Genes for prostaglandin synthesis, transport and inactivation are differentially expressed in human uterine tissues, and the prostaglandin F synthase AKR1B1 is induced in myometrial cells by inflammatory cytokines

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Submitted on February 18, 2010; resubmitted on June 2, 2010; accepted on June 24, 2010

ABSTRACT: Prostaglandins (PGs) are important factors in the physiology of human parturition and the control of uterine contractility. We have characterized the expression of 15 genes from all stages of the PG pathway in human pregnant and non-pregnant (NP) myometrium and in other uterine tissues at delivery, and the results show patterns indicative of different capacities for PG synthesis and catabolism in each tissue. In placenta, the PG synthase expression profile favours production of PGD2, PGE2 and PGF2, with high levels of PG transporters and catabolic PG dehydrogenase suggesting rapid PG turnover. Choriodecidua is primed for PGE2, PGF2 and PGD2 production and high PG turnover, whereas amnion expresses genes for PGE2 synthesis with low levels of PG transporters and dehydrogenase. In umbilical cord, PGI2 synthase is highly expressed. In pregnant myometrium, PGI2, PGD2 and PGF2 synthases are highly expressed, whereas PG dehydrogenase is underexpressed. Myometrium from women with spontaneous or induced labour had higher expression of the PGH2 synthase PTGS2 than tissue from women not-in-labour. Myometrium from NP women had lower levels of PG synthases and higher levels of PG dehydrogenase than pregnant myometrium. Discriminant function analysis showed that expression of selected genes in myometrium could distinguish groups of women with different modes of labour from each other and from NP women. In cultured myometrial cells, there was a dose-dependent stimulatory effect of interleukin 1β and tumour necrosis factor α on PTGS2, PTGES and AKR1B1 (PGF synthase) expression.

Key words: cytokines / gene expression / pregnancy / prostaglandins / uterus

Introduction

The uterus is a remarkable organ, growing throughout 40 weeks of pregnancy to accommodate the developing fetus, before the onset of coordinated contractions in the muscular wall (myometrium), along with rupture of the fetal membranes and dilatation and remodeling of the cervix combine to force the fetus and placenta through the birth canal. However, although 5–10% of births are preterm (occurring prior to 37 weeks of gestation), these are associated with 70–80% of neonatal mortality and morbidity (Goldenberg et al., 2008). Preterm births are either spontaneous or iatrogenic, with intervention for underlying conditions such as preeclampsia and intrauterine growth restriction (Moutquin, 2003). Spontaneous preterm labour (SPL) follows a number of pathophysiological conditions including infection, premature rupture of the membranes and intrauterine bleeding, or it can be idiopathic, with no apparent pathology but...
with increased sensitivity of the uterus to stimulatory agonists (López Bernal, 2003). Incomplete knowledge of the mechanisms of normal human labour at term hinders attempts to prevent preterm birth. Roles in term and preterm activation of the uterus have been proposed for several hormones including oxytocin, progesterone, corticotrophin releasing factor and prostaglandins (PGs; Smith et al., 2002; Arthur et al., 2007; Olson and Aamann, 2007).

Series 2 PGs are eicosanoids derived from a 20-carbon fatty acid (arachidonic acid), which is released from cell membrane phospholipids by the actions of phospholipases. Primary PG synthases are cyclooxygenases enzymes that catalyse the conversion of arachidonic acid to PGH2. Terminal PG synthases convert PGH2 to structurally similar but functionally diverse PGs that have critical roles in reproductive processes, including myometrial quiescence and activation and cervical ripening (Keirse, 1978; Hertelendy and Zakar, 2004). In women, parturition is associated with increased intrauterine PG release as demonstrated by the rising PGE2 and PGF2α, levels in amniotic fluid with progressive cervical dilation (Keirse, 1978) and the abrupt rise in PG metabolites in the maternal circulation (Sellers et al., 1982) and urine (Granstrom and Kindahl, 1976) in women in active labour.

In human myometrium, PGs affect smooth muscle tone with PGD2 and PGI2 considered to promote relaxation, whereas PGE2 and PGF2α promote contraction (Senior et al., 1993). This view is a simplification, as the effects of PGs depend on the expression of their receptors and coupling to intracellular signalling pathways. For instance, the inhibitory PGI2 has recently been shown to up-regulate the myometrial expression of contraction-associated proteins (Fetalvero et al., 2008). PGE2 can have stimulatory or inhibitory activity depending on the relative expression of the four PGE receptor (PTGER 1–4) types, which are linked to different signalling pathways.

Previous studies on the role of PGs in parturition have focused on the release of arachidonate by phospholipases or the role of PG H2 synthase (PTGSI and PTGS2) activities in placenta, fetal membranes, decidua and myometrium (Keelan et al., 2003; Olson, 2003; Hertelendy and Zakar, 2004) and this has provided much valuable information. PTGSI is considered to mainly have constitutive expression in uterine tissues, whereas PTGS2 is an inducible enzyme. In some species, parturition is associated with increased intrauterine PTGS2 expression and increased PG release (Dong et al., 1996; Gibb et al., 2000; Wu et al., 2001). However, detailed temporal studies in sheep (Palliser et al., 2004) suggest that a critical step for the onset of labour is the increase in intrauterine PGF2α release with relatively constant PGE2 production. In other words, the late pregnancy increase in PTGS2 may not be sufficient to trigger labour, unless accompanied by preferential activation of PGF synthase over PGE synthase, resulting in an increased intrauterine PGE2/PGF ratio. These observations remain controversial (Wu et al., 2001), although they agree well with the previous results in human decidual cells where the ratio of PGF/PGE and PGE/PGF release increased significantly with the onset of labour (Norwitz et al., 1992) and with recent data that suggest that increased PGF2α levels in amniotic fluid precede and predict term and preterm labour (Lee et al., 2009).

In humans, there are two PGD synthases, PTGDS, previously known as lipocalin-type PGD synthase, and HPGDS, or hematopoietic PGD synthase (Helliwell et al., 2004). There are three enzymes with PGE synthase activity (PGES), which is the conversion of PGH2 to PGE2 (Fig. 1); PTGES (mPGES-1), PTGES2 (mPGES-2) and PTGES3 (cPGES). There are also three enzymes with PGF synthase activity (PGFS), although each of these enzymes also carries out a number of other reduct reactions. AKR1C3 is an aldo-keto reductase capable of converting PGH2 to PGF2a, and PGD2 to 9α-11β-PGF2α, but also active on a range of steroid intermediates; AKR1B1 (aldo reductase) is an aldo-keto reductase that was recently shown to convert PGH2 to PGF2α (Fortier et al., 2008; Kabututu et al., 2009), while its initially described and accepted primary activity is to reduce glucose to sorbitol under high glucose concentrations associated with diabetes. CBR1 is a carbonyl reductase that converts PGE2 to PGF2α, and that is also capable of converting PGs to their inactive metabolites through a 15-hydroxyprostaglandin dehydrogenase activity. There is only one known human PGI2 synthase, PTGIS (prostacyclin synthase). Owing to their anionic charge, the influx and release of PGs through the cell membrane requires specialized transport proteins such as the solute carrier organic anion transporter member 2A1 (SLCO2A1, also known as PG transporter) and human ATP-binding cassette transporter member 4 (ABCC4), which are currently considered to be responsible for uptake and release, respectively, of PGs from the cell. SLCO2A1 is expressed in many tissues and actively transports several substrates including PGE2, PGF2α, and PGD2, driven by the exchange of lactate (Chan et al., 2002); alterations in lactate metabolism may be a cause of abnormal uterine contractility (Anai et al., 2002). The PG transporter is involved in the synchronization of PGF2α- and PGF2α-modulated calcium waves in human myometrial cells (Young et al., 2002). In human endometrium, SLCO2A1 expression is regulated during the menstrual cycle (Kang et al., 2005) and increases following the decidualization of stromal cells (Kang et al., 2006). Kinetic studies in several cell types have demonstrated that ABCC4 functions as a PG efflux transporter with physiological affinity for PGE2, PGF2α, and thromboxane B2 (Rius et al., 2005). The activity of ABC4 is inhibited by indomethacin (Reid et al., 2003). Attenuation of PG signalling occurs by the coupling of PG uptake and catabolism by the 15-OH PG dehydrogenase enzyme, HPGD (previously PGDH; Schuster, 1998).

In late pregnancy, the myometrium is in contact with the maternal decidua, which is fused to the outer fetal membrane, the chorion, within which lies the amnion. The nutrient exchange organ, the placenta, has decidua at the point of contact with the uterine wall, with fetal trophoblastic villi projecting into maternal blood and carrying the fetal circulation via the umbilical cord to the fetus. All the parts of this system may produce PGs with autocrine or paracrine activity. No previous study has attempted to characterize the relative expression of the different elements of the PG pathway in these uterine tissues. The identification of terminal PG synthases active in human uterine tissues might suggest utero-specific strategies for tocolytic therapy by selective terminal PG synthase inhibition, with potential to improve efficacy and minimize side effects compared with current tocolytics.

The aim of the research was to study the distribution of enzymes of the PG pathway in intrauterine tissues. The results demonstrate that each tissue has a particular potential for PG metabolism. We have identified changes in myometrial gene expression in pregnancy and in association with term or preterm labour. Specific patterns of
myometrial gene expression in different groups of women allow the separation of women into pregnant and non-pregnant (NP) groups and into groups with different modes of onset of labour by discriminant function analysis. In view of the possible role of inflammatory mediators in the mechanism of labour, we have also investigated the effects of pro-inflammatory cytokines in myometrial cells, identifying up-regulation of PTGS2, PTGES and AKR1B1.

**Materials and Methods**

**Collection of tissue**

All women gave written informed consent according to the requirements of the North Somerset and South Bristol Research Ethics Committee. NP myometrium was obtained from premenopausal women undergoing hysterectomy for benign gynaecological disorders [number of patients \( n = 24 \)]. Pregnant myometrium was collected at Caesarean section from the following groups of women: term (>37 weeks gestation) not-in-labour (TNIL), at elective Caesarean sections \( n = 26 \), mean gestational age \( \text{g.age} = 39 \) weeks. Spontaneous term labour (STL), at emergency Caesarean sections for fetal distress and/or failure to progress \( n = 24 \), g.age = 40 weeks. Term following induction of labour (IOL) with intravaginal PGE pessary and intravenous oxytocin infusion; emergency Caesarean sections in this group were for failure to progress or fetal distress \( n = 14 \), g.age = 41 weeks. Preterm (25–36 weeks gestation) not-in-labour (PNIL); Caesarean sections in this group were indicated for maternal or fetal complications \( n = 16 \), g.age = 32 weeks. Spontaneous preterm labour (SPL), at emergency Caesarean sections for fetal distress \( n = 22 \), g.age = 33 weeks. Myometrial tissue free of decidua or serosa was removed from the upper border of the lower uterine segment incision. After delivery, samples of placenta and attached fetal membranes and cord were also taken for comparisons. The women were of mixed parity and all delivered live infants with no signs of infection. Tissue samples were dissected immediately after delivery, washed in sterile saline, dried, snap-frozen and stored in liquid nitrogen; in some instances, myometrial tissue was kept fresh for the preparation of cell cultures.
**Myometrial cell culture**

Myometrial tissues from the TNIL group were washed in saline solution and digested in serum-free Dulbecco’s modified Eagle’s medium (DMEM) containing 3000 U/ml collagenase (type II), 0.3 U/ml dispase, 30 U/ml DNase I and 0.09 U/ml elastase at 37°C for 4.5 h. Liberated smooth muscle cells were then grown in adherent cell culture with DMEM containing 10% fetal calf serum, 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C in 95% air, 5% CO₂. Cells were split into 12-well plates, grown to 70–80% confluence and the medium changed to serum-free DMEM, containing 0.2% lactalbumin hydrolysate, 100 U/ml penicillin and 100 µg/ml streptomycin. At the point of assay, cells were at passage seven or lower. After 1 h, either lipopolysaccharide (LPS) or cytokines interleukin 1 (IL1β) and tumour necrosis factor (TNFα), diluted in DMEM, were added, and cells were incubated at 37°C for 24 h. The medium was then removed, cells washed with phosphate-buffered saline and lysed by scraping into RIPA buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% NP40, 0.5% Triton X-100, 1% SDS). Lysates were cleared by centrifugation at 10 000 g for 20 min, and protein content of supernatants quantitated by the bicinchoninic acid (BCA) assay (Thermo Scientific, Rockford, IL, USA) and analysed by western blotting.

**Quantitative real-time PCR**

Total RNA was extracted from 100 mg tissue samples by the guanidine isothiocyanate/phenol method using 1 ml TRIzol (Invitrogen, Carlsbad, CA, USA), giving yields of 10–150 µg. RNA was quantified using a Gene Quant II spectrophotometer (GE Healthcare, Little Chalfont, Bucks, UK). Two micrograms of total RNA were used as a template for cDNA synthesis primed by random primers using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). cDNAs were diluted 4-fold and 2 µl used as template for quantitative PCR (qPCR) with the Power SYBR Green PCR Master Mix (Applied Biosystems), with reaction volumes of 20 µl, forward and reverse primer concentrations of 75 nM and 45 cycles of 95°C for 15 s and 60°C for 60 s, followed by a dissociation stage, using a 7500 Real-Time PCR System (Applied Biosystems). To minimize the effect of inter-run variation in C_target, values, plates were set up using the ‘sample maximization’ method, so that the samples for each gene analysed were run on as few plates as possible. In addition, inter-run calibrators were included on each plate. Results were analysed using the Sequence Detection Software v1.2.3, and control C_target values for each sample were calculated by taking the geometric mean of the C_target values for the endogenous control genes POLR2A and ARHGDI. For each sample, the control C_target value was subtracted from the C_target values for target genes to give ΔC_target values. Mean ΔC_target values were calculated using the arithmetical mean of all ΔC_target values for a gene. ΔΔC_target values were calculated by subtraction of the mean ΔC_target from each sample’s ΔC_target. Relative expression levels were calculated by $e^{\Delta\Delta C_{target}}$ and then normalized to reflect overall gene expression levels, as determined by the mean C_target for each gene. For all subsequent statistical analyses, these normalized e values were log_{10}-transformed. Sample maximization, multiple endogenous control genes, and mean ΔC_target for the calculation of ΔΔC_target are all intended to decrease error in qPCR quantification (Vandesompele et al., 2002; Hellemans et al., 2007). Sequences for all primers used in this study are given in Table I. The positions of the different enzymes in the PG pathways are illustrated in Fig. 1. OXTR (oxytocin receptor) levels were measured in myometrial samples, to compare expression of a gene involved in pregnancy and labour but not PG metabolism.

**Western blotting**

Total protein extracts were prepared by homogenization of 250 mg tissue samples in 0.8 ml RIPA buffer [50 mM Tris–HCl, 150 mM NaCl, 1 mM MgCl₂, 0.5% Triton X-100, 1% SDC] for 20 min, and protein content of supernatants quantitated by the bicinchoninic acid (BCA) assay (Thermo Scientific, Rockford, IL, USA) and analysed by western blotting.

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**Table I Genes studied and primer sequences used for real-time RT–PCR.**

<table>
<thead>
<tr>
<th>Gene nomenclature</th>
<th>Accession number</th>
<th>Forward primer* (5’–3’</th>
<th>Reverse primer* (5’–3’</th>
</tr>
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<tbody>
<tr>
<td>PLA2G4A</td>
<td>NM_024420</td>
<td>(205) AATGTCAATTATAGATCCTACC</td>
<td>(486) GCATCCATAAAGTAATCTCC</td>
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<tr>
<td>PTGS1</td>
<td>NM_000962</td>
<td>(123) CAGCACGCCCAGCGATGAG</td>
<td>(355) ACAGGCCCAGGGATGTGC</td>
</tr>
<tr>
<td>PTGS2</td>
<td>NM_000963</td>
<td>(90) CTCAGACAGCAAAAGCTACC</td>
<td>(461) ATGTGATCTGATGCTCAAC</td>
</tr>
<tr>
<td>AKR1B1</td>
<td>NM_001628</td>
<td>(71) AGCCATGCAAAGCGCTCCTC</td>
<td>(317) GCACCATGCTTGGTCAAC</td>
</tr>
<tr>
<td>AKR1C3</td>
<td>NM_003739</td>
<td>(378) CCTCGAGCTGCTGACCAAC</td>
<td>(448) CCTCACGTCCTGTTAGAC</td>
</tr>
<tr>
<td>CBR1</td>
<td>NM_001757</td>
<td>(50) AGAGATGCGCTGCCAACAC</td>
<td>(542) AGTCTACACTCCCTCC</td>
</tr>
<tr>
<td>PTGES1</td>
<td>NM_001626</td>
<td>(29) GAGAGTGCTACCTGACCAAG</td>
<td>(520) GCTGCTGTACAGGTGTC</td>
</tr>
<tr>
<td>PTGES2</td>
<td>NM_004878</td>
<td>(46) AGCCCGGCAGGCTTGGG</td>
<td>(439) GAAGATGCTTACCTCCTG</td>
</tr>
<tr>
<td>PTGES3</td>
<td>NM_005601</td>
<td>(88) CGAGGAAATGGCTACTCTAC</td>
<td>(1641) TGCTCCCTGCTGTCCTG</td>
</tr>
<tr>
<td>PTGIS</td>
<td>NM_000961</td>
<td>(71) GCATAAACAAGAATTCACC</td>
<td>(280) CTGTTGAAAGTAGCTTAC</td>
</tr>
<tr>
<td>PTGDS</td>
<td>NM_001626</td>
<td>(3) CTGAGCCTAGCATGGAAC</td>
<td>(232) AAGTCTGCTCAGTGCT</td>
</tr>
<tr>
<td>HPGD</td>
<td>NM_001626</td>
<td>(79) NGGACCAATGGCTTGGG</td>
<td>(328) GACATTGCTGATTGCTC</td>
</tr>
<tr>
<td>HPGD</td>
<td>NM_001626</td>
<td>(247) CAACTACCTCAGGAACC</td>
<td>(3758) CTCACTGTTGCTGACCAC</td>
</tr>
<tr>
<td>SLC20A1</td>
<td>NM_005630</td>
<td>(1479) TGTGCTGAGCCCTTCTCTC</td>
<td>(1624) KCATCCTGAGAGCATGTA</td>
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<tr>
<td>ABC4</td>
<td>NM_005845</td>
<td>(358) ACCTGACGGCGGCACCTGG</td>
<td>(628) GACATTGCTGAGCTGCC</td>
</tr>
<tr>
<td>OXTR</td>
<td>NM_000916</td>
<td>(4453) GCACACGTCCAAATGACAT</td>
<td>(4719) GCACCCCTGCTCCATAAAC</td>
</tr>
</tbody>
</table>

*Corresponding position in gene sequence of 5′-terminal nucleotide of each primer is indicated in parentheses preceding primer sequence.
EDTA, 0.5% (v/v) NP40, 0.25% (v/v) Triton X-100, pH 7.5] using a rotor–stator homogenizer with three 15 s pulses. Extracts were clarified by centrifugation at 10 000g for 20 min at 4 °C, and protein concentration of the supernatants determined by the BCA assay. Extracts were diluted to give 50 μg protein in 30 μl of 1× Laemmli buffer [62.5 mM Tris–HCl, 2% (w/v) SDS, 10% (v/v) glycerol, 0.01% (v/v) bromophenol blue, 2.5% (v/v) β-mercaptoethanol, pH 6.8], heated to 100°C for 3 min, and proteins separated on 12% acrylamide tris-glycine SDS–PAGE gels. A pooled sample consisting of a combination of protein extracts from each of the tissues was used as a standard sample that was loaded onto each SDS–PAGE gel used in the study. Proteins were transferred to PVDF membranes that were then air-dried, re-wet with methanol, blocked in WesternBlaze (Invitrogen) blocking buffer for 30 min and incubated with primary antibodies in WesternBreeze antibody dilution buffer overnight at 4°C. After washing, membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 2 h at room temperature, then washed and covered for 5 min with Immobilon Western Chemiluminescent HRP Substrate (Millipore, Billerica, MA, USA). Excess substrate was removed with blotting paper; membranes were encased in SaranWrap and exposed to Hyperfilm ECL (GE Healthcare) at room temperature, then washed and covered for 5 min with Immobilon-P PVDF membranes that were then air-dried, re-wet with methanol, incubated with primary antibodies in WesternBreeze antibody dilution buffer overnight at 4°C. For a subset of eight of the PG pathway gene products and the Rho GDP-dissociation inhibitor 1 protein (ARHGDIA) control, relative levels were also determined by western blotting, and these levels are shown in Fig. 2B. Representative western blots are presented in Fig. 3. The samples used for protein measurement and those used for mRNA quantification form overlapping, but not identical groups. Pearson’s correlation coefficients, r (and 95% confidence intervals), between protein and mRNA levels for samples with matched protein and mRNA data (approximately one-third of all samples) were: PTGS1, r = 0.72 (95% CI: 0.60–0.81); PTGS2, r = 0.37 (95% CI: 0.14–0.56); PTGES, r = 0.24 (95% CI: 0.00–0.45); AKR1B1, r = 0.73 (95% CI: 0.61–0.82); AKR1C3, r = 0.32 (95% CI: 0.11–0.51); CBR1, r = 0.47 (95% CI: 0.27–0.63); SLCO2A1, r = 0.40 (95% CI: 0.19–0.57); HPGD, r = 0.80 (95% CI: 0.70–0.87).

Statistical analysis

Differences between the means of the groups were assessed using one-way ANOVAs (univariate analyses). ANOVAs were first used to compare different tissues, followed by Tukey’s multiple comparison procedure to compare all possible pairs of tissues.

ANOVAs were then used to explore differences in myometrium between the subgroups of women defined above (NP, PNIL, SPL, TNIL, STL and IOL). These ANOVAs were followed by a series of pre-specified contrasts that involved pooling of some patient groups: P (pregnant) = PNIL + SPL + TNIL + STL + IOL; PT (preterm) = PNIL + SPL; T (term) = TNIL + STL + IOL; NIL (not-in-labour) = PNIL + TNIL; SL (spontaneous labour) = SPL + STL. The contrasts were: NP versus P, PT versus T, PNIL versus TNIL, SPL versus STL, NIL versus SL, PNIL versus SPL, TNIL versus STL, NIL versus IOL, SL versus IOL and STL versus IOL. These comparisons and contrasts between means were considered significant when P-values were <0.01.

Following on from these univariate analyses, genes whose expression varied significantly between the groups of myometrial samples (NP, PNIL, SPL, TNIL, STL and IOL) on univariate analysis were analysed together using a linear discriminant analysis (multivariate analysis) to further explore the differences between the groups with respect to the set of gene expressions.

Correlation between gene expression and protein levels was determined by calculation of Pearson’s correlation coefficients for genes with both mRNA and protein quantification data. Calculations were performed in Microsoft Excel using log10-transformed data.

Other software used included Prism v4.00 (GraphPad Software, La Jolla, CA, USA) to plot the figures and SPSS v14.0 (SPSS Inc., Chicago, IL, USA) to conduct ANOVA and discriminant analyses.

Results

Differential expression of PG pathway-related genes in uterine tissues

Relative levels of mRNA in each pregnant uterine tissue are shown in Fig. 2A. With the extreme sensitivity of qPCR, the expression of all the genes was detected in all the tissues, although at very different levels. Each tissue had a characteristic expression profile: myometrium was characterized by high expression of PTGS1, PTGDS, AKR1C3, CBR1 and PTGIS and synthases and SLCO2A1 and ABC4 transporters, with relatively low expression of PLA2G4A, PTGS2, HPGDS, PTGES3, AKR1B1 and HPGD; placenta had high PTGS1, HPGDS, PTGDS, PTGES3, AKR1B1, SLCO2A1, ABC4 and HPGD; amnio had high PTGS2, PTGES and PTGES3; cord had high PTSG1, PTGS2, PTGES, CBR1, SLCO2A1 and HPGD.

Protein expression

For a subset of eight of the PG pathway gene products and the Rho GDP-dissociation inhibitor 1 protein (ARHGDIA) control, relative levels were also determined by western blotting, and these levels are shown in Fig. 2B. Representative western blots are presented in Fig. 3. The samples used for protein measurement and those used for mRNA quantification form overlapping, but not identical groups. Pearson’s correlation coefficients, r (and 95% confidence intervals), between protein and mRNA levels for samples with matched protein and mRNA data (approximately one-third of all samples) were: PTGS1, r = 0.72 (95% CI: 0.60–0.81); PTGS2, r = 0.37 (95% CI: 0.14–0.56); PTGES, r = 0.24 (95% CI: 0.00–0.45); AKR1B1, r = 0.73 (95% CI: 0.61–0.82); AKR1C3, r = 0.32 (95% CI: 0.11–0.51); CBR1, r = 0.47 (95% CI: 0.27–0.63); SLCO2A1, r = 0.40 (95% CI: 0.19–0.57); HPGD, r = 0.80 (95% CI: 0.70–0.87).

Effect of pregnancy on myometrial gene expression

A comparison of NP and pregnant myometrial samples showed remarkable differences in gene expression (Fig. 4). Several genes (PTGS1, PTGS2, HPGDS, PTGES3, PTGIS, SLCO2A1, ABC4 and OXTR) were significantly up-regulated in pregnancy. Some genes (PLA2G4A, PTGDS, PTGES2, AKR1B1, AKR1C3 and CBR1) showed no significant difference between pregnant and NP tissue, whereas HPGD was down-regulated in pregnant myometrium. The measurement of myometrial protein gave similar results, with PTGS1 levels increased in pregnancy and no change in AKR1C3 or CBR1 (Fig. 4C). These proteins were all highly expressed in myometrium and readily characterized by western blotting. Other proteins (PTGS2, PTGES, AKR1B1, SLCO2A1 and HPGD) had very low
Figure 2. Expression of PG pathway genes in pregnant human uterine tissues. M, myometrium; P, placenta; Cd, choriodecidua; A, amnion; C, umbilical cord. (A) Relative levels of mRNA, calculated by the \( \Delta \Delta C_t \) method using the results of qRT–PCR, as described in the ‘Materials and Methods’ section. Numbers of samples: M = 38; P = 31; Cd = 31; A = 23; C = 25. (B) Protein levels, determined by western blotting, presented in arbitrary densitometric units (ADU). Numbers of samples: M = 38; P = 29; Cd = 28; A = 15; C = 26. For each gene/protein, levels in different uterine tissues were compared by ANOVA, resulting in 10 pairwise comparisons summarized above each graph, the key to which is shown in (C). This gives the tissues involved in each pairwise comparison, and also the colour code used to summarize the \( p \)-values returned by Tukey’s multiple comparison procedure. Values are log_{10}-transformed and shown as mean ± SD.
levels of expression in the myometrial samples, as demonstrated in Fig. 3. We were unable to obtain sufficient quantitative data on these proteins to compare their levels of expression in the different myometrial groups.

Effect of mode of onset of labour

For most genes, the level of mRNA expression in pregnant myometrium was similar whether the samples were obtained from women in-labour or not-in-labour (Fig. 4). Differences between in-labour and not-in-labour myometrium were seen for PTGS2, which had significantly higher expression in both spontaneous and induced labour, whereas OXTR had the opposite pattern, with lower levels in in-labour samples, although this was not statistically significant.

Linear discriminant function analysis

In univariate ANOVAs, the following PG pathway genes showed significant differences in myometrial expression between the six groups of women (NP, PNIL, SPL, TNIL, STL and IOL): ABCC4 (P < 0.001), AKR1B1 (P = 0.039), SLC20A1 (P = 0.01), PTGS1 (P = 0.01), PTGS2 (P < 0.001), PTGES3 (P < 0.001), PTGES (P < 0.001), HPGD (P < 0.001) and HPGDS (P = 0.010). These genes were further analysed using a linear discriminant analysis.

Discriminant analysis calculates a set of up to five uncorrelated functions to separate out the six defined groups, where each function is a weighted sum of the individual gene expressions. The weights for the first function are calculated in such a way as to maximally separate out the six groups; the weights for the second function maximally separate the groups with respect to any differences that remain, and so on.

Six genes showed no significant differences in univariate ANOVAs (AKR1C3 (P = 0.11), CBR1 (P = 0.83), PTGES (P = 0.59), PTGES2 (P = 0.65), PLA2G4A (P = 0.15), PTGD5 (P = 0.07)). When added individually to the discriminant analysis, these genes did not significantly improve the discrimination (data not shown), and they are therefore not included in the analysis.

Figure 5 is a plot of individual sample scores of the first and the second functions for the 45 myometrial samples, as well as the group mean values (centroids) of the scores. The weights used to calculate the function scores are shown in the figure legend. The remaining three functions did not give any further significant group separation and so this two-dimensional figure gives a good representation of the group structure.

From this figure, it can be seen that Function 1 reveals the clearest differences between the groups with the separation of NP, not-in-labour and spontaneous labour samples. Function 2 illustrates the group differences not shown by Function 1, by clearly separating not-in-labour from in-labour samples. There is little separation of the two not-in-labour groups from each other, or of the spontaneous labour groups, which are slightly removed from the IOL group. This demonstrates that there are consistent patterns in the expression of these genes within the myometrium of women in each patient group.

Effect of pro-inflammatory cytokines on gene expression

We tested the effects of IL1β, TNFα and LPS on PG pathway enzyme expression in cultured myometrial cells (Fig. 6). A combination of IL1β and TNFα had a strong dose-dependent stimulatory effect on the expression of PTGS2 and PTGES proteins (Fig. 6, combined experimental data in A and C, representative western blots in a and c). The stimulatory effect increased exponentially with treatments ranging from 40 to 1000 pM cytokines. A clear stimulatory effect was also observed on AKR1B1 production, which showed a lower level of stimulation, but a greater sensitivity, starting at 8 pM and reaching a plateau at 200 pM cytokine concentration [Fig. 6B (data) and b (western blots)]. LPS had no significant effect under the conditions used (up to 1 μg/ml). The cytokines had no effects on the production of PTGS1, AKR1C3, CBR1 (representative western blots shown in Fig. 6d, e and f), SLC20A1, HPGD or ARHGDIA (data not shown).

Discussion

This paper provides a comprehensive description of enzymes involved in PG synthesis, transport and metabolism in human uterine tissues and shows for the first time the presence of the terminal PGF synthases AKR1B1, AKR1C3 and CBR1, and the PG transporters SLC20A1 and ABCC4 in human myometrium. Previous studies have demonstrated essential roles for PGs in the uterus in pregnancy and labour (Keirse, 1978; Keelan et al., 2003; Olson and Ammann, 2007). To investigate this further, we set out to analyse the expression of genes involved throughout the entire PG metabolic pathway to enable us to identify those genes responsible for the observed patterns of uterine PG production, and so to reveal targets for clinical intervention or markers indicating premature labour.

We identified expression of 15 genes in intraterine tissues. The products of these genes are: PLA2G4A, a phospholipase responsible for release of the eicosanoid precursor arachidonic acid from membrane phospholipids; PTGS1 and PTGS2, which convert arachidonic acid to PGH₂, the common substrate for the different terminal synthases including PGD₂ (PTGDS, HPGD), PGE₂ (PTGES, PTGES2, PTGES3), PGF₂a (AKR1B1, AKR1C3, CBR1) and PGI₂
Figure 4 Expression of PG pathway genes in human myometrium. NP, non-pregnant; PNIL, preterm not-in-labour; SPL, spontaneous preterm labour; TNIL, term not-in-labour; STL, spontaneous term labour; IOL, induction of labour. (A) Relative levels of mRNA, calculated by the ΔΔCt method using the results of qRT–PCR, as described in the ‘Materials and Methods’ section. Numbers of samples: NP = 10; PNIL = 7; SPL = 6; TNIL = 7; STL = 7; IOL = 8. (B) Relative levels of OXTR mRNA in myometrium, measured as in (A). (C) Protein levels of PTGS1, AKR1C3 and CBR1, determined by western blotting, presented in arbitrary densitometric units (ADU). Numbers of samples: NP = 18; PNIL = 8; SPL = 9; TNIL = 14; STL = 8; IOL = 6. For each gene/protein, one-way ANOVA was used to make comparisons between pairs of individual or combined patient groups, as described in the ‘Materials and Methods’ section. These comparisons are summarized above each graph, with a key shown in (D) giving the tissues involved in each comparison and the colour code used to summarize the P-values for the differences between the means. Combinations of patient groups: P, pregnant (PNIL, SPL, TNIL, STL and IOL); PT, preterm (PNIL and SPL); T, term (TNIL, STL and IOL); NIL, not-in-labour (PNIL and TNIL); SL, spontaneous labour (SPL and STL). All values are log10-transformed and shown as mean ± SD.
ABCC4 and SLCO2A1 transport PGs across cell membranes and HPGD catalyses the first step in the degradation of PGs. This study is the first direct comparison of these genes in intrauterine tissues.

Patterns of expression shared by different genes were identified, suggesting common mechanisms of tissue-specific transcriptional regulation. The expression patterns of PTGS1 and PTGIS, and to a lesser extent those of PTGS2 and PTGES, were alike, reflecting previously observed regulatory associations (Murakami et al., 2000; Korita et al., 2004). Although PTGS1 is considered a constitutively-expressed gene, we observed differential expression in the uterus, with the highest levels in myometrium and umbilical cord, both of which have high smooth muscle content. Even within tissues, expression of PTGS1 is not uniform, and it undergoes a considerable increase in pregnant myometrium, confirming previous observations (Korita et al., 2002).

The observed patterns of expression imply that each tissue in the pregnant uterus has a distinct potential for PG synthesis and catabolism; however, confirmation of this would require direct measurement of PG turnover in each tissue. The myometrium, a highly vascular smooth muscle tissue, showed high expression of genes encoding PGIS, one PGDS and two PGFS, and intermediate or low expression of one PGDS, three PGES and one PGFS. Previous observations in human myometrium described the presence of PGES (Astle et al., 2007) and PTGIS (Moonen et al., 1984) enzymes, PGD synthase activity (Rees and Kelly, 1986) and the capacity for PGE₂, PGF₂α, and PG₂ production (Robinson et al., 1979; Bamford et al., 1980; Hertelendy and Zakar, 2004). Our observations agree with lipidomic measurements of prostanoids in human myometrium demonstrating high levels of PGI₂, intermediate levels of PGD₂ and PGF₂α, and lower levels of PGE₂ (Durn et al., 2010). Contrasting effects on myometrial activity have been reported for these PGs—PGI₂ is relaxatory but has also been shown to potentiate the contractile response to oxytocin (Fetalvero et al., 2008) and PGD₂ is relaxatory and can have anti-inflammatory effects through conversion to cyclopentenone J₂-series prostanoids (Shibata et al., 2002). However, PGD₂ can also be converted to the contractile (Senior et al., 1993) and pro-inflammatory (Sandig et al., 2006) 9α-11β-PGF₂ by AKR1C3, which we have shown to be highly expressed in the myometrium. PGE₂ can have stimulatory or inhibitory effects on myometrial activity depending on the profile of receptor subtype expression (Astle et al., 2005; Grigsby et al., 2006) and can be converted to PGF₂α by the 9-ketoreductase activity of CBR1. PGF₂α, which acts through a single receptor to mobilize intracellular calcium, stimulates myometrial activity (Senior et al., 1993; Carrasco et al., 1996). We have shown that there are three PGF synthase isoforms in myometrium that are capable of producing uterotonic PGF; of these, AKR1C3 has the highest expression in myometrium relative to other uterine tissues.

Figure 5 Linear discriminant analysis, using qPCR results to separate out the groups of myometrial tissue. Each data point represents the weighted sums of the expression values of a single myometrial sample calculated using two uncorrelated functions, with Function 1 \([-0.08(ABCC4) + 0.17(ABCC4) + 0.87(SLCO2A1) + 0.70(PTGS1) + 0.29(PTGS2) - 0.22(PTGES3) + 0.34(PTGIS) - 1.23(HPGD) - 0.59(HPGDS) + 0.94] \) plotted on the x-axis, and Function 2 \([2.11(ABCC4) + 0.01(ABCC4) - 0.64(SLCO2A1) - 0.93(PTGS1) + 0.72(PTGS2) - 0.89(PTGES3) + 0.57(PTGIS) + 0.95(HPGD) - 1.49(HPGDS) + 0.53] \) plotted on the y-axis. The figure also shows the group centroids for each of the six sample groups, which are calculated by applying the functions to the mean values of gene expression in each group. NP, non-pregnant; PNIL, preterm not-in-labour; SPL, spontaneous preterm labour; TNIL, term not-in-labour; STL, spontaneous term labour; IOL, induction of labour.
AKR1B1 has relatively low expression in myometrium, but in contrast to AKR1C3 and CBR1, it is induced by inflammatory cytokines in myometrial cells.

Pregnant myometrium had high PG transporter and low PG dehydrogenase levels, suggesting uncoupling of PG uptake and catabolism, although the contribution of the highly expressed CBR1 to PG dehydrogenase activity in the human myometrium is unknown.

In the placenta, the PG synthase expression profile appears to favour production of PGD2, PGE2 and PGF2α, with high levels of both PG transporters and PG dehydrogenase suggesting a rapid turnover of PGs, with active release, uptake and degradation. Choriodecidual appears primed for PGE2, PGF2α and PGD2 production with rapid PG turnover. The high level expression of the catabolic system of PG transporter and dehydrogenase in the choriodecidual and placenta implies that this system acts as a barrier separating the maternal and fetal PG environments (Keirse and Turnbull, 1975; Sangha et al., 1994). The amnion specifically expresses genes for PGE2 synthesis, with low levels of PG transporters and dehydrogenase. Umbilical cord has the capacity for high levels of PG synthesis via both PTGS1 and PTGS2, apparently directed primarily to production of PGE2, with some capacity for PGD2, PGE2 and PGF2α, synthesis, PG uptake and catabolism. These are speculative assessments of physiological potential based on mRNA and protein measurements rather than the direct observation of enzyme activity, but they agree with the known patterns of PG release in these tissues (Keirse, 1978; Norwitz et al., 1992; Keelan et al., 2003; Olson, 2003).

Expression of the PG pathway genes was further analysed by comparison of myometrial taken from NP patients with that from pregnant patients delivering preterm or full-term in the presence or the absence of spontaneous labour or with IOL. Differential regulation of a number of genes was seen in pregnancy, which should lead to increased PG synthesis and signalling in pregnant myometrium. PTGS1, HPGD, PTGES3, PTGIS, ABCG4 and SLCO2A1 increased in all pregnant groups, whereas for PTGS2, an overall elevation of expression in pregnancy appeared to be the result of a specific increase in in-labour samples. HPGD decreased significantly in all pregnant groups, suggesting that pregnancy is associated with an increase in the functional availability of myometrial PGs.

Among the pregnant myometrial sample groups, the only significant difference was that PTGS2 was higher in in-labour samples compared with not-in-labour. Previous descriptions of PTGS2 expression in pregnant human myometrium contrast greatly, variously indicating an increase at term prior to the onset of labour (Slater et al., 1999), a decrease or no change with labour (Zuo et al., 1994; Moore et al., 1999; Giannoulias et al., 2002), or an increase associated with labour (Erkinheimo et al., 2000; Choi et al., 2007), which is the observation supported by our data. As PTGS2 and PTGES may act in concert (Murakami et al., 2000), the elevation of PTGS2 that we have observed in myometrium may explain an increase in myometrial PGE2 levels seen in preterm and term labour (Durn et al., 2010), which may lead to dilatation of the cervix and relaxation of the birth canal. Our data show that the spontaneous onset of labour is associated with increased PTGS2 gene expression and suggest that the mechanism of labour is similar at term and preterm. Moreover, when labour was induced, PTGS2 levels were also significantly increased and this would suggest that the increase in PTGS2 is a consequence of the process of labour, rather than part of the initiating mechanism. Previous studies measuring myometrial PTGS2 expression have not compared spontaneous labour with IOL samples (Slater et al., 1999; Sooranna et al., 2006; Astle et al., 2007; Tattersall et al., 2008). The controversy as to whether changes in PTGS2 expression are cause or consequence of labour remains unresolved as it is not possible

**Figure 6** Changes in protein expression in primary myometrial cell cultures in response to cytokine treatment. Human myometrial cells were cultured as described in the Materials and Methods section and treated with a combination of the cytokines IL1β and TNFα (IL1/TNF) or with LPS for 24 h. Protein levels were determined by western blotting, and normalized to the untreated controls. ADU, arbitrary densitometric units. For PTGS2 (A), AKR1B1 (B) and PTGES (C), a change in expression on cytokine treatment was seen, and graphs summarize the results from three separate experiments with cells derived from different women. Asterisks indicate a significant difference in protein levels compared with the untreated control, as determined by ANOVA: *p < 0.01, **p < 0.001. For PTGS2 (a), AKR1B1 (b), PTGES (c), PTGS1 (d), AKR1C3 (e) and CBR1 (f), a representative set of blots from a single experiment is shown. For abbreviations see Fig. 1 legend.
to obtain serial samples of tissue from the same women. Some studies have analysed paired myometrial samples from the uterine fundus and the lower uterine segment and most changes in gene expression associated with labour, including changes in PTGS2 expression, are more significant in the lower segment than in the upper parts of the pregnant uterus (Sparey et al., 1999; Havelock et al., 2005; Tattersall et al., 2008). It has been suggested that this pattern of PTGS2 expression parallels that of leukocyte migration into the uterus during labour (Thomson et al., 1999) and is part of changes in genes involved in acute phase responses that increase in myometrium due to factors unrelated to labour (e.g. trauma of the surgical procedure, underlying pathology, hypoxia; Havelock et al., 2005). The leukocytic inflammatory infiltration of the myometrium is more common in advanced labour than in early labour and seems to be a consequence of the labour process, rather than its cause (Keski-Nisula et al., 2000). Thus, any increase in PTGS2 in in-labour myometrium is likely to be due to a secondary inflammatory response, rather than a primary physiological change before labour (Havelock et al., 2005).

Moreover, an initial increase in PTGS2 expression in early spontaneous or induced labour may be reinforced by a secondary increase driven by inflammatory or hypoxic changes in mid and late labour.

Oxytocin receptor gene expression was measured in the myometrial samples and showed a contrasting pattern to PTGS2—although OXTR expression increased in pregnancy, it showed a relative decrease in in-labour compared with not-in-labour samples. Previous studies described an increase in OXTR expression through pregnancy to term, with no effect of labour (Wathes et al., 1999). Our data suggest that OXTR expression, and therefore sensitivity to the contractile influence of oxytocin, is maximal prior to the onset of labour. Parturition leads to down-regulation of OXTR expression in lower segment myometrium in advanced labour, probably as a mechanism to avoid tetanic contractions that would compromise the fetus (Phaneuf et al., 1998).

The differences in gene expression between pregnant and NP myometrium are the consequence of many weeks of exposure to steroids, growth factors and other hormones and are reflected by structural changes in myometrial tissue (hypertrophy, hyperplasia, increased vascularization), whereas changes around parturition occur over a relatively short period of a few hours. Our data show that a complex system of myometrial PG pathway genes exists in the tissue in late pregnancy ready to respond and participate in the mechanism of labour. Linear discriminant analysis was used to further analyse the expression results of those genes that showed differences between the myometrial sample groups in ANOVAs; it was used to define weighted functions that, when applied to expression values of multiple genes for each sample, clearly separated NP, not-in-labour, spontaneous labour and, to a lesser extent, induced-labour sample groups. There was no clear effect of gestational age, either in the not-in-labour or in the spontaneous labour groups. IOL by the administration of PGE and oxytocin resulted in a similar, but not identical, pattern of myometrial PG pathway gene expression to the patterns seen in spontaneous preterm and term labour. This analysis provided a method for assessing the overall differences between the myometrial samples; it showed that expression of some PG pathway genes allows discrimination between different groups and reinforces the view that the regulation of PG genes is functionally correlated.

Parturition has similarities with inflammatory responses, and it is known that pro-inflammatory cytokines stimulate PTGS2 and PTGES expression in human myometrial cells and that this is accompanied by increased release of PGE2, PGF2α and 6-keto PGF1α (Bartlett et al., 1999; Erkinheimo et al., 2000; Rauk and Chiao, 2000; Hertelendy et al., 2002; Sooranna et al., 2006; Astle et al., 2007). Our results showing stimulation of primary myometrial cell cultures with IL1β and TNFα support this, and we have made the novel observation that the PGF synthase AKR1B1 is also induced, and although the maximal response was not of the same magnitude as that obtained for PTGS2 and PTGES, significant stimulation was observed at low cytokine concentrations. It is possible that in the initial phases of inflammatory infiltration, or when the release of cytokines is modest, PGF2α production by AKR1B1 in myometrial cells is dominant, whereas with more severe inflammation and cytokine release, PGE2 production by PTGES may become superimposed. If this cytokine-dependent mechanism operates in the myometrium in vivo, it does not appear to be responsible for the increase in PTGS2 expression that we have seen in preterm and term labour, as there is no corresponding increase in PTGES or AKR1B1 expression. It should be noted that, although primary myometrial cell culture is a commonly used experimental model, cells in culture may be subject to loss of differentiation and alteration of phenotype, and so may not accurately reflect physiological responses seen in vivo. Moreover, our study did not include patients with infection-associated preterm labour or uterine inflammation, in whom this stimulation is more likely to occur.

This study has revealed the patterns of expression of PG pathway genes that reflect the physiological roles of the different uterine tissues. It has demonstrated for the first time differential expression of a number of PG pathway genes in human uterine tissues and represents an integrated approach where net PG output has been addressed by measuring genes involved at all steps from liberation of substrate, catalytic conversion, transport and catabolism. Each tissue has been shown to have a distinct potential for production of particular PGs. In the myometrium, several PG pathway genes have been shown to be affected by pregnancy, but only PTGS2 displayed regulation associated with labour. Induction of the PGF synthase AKR1B1 (but not AKR1C3 or CBR1) in myometrial cells has been shown in response to cytokine stimulation. The conserved patterns of expression that were observed in different PG pathway genes in human uterine tissues suggest common mechanisms of transcriptional regulation of these genes. The description of the dominant terminal PG synthases in these tissues increases the choice of utero-specific targets for pharmacological inhibition of uterine contractility in the management of preterm labour.

**Authors’ roles**

R.J.P.: experimentation, analysis and manuscript preparation; H.A.-Z.: contribution to experiments; L.P.H.: statistical advice and data analysis; M.A.F. and A.L.B.: design of study and preparation of manuscript.

**Acknowledgements**

We are grateful to research midwives Anne Duffner and Alison Kirby for obtaining consent from women at St Michael’s Hospital and organizing the collection of samples.
Funding
This work was supported by Wellbeing of Women (RG825).

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