

Bile acids and ursodeoxycholic acid alter gene expression in primary myometrial cells

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Abstract

Intrahepatic cholestasis of pregnancy (ICP), characterised by elevated maternal serum bile acids (BAs), increases risk of preterm labour that may be mitigated by ursodeoxycholic acid (UDCA) treatment. How different BA species, including UDCA, affect myometrial function remains imperfectly understood. Primary human myometrial cells were isolated from women with uncomplicated pregnancy and treated with taurocholic acid (TCA, 150 µmol/l), tauro-UDCA (TUDCA, 150 µmol/l) or both TCA and TUDCA (75 µmol/l each); some were stimulated oxytocin (10 nmol/l, 6 h). Gene expression was determined using real-time quantitative PCR, and IL-6 protein in supernatant using ELISA. TCA increased *OXTR* mRNA expression after 4, 10, 18 and 24 h. Following overnight BA pre-treatment, 6-hour combined treatment with TCA and TUDCA led to highest *OXTR* ($p < 0.01$ vs. control) and increased oxytocin-induced *COX-2* ($p < 0.05$) and *IL-6* mRNA ($p = 0.06$) expression. IL-6 protein was increased by TCA but not TUDCA. This indicates elevated BAs in maternal serum may alter expression of oxytocin-responsive genes by upregulating *OXTR* expression. Combined exposure to high TCA and TUDCA increased expression of *OXTR*, *COX-2* and *IL-6*, but not IL-6 protein, while TUDCA alone had no effect. Altered *OXTR* expression and downstream gene expression changes may contribute to ICP-associated myometrial contractility and preterm birth risk, meriting further study.

Keywords Preterm labour · Bile acids · Ursodeoxycholic acid · Oxytocin · Myometrium

Introduction

Intrahepatic cholestasis of pregnancy (ICP) is the most common gestational liver disease. As shown in international cohorts, risk of spontaneous preterm labour (PTL) increases in women with severe ICP, defined by maternal total serum bile acid (BA) concentrations of ≥ 40 µmol/l^{1–3}. The mechanism for ICP-induced PTL is unknown, although there is evidence that BAs can affect myometrial contractile activity. This risk of PTL markedly increases in ICP-affected women with singleton pregnancies who have total serum BA concentrations of ≥ 100 µmol/l³. Chronic infusion of the BA cholic acid (CA) in sheep resulted in 100% of lambs being spontaneously delivered prematurely⁴, implying a causal role for BAs in this process. BAs may act by increasing myometrial

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sensitivity to oxytocin. Myometrial contractile responses to oxytocin are greater in ICP patients compared to control women both in vitro and in vivo, and enhanced contractility was also observed in human myometrial strips pre-treated with CA^{5,6}. This may occur at least partly through increased protein and mRNA expression of the oxytocin receptor (*OXTR*) which was shown in CA-treated human primary myometrial cells⁶. Studies in human placental tissue also suggest BA exposure may induce pathological placental oxidative stress and inflammation, possibly contributing to the induction of PTL^{7,8}. As preterm birth (PTB) is the leading cause of infant mortality and increases the risk of developing a disability in later life^{9,10}, it is vital that its aetiology is better understood.

ICP is commonly treated with ursodeoxycholic acid (UDCA). Although evidence from the largest randomised controlled trial to date did not show a statistical difference in a composite result of fetal adverse outcomes that included the incidence of spontaneous PTB¹¹, a subsequent individual participant data meta-analysis of randomised control trials found that UDCA is associated with a reduction PTB in singleton pregnancies with serum BA concentrations $\geq 40 \mu\text{mol/l}$ ¹². UDCA was also able to ameliorate some of the aforementioned cellular stress processes linked to PTL in human placentas^{7,8} and alters the maternal BA pool favouring less toxic BAs¹². However, the exact mechanisms behind its protective effect on PTL in ICP remain unknown.

The aim of this study was therefore to investigate how elevated BA concentrations and UDCA treatment affect the gene expression profile of primary human myometrial cells.

Results

Characterisation of the model

Primary myometrial cells were isolated from uncomplicated pregnancies at term, as previously reported¹³, and used at passages 3–6. The cells showed high mRNA expression for the smooth muscle cell marker *α SMA* and the fibroblast marker *VIM* encoding vimentin, indicating these cells are likely myofibroblastic in nature. The mRNA expression of *OXTR* and *DES* (encoding the smooth muscle marker desmin) showed the second highest expression alongside inflammatory markers *IL-6*, *CXCL8* and *MCP-1*. The cells also expressed receptors and catalytic enzymes for reproductive hormone signalling as expected (Supplementary Table 1). The model therefore was considered suitable to investigate transcriptional and inflammatory responses. Cells from most donors were oxytocin-responsive, showing a significant induction of *COX-2* mRNA after 6 h, but not 24 h of culture (Fig. 1A), consistent with previous work in this model¹³ and published literature regarding desensitisation of myometrial cells to oxytocin following prolonged treatment¹⁴. The response was variable, with some donors showing

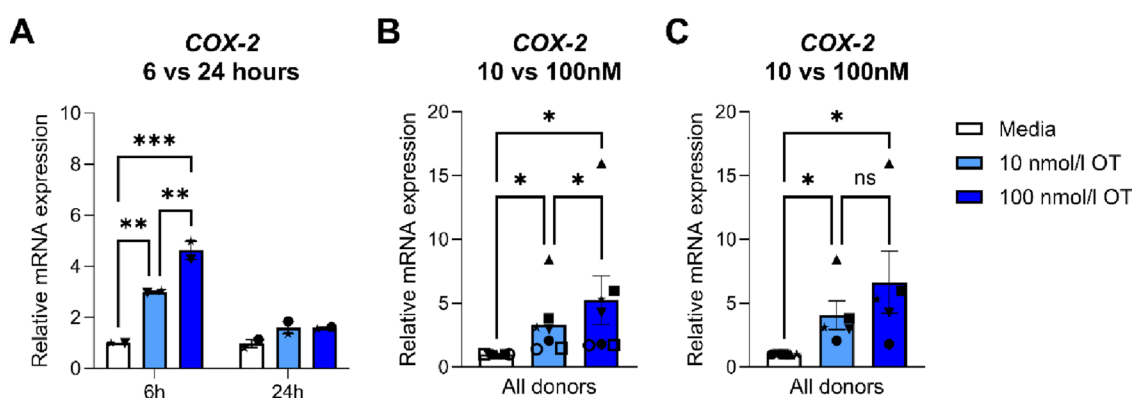


Fig. 1 COX-2 induction following oxytocin treatment at 10 or 100 nmol/l, (A) in response to 6 versus 24 h treatment ($n=2$ donors), or in response to 6 h treatment in (B) all donors including non-responders (open symbols, $n=8$ donors) and (C) in responders only (closed symbols, $n=5$ donors). $*p<0.05$, $**p<0.01$, $***p<0.001$ using repeated measures two-way ANOVA (A) or lognormal repeated measures one-way ANOVA (B-C). Each symbol reflects one donor.

less than 1.5-fold responses in *COX-2* expression after oxytocin stimulation, compared to over 3-fold in others (Fig. 1B). Because of the known reduction in *OXTR* mRNA during labour¹⁴, it is possible that some women had already begun to exhibit early labour-associated changes at the time of sampling. Myometrial cells from women in labour show vastly different expression of labour-associated proteins such as cytokines¹⁵. Therefore, only cells from donors that had retained their oxytocin responsiveness (showing > 2-fold induction of *COX-2* following oxytocin treatment) were considered sufficiently far removed from labour to be used in follow-up experiments. In this group, increasing the oxytocin concentration to 100 nmol/l had no additional effect on *COX-2* expression (Fig. 1C), hence for future experiments 10 nmol/l oxytocin treatment was used.

Expression of the oxytocin receptor is increased by taurocholic acid treatment

Cells were exposed to 150 $\mu\text{mol/l}$ taurocholic acid (TCA), the most commonly elevated BA species in ICP pregnancy¹⁶, for 4 to 24 h. Expression of *OXTR* progressively increased with time until 10 h in both groups, after which it decreased back to baseline levels. TCA treatment significantly increased *OXTR* mRNA expression at 4, 10, 18 and 24 h, while expression at 6 and 8 h was not significantly different to controls (Fig. 2A). When expressed as a fold-change compared to the media control taken at each timepoint, the same was seen (Fig. 2B). The highest relative increase compared to control cells occurred at 18 h (1.5-fold, Fig. 2B).

Combined TCA and TUDCA treatment increases expression of *OXTR* and oxytocin-responsive genes

Because 18 h incubation showed the strongest and most consistent increase in *OXTR* with TCA, and to mimic prolonged exposure to BAs as seen during ICP-complicated pregnancy, we looked at the expression of *OXTR* and oxytocin-responsive genes following overnight BA treatment. We investigated the effects of high levels of TCA and tauroursodeoxycholic acid (TUDCA), the tauro-conjugated form of UDCA. We also included a combined TCA + TUDCA group that reflects treated ICP in which over half of the serum BA pool is replaced by UDCA¹⁷. Total BA concentrations were kept constant at 150 $\mu\text{mol/l}$ as total BA levels do not tend to change with UDCA treatment. Following overnight treatment, half of the cells were exposed to a 6-hour oxytocin $\pm$ BA stimulus while the other half was treated with BAs only. There was no transcriptional evidence of bile acid toxicity at the concentrations used (Supplementary Fig. 1).

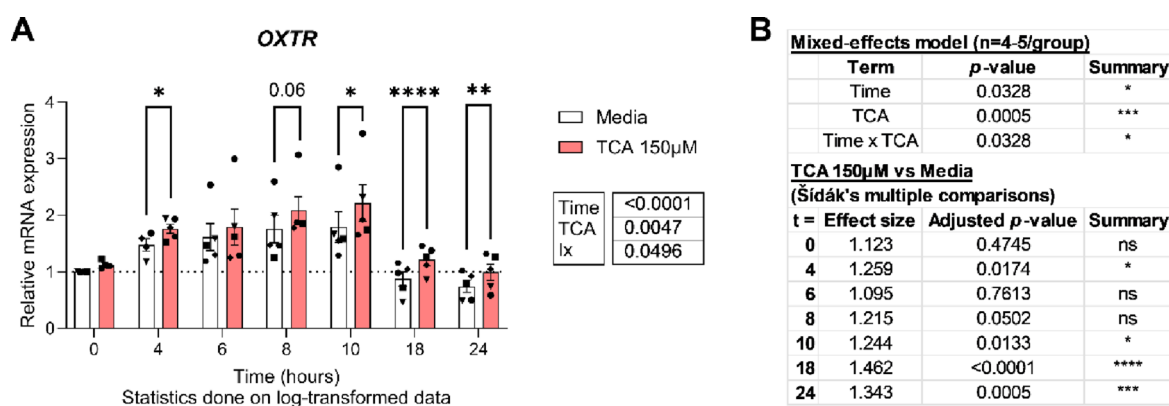


Fig. 2 Expression of oxytocin receptor (*OXTR*) mRNA following treatment with 150 $\mu\text{mol/l}$ taurocholic acid ($n=5$ donors, with $n=4-5$ for each condition due to random missing data, TCA). (A) Expression compared to media control at $t=0$ h or (B) compared to the media control at each timepoint. (A) * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$ using repeated measures mixed effects modelling testing for effects of time, TCA and the interaction between them with Sidak's multiple comparison. (B) * $p<0.05$, *** $p<0.001$, **** $p<0.0001$ using mixed effects modelling testing for effects of time, TCA and the interaction between them with Sidak's multiple comparison. Each symbol reflects a separate donor.

There was a significant main effect of BA exposure on *OXTR* expression, driven by an increase in the TCA+TUDCA group when compared to the no bile acid control, TUDCA alone (significant) and TCA (trend) groups (Fig. 3A). The TUDCA alone group showed a decrease in *OXTR* expression compared to the TCA alone group (Fig. 3A). There was no effect of oxytocin to regulate its own expression. There was a weak ($p < 0.2$) interaction between oxytocin and BA exposure, and post hoc analysis showed that the TCA+TUDCA group may specifically increase *OXTR* expression without an oxytocin stimulus (Fig. 3A).

Next, we investigated whether the changes in *OXTR* expression were accompanied by changes in expression of oxytocin-responsive genes in primary myometrium cells. As expected, oxytocin treatment significantly increased mRNA expression of *COX-2*, the cytokine *IL6* that acts as a pro-inflammatory signal in labour, and the chemokine *MCP1* (Fig. 3B-D). Prolonged exposure to both TCA+TUDCA significantly increased *COX2* mRNA expression upon oxytocin stimulation and resulted in a trend for increased *IL6* expression in primary myometrial cells (regardless of oxytocin), when compared to no BA control (Fig. 3C-D). In contrast, TUDCA alone may also increase the expression of *COX2* compared to controls in absence of oxytocin treatment, despite having lower *OXTR* expression than in the TCA+TUDCA and TCA groups (Fig. 3B). There was no BA effect on *MCP1* expression (Fig. 3D).

Bile acids alter oxytocin-induced IL-6 production

Because BAs tended to affect *IL6* mRNA, IL-6 protein levels were measured in the supernatant from oxytocin-exposed cells and the unstimulated media control. Oxytocin significantly promoted IL-6 release from the primary myometrial cells after 6 h of culture (Fig. 4A, approximately 1.99 ± 0.23 -fold, by 108 ± 29 pg/ml). BA exposure

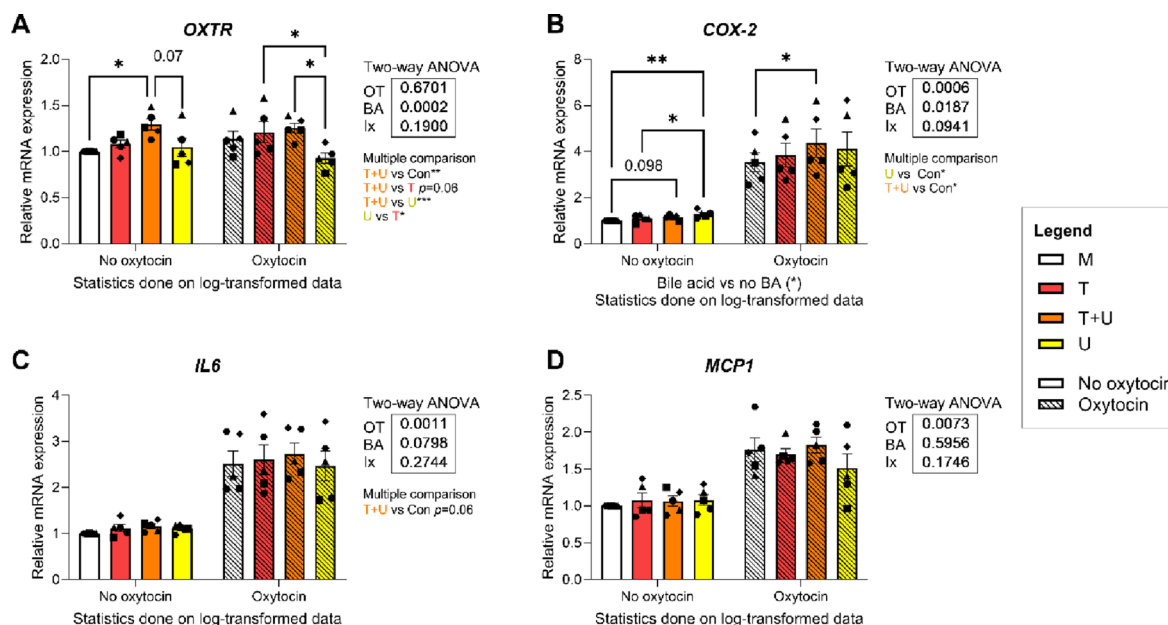
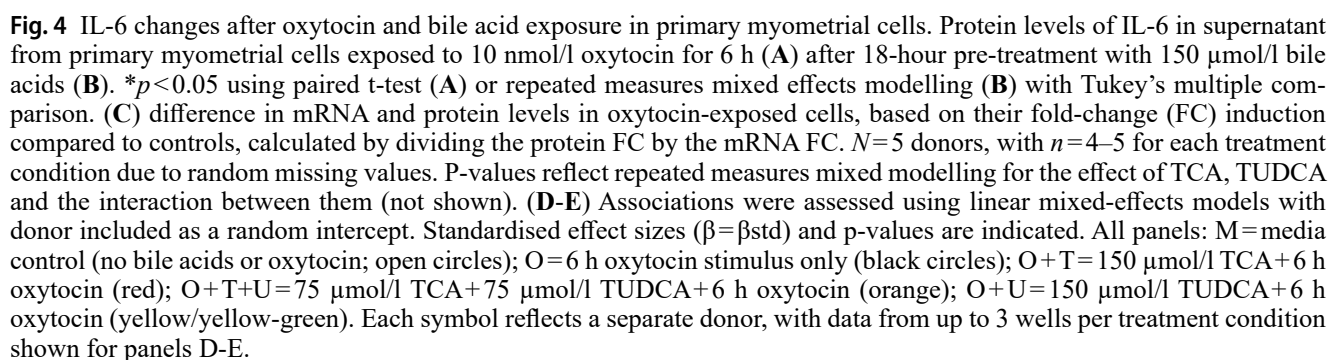


Fig. 3 Expression of genes related to oxytocin signalling following bile acid prolonged bile acid exposure ($n = 5$ donors, with $n = 4-5$ for each experimental condition due to random missing data). Expression of levels of (A) oxytocin receptor (*OXTR*), (B) cyclo-oxygenase 2 (*COX-2* encoded for by the *PTGS2* gene), (C) interleukin 6 (*IL6*) and (D) monocyte chemoattractant protein 1 (*MCP1*) mRNA following overnight treatment with 150 μ mol/l bile acids (BAs) [taurocholic acid (TCA) and/or tauroursodeoxycholic acid (TUDCA)] with (dashed bars) or without (open bars) 6-hour oxytocin stimulus (OT, 10 nmol/l). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ using repeated measures two-way ANOVA testing for effects of OT, BAs and the interaction between them with Tukey's multiple comparison. Multiple comparisons reflect BA group differences independent of oxytocin treatment (main group effect; where 'vs Con' = compared to media control and oxytocin groups combined). M = media control (no bile acids or oxytocin); T = 150 μ mol/l TCA; T + U = 75 μ mol/l TCA + 75 μ mol/l TUDCA; U = 150 μ mol/l TUDCA.



also significantly affected release. TCA treatment increased oxytocin-stimulated IL-6 release when compared to oxytocin alone by 27 ± 5 pg/ml (Fig. 4B). IL-6 protein levels in the combined TCA+TUDCA group were not significantly different from either the TCA or oxytocin alone groups. TUDCA decreased IL-6 release compared to TCA alone (-52 ± 7 pg/ml) and compared to combined TUDCA and TCA treatment (-32 ± 5 pg/ml). Since this was a different pattern than what was observed for *IL6* mRNA expression, we investigated the difference in magnitude between IL-6 mRNA and protein induction, which tended to be affected by what BA was present. Although not statistically significant, TCA tended to increase the protein/mRNA induction ratio, whereas TUDCA tended to decrease it (Fig. 4C).

IL6 mRNA was the strongest predictor for IL-6 protein release independent of donor (Fig. 4D, Supplementary Table 2). In addition, *COX-2* mRNA predicted levels of IL-6 protein and *IL6* mRNA; the relationship was attenuated (*IL6* mRNA) or lost (IL-6 protein) when only samples with oxytocin were included (Fig. 4E & Supplementary Table 2), suggesting mechanisms other than OXTR may regulate oxytocin-induced IL-6 protein release. Accordingly, although *OXTR* mRNA had some ability to predict *IL6* mRNA, there was no meaningful correlation between *OXTR* mRNA and IL-6 protein (Supplementary Table 2).

Bile acid receptor expression in myometrial cells

BAs can signal through the nuclear farnesoid X receptor (FXR) and membrane-bound Takeda G protein-coupled receptor 5 (TGR5)¹⁸. Expression of *NR1H4*, encoding FXR, was not reliably detected in any of the donors (Supplementary Table 1). The gene encoding TGR5, *GPBAR1*, was detectable at extremely low levels (0.1% of the expression level for housekeeping genes). BAs have been shown to activate muscarinic receptors in cardiomyocytes¹⁹. The muscarinic M2 receptor was present at low but consistently detectable levels, while the muscarinic M3 receptor was not reliably expressed. The sulphonylurea receptor, activation of which can occur downstream of TGR5 signalling¹⁸, was not detected in any of the cells (Supplementary Table 1).

Discussion

This study investigated the effect of BAs on primary myometrial cells. Consistent with previous observations using cholic acid⁶, we found that TCA, the species most elevated in ICP, increased mRNA expression of oxytocin receptor in a time-dependent manner, with the strongest increase of 1.5-fold occurring with prolonged exposure (18 h). The highest induction of *OXTR* mRNA was seen with overnight combined TCA and TUDCA treatment, and this group also showed the highest expression of *COX-2* and *IL6* mRNA following a 6-hour oxytocin challenge. In contrast, oxytocin-induced IL-6 protein release was increased by TCA but not by combined TCA and TUDCA (pre-)treatment. TUDCA treatment on its own did not affect *OXTR* expression, nor IL-6 mRNA/release, when compared to oxytocin controls. TUDCA also decreased *OXTR* mRNA and IL-6 protein when compared to the other BA groups.

The model of primary myometrial isolation and culture has been described previously, and is considered the *in vitro* model most reflective of a transcriptional signature in human myometrial tissue²⁰. The cells expressed key hormone receptors and expected markers of myocytic and fibroblastic cells, consistent with a myofibroblastic phenotype in culture. Together with their retained transcriptional response to oxytocin stimulation, this makes them a good model to study the effect of BAs and UDCA on labour-associated gene regulation and inflammatory signalling in human myometrium. In accordance with the literature, stimulation with 10 nmol/l oxytocin for 6 h increased expression of *COX-2*, *IL6* and *MCP-1* mRNA as well as IL-6 protein release¹³.

TCA consistently increased the expression of *OXTR* compared to the media control. Our findings are consistent with earlier work undertaken in primary myometrial cells and myometrial strips, the latter of which also showed more forceful oxytocin-induced contraction after incubation with cholic acid⁶. As the same was seen in myometrial strips from women with ICP⁶, ours and published work combined suggest enhanced myometrial oxytocin

sensitivity may be one mechanism through which ICP confers an increased risk of PTL. The greatest impact on *OXTR* mRNA, a 1.5-fold induction, was seen after 18 h of treatment. The importance of prolonged BA exposure in these experiments is of note because serum BAs are elevated for several weeks before labour in women with ICP²¹. Although small, such an increase may have great clinical impact, as *OXTR* protein expression in human myometrial/decidual homogenates was shown to only increase about 2.5-fold before and after the onset of term labour²², and mRNA levels in the myometrium may remain unchanged²³. As changes in *OXTR* protein levels, binding and activity are also involved in oxytocin signalling, further work is required to determine the impact of BAs on these aspects in the myometrium.

There was no autoregulation of *OXTR* mRNA in response to oxytocin, which is to be expected with short-term treatment. *OXTR* mRNA expression in absence of stimulation increased slightly (less than 2-fold) over time up to 10 h, after which expression dropped to below baseline. Oxytocin receptor desensitisation, including mRNA expression, was previously shown to occur after 12 h of oxytocin stimulation¹⁴. Based on the similarity in timing, our treatment media may have activated pathways similar to oxytocin signalling or other labour inducing factors. Although we serum-starved cells prior to treatment, others have shown that concentrations as little as 0.1% fetal bovine serum (FBS) elicited a small increase *OXTR* mRNA expression in primary myometrial cells after 8 h of culture, with an approximately 4- and 5-fold increase in *OXTR* mRNA and oxytocin binding shown at 1% FBS²⁴, respectively. Therefore, it is possible that serum components in the treatment media were responsible for the time-dependent changes in *OXTR* expression observed.

Despite *OXTR*, *COX-2* and *IL6* mRNA levels being highest in the combined TCA + TUDCA group, oxytocin-induced IL-6 protein was increased in TCA-treated cells only. These findings argue against a simple linear relationship between *OXTR* transcription and downstream inflammatory protein release. An increase in *OXTR* mRNA expression may not reflect an increase in oxytocin receptor protein levels, activity or receptor sensitivity, even though *COX-2* mRNA expression was increased in the TCA + TUDCA group. Our correlation findings suggest *COX2* and *OXTR* mRNA expression positively correlated with *IL6* mRNA, including under oxytocin stimulation, but the relationships with IL-6 protein release are lost when only oxytocin-stimulated samples are studied. Combined, this suggests that changes in IL-6 release could be in part related to increased oxytocin signalling by BAs, but the magnitude of the response likely depends on additional mechanisms independent of *OXTR* expression and/or *IL6* mRNA translation. Further work is required to determine the relative effects of the different bile acid species. Interestingly, IL-6 itself can induce *OXTR* mRNA expression in human myometrial cells²⁵, suggesting our correlations could be in fact bi-directional. When comparing mRNA and protein levels for IL-6, the greatest discordance between protein and mRNA was seen with TUDCA treatment alone, while TCA treatment showed IL-6 protein release more similar to the induction of its mRNA. These findings highlight the possibility that bile acid species may differentially influence the relationship between IL-6 transcription and protein accumulation. This could suggest a possible effect of different BAs to interfere with mRNA translation or protein stability (the latter of which has been shown in mouse intestinal tissues²⁶, with potential opposing effects of TCA and TUDCA. For example, BAs could potentially interact with microRNAs implicated in the onset of spontaneous and oxytocin-induced labour, some of which can target interleukins and other inflammatory mediators²⁷. Our findings suggest that regulation of inflammatory signalling by bile acids is not fully explained by *OXTR* mRNA expression alone, suggesting additional mechanisms downstream of/independent from this receptor may contribute to the final inflammatory response. Similarly, the mechanisms promoting IL-6 protein synthesis and release from previously synthesised stores may not be the same, and may be regulated by different receptors that can be differentially activated by the various bile acid species. Lastly, UDCA treatment is known to reduce toxicity of the bile acid pool in women with ICP by decreasing the relative proportion of hydrophobic bile acids like TCA¹⁶. Although we did not find differences in expression of cellular stress and apoptosis markers in vitro, reduced bile acid toxicity may be involved in prevention of PTL in vivo.

The molecular pathways by which BAs increase *OXTR* mRNA and affect IL-6 levels are unknown. Signalling through the BA receptors FXR and TGR5 is unlikely as their expression was undetectable or very low. Alternatively, BAs may affect stability of the mRNA as some have proposed that this is how *OXTR* expression is

regulated in the human myometrium²⁴. Instead of the classic BA receptors, the muscarinic M2 receptor may be involved as this receptor was shown to mediate the negative chronotropic effect of conjugated BAs on neonatal rat cardiomyocytes, in which TCA and TUDCA have full and partial agonist activity for M2^{19,28}. M2 receptors have been shown to inhibit the relaxatory effect of cyclic AMP in rodent uterine strips²⁹, suggesting relative activation or inhibition of this receptor may enhance or dampen (oxytocin-)stimulated contractions in the human myometrium as well. Another proposed mechanism is activation of K-ATP channels by UDCA, which has been linked to hyperpolarisation in cardiac cells³⁰ and may thus protect against the depolarising effect of oxytocin and prostaglandins on the myometrium, a process strongly implicated in human labour³¹. However, these mechanisms remain speculative. Bile acid affinity for different receptors may be tissue-specific¹⁹, therefore the comparative effects of TCA and TUDCA on M2 receptors, K-ATP channel activation and membrane polarisation would need to be confirmed in human primary myometrial cells.

This work primarily set out to explore the effect of different bile acid species on oxytocin receptor expression in human myometrium cells. Consequently, the study focused on gene expression and cytokine release endpoints, and did not assess OXTR protein abundance or functional outputs such as receptor signalling, calcium handling or myometrial contractility. In addition, only a single overall bile acid concentration (150 $\mu\text{mol/l}$, chosen to model severe ICP) was studied. While the combined TCA+TUDCA condition was designed to reflect the altered BA composition observed in UDCA-treated ICP (in whom UDCA-derived bile acids comprise a substantial proportion of the circulating bile acid pool while total bile acid concentrations remain elevated), the effects of TUDCA addition cannot be separated from those resulting from a reduction in TCA concentration and therefore should not be interpreted as evidence of synergistic signalling. Finally, although the use of independent donor-derived primary cells is a strength of the study, the number of donors was limited by the practical constraints associated with obtaining oxytocin-responsive human pregnancy tissues. Replication in larger cohorts is required to establish the broader generalisability of these findings.

Consistent with previous studies, TCA treatment increased mRNA expression of the oxytocin receptor in primary human myometrial cells, supporting the hypothesis that bile acids may contribute to the increased risk of spontaneous PTL in ICP by influencing oxytocin-responsive pathways. Combined TCA and TUDCA treatment (mimicking UDCA-treated ICP) further increased *OXTR* expression, but the molecular mechanism and functional consequences of this interaction require further exploration. TUDCA treatment alone did not increase *OXTR* expression or IL-6 production in myometrial cells, suggesting that further studies examining its potential effects on labour-associated pathways and PTL prevention is warranted, including in women without ICP.

Methods

Cell culture

Women with uncomplicated singleton pregnancies were recruited prior to undergoing an elective caesarean section procedure at 38–40 weeks' gestation due to breech presentation or previous caesarean section at Queen Charlotte's and Chelsea Hospital and Chelsea and Westminster Hospital. The study was approved by the London-Harrow Research Ethics Committee (19/LO/1657) and performed in accordance with the Helsinki Declaration. All participants provided written informed consent. A small biopsy (1 cm) of the lower segment of the uterus was taken with informed consent and primary cells were isolated as previously described¹³. Cells were cultured in high glucose full Dulbecco's Modified Eagle Medium (DMEM, Sigma, D5671) containing 10% FBS (Sigma, F7524), 1% L-Glutamine (vol/vol, Sigma, G7513) and 1% penicillin and streptomycin (vol/vol, Sigma, P0781) at 37 °C and 5% carbon dioxide until passage 2–5. For passaging, flasks were washed in phosphate buffered saline and trypsinised in 0.05% Trypsin-EDTA (Sigma, T4049) for 5 min before inhibition by full DMEM.

Drug treatments

Cells were used for experiments at passages 3–6. One passage per donor (i.e. from each myometrial biopsy) was used for each individual experiment; but different passages may be used for different experiments, providing they did not exceed 6. Three wells seeded from the same passage were used for each treatment condition, with each donor counting as a biological replicate ($n=5$ for each experiment, except for Fig. 1A).

When cells reached 70–80% confluency, they were seeded in 6-well tissue culture plates at 1/24th of a flask (approx. 300,000 cells) per well and left to adhere for at least 18 h. As serum is known to contain components that can interfere with oxytocin signalling²⁴, and phenol-red may interfere with colorimetric assays for protein measurement, cells were serum starved with white media (D5921, Sigma) supplemented with 1% FCS, 1% L-Glutamine and 1% penicillin and streptomycin for either 6 (overnight experiments) or 24 h before treatment. This media was also used during cell treatment (1.5 mL/well). Cells were treated with the bile acids TCA (Sigma, T4009) and/or TUDCA (Sigma, T0266) at a total bile acid concentration of 150 $\mu\text{mol/l}$ (150 $\mu\text{mol/l}$ per bile acid if mono-treatment, 75 $\mu\text{mol/l}$ each for combination treatments) between 4 and 24 h. This concentration was selected to model severe ICP (total serum bile acids ≥ 100 $\mu\text{mol/l}$), the patient group at highest risk of spontaneous preterm birth. Cells were treated with oxytocin (Sigma, O6379) for 6 h at 10 nmol/l. At the end of the experiment, supernatant was collected, spun for 10 min at 1,000 g at 4 °C, and the top liquid was stored at – 20 °C prior to protein analysis. Cells were lysed within the plate using 350 μL RLT Plus Lysis buffer (QIAGEN, part of RNeasy Plus Micro Kit 74034), collected using a cell scraper and the lysate was snap frozen on dry ice before storage at – 70 °C until processing.

Gene expression

RNA was extracted from frozen lysates using the RNeasy Plus Micro Kit (QIAGEN, 74034) according to the manufacturer's instructions. RNA quantity and quality was assessed using a NanoDrop spectrometer and 1000ng RNA per sample was used to generate cDNA using the High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (ABI Applied Biosystems, 4374966), where possible. Gene expression was determined by quantitative real-time polymerase chain reaction (qRT-PCR) using the PowerUp SYBR Green Master Mix (ABI Applied Biosystems, A25777) on a StepOnePlus machine (ABI Applied Biosystems). Data were analysed using the comparative CT method and normalised against the geometric mean of the housekeepers *CYC1*, *GAPDH* and *YWHAZ* that are shown to perform well in myometrial cells³². To determine the *BAX/BCL2* ratio, ΔCT was calculated as $\Delta\text{CT}_{\text{BAX}} - \Delta\text{CT}_{\text{BCL2}}$. Data are shown as fold-changes compared to the media only control group for all genes. To determine expression levels of genes of interest, average CT values were calculated from the experiments or CT values were measured in a pooled sample at the same dilution (1:40). Primer sequences are shown in Table 1. qPCR assays were run in technical triplicates for each sample.

Protein measurement

Concentrations of IL-6 in supernatant were determined by enzyme-linked immunosorbent assay (ELISA) according to manufacturer's instructions (R&D systems, D6050B). ELISA assays were run in technical duplicates for each sample.

Statistical analysis

All gene expression data followed a log-normal distribution as assessed using GraphPad Prism (version). Data were analysed using paired t-tests, repeated measures one- or two-way ANOVA with Tukey's multiple comparison where applicable, or mixed effects models with Sidak's multiple comparison in case of random missing data-points. Associations between variables were assessed using linear mixed-effects models (lmer, lme4 package, R),

Table 1 Primer sequences for qRT-PCR.

Gene name (qPCR)	Encodes	Forward 5'- to 3'	Reverse 5'- to 3'
<i>ABCC8</i>	SUR1	CTATGGGATGCTGCTCCTCG	GAGCCGTTGGTAGTTGGTGA
<i>ACTA2</i>	α -SMA	GAGTTACGAGTTGCCTGATGG	GAATCCATCCCAGTGAAGGA
<i>BAX</i>	BAX	CCCGAGAGGTCTTTTCCGAG	CCAGCCCATGATGGTTCTGAT
<i>BCL2</i>	BCL2	TCCGCATCAGGAAGGCTAGA	AGGACCAGGCCTCCAAGCT
<i>CAT</i>	Catalase	GCTCTTCTGGACAAGTACAATG CTG	TTACACGGATGAACGCTAAGCT TC
<i>CCL2</i>	MCP-1	AAGCTCGCACTCTCGCCTCC	GGGCATTGATTGCATCTGGCTGAG
<i>CHRM2</i>	M2	GATGGCCTGGAGCACAACA	GCTGCTTAGTCATCTTCACAATC
<i>CHRM3</i>	M3	GCACTTGTGTTCTGATTAGTGGC	CAAGAGACCAGCTCCACCTGT
<i>CXCL8</i>	IL-8	GCCTTCCTGATTTCTGCAGC	CGCAGTGTGGTCCACTCTCA
<i>CYC1</i>	CYC1	GAGGTGGAGGTTCAAGACGG	TAGCTCGCACGATGTAGCTA
<i>DES</i>	Desmin	CATCGCGCTAAGAACATTT	GCCTCATCAGGGAATCGTTA
<i>GAPDH</i>	GAPDH	CGACCACTTGTCAAGCTCA	AGGGAGATTACAGTGTGGTG
<i>GJA1</i>	CX43	GGAGTTCAATCACTTGGCGT	ACACCTTCCCTCCAGCAGTT
<i>GPBAR1</i>	TGR5	CACTGTTGTCCCTCCTCTCC	ACACTGCTTTGGCTGCTTG
<i>IL6</i>	IL6	ATGCTTCCAATCTGGATTCAATG	CTCAAACCTCCAAAAGACCAGTG ATG
<i>NR1H4</i>	FXR	AGGATTTCAAGACTTTGGACCAT GA	TGCCCAGACGGAAGTTTCTTATT
<i>OXTR</i>	OXTR	TGTCGCTCCACCTACCT	CTCGCGCAGAGAGAAGATGTG
<i>PR-b</i>	PR	AACTCAGCGAGGGACTGAGA	GAGGACTGGAGACGCAGAGT
<i>PR-total</i>	PR	AGCCCACAATACAGCTTCGAG	TTTCGACCTCCAAGGACCAT
<i>PTGER1</i>	EP1	TCGCTTCGGCCTCCACCTTCTTG	CGTTGGGCTCTGGTTGTGCTTAG
<i>PTGER2</i>	EP2	CCACCTCATTCTCCTGGCTA	AGGTCCCATTTTTCCTTTTCG
<i>PTGER3</i>	EP3	ATCATGTGCGTGCTGTCTG	TGCAGTGCTCAACTGATGTCT
<i>PTGER4</i>	EP4	CTCCCTGGTGGTGCTCAT	GGCTGATATAACTGGTTGACGA
<i>PTGES</i>	PTGES	GGCTATACCTGGGGACTTGATG	CAGGAATCCAAGGGGCTAAGA
<i>PTGFR</i>	PTGFR	CCTTGCCATCGCCATTCTCAT	CCAGAAAACACCATGCAGATACC
<i>PTGS2</i>	COX2	TGCCCAGCACTTCACCCATCA	AGTCCACTCCATGGCCCAGTCC
<i>TP53</i>	p53	CCCCTCCATCCTTTCTTCTC	ATGAGCCAGATCAGGGACTG
<i>VIM</i>	Vimentin	GGCAGTCTTGACCTTGAAC	GTTCTGAATCTGAGCCTGC
<i>YWHAZ</i>	YWHAZ	ACGACGTCCCTCAAACCTTG	AAGATGACCTACGGGCTCCT

with donor included as a random intercept to account for clustering of repeated measurements and technical replicates within donors. Standardised regression coefficients (β_{std} , may be denoted as β) and associated p-values are reported. β_{std} denotes the standardised regression coefficient, allowing direct comparison of association strengths across variables measured on different scales.

All experiments (with exception of Fig. 1A) were performed at $n=5$. N-numbers lower than 5 indicate a random missing datapoint due to loss of sample or exclusion after quality control.

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Data availability Data will be made available upon reasonable request.

Declarations

Competing interests The authors declare no competing interests.

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