



Regulation of 20 α -Hydroxysteroid Dehydrogenase Expression in Term Pregnant Human Myometrium Ex Vivo

Marina Paul^{1,2,3} · Anna Paredes Barreda^{2,4,5} · Amy Gregson^{2,4,5} · Richard Kahl^{2,4,5} · Madeline King^{2,4,5} · Waleed M. Hussein⁶ · Frederick R. Walker^{1,2,3} · Roger Smith^{2,4,5,7} · Tamas Zakar^{2,4,5,7} · Jonathan W. Paul^{2,4,5}

Received: 19 February 2023 / Accepted: 16 August 2023 / Published online: 30 August 2023
© The Author(s) 2023

Abstract

Metabolic inactivation of progesterone within uterine myocytes by 20 α -hydroxysteroid dehydrogenase (20 α -HSD) has been postulated as a mechanism contributing to functional progesterone withdrawal at term. In humans, 20 α -HSD is encoded by the gene *AKR1C1*. Myometrial *AKR1C1* mRNA abundance has been reported to increase significantly during labor at term. In spontaneous preterm labor, however, we previously found no increase in *AKR1C1* mRNA level in the myometrium except for preterm labor associated with clinical chorioamnionitis. This suggests that increased 20 α -HSD activity is a mechanism through which inflammation drives progesterone withdrawal in preterm labor. In this study, we have determined the effects of various treatments of therapeutic relevance on *AKR1C1* expression in pregnant human myometrium in an ex vivo culture system. *AKR1C1* expression increased spontaneously during 48 h culture ($p < 0.0001$), consistent with the myometrium transitioning to a labor-like phenotype ex vivo, as reported previously. Serum supplementation, prostaglandin F_{2 α} , phorbol myristate acetate, and mechanical stretch had no effect on the culture-induced increase, whereas progesterone ($p = 0.0058$) and cAMP ($p = 0.0202$) further upregulated *AKR1C1* expression. In contrast, culture-induced upregulation of *AKR1C1* expression was dose-dependently repressed by three histone/protein deacetylase inhibitors: trichostatin A at 5 ($p = 0.0172$) and 25 μ M ($p = 0.0115$); suberoylanilide hydroxamic acid at 0.5 ($p = 0.0070$), 1 ($p = 0.0045$), 2.5 ($p = 0.0181$), 5 ($p = 0.0066$) and 25 μ M ($p = 0.0014$); and suberoyl bis-hydroxamic acid at 5 ($p = 0.0480$) and 25 μ M ($p = 0.0238$). We propose the inhibition of histone/protein deacetylation helps to maintain the anti-inflammatory, pro-quiescence signaling of progesterone in pregnant human myometrium by blocking its metabolic inactivation. Histone deacetylase inhibitors may represent a class of agents that preserve or restore the progesterone sensitivity of the pregnant uterus.

Keywords Myometrium · Progesterone · *AKR1C1* · 20 α -HSD · Histone deacetylase inhibitors · Trichostatin A

✉ Jonathan W. Paul
jonathan.paul@newcastle.edu.au

Marina Paul
marina.paul@newcastle.edu.au

Anna Paredes Barreda
anna.paredesbarreda@uon.edu.au

Amy Gregson
amy.gregson@newcastle.edu.au

Richard Kahl
richard.kahl@newcastle.edu.au

Madeline King
madeline.king@uon.edu.au

Waleed M. Hussein
w.hussein@uq.edu.au

Frederick R. Walker
rohan.walker@newcastle.edu.au

Roger Smith
roger.smith@newcastle.edu.au

Tamas Zakar
tamas.zakar@newcastle.edu.au

¹ School of Biomedical Sciences and Pharmacy, College of Health, Medicine and Wellbeing, University of Newcastle, Callaghan, NSW 2308, Australia

² Hunter Medical Research Institute, New Lambton Heights, NSW 2305, Australia

³ Centre for Rehab Innovations, University of Newcastle, Callaghan, NSW 2308, Australia

⁴ School of Medicine and Public Health, College of Health, Medicine and Wellbeing, University of Newcastle, Callaghan, NSW 2308, Australia

⁵ Mothers and Babies Research Program, New Lambton Heights, NSW 2305, Australia

⁶ Institute for Molecular Bioscience, The University of Queensland, Brisbane, St. Lucia, QLD 4072, Australia

⁷ John Hunter Hospital, New Lambton Heights, NSW 2305, Australia

Introduction

The steroid hormone progesterone (P4) is essential for establishing and maintaining pregnancy [1–3]. The withdrawal of P4 action signals the end of pregnancy and in most mammalian species this occurs through a rapid fall in circulating levels of P4, which precipitates labor [4–8]. However, in humans and higher primates, circulating P4 levels remain elevated up to and during labor [9–11]. Nonetheless, blocking the actions of P4 by progesterone antagonists (e.g., RU486) promotes cervical ripening and labor in humans [12, 13]. This indicates that P4 action is essential in maintaining human pregnancy and suggests that P4 withdrawal at parturition occurs functionally rather than due to a systemic decline in the hormone level. In 2016, Nadeem et al. [14] provided evidence that functional P4 withdrawal results from the combination of signaling changes mediated by the nuclear P4 receptors, PR-A and PR-B, and the increased metabolic inactivation of P4 in the uterine tissue. During pregnancy, P4-liganded PR-B associates with Jun/Jun homodimers and the repressor complex, P54nrb/Sin3A/HDAC, to repress the transcription of *GJAI*, which encodes the key contraction-associated protein (CAP), connexin-43 [14]. During labor, PR-A, the truncated PR isoform, dissociates from P4 and interacts with Fos/Jun heterodimers, which activates the transcription of *GJAI*, thereby promoting term labor [14]. The reduction of P4 abundance and the appearance of unliganded PR-A in the myocyte nuclei were associated with the increased expression of the P4 metabolizing enzyme, 20 α -hydroxysteroid dehydrogenase (20 α -HSD), within uterine myocytes.

20 α -HSD, a member of the aldo–keto reductase (AKR) superfamily, catalyzes the conversion of P4 to its inactive metabolite, 20 α -hydroxyprogesterone (20 α -OHP) [15]. The enzyme is encoded by the *AKR1C1* gene, which, in humans, is located on chromosome 10p15-p14. Prior research has shown that *AKR1C1* mRNA abundance and 20 α -HSD protein levels significantly increased in human myometrium during term labor [14, 16]. Recently, Nadeem et al. [17] found upregulation of *AKR1C1* during labor in human and mouse myometrium as well as in mouse models of preterm labor (lipopolysaccharide (LPS)- and RU486-model). Furthermore, mechanistic studies with immortalized cells have shown that nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) / activator protein 1 (AP-1) transcription factors mediated effects of the pro-inflammatory mediators, LPS and 12-*O*-tetradecanoylphorbol-13-acetate (TPA), respectively, on 20 α -HSD gene transcription [17]. The increase in 20 α -HSD led to local P4 withdrawal, which was concomitant with the upregulated transcription of CAPs, especially *GJAI*, thereby promoting myometrial cell contractility and labor [17]. In our recent study of

pregnant human myometrium, we confirmed that myometrial *AKR1C1* expression (mRNA abundance) was upregulated during labor onset at term [18]. We also examined *AKR1C1* expression within preterm human myometrium but found no increase in *AKR1C1* mRNA abundance in association with preterm labor [18]. *AKR1C1* expression was, however, significantly upregulated in the myometrium of women who were in preterm labor and had clinical chorioamnionitis, suggesting that increased *AKR1C1* expression and 20 α -HSD activity may be a mechanism through which inflammation drives progesterone withdrawal in preterm labor [18]. Taken together, these findings highlight the involvement of 20 α -HSD in the timing of parturition and indicate a link between uterine inflammation and local P4 withdrawal that triggers labor. It is therefore of paramount importance to understand how 20 α -HSD is regulated in human myometrium.

In the present study, we determined the effects of hormones, mechanical stretch, and pharmacologic treatments on myometrial *AKR1C1* expression utilizing our ‘ex vivo labor model’ of the human myometrium [19, 20]. We developed this model by culturing non-laboring term pregnant human myometrial tissue samples for 48 h, during which time the expression of the contraction-associated genes, *ESRI*, *PTGS2*, *OXTR*, and *PGR*, changed in a manner that is consistent with the transition to a pro-contractile, labor-like phenotype [19, 20]. We have demonstrated previously that treatments with steroid hormones or activators or inhibitors of signaling pathways modify contraction-associated gene expression in this ex vivo labor model [19, 20]. Notably, trichostatin A (TSA), a histone deacetylase (HDAC) inhibitor (HDACi), repressed the spontaneous upregulation of *PR-A* while having no effect on *PR-B* expression, thus maintaining a low *PR-A/PR-B* expression ratio that is characteristic of non-laboring myometrium and believed to be consistent with the maintenance of progesterone responsiveness [20]. Here, we show that *AKR1C1* mRNA levels increase spontaneously in our ex vivo labor model, consistent with other contraction-associated genes, and that three HDACis, TSA, suberoylanilide hydroxamic acid (SAHA), and suberoyl bis-hydroxamic acid (SBHA), repress the upregulation of *AKR1C1* expression, potentially blocking the metabolic arm of functional progesterone withdrawal.

Materials and Methods

Myometrial Tissue Acquisition

The study was approved by the Hunter and New England Area Human Research Ethics Committee (2019/ETH12330). Human myometrial samples were obtained after informed written consent of the donors from the upper lip of the incision in the lower uterine segment during cesarean section

Table 1 cDNA primer sequence – *AKRIC1*

Primer	Primer sequence	Amplicon size	GeneBank#
<i>AKRIC1</i>	F: AGTTCACCGCTCGCATAA R: GGCTGTAGATAGGCTTAGTGT	60	NM_001353.6

Abbreviation: *AKRIC1* Aldo–keto reductase family 1 member C1

(CS) of singleton term pregnancies (38.3 – 39.5 weeks gestation). The patient body mass index (BMI) range was 20.3 – 33.6, and none of the patients was in labor. The indications for elective CS were previous CS, placenta previa, fetal distress, or breech presentation. Women were excluded if they were given steroids prenatally. Myometrial samples were placed on ice in a serum-free Dulbecco's Modified Eagle Medium (DMEM) with high glucose, 2 mM L-Glutamine, 1 mM Sodium Pyruvate, 40 µg/mL Gentamicin, and 10 mM HEPES, then transferred to the laboratory for explant culture.

Myometrial Tissue Culture and Treatments

Approximately 100 mg of tissue from each sample was immediately snap-frozen using liquid nitrogen to serve as a gene expression baseline (0 h). The remaining tissue was dissected into approximately 2 × 2 × 2 mm pieces and washed in serum-free DMEM. To determine treatment effects, myometrial samples were incubated for 48 h in a 37 °C, 95% air/5% CO₂ humidified incubator in the presence of 5% (v/v) charcoal-stripped, heat-inactivated fetal bovine serum (CSS)-supplemented media (Gibco), P4 (Sigma; 500 nM), estradiol (E2; Sigma; 100 or 400 nM), 8-Bromoadenosine 3',5'-cyclic monophosphate (8-Br-cAMP; Sigma; 250 µM), phorbol 12-myristate 13-acetate (PMA; Cayman Chemical Company; 0.1 and 1 µM), prostaglandin F_{2α} (PGF_{2α}; Cayman Chemical Company; 1, 10, and 100 nM), TSA (Bio-Scientific Pty Ltd; 0.5, 1, 2.5, 5, or 25 µM), SAHA (Cayman Chemical Company; 0.5, 1, 2.5, 5, or 25 µM), or SBHA (Cayman Chemical Company; 0.5, 1, 2.5, 5, or 25 µM). The vehicle was DMSO (0.1% v/v). For the stretch experiments, stainless steel weights were attached to the myometrial tissue strips (2 × 2 × 10 mm) using a nylon thread, and the strips were suspended in 30 mL of culture media in 50 mL tubes as described previously [19, 20]. Media (including treatments) were refreshed after 24 h. After 48 h of incubation, the tissues were snap-frozen using liquid nitrogen and stored at -80 °C for subsequent analyses.

RNA Extraction, Reverse Transcription, and Real-time Quantitative PCR

RNA was extracted using TRizol Reagent (Thermo Fisher Scientific) according to the manufacturer's protocol.

Homogenization of tissue in TRizol Reagent was performed using a Precellys24 homogenizer (5,000 rpm for 3 × 30 s, with 20 s intervals) (Bertin Instruments). Following extraction, RNA samples were further purified using the TURBO DNA-free kit (Thermo Fisher Scientific). RNA concentration (absorbance at 260 and 280 nm) and purity were assessed using a ND-1000 spectrophotometer and RNA integrity was checked by agarose gel electrophoresis before and after DNase treatment. Each RNA sample (0.5 µg of total RNA) was spiked with 0.5 × 10⁷ copies of Alien RNA (Integrated Sciences Pty) and reverse-transcribed using the SuperScript III First-Strand Synthesis System with random hexamer primers (Thermo Fisher Scientific). The Alien RNA transcript is an in vitro transcribed RNA molecule that is non-homologous to any known nucleic acids and, as such, was used as a reference gene for these studies [18–21]. Quantitative RT-PCR was performed using QuantStudio 6 Flex Real-Time PCR (Applied Biosystems). No-reverse transcription negative controls were prepared for each sample. The final volume of each PCR reaction was 20 µL, containing 10 µL of 2 × SYBR Green PCR Master Mix (Thermo Fisher Scientific), master mix cDNA template (corresponding to 10 ng of reverse-transcribed RNA), *AKRIC1* cDNA-specific forward and reverse primers (500 nM). For the reference gene (Alien), the final volume was 20 µL with 1.0 µL of 2.5 µM of Alien Primer Mix (with proprietary sequence), 10 µL of 2 × SYBR Green PCR, the same amount of cDNA as the target genes. No-template control samples were included in each PCR plate to detect any contamination and primer-dimers. The thermal sequence and cycling conditions were as recommended by the manufacturer (Applied Biosystems). Melt curves were determined in each PCR reaction to ascertain the homogeneity of amplified products. *AKRIC1* cDNA primers (Table 1) (Sigma) were designed using Primer-BLAST, optimized, and validated by confirming that single amplicons of appropriate size were generated.

Protein Extraction, One-dimensional (1D) SDS-PAGE and Immunoblotting

Protein was extracted into sodium dodecyl sulfate (SDS) extraction buffer (2% SDS, 50 mM Tris pH 6.8, 5 mM EDTA) supplemented with PhosSTOP phosphatase inhibitor (Roche) and Complete Mini Protease Inhibitor (Roche). Tissue was homogenized in SDS extraction buffer using a Precellys24 homogenizer (6,500 rpm for 3 × 60 s, with 20 s

intervals), after which homogenates were incubated on a rotary mixer for 1 h at 4 °C. Homogenates were then centrifuged at 15,500 g for 15 min at 4 °C (Beckman Coulter Microfuge 20R) and supernatants were collected. Protein concentration was determined using a BCA Protein Assay Kit (Thermo Fisher Scientific).

Myometrial protein extracts (up to 50 µg per lane, due to low AKR1C1 abundance in pregnant human myometrium) were loaded onto 4–12% NuPAGE gels and separated using a Novex Mini-Cell system at constant voltage (200 V for 50 min; Invitrogen). S9 Fraction from Human Liver Extract (Sigma Aldrich, cat# S2442; 0.5 µg/lane) and SDS protein extract from term human placenta (up to 50 µg/lane) were included as positive and negative controls for AKR1C1 detection, respectively. Following 1D SDS-PAGE, proteins were transferred to Hybond-C nitrocellulose (Amersham Biosciences) using the XCell II Blot Module (Invitrogen). Total protein was stained using Ponceau S and imaged using an Amersham Imager 600 (GE Healthcare).

During immunoblotting, all incubations were performed on a rocking platform. Membranes were blocked in 5% skim milk powder in tris-buffered saline (TBS; 500 mM NaCl, 20 mM Tris) for 1 h at room temperature. The blocking solution was decanted and the primary antibody was applied in 5 mL 1% skim milk powder in TBS-Tween-20 (TBS-T; 500 mM NaCl, 20 mM Tris, 0.01% Tween-20) overnight at 4 °C. Antibodies against AKR1C1 (GeneTex, cat# GTX105620, rabbit polyclonal) and a β -actin (Abcam, cat# ab8226, mouse monoclonal) were applied at 1:1,000 dilution. Blots were subjected to 4 × 5 min washes with 100 mL of TBS-T. Washed blots were then incubated in horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (Cell Signaling Technology, cat# 7074) or anti-mouse IgG (Cell Signaling Technology, cat# 7076) secondary antibody as appropriate, applied at 1:2500 dilution in 5 mL of 1% skim milk powder in TBS-T for 1 h at room temperature. Blots were washed for 3 × 5 min in 100 mL of TBS-T before immunoreactive products were detected using Immobilon Forte (high sensitivity) Western HRP substrate (Merck Millipore) and visualized using the Amersham Imager 600 (GE Healthcare). To ensure internal controls, blots were first probed with the AKR1C1 antibody before being stripped and re-probed with the antibody against the total proteins. Membranes were stripped by 2 × 5 min incubations in 100 mL of 0.2 M NaOH. Stripped blots were then washed for 3 × 3 min in TBS-T before being re-probed according to the outlined regimen.

Data and Statistical Analysis

All mRNA abundance data were expressed relative to the Alien reference RNA. The relative mRNA abundance was calculated using the delta Ct (Δ Ct) method [22].

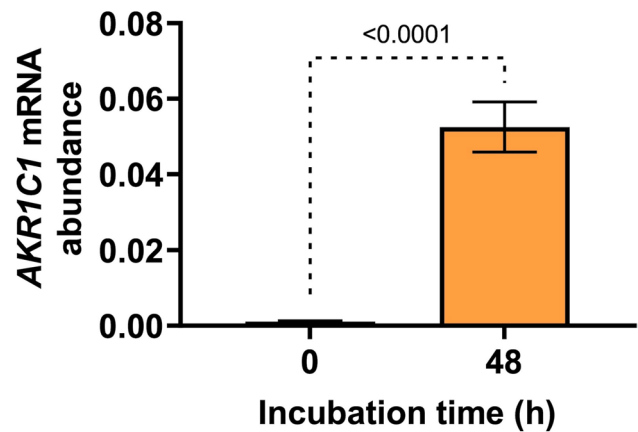


Fig. 1 Upregulation of *AKR1C1* expression in term pregnant non-laboring human myometrium cultured for 48 h ex vivo. Relative mRNA abundance of *AKR1C1* was measured either immediately after biopsy (0 h) or following 48 h incubation ex vivo ($n=31$). *AKR1C1* mRNA abundance is expressed relative to Alien reference. Data were checked for distribution using the Shapiro–Wilk normality test, logarithmically transformed to approach normal distribution, and analyzed by paired *t*-test. Data are mean \pm SEM. A significant *p*-value is indicated

Densitometric analysis of immunoreactive protein bands was performed using Amersham Imager 600 analysis software (GE Healthcare). The protein abundance data (optical density (OD; arbitrary units)) were expressed relative to β -actin and for each replicate, relative AKR1C1 protein levels (AKR1C1/ β -actin) were normalized to Fresh (0 h) samples. Statistical analyses were conducted with GraphPad Prism software and confirmed using STATA. All mRNA and protein relative abundance values were checked with the Shapiro–Wilk distribution test and not normally distributed data were logarithmically transformed to approach normal distribution. Graphical data are presented as mean \pm SEM. For comparison between the two groups, a Student’s *t*-test was used. For comparisons of multiple groups and interactions, analysis of variance and covariance (ANOVA) was performed. *P*-values < 0.05 were considered statistically significant.

Results

AKR1C1 Expression Changes During Ex Vivo Culture

Myometrial tissues were incubated for 48 h in serum-free media to determine gene expression changes while cultured ex vivo. Consistent with our previous findings of spontaneously upregulated expression of pro-contractile genes [19, 20], myometrial *AKR1C1* mRNA abundance was significantly increased following 48 h incubation ($p < 0.0001$) (Fig. 1).

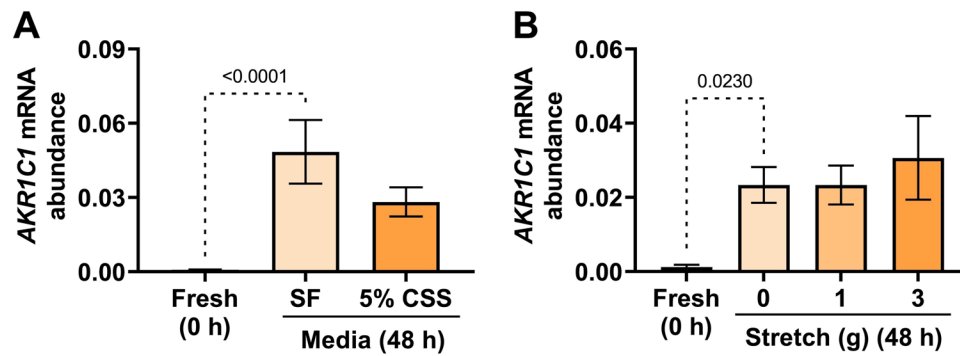


Fig. 2 Effect of serum and stretch on the upregulation of myometrial *AKR1C1* expression ex vivo. *AKR1C1* mRNA abundance was measured in (A) term not-in-labor myometrial biopsies incubated for 48 h in either serum-free media ($n = 13$) or media supplemented with 5% CSS ($n = 15$), and in (B) term non-laboring myometrial strips that were incubated for 48 h while applying 0, 1, and 3 g of stretch

($n = 3$). *AKR1C1* mRNA abundance is expressed relative to Alien reference. Data were checked for by the Shapiro–Wilk normality test, logarithmically transformed to approach a normal distribution, and then analyzed by 1-way ANOVA with Dunnett’s multiple comparisons test relative to SF (Panel A) and 0 g Stretch (Panel B). Data are mean \pm SEM. Significant p -values are indicated

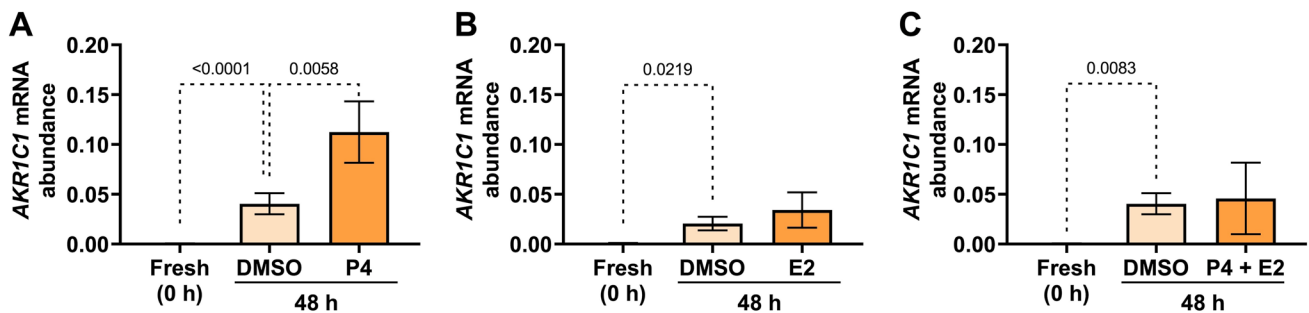


Fig. 3 Effect of progesterone (P4) and estradiol (E2) on the upregulation of myometrial *AKR1C1* expression ex vivo. *AKR1C1* mRNA abundance was measured in term non-laboring myometrial explants following 48 h incubation in the presence of (A) P4 alone (500 nM) ($n = 3$), (B) E2 alone (100 nM) ($n = 3$), or (C) P4 (500 nM)+E2 (400 nM) in combination ($n = 3$). *AKR1C1* mRNA abundance is

expressed relative to Alien reference. Data were checked for normality using the Shapiro–Wilk normality test, logarithmically transformed to approach normal distribution, and then analyzed by 1-way ANOVA with multiple comparisons (Dunnett’s relative to DMSO in all panels). Data are mean \pm SEM. Significant p -values are indicated

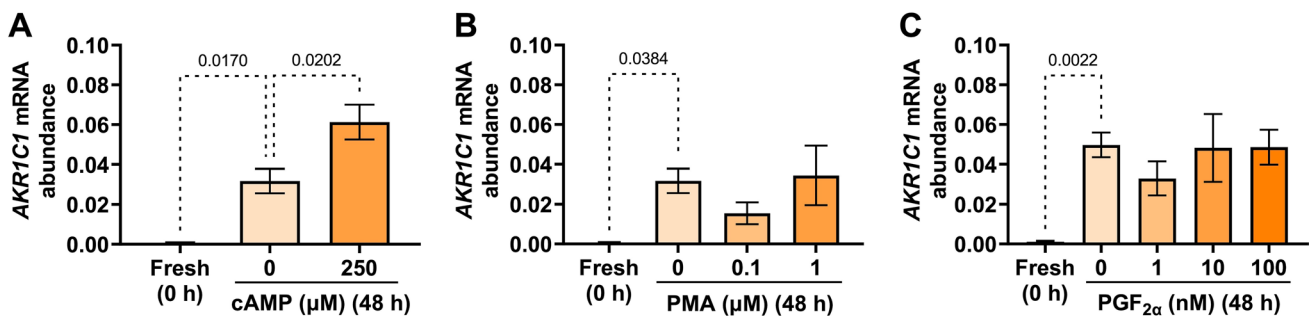


Fig. 4 Effect of 8-Br-cAMP (cAMP), phorbol myristate acetate (PMA), and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) on the upregulation of myometrial *AKR1C1* expression ex vivo. *AKR1C1* mRNA abundance was measured in term non-laboring myometrial explants following 48 h incubation in the presence of (A) 8-Br-cAMP (250 μ M) ($n = 3$), (B) the PKC activator, PMA (0.1, 1.0 μ M) ($n = 3$), or (C) the prostaglan-

din, $PGF_{2\alpha}$ (1, 10, 100 nM) ($n = 3$). *AKR1C1* mRNA abundance is expressed relative to Alien reference. Data were checked for normality using the Shapiro–Wilk normality test and then analyzed by 1-way ANOVA with multiple comparisons (Sidak’s relative to 0 in all panels). Data are mean \pm SEM. Significant p -values are indicated

Treatment Effects on *AKR1C1* Expression

Considering that the spontaneous upregulation of *AKR1C1* mRNA abundance in culture may model labor-associated changes, we examined whether culture conditions and various treatments can influence the *AKR1C1* expression change *ex vivo*.

Supplementing the culture media with 5% (v/v) CSS resulted in no significant change in *AKR1C1* mRNA abundance following 48 h incubation compared to serum-free media ($p=0.1312$) (Fig. 2A). Stretch has been implicated in the regulation of myometrial gene expression during pregnancy [23–26]; however, applying 1 or 3 g of stretch to myometrial tissue strips over 48 h incubation had no effect on the culture-induced change in *AKR1C1* expression, compared to non-stretched (0 g) tissue strips (Fig. 2B).

Supplementing the media with 500 nM P4 significantly enhanced the culture-induced increase in *AKR1C1* mRNA level compared to vehicle (DMSO)-treated tissues ($p=0.0058$) (Fig. 3A), whereas supplementing media with E2 alone (100 nM) ($p=0.7349$) (Fig. 3B) or 500 nM P4 + 400 nM E2 ($p=0.7923$) in combination had no effect (Fig. 3C).

Adding 250 μM 8-Br-cAMP to the media significantly enhanced the culture-induced increase in *AKR1C1* mRNA abundance following 48 h incubation, relative to vehicle-treated tissues ($p=0.0202$) (Fig. 4A), while supplementing media with PMA (0.1 and 1.0 μM) ($p=0.3097$ and $p=0.9884$) or $\text{PGF}_{2\alpha}$ (1, 10, 100 nM) ($p=0.3184$, $p=0.9997$, and $p=0.9999$) did not significantly enhance or repress the culture-induced increase in *AKR1C1* expression, relative to vehicle controls (Fig. 4B and 4C, respectively).

Supplementing media with TSA, SAHA, or SBHA (0.5, 1, 2.5, 5, 25 μM) repressed the culture-induced increase in *AKR1C1* mRNA abundance following 48 h

incubation. Relative to vehicle (DMSO)-treated controls, TSA significantly repressed *AKR1C1* upregulation at 5 ($p=0.0172$) and 25 μM ($p=0.0115$) (Fig. 5A). Consistent results were observed for SBHA, which also significantly repressed *AKR1C1* upregulation at 5 ($p=0.0480$) and 25 μM ($p=0.0238$) (Fig. 5C), whereas SAHA significantly repressed *AKR1C1* upregulation, compared to vehicle treatment, at 0.5 ($p=0.0070$), 1 ($p=0.0045$), 2.5 ($p=0.0181$), 5 ($p=0.0066$) and 25 μM ($p=0.0014$) (Fig. 5B).

AKR1C1 Protein Levels

Western blotting of pregnant human myometrial protein extracts revealed that the GeneTex anti-*AKR1C1* antibody was not specific for *AKR1C1* in that the antibody cross-reacted with a triplicate of protein bands in the vicinity of the expected molecular weight of *AKR1C1* (37 kDa) (Fig. 6). The dominant (middle) band out of the three was further analyzed; however, it remains to be confirmed whether the dominant band is indeed *AKR1C1*. Nonetheless, the analysis revealed that the intensity of the dominant protein band significantly increased following 48 h incubation ($p=0.0042$ in Fig. 6C, $p=0.0007$ in Fig. 6F, and $p=0.0120$ in Fig. 6I). Supplementing media with TSA at 5 ($p=0.0060$) and 25 μM ($p=0.0107$) repressed this culture-induced increase of band intensity ($n=4$) (Fig. 6C). Treatment with SAHA also repressed the culture-induced increase of protein band intensity at both 5 ($p=0.0018$) and 25 μM ($p=0.0011$) ($n=5$) (Fig. 6F), while SBHA significantly repressed the culture-induced increase of the protein band at 25 μM ($p=0.0141$) ($n=3$) (Fig. 6I).

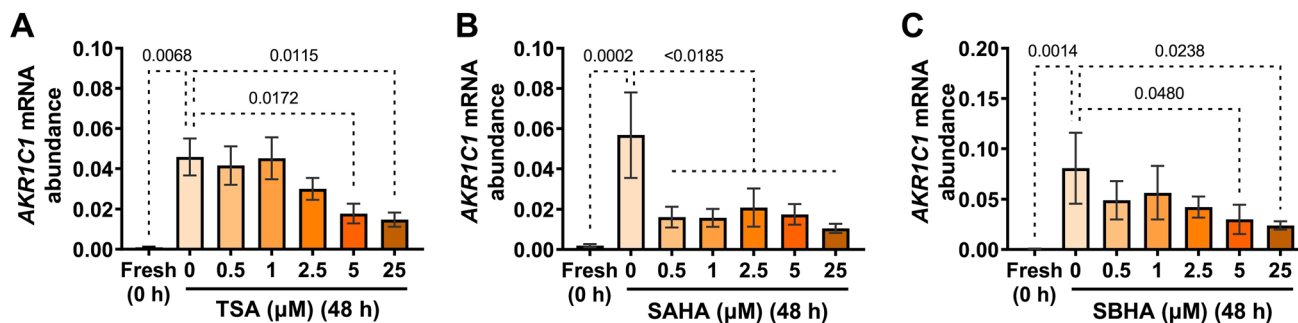


Fig. 5 Effect of histone deacetylase inhibitors (TSA, SAHA, and SBHA) on the upregulation of myometrial *AKR1C1* expression *ex vivo*. *AKR1C1* mRNA abundance was measured in term non-laboring myometrial explants following 48 h incubation in the presence of (A) TSA (0.5, 1, 2.5, 5, 25 μM) ($n=8$), (B) SAHA (0.5, 1,

2.5, 5, 25 μM) ($n=5$) or (C) SBHA (0.5, 1, 2.5, 5, 25 μM) ($n=4$). *AKR1C1* mRNA abundance is expressed relative to Alien reference. Data were checked using the Shapiro–Wilk normality test and analyzed by 1-way ANOVA with multiple comparisons (Dunnett's relative to 0). Data are mean \pm SEM. Significant p -values are indicated

Discussion

Prior studies have shown that *AKR1C1* mRNA abundance and 20 α -HSD protein levels increase with labor onset in pregnant human myometrium at term [14, 16, 17]. Recently, we reported an average sixfold increase in myometrial *AKR1C1* mRNA abundance in association with labor onset at term [18]. Whilst the regulation of *AKR1C1* has been studied in human telomerase-immortalized myometrial (hTERT-HM) cells [17], it remains crucial to examine *AKR1C1* regulation in human myometrial tissue in short-term cultures reflecting the in vivo state more closely. Accordingly, we utilized our ex vivo labor model in which key labor-associated genes (*ESR1*, *PTGS2*, *OXTR*, and *PGR*) undergo culture-induced expression changes within 48 h that are consistent with transitioning to a labor-like phenotype [19, 20]. Within this model, we observed a significant increase in *AKR1C1* mRNA abundance across the 48 h culture period (Fig. 1). This increase reflects the documented upregulation of *AKR1C1* expression that occurs in association with term labor [18] and further supports that non-laboring myometrium transitions toward a labor-like phenotype during ex vivo culture. The model enabled us to assess whether treatments known to influence myometrial activity affected the culture-induced increase in *AKR1C1* expression and contribute to enhancing or repressing the ex vivo transition toward a labor-like phenotype.

Upon examining the effects of various pro-quiescence and pro-contraction agents, we found that treatment with P4 significantly upregulated *AKR1C1* expression beyond the culture-induced change (Fig. 3A). The possibility that P4 can upregulate its own metabolism and exert negative feedback on target tissue P4 sensitivity is intriguing, as P4 can be anticipated intuitively to repress myometrial *AKR1C1* expression for maintaining high P4 levels throughout pregnancy. Clearly, E2 blocks this effect. One may also consider that *AKR1C1* and its paralogues have loose steroid selectivity and can be involved in the reductive metabolism of intrauterine androgens and estradiol, in addition to P4 [27, 28]. To assume that the sole function of *AKR1C1* is to control P4 levels is a simplification, and further studies are required to precisely delineate the role AKR1C family members have in shaping the steroid environment of the myometrium.

AKR1C1 mRNA expression was also upregulated by cAMP (Fig. 4A), which is a powerful suppressor of myometrial contractions. However, genomic cAMP signaling has been shown to exert both pro-relaxatory and pro-contraction roles [29, 30] in that prolonged cAMP exposure can activate pro-contraction mitogen-activated protein kinase (MAPK) signaling [31] and upregulate pro-contraction genes (reviewed by Butler et al. [30]). It is plausible

that cAMP may drive *AKR1C1* expression in pregnant human myometrium as part of a pro-contraction action. More in-depth analyses, such as time-dependency studies, are required to determine whether cAMP exerts differential effects on *AKR1C1* expression following short- or prolonged exposure and determine the underlying molecular mechanism(s).

Recently, Nadeem et al. [17] reported that treating hTERT-HM cells with the pro-inflammatory mediator TPA, also known as PMA (20 ng/mL; 32.4 nM), induced *AKR1C1* expression and increased AKR1C1 protein levels. At odds with this report, we observed no effect of TPA/PMA (0.1 and 1 μ M) on myometrial *AKR1C1* expression (Fig. 4B). The difference may be attributable to the different model systems used (immortalized myometrial cell line vs primary myometrial tissue) or the different TPA/PMA concentrations applied. The concentrations of PMA used in this study (0.1 and 1 μ M) were effective in our former study at significantly increasing the *PR-A/PR-B* ratio in human myometrial explants ex vivo, compared to 48 h vehicle-treated tissue [20], but in this study, the same PMA concentrations did not affect *AKR1C1* expression.

Within our ex vivo labor model, TSA, SAHA, and SBHA each dose-dependently inhibited the culture-induced increase in *AKR1C1* expression (Fig. 5) and significantly repressed upregulation of the dominant immunoreactive protein band detected at the expected molecular weight for AKR1C1 (Fig. 6). This is consistent with a pro-relaxation/pro-quiescence effect, as repressing *AKR1C1* expression and 20 α -HSD levels are anticipated to contribute toward preventing P4 metabolism in the myometrium, thus promoting the maintenance of P4 signaling through PR-B. The precise mechanism by which HDACis inhibit *AKR1C1* expression is yet to be determined; however, it may be through HDACis affecting the acetylation of transcription factors that regulate *AKR1C1* expression or by effects on chromatin structure (nucleosome acetylation) within gene regulatory regions [32].

Previously, we have demonstrated that TSA exerts a pro-quiescence effect on pregnant human myometrium by repressing upregulation of *PR-A*, but not *PR-B*, to maintain a low *PR-A/PR-B* ratio. Our team was the first to suggest that the “*efficacy of progesterone administration may be enhanced if an agent such as TSA could be administered to preserve or even restore progesterone sensitivity in women with threatened preterm labor*” [20]. Here, our data demonstrating that HDACis exert an additional pro-quiescence effect of repressing *AKR1C1* upregulation in pregnant human myometrium further supports the possibility that co-administration of a HDACi may enhance the effectiveness of P4 therapy. In agreement with this, Zierden et al. [33] showed that administration of a TSA nanosuspension in combination with a nanosuspension of P4 was effective

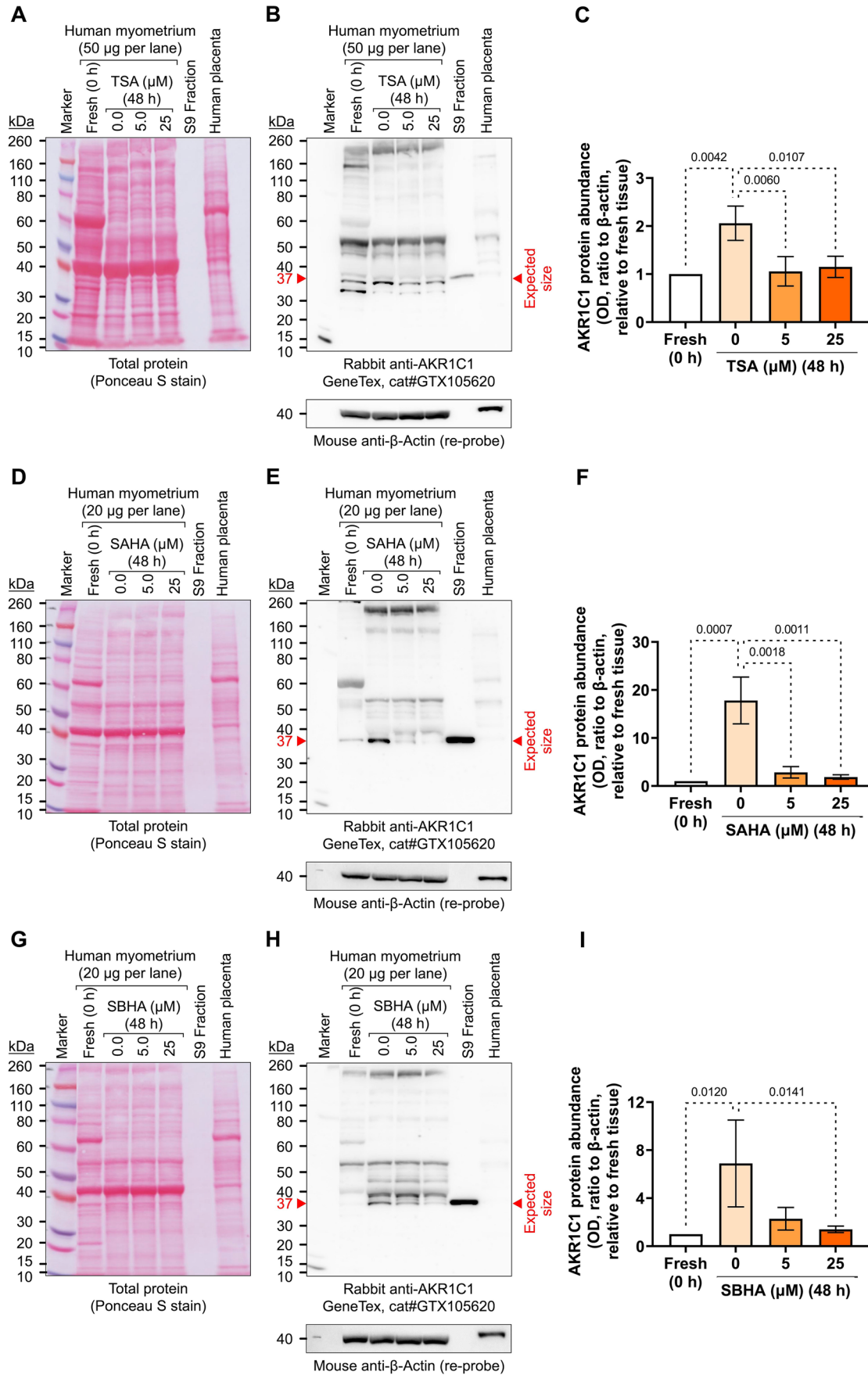


Fig. 6 Effect of TSA, SAHA, and SBHA on the upregulation of myometrial AKR1C1 protein abundance *ex vivo*. Protein extracts from fresh (0 h) term non-laboring myometrium or myometrial explants incubated for 48 h in the presence of 0, 5, or 25 μM TSA ($n=4$) (panels A – C), SAHA ($n=5$) (panels D – F), or SBHA ($n=3$) (panels G – I) were separated by SDS-PAGE (20–50 $\mu\text{g}/\text{lane}$) and then transferred to a nitrocellulose membrane. S9 Fraction from human liver extract (0.5 $\mu\text{g}/\text{lane}$) and human placenta extract (20–50 $\mu\text{g}/\text{lane}$) were included as positive and negative controls, respectively. Total protein transfer was visualized by Ponceau S staining and then imaged (panels A, D, G). Membranes were then probed for AKR1C1 detection using GeneTex (cat# GTX105620) rabbit anti-AKR1C1 polyclonal antibody (1:1000) and anti-rabbit-HRP secondary antibody (1:2000). Representative images show immunoreactive bands detected using Immobilon Forte chemiluminescence reagent (panels B, E, H). Blots were then stripped and re-probed using mouse anti- β -actin (1:1000) and anti-mouse-HRP (1:2000). The molecular weight marker was Novex™ Sharp Pre-stained Protein Standard. The optical density of the dominant immunoreactive band corresponding to the expected molecular weight of AKR1C1/20 α -HSD (37 kDa) was measured and protein abundance expressed relative to β -actin and normalized to fresh (0 h) myometrial explants (panels C, F, I). Data were checked using the Shapiro–Wilk normality test and analyzed by 1-way ANOVA with multiple comparisons (Dunnett's relative to 0). Data are mean \pm SEM. Significant *p*-values are indicated

at significantly reducing the rate of inflammation-induced preterm birth in mice, whereas the nanosuspension of P4 alone had no significant effect. Moreover, the TSA + P4 combination therapy led to the birth of neurotypical offspring [33], which is consistent with TSA having been shown to extend mouse pregnancy with no obvious impacts on litter size or fetal viability [34]. New developments in drug delivery techniques, such as mucus penetrating nanoparticles [33, 35] and uterine-targeted nanoliposomes [36], may therefore allow the selective deployment of HDACis to the myometrium for maintaining P4 sensitivity and achieving tocolysis while reducing the likelihood of maternal or fetal side-effects. This work, showing that HDACis have the pro-relaxatory effect of suppressing myometrial *AKR1C1* upregulation *ex vivo*, adds to prior work showing that TSA regulates the *PR-A/PR-B* ratio [20], and that vaginal administration of P4 combined with TSA can prevent preterm birth in mice [33]. Further research will elucidate the molecular mechanisms by which HDACis regulate the genes controlling P4 action in the uterus, which may include affecting the acetylation of transcription factors that regulate these genes, or altering histone acetylation, thus changing chromatin accessibility at gene regulatory regions in the myometrium [33].

A strength of this study is that we have used an ‘*ex vivo* labor’ model of the human myometrium, which is uniquely informative about the responses of the tissue to treatments that influence the labor-associated uterine phenotype transition. A caveat is the limited reliability of our protein data.

Extensive efforts were made to evaluate AKR1C1 protein levels in extracts of pregnant human myometrium via Western blotting; however, this proved difficult due to the available anti-AKR1C1 antibodies cross-reacting with a triplicate of protein bands within the vicinity of the expected molecular weight for human AKR1C1. It is possible that the triplicate bands correspond to AKR1C1 (37 kDa), AKR1C2 (36 kDa), and AKR1C3 (34 kDa), given that in humans, AKR1C1 shares 97.8% and 87.9% sequence homology with AKR1C2 and AKR1C3, respectively. The lack of reliable antibodies for distinguishing between aldo-keto reductase family members has been noted by others [37], and due to this limitation, the precise identity of the triplicate gene products remains currently unknown [18]. A further limitation is that we did not assess the downstream effects of AKR1C1 expression changes, including P4 metabolic activity, AKR1C1 enzyme activity, and canonical P4-responsive marker gene products (e.g., *FKBP5* mRNA). A full characterization of the *ex vivo* model will include these aspects as well as the contribution of other steroid metabolic enzymes in the various myometrial tissue compartments potentially affecting local P4 availability. We also note that myometrial tissue is heterogeneous, meaning that low levels of non-myometrial cell types may be present within the explants, and the presence of these cells has the potential to impact both the mRNA and protein data. Nonetheless, it is relevant to consider that P4, being a steroid, may diffuse within the tissue, and AKR1C1 in any tissue component may affect P4 availability locally.

In summary, we have demonstrated that myometrial *AKR1C1* mRNA expression, and the dominant immunoreactive band representing AKR1C1/20 α -HSD protein abundance, significantly increased in the term, not-in-labor pregnant human myometrium cultured *ex vivo*; which is consistent with the transition to a pro-labor phenotype and the upregulation of other labor-associated genes in our *ex vivo* labor model. The culture-induced increase in *AKR1C1* expression was significantly exacerbated by P4 and cAMP, while the HDACis, TSA, SAHA, and SBHA, dose-dependently repressed the *ex vivo* upregulation of myometrial *AKR1C1* mRNA and AKR1C1/20 α -HSD protein levels, indicating that protein acetylation plays a role in myometrial *AKR1C1* expression control. Moreover, these findings indicate that suppression of AKR1C1/20 α -HSD mediates, in part, the pro-relaxation effects of HDACis regulating P4 sensitivity in human myometrium, complementing the previously described ability of these drugs to selectively repress labor-promoting *PR-A* expression in this tissue. Our data further highlight the potential of HDACi compounds to be clinically useful for pregnancy maintenance in a setting where they can be safely deployed.

Glossary of Terms

<i>1D</i>	One-dimensional
<i>20α-HSD</i>	20 α -Hydroxysteroid dehydrogenase
<i>20α-OHP</i>	20 α -Hydroxyprogesterone
<i>8-Br-cAMP</i>	8-Bromoadenosine 3',5'-cyclic monophosphate
<i>AKR</i>	Aldo-keto reductase
<i>AKR1C1</i>	Aldo-keto reductase family 1 member C1 gene
<i>AKR1C2</i>	Aldo-keto reductase family 1 member C2 gene
<i>AKR1C3</i>	Aldo-keto reductase family 1 member C3 gene
<i>ANOVA</i>	Analysis of variance
<i>BCA</i>	Bicinchoninic acid
<i>BMI</i>	Body mass index
<i>cAMP</i>	Cyclic adenosine monophosphate
<i>CAP</i>	Contraction-associated protein
<i>CS</i>	Cesarean section
<i>CSS</i>	Charcoal-stripped fetal bovine serum
<i>Cx43</i>	Connexin 43
<i>EDTA</i>	Ethylenediaminetetraacetic acid
<i>DME</i>	Dulbecco's modified eagle medium
<i>E2</i>	Estradiol
<i>GJA1</i>	Gap junction alpha-1 protein
<i>HDAC</i>	Histone deacetylase
<i>HDACi</i>	Histone deacetylase inhibitor
<i>HRP</i>	Horseshoe peroxidase
<i>hTERT-HM</i>	Human telomerase immortalized myometrial cells
<i>LPS</i>	Lipopolysaccharide
<i>MAPK</i>	Mitogen-activated protein kinase
<i>mRNA</i>	Messenger ribonucleic acid
<i>NF-κB</i>	Nuclear factor kappa-light-chain-enhancer of activated B cells
<i>OXTR</i>	Oxytocin receptor gene
<i>P4</i>	Progesterone
<i>PBS</i>	Phosphate-buffered solution
<i>PGF_{2α}</i>	Prostaglandin F _{2α}
<i>PMA</i>	Phorbol myristate acetate
<i>PR</i>	Progesterone receptor
<i>RT-PCR</i>	Real-time quantitative polymerase chain reaction
<i>PTGS2</i>	Prostaglandin-endoperoxide synthase 2 gene
<i>RNA</i>	Ribonucleic acid
<i>SAHA</i>	Suberoylanilide hydroxamic acid
<i>SBHA</i>	Suberoyl bis-hydroxamic acid
<i>SDS</i>	Sodium dodecyl sulfate
<i>SDS-PAGE</i>	Sodium dodecyl sulfate–polyacrylamide gel electrophoresis
<i>TBS</i>	Tris buffered saline
<i>TBS-T</i>	Tris buffered saline tween-20
<i>TPA</i>	12-O-Tetradecanoylphorbol-13-acetate
<i>TSA</i>	Trichostatin A

Acknowledgements The authors wish to thank the obstetricians from the John Hunter Hospital, NSW, our research midwife, Anne Wright, and the research participants who donated samples toward this study.

Authors' contributions Conceptualization: MP, JWP. Sample collection: MP, APB, RK, JWP. Data curation: MP, APB, AG, RK, JWP. Formal analysis: MP, TZ, JWP. Funding acquisition: JWP, WMH, TZ. Manuscript drafting: MP, JWP. Review & editing: TZ, APB, AG, RK, MK, WMH, FRW, RS.

Funding Open Access funding enabled and organized by CAUL and its Member Institutions. This work was supported by National Health and Medical Research Council, Australia (NHMRC) funding awarded to JWP, WMH and TZ (GNT1162684 and GNT2012583). The funding providers had no involvement in the study or production of this article. Facilities and infrastructure were provided by the University of Newcastle and Hunter Medical Research Institute (HMRI).

Data availability Data available on request due to privacy/ethical restrictions.

Code availability Not applicable.

Declarations

Ethics approval The study was approved by the Hunter and New England Area Human Research Ethics Committee (2019/ETH12330).

Consent to participate All participants gave informed written consent.

Consent for publication Not applicable.

Competing interests The authors declare that they have no competing interests.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by/4.0/>.

References

1. Arck P, Hansen PJ, Mulac Jericevic B, Piccinni MP, Szekeres-Bartho J. Progesterone during pregnancy: endocrine-immune cross talk in mammalian species and the role of stress. *Am J Reprod Immunol.* 2007;58(3):268–79. <https://doi.org/10.1111/j.1600-0897.2007.00512.x>.
2. Astle S, Slater DM, Thornton S. The involvement of progesterone in the onset of human labour. *Eur J Obstet Gynecol Reprod Biol.* 2003;108(2):177–81. [https://doi.org/10.1016/s0301-2115\(02\)00422-0](https://doi.org/10.1016/s0301-2115(02)00422-0).
3. Csapo A. Progesterone block. *Am J Anat.* 1956;98(2):273–91. <https://doi.org/10.1002/aja.1000980206>.
4. Allport VC, Pieber D, Slater DM, Newton R, White JO, Bennett PR. Human labour is associated with nuclear factor-kappaB activity which mediates cyclo-oxygenase-2 expression and is involved

- with the “functional progesterone withdrawal.” *Mol Hum Reprod.* 2001;7(6):581–6. <https://doi.org/10.1093/molehr/7.6.581>.
5. Liggins GC. Initiation of labour. *Biol Neonate.* 1989;55(6):366–75. <https://doi.org/10.1159/000242940>.
 6. Liggins GC, Fairclough RJ, Grieves SA, Kendall JZ, Knox BS. The mechanism of initiation of parturition in the ewe. *Recent Prog Horm Res.* 1973;29:111–59. <https://doi.org/10.1016/b978-0-12-571129-6.50007-5>.
 7. Mesiano S. Myometrial progesterone responsiveness and the control of human parturition. *J Soc Gynecol Investig.* 2004;11(4):193–202. <https://doi.org/10.1016/j.jsig.2003.12.004>.
 8. Young IR. The comparative physiology of parturition in mammals. *Front Horm Res.* 2001;27:10–30. <https://doi.org/10.1159/000061036>.
 9. Walsh SW, Stanczyk FZ, Novy MJ. Daily hormonal changes in the maternal, fetal, and amniotic fluid compartments before parturition in a primate species. *J Clin Endocrinol Metab.* 1984;58(4):629–39. <https://doi.org/10.1210/jcem-58-4-629>.
 10. Tulchinsky D, Hobel CJ, Yeager E, Marshall JR. Plasma estrone, estradiol, estriol, progesterone, and 17-hydroxyprogesterone in human pregnancy. I. Normal pregnancy. *Am J Obstet Gynecol.* 1972;112(8):1095–100. [https://doi.org/10.1016/0002-9378\(72\)90185-8](https://doi.org/10.1016/0002-9378(72)90185-8).
 11. Boroditsky RS, Reyes FI, Winter JS, Faiman C. Maternal serum estrogen and progesterone concentrations preceding normal labor. *Obstet Gynecol.* 1978;51(6):686–91.
 12. Avrech OM, Golan A, Weinraub Z, Bukovsky I, Caspi E. Mifepristone (RU486) alone or in combination with a prostaglandin analogue for termination of early pregnancy: a review. *Fertil Steril.* 1991;56(3):385–93. [https://doi.org/10.1016/s0015-0282\(16\)54527-0](https://doi.org/10.1016/s0015-0282(16)54527-0).
 13. Mesiano S, Welsh TN. Steroid hormone control of myometrial contractility and parturition. *Semin Cell Dev Biol.* 2007;18(3):321–31. <https://doi.org/10.1016/j.semcdb.2007.05.003>.
 14. Nadeem L, Shynlova O, Matysiak-Zablocki E, Mesiano S, Dong X, Lye S. Molecular evidence of functional progesterone withdrawal in human myometrium. *Nat Commun.* 2016;7:11565. <https://doi.org/10.1038/ncomms11565>.
 15. Runnebaum B, Zander J. Progesterone and 20 alpha-dihydroprogesterone in human myometrium during pregnancy. *Acta Endocrinol Suppl (Copenh).* 1971;150:3–45.
 16. Williams KC, Renthal NE, Condon JC, Gerard RD, Mendelson CR. MicroRNA-200a serves a key role in the decline of progesterone receptor function leading to term and preterm labor. *Proc Natl Acad Sci U S A.* 2012;109(19):7529–34. <https://doi.org/10.1073/pnas.1200650109>.
 17. Nadeem L, Balendran R, Dorogin A, Mesiano S, Shynlova O, Lye SJ. Pro-inflammatory signals induce 20alpha-HSD expression in myometrial cells: A key mechanism for local progesterone withdrawal. *J Cell Mol Med.* 2021;25(14):6773–85. <https://doi.org/10.1111/jcmm.16681>.
 18. Paul M, Zakar T, Phung J, Gregson A, Barreda AP, Butler TA, Walker FR, Pennell C, Smith R, Paul JW. 20alpha-Hydroxysteroid Dehydrogenase Expression in the Human Myometrium at Term and Preterm Birth: Relationships to Fetal Sex and Maternal Body Mass Index. *Reprod Sci.* 2023. <https://doi.org/10.1007/s43032-023-01183-2>.
 19. Ilicic M, Butler T, Zakar T, Paul JW. The expression of genes involved in myometrial contractility changes during ex situ culture of pregnant human uterine smooth muscle tissue. *J Smooth Muscle Res.* 2017;53:73–89. <https://doi.org/10.1540/jsmr.53.73>.
 20. Ilicic M, Zakar T, Paul JW. Modulation of Progesterone Receptor Isoform Expression in Pregnant Human Myometrium. *Biomed Res Int.* 2017;2017:4589214. <https://doi.org/10.1155/2017/4589214>.
 21. Huggett J, Dheda K, Bustin S, Zumla A. Real-time RT-PCR normalisation; strategies and considerations. *Genes Immun.* 2005;6(4):279–84. <https://doi.org/10.1038/sj.gene.6364190>.
 22. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods.* 2001;25(4):402–8. <https://doi.org/10.1006/meth.2001.1262>.
 23. Loudon JA, Sooranna SR, Bennett PR, Johnson MR. Mechanical stretch of human uterine smooth muscle cells increases IL-8 mRNA expression and peptide synthesis. *Mol Hum Reprod.* 2004;10(12):895–9. <https://doi.org/10.1093/molehr/gah112>.
 24. Sooranna SR, Engineer N, Loudon JA, Terzidou V, Bennett PR, Johnson MR. The mitogen-activated protein kinase dependent expression of prostaglandin H synthase-2 and interleukin-8 messenger ribonucleic acid by myometrial cells: the differential effect of stretch and interleukin-1beta. *J Clin Endocrinol Metab.* 2005;90(6):3517–27. <https://doi.org/10.1210/jc.2004-1390>.
 25. Sooranna SR, Lee Y, Kim LU, Mohan AR, Bennett PR, Johnson MR. Mechanical stretch activates type 2 cyclooxygenase via activator protein-1 transcription factor in human myometrial cells. *Mol Hum Reprod.* 2004;10(2):109–13. <https://doi.org/10.1093/molehr/gah021>.
 26. Terzidou V, Sooranna SR, Kim LU, Thornton S, Bennett PR, Johnson MR. Mechanical stretch up-regulates the human oxytocin receptor in primary human uterine myocytes. *J Clin Endocrinol Metab.* 2005;90(1):237–46. <https://doi.org/10.1210/jc.2004-0277>.
 27. Penning TM, Burczynski ME, Jez JM, Hung CF, Lin HK, Ma H, Moore M, Palackal N, Ratnam K. Human 3alpha-hydroxysteroid dehydrogenase isoforms (AKR1C1-AKR1C4) of the aldo-keto reductase superfamily: functional plasticity and tissue distribution reveals roles in the inactivation and formation of male and female sex hormones. *Biochem J.* 2000;351(Pt 1):67–77. <https://doi.org/10.1042/0264-6021:3510067>.
 28. Steckelbroeck S, Jin Y, Gopishetty S, Oyesanmi B, Penning TM. Human cytosolic 3alpha-hydroxysteroid dehydrogenases of the aldo-keto reductase superfamily display significant 3beta-hydroxysteroid dehydrogenase activity: implications for steroid hormone metabolism and action. *J Biol Chem.* 2004;279(11):10784–95. <https://doi.org/10.1074/jbc.M313308200>.
 29. Li JKH, Lai PF, Tribe RM, Johnson MR. Transcription factors regulated by cAMP in smooth muscle of the myometrium at human parturition. *Biochem Soc Trans.* 2021;49(2):997–1011. <https://doi.org/10.1042/BST20201173>.
 30. Butler TA, Paul JW, Smith R. Non-conventional signalling in human myometrium by conventional pathways: looking back for a synergistic future. *Curr Opin Physiol.* 2020;13:145–54. <https://doi.org/10.1016/j.cophys.2019.11.010>.
 31. Chen L, Sooranna SR, Lei K, Kandola M, Bennett PR, Liang Z, Grammatopoulos D, Johnson MR. Cyclic AMP increases COX-2 expression via mitogen-activated kinase in human myometrial cells. *J Cell Mol Med.* 2012;16(7):1447–60. <https://doi.org/10.1111/j.1582-4934.2011.01413.x>.
 32. Ilicic M, Zakar T, Gregson A, Hussein WM, Smith R, Paul JW. Histone Deacetylase Inhibitors: Providing New Insights and Therapeutic Avenues for Unlocking Human Birth. *Reprod Sci.* 2022;29(11):3134–46. <https://doi.org/10.1007/s43032-021-00778-x>.
 33. Zierden HC, Ortiz JI, DeLong K, Yu J, Li G, Dimitrion P, Bensouda S, Laney V, Bailey A, Anders NM, Scardina M, Mahendroo M, Mesiano S, Burd I, Wagner G, Hanes J, Ensign LM. Enhanced drug delivery to the reproductive tract using nanomedicine reveals therapeutic options for prevention of preterm birth. *Sci Transl Med.* 2021;13:576. <https://doi.org/10.1126/scitranslmed.abc6245>.
 34. Condon JC, Jeyasuria P, Faust JM, Wilson JW, Mendelson CR. A decline in the levels of progesterone receptor coactivators in the pregnant uterus at term may antagonize progesterone receptor function and contribute to the initiation of parturition. *Proc Natl Acad Sci U S A.* 2003;100(16):9518–23. <https://doi.org/10.1073/pnas.1633616100>.

35. Ensign LM, Tang BC, Wang YY, Tse TA, Hoen T, Cone R, Hanes J. Mucus-penetrating nanoparticles for vaginal drug delivery protect against herpes simplex virus. *Sci Transl Med*. 2012;4(138):138ra79. <https://doi.org/10.1126/scitranslmed.3003453>.
36. Paul JW, Hua S, Ilicic M, Tolosa JM, Butler T, Robertson S, Smith R. Drug delivery to the human and mouse uterus using immunoliposomes targeted to the oxytocin receptor. *Am J Obstet Gynecol*. 2017;216(3):283 e1-283 e14. <https://doi.org/10.1016/j.ajog.2016.08.027>.
37. Roberson AE, Hyatt K, Kenkel C, Hanson K, Myers DA. Interleukin 1 β regulates progesterone metabolism in human cervical fibroblasts. *Reprod Sci*. 2012;19(3):271–81. <https://doi.org/10.1177/1933719111419246>.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.