocytes in primary culture as opposed to the in vivo situation where their function is modulated by other cellular and connective tissue components of the myometrium.

We found that IL-8 is increased in both PTL and TL consistent with the published data (Winkler et al. 1999, Elliott et al. 2000, Chan et al. 2002, Osman et al. 2003). The multiple regression analysis found associations between IL-8 mRNA levels and those of PGHS-2, IL-1β and IL-6, but in vitro, uterine myocyte expression of IL-8 was increased by IL-1β and TNF-α, but not IL-6. Interestingly, neither PGE2 nor PGF2α affected IL-8 mRNA expression significantly, suggesting that the association between IL-8 and PGHS-2 mRNA levels seen in the multiple regression analysis may reflect a common regulation of both genes by inflammatory cytokines. We also found that IL-8 increased with advancing gestation in non-labour samples albeit weakly, and previously we found that IL-8 mRNA expression and synthesis were increased by mechanical stretch of uterine myocytes in vitro (Loudon et al. 2004). Together, these data suggest that myometrial IL-8 mRNA expression may be physiologically up-regulated by increasing uterine wall tension secondary to the growth of the pregnancy. The increased IL-8 synthesis could then provoke an inflammatory infiltration of the myometrium resulting in the release of IL-1β which stimulates further myometrial synthesis of IL-8, so establishing a positive feedback loop culminating in the onset of labour.

It is possible that the differences we found between PTL and TL are the result of confounding factors, but the groups were similar in most respects and particularly in terms of cervical dilatation, gravidity and parity. Some discrepancy is inevitable between groups, for example, the term group it is more likely have an LSCS for failure to progress and less likely to have an LSCS for malpresentation than the PTL group. The causes of PTL were uncertain for the majority, two having multiple pregnancies and one having an abruption with a non-reassuring fetal heart pattern. There were differences in the racial distribution between groups, the TL group contained relatively more African Americans than the PTNL group, but gene expression in these groups was not directly compared. Certainly, in general terms, labour of an infective aetiology would be expected to be over-represented in the PTL group, but such a cause would also be expected to be associated with an overexpression of inflammatory genes. However, in our study, it appeared the converse was true and inflammatory gene expression was lower in PTL. PTL in multiple pregnancies might be expected to overexpress PGHS-2 and IL-8 as these are the most consistent stretch-responsive genes (Sooranna et al. 2005); again, this was not the picture we observed. The relatively greater variation in mRNA levels of the genes analysed may have masked significant changes between groups, only a larger study of clinically well-defined cases will allow us to clarify the molecular mechanisms responsible for the onset of PTL of different causes.

The inflammatory cytokines, IL-1β, IL-6 and TNF-α are thought to be released by activated neutrophils and to promote the onset of labour by increasing PGHS-2...
Our observation of a lack of change in TNF-α mRNA expression with the onset of labour is consistent with the literature. However, in the mouse model, TNF-α seems to have an important role, since knocking out either IL-1 or TNF receptors alone failed to prevent infection-induced PTL, but removing both IL-1 and TNF-α receptors significantly reduces infection-induced PTL (Hirsch et al. 2006). These data imply that the combination of IL-1 and TNF-α plays a critical role in the process of infection-induced PTL in the mouse. In the primate, intra-amniotic infusions of IL-1β stimulated the most intense contraction pattern, while TNF-α infusions induced labour in two cases and moderate contractions in three others (Sadowsky et al. 2006). The exact roles and relative importance of the inflammatory cytokines in human labour are not certain, but the evidence suggests that they are very likely to be involved in the onset of labour.

In this study, the expression of IL-8, PGHS-2, IL-1β and CX-43 mRNA was greater in the lower segment than in their paired US samples; only OXTR mRNA expression was greater in the US than in the lower segment samples. These data are consistent with the literature with regard to OXTR (Blanks et al. 2003), but not for CX-43, the expression of which was previously reported to be greater in the US (Sparey et al. 1999). We have previously reported that cPLA2, sPLA2, PGHS-2, PGES-1 and PGES-2 mRNA expression were greater in the lower than in the US samples (Sooranna et al. 2006) in agreement with Sparey et al. (1999) in the case of PGHS-2. Our observation that the mRNA expression of the inflammatory cytokine IL-1β is greater in the lower than in the US is consistent with the more marked inflammatory infiltration found in the lower segment (Thomson et al. 1999). The lower segment starts to form at the end of the second trimester reflecting increased tension in this portion of the uterus. As discussed above, it is possible that the increase tension induces IL-8 production initiating the process that results in the onset of labour. Certainly, the increase in gene expression associated with labour appears to greatest in the lower segment, suggesting that changes in the lower segment drive the process, producing uterotonins that promote the contractile activity of the US.

These data suggest that myometrial gene expression is different in PTL and TL. Clearly, PTL occurs for many reasons, such as infection, ruptured membranes and cervical insufficiency, and our approach to compare all cases of PTL to TL does not allow us to discriminate. Furthermore, we have only analysed genes directly related to labour or inflammation and other sets of genes may behave differently. Only by studying accurately phenotyped samples obtained from women in PTL will we be able to define whether some subsets of PTL are in fact accelerated TL while other types of PTL are distinct from TL.

**Materials and Methods**

**Tissue collection and preparation**

All procedures involving human myometrial tissues were conducted in compliance with the Institution Review Board of the University of Cincinnati (Cincinnati, OH, USA). Informed consent was obtained from all women prior to any tissue collection. Paired upper and lower segment human myometrial samples were obtained from the following four groups of women (mean gestational age ± s.d. in each case): at PTNL (31.5 ± 3.5 weeks; mean ± s.d.; n = 9), PTL (32.3 ± 4.1 weeks; n = 9), TNL (38.0 ± 1.2 weeks; n = 8) and TL (39.4 ± 0.5 weeks; n = 10; Table 2). Labour was defined as the presence of regular uterine contractions (every 3–4 min) resulting in cervical effacement and dilation. Myometrial samples were removed from the upper margin of the incision made in the lower uterine segment and for the US, an Allis clamp was used to group a small segment (1.0 × 0.5 cm) of myometrium below the fundus and tissue excised using Mayo scissors (this included the serosal surface but not the endometrium). Homeostasis was obtained using a single figure of size 8 suture. Tissues were flash frozen in liquid nitrogen prior to storage at −80 °C. The indications for caesarean section included: failure to progress (n = 4), fetal distress (n = 8), previous uterine surgery (n = 12), malpresentation (n = 5), severe pre-eclampsia (n = 5), placenta praevia (n = 1) and gestational diabetes (n = 1).

**Quantitative RT-PCR**

Total RNA was extracted and purified from the upper and lower segment myometrial samples using the Tri-reagent method (Trizol, Sigma–Aldrich Co. Ltd). After quantification, 2.0 μg RNA was pretreated with DNase I (Amp Grade, Invitrogen Ltd) and then reverse transcribed with Oligo dT random primers using SuperScript II reverse transcriptase (Invitrogen Ltd). Total RNA was extracted and purified from myometrial cells grown on 6-well plates using the RNAeasy mini kit from Qiagen Ltd. After quantification, 1.0 μg was reverse transcribed with oligo dT random primers using MuLV reverse transcriptase (Applied Biosystems Ltd, Warrington, UK).

Paired oligonucleotide primers for amplification of pro-labour genes were designed using Primer Designer (Scientific and Educational Software, Durham, NC, USA) against the sequence downloaded from GenBank. The primer sets used (Table 3) produced amplicons of the expected size and flanked intron/exon junctions. Assays were validated for all primer sets by confirming that single amplicons of appropriate size and sequence were generated. Quantitative PCR was performed in the presence of SYBR Green (Qiagen Ltd), and amplicon yield was monitored during cycling in a RotorGene Sequence Detector (Corbett Research Ltd, Mortlake, Sydney, Australia). Pre-PCR cycle was 10 min at 95 °C followed by up to 45 cycles at 95 °C for 20 s, 58–60 °C for 20 s and 72 °C for 20 s followed
Table 2 The demographic data of the study subjects.

<table>
<thead>
<tr>
<th></th>
<th>PTNL (n=9)</th>
<th>PTL (n=9)</th>
<th>TNL (n=8)</th>
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<td>5 (1-10)</td>
<td>0 (0-3)</td>
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</tr>
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</table>

Reason for LSCS
- FTP
- NRFHFT
- PUS
- Malpresentation
- PET
- Placenta praevia
- GDM

PTNL, preterm no labour; PTL, preterm labour; TNL, term no labour; TL, term labour; FTP, failure to progress; NRFHFT, non-reassuring fetal heart trace; PET, pre-eclampsia; GDM, gestational diabetes. *P<0.05 and †P<0.001.

by an extension at 72 °C for 15 s. A melt over the temperature range of 72–99 °C rising by 1 °C steps with a wait for 15 s on the first step followed by a wait of 5 s for each subsequent step completes the procedure. The cycle at which the fluorescence reached a preset threshold (cycle threshold) was used for quantitative analyses. The cycle threshold in each assay was set at a level where the exponential increase in amplicon abundance was approximately parallel between all the samples. The r² values and efficiencies for the primer pairs are given in Table 3. The mRNA data were expressed relative to the amount of the constitutively expressed housekeeping genes β-actin and GAPDH for myometrial tissue samples and primary myometrial cell cultures respectively. Different housekeeping genes were used as we have found that in primary uterine myocytes cell culture GAPDH showed less variation and that in myometrial samples β-actin showed less variation (N Engineer, SR Sooranna, S Chalkley, L Myatt & MR Johnson, unpublished observations).

Western analysis

Myometrial tissues were homogenised in five volumes of a buffer containing 20 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 2 mM dithiotreitol, 1 mM Na3VO4, 1 mM phenylmethylsulphonyl fluoride and 1 μg/ml leupeptin. After centrifugation for 1 min at 12 000 g, homogenates were aliquoted and frozen at −80 °C. Protein concentrations were determined by Protein assay (Bio-Rad Laboratories) and BSA reference standards. Electrophoresis was carried out using 15 μg aliquots of protein samples in 2× loading buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue and 0.125 mol/l Tris–HCl, pH 6.8). Samples were boiled for 5 min, quenched on ice and subsequently run on precast 10% SDS-polyacrylamide gels (Bio-Rad Laboratories).

Western blotting was carried out following electrophoretic transfer in 25 mmol/l Tris (pH 8.3), 192 mmol/l glycine and 20% v/v methanol on to Hybond ECL nitrocellulose membrane (Amersham Life Science). Membranes were blocked in 5% milk protein in 0.1% Tween–PBS for 1 h at room temperature. Mouse MAB to β-actin was purchased from Sigma–Aldrich Co. Ltd and goat polyclonal antibodies to OXTR (two antibodies, one directed to the C-terminus and the other to the N-terminus) CX-43 (both phospho and non-phospho forms) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Polyclonal antibody against human PGHS-2 was purchased from Cayman Chemical (Ann Arbor, MI, USA). Specific rabbit polyclonal antibodies directed against the phospho- or total CX-43 (New England Biolabs Ltd, Hitchin, UK). The antibodies were used at a dilution of 1:1000 and incubated overnight at 4 °C. Membranes were washed with 0.1% Tween–PBS and then incubated with anti-goat immunoglobulin G-horseradish peroxidase secondary antibody at a dilution of 1:2000 for 1 h at room temperature. ECL Western blotting detection was carried out using standard protocols (Amersham Life Science). Protein band size was determined using Rainbow coloured protein molecular weight markers (Amersham Life Science). Antibody specificity was confirmed using positive controls. Exposure of cells to u.v. B irradiation (wavelength 356 nm) for 30 min was used as a positive control. Western autoradiographs were quantified by digital densitometry using the Image Master VDS gel documentation system and Image Master VDS Software (Amersham Life Science). Protein bands were digitised, ensuring that the range of pixel densities did not extend to either the minimum or maximum values. Mean pixel density for each band was assessed using a sample gate of the same size. To allow comparisons between blots prepared on different occasions, a single control sample was included on each blot. Final pixel density was adjusted to ensure that this control sample carried the same value for each blot.

Cell culture

Biopsies (0.5 cm³) of term human myometrium were collected at the time of caesarean section (LSCS) from women not in...
labour and stored in DMEM containing 100 mU/ml penicillin and 100 μg/ml streptomycin. Samples were stored at 4 °C for no more than 3 h prior to cell preparation for culture. The indications for LSCS in this group of women were previous LSCS, breech presentation and maternal request. All specimens were obtained after patient consent, and the Riverside Research Ethics Committee approved the study.

Primary human uterine myocytes were isolated using a mixture of collagenases and cultured in DMEM 7.5% fetal calf serum (FCS), 100 munits/ml penicillin and 100 100 μg/ml streptomycin in T75 in an atmosphere of 5% CO₂; 95% air at 37 °C (Sooranna et al. 2005). Myometrial cells grown in this manner have previously been characterised (Sooranna et al. 2005). Cells from passages 1 to 4 were trypsinised in 0.25% trypsin containing 0.02% EDTA in PBS and cultured in 6-well plates. When cells were 80–90% confluent (days 3–4), old medium was removed and replaced with 1.5 ml fresh medium supplemented with 7.5 mM HEPES with 1% FCS overnight. After 16–18 h, cells were incubated for 1, 6 and 24 h with 1 ng/ml IL-1β, 1 ng/ml TNF-α, 1 ng/ml IL-6, 1 ng/ml IL-8, 10 nM PGE₂, 10 nM PGF₂α or 100 nM OXT after which media was removed and cells frozen in liquid nitrogen and stored at −80 °C until extraction of RNA.

### Statistical analysis

Demographic data were analysed using the Mann–Whitney U test for continuously distributed data and a Fisher’s exact test for categorical data as appropriate. The myometrial data were not normally distributed and were expressed as median and range. The data were analyzed with a Kruskall–Wallis test and post-testing with Mann–Whitney U test for independent samples. The effect of gestational age on gene expression was assessed using a Spearman test for non-parametric data. Multiple regression analysis was used to assess whether there was any relationship between individual gene expression, gestational age, labour status and site of tissue biopsy (upper or lower segment). In cell culture studies, the data were not normally distributed and were expressed as median and range. The data were analyzed with a Friedman test (non-parametric repeated measures ANOVA) and post-testing with Mann–Whitney U test.

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