Pro-inflammatory signals induce 20α-HSD expression in myometrial cells: A key mechanism for local progesterone withdrawal

Lubna Nadeem1 | Rathesh Balendran1 | Anna Dorogin1 | Sam Mesiano2 | Oksana Shynlova1,3,4 | Stephen J. Lye1,3,4

Abstract

Metabolism of progesterone (P4) by the enzyme 20α hydroxysteroid dehydrogenase (20α-HSD) in myometrial cells is postulated to be a mechanism for P4 withdrawal, which occurs concomitant to uterine inflammation (physiologic or infection-induced) and associated activation of transcription factors: NF-κB and AP-1, common to term and preterm labour. We found that 20α-HSD protein is significantly increased in human myometrium during term labour, and in mouse uterus during term and preterm labour. Treatment of human myometrial cells with the pro-inflammatory mediators, lipopolysaccharide (LPS, mimicking infection) and 12-O-tetradecanoylphorbol-13-acetate (TPA, mimicking inflammation), induced 20α-HSD gene expression and increased 20α-HSD protein abundance. LPS treatment decreased P4 release into the culture medium and resulted in up-regulation of GJA1 in the hTERT-HM cells. The NF-κB /AP-1 transcription factors mediated effects of LPS and TPA on 20α-HSD gene transcription. Both pro-inflammatory stimuli induced 20α-HSD promoter activity in LPS/TPA-treated cells which was significantly attenuated by inhibition of NF-κB (JSH: 20 µM) or AP-1 signalling (T5224: 10 µM). Deletion of NF-κB consensus sites abrogated LPS-mediated promoter induction, while removal of AP-1 sites reversed the TPA-mediated induction of 20α-HSD promoter. We conclude that inflammatory stimuli (physiologic or pathologic) that activate NF-κB or AP-1 induce 20α-HSD transcription and subsequent local P4 withdrawal resulting in up-regulation of GJA1 and activation of myometrium that precedes labour.

Keywords

20α-HSD, AP-1, labour, myometrium, NF-κB, preterm birth, progesterone metabolism
1 | INTRODUCTION

The steroid hormone progesterone (P4) is essential for the establishment and maintenance of pregnancy, and in all eutherian species examined so far, P4 withdrawal by decline in P4 levels or disruption of P4 signalling initiates labour. Human parturition that occurs without a systemic decline in maternal P4 levels is speculated to be triggered by local P4 withdrawal in the uterine tissues.\textsuperscript{1,2} Local P4 withdrawal can occur by changes in signalling mediated by the nuclear P4 receptor (PR) isoforms, PR-A and PR-B or by increased metabolism of P4 to a PR-inactive form in uterine target cells. Our previous data suggest a combination of these mechanisms whereby local metabolism/inactivation of P4 within the myometrium leads to the loss of P4/PR-B function and dominance of ligand-independent PR-A activity that induces expression of genes encoding contraction-associated proteins (CAPs) that augment myometrial contractility.\textsuperscript{2} Local intracrine P4 withdrawal can be mediated by the P4-metabolizing enzyme, 20α-hydroxysteroid dehydrogenase (20α-HSD),\textsuperscript{1,2} which belongs to the aldo-keto reductase (AKR) superfamily and acts on a variety of substrates, including steroid hormones and endogenous prostaglandins.\textsuperscript{3,4} In particular, human 20α-HSD (encoded by AKR1C1, murine homologue: Akr1c18) catalyses the conversion of P4 into 20α-hydroxyprogesterone (20α-OHP), a relatively weak progesterin compared with P4.\textsuperscript{5} It has been reported earlier that compared to early gestation (13th week), the P4/20α-OHP ratio is decreased in term (40-42nd week) human myometrium suggesting increased P4 metabolism with advancing gestation.\textsuperscript{5}

The association of 20α-HSD with labour is evident from studies in rodents. In rats, expression of 20α-HSD in ovaries/corpus luteum negatively correlated with the circulating P4 levels, with luteolysis being associated with increased 20α-HSD expression and the fall in P4 that initiates labour.\textsuperscript{6,7} In mice, the knockout of the transcription factor (TF) STAT5b caused early abortion due to increased expression and activity of 20α-HSD in corpus luteum and subsequent P4 withdrawal.\textsuperscript{8,9} Moreover, 20α-HSD gene knockout in mice increased P4 levels and delayed parturition,\textsuperscript{9,10} suggesting a central role of the enzyme in labour onset. This may be critical in facilitating local P4 withdrawal in the myometrium to trigger labour. In lower mammals despite the decline in peripheral P4, maternal P4 levels at term remain well above the dissociation constant (Kd) for binding to PRs.\textsuperscript{11}

Similarly in humans, increased expression and abundance of myometrial 20α-HSD\textsuperscript{1} has been associated with local withdrawal of P4 and P4/PR function in myometrium.\textsuperscript{1,2} It is plausible that PTB is caused by premature induction of 20α-HSD activity in myometrial cells, thus understanding how 20α-HSD is regulated is of paramount importance.

The mechanisms by which 20α-HSD is up-regulated in term myometrium\textsuperscript{1,2} in the presence of high P4 levels is unclear since its transcription is suppressed by P4/PR signalling during gestation.\textsuperscript{12,13} A possible explanation is the presence of other factors that induce 20α-HSD expression in the term myometrium. Physiologic inflammation\textsuperscript{14} as a result of maternal and foetal causes, such as mechanical stretch\textsuperscript{15} and foetal lung maturation,\textsuperscript{16–18} that increase cytokines/chemokine levels and corresponding immune cells infiltration might be one such factor.\textsuperscript{19,20} There is ample evidence that in both mice and humans, pro-inflammatory transcription factors (TFs), AP-1\textsuperscript{21,22} and NF-κB,\textsuperscript{16} are induced in the myometrium during term and preterm labour. In humans, cervical expression of AP-1 and NF-κB has shown to be increased prior to parturition.\textsuperscript{23} Since systemic P4 levels remain elevated in humans prior to and during labour, which negatively regulates 20α-HSD expression [reviewed in\textsuperscript{24}], we hypothesized that uterine inflammation causes 20α-HSD induction in myometrium and that the pro-inflammatory TFs, AP-1 and/or NF-κB, play a role in the transcriptional regulation of the gene encoding 20α-HSD.

In this study, we examined the myometrial expression of 20α-HSD in pregnant women and mice, in association with term and preterm labour and determined whether it is affected by pro-inflammatory stimuli and by the activity of NF-κB or AP-1 TFs. We found that myometrial levels of 20α-HSD increase in association with term and preterm labour and that the activation of NF-κB or AP-1 induces AKR1C1 transcription. Our findings suggest a functional link between uterine inflammation and local P4 withdrawal that triggers labour.

2 | MATERIALS AND METHODS

2.1 | Ethical approval for human and murine studies

The study involving human tissues was approved by the Research Ethics Board of Mount Sinai Hospital, Toronto, Canada (REB #18-0168-A). All patients provided a written consent to participate in the study. All murine experiments were approved by the institutional Animal Care Committee (AUP # 21-0164H). Hsd:ICR (CD-1) outbred timed pregnant dams were purchased from Harlan Laboratories (http://www.harlan.com/) and housed under specific pathogen-free conditions at the Toronto Centre for Phenogenomics (TCP) on a 12L:12D cycle and were administered food and water ad libitum.

2.2 | Murine models of labour; Term labour (TL) model

Female mice were mated overnight with males, and the day of vaginal plug detection was designated gestational day (GD) 0.5 of pregnancy. The average time of delivery was the early morning of GD19. Mice were killed by carbon dioxide inhalation, and uteri were collected at 10 AM on all days with the exceptions of the labour sample (TL) that was collected once the animals had delivered at least one pup from average number of 14 in two uterine horns. Myometrial tissue was collected from 4 animals on GD8 (early gestation), GD15 (mid-gestation), GD19 (term not in labour, TNIL), and GD19/20 (term labour, TL).
2.3 | Preterm labour (PTL) models

2.3.1 | LPS-induced PTL

The lipopolysaccharide (LPS) used for this study was isolated from Escherichia coli, serotype O55:B5 (Sigma-Aldrich). On GD15, mice underwent mini-laparotomy under general anaesthesia (isoflurane) with intraperitoneal injection of 125 μg LPS in 100 μL of sterile saline (LPS group) or 100 μL sterile saline (Sham group) or vehicle (100 μL corn oil containing 10% EtOH, vehicle group) (n = 4 per group). PTL occurred in 24 ± 2 hours post-LPS injection. Myometrial samples were collected from LPS group during PTL after delivery of at least one pup, and 24 hours after RU486 treatment. Myometrial samples were collected from RU486 group or vehicle (100 μL corn oil containing 10% EtOH, vehicle group) or vehicle (100 μL sterile saline (Sham group) between two amniotic sacs close to the cervix. Animals (n = 3 per group) were killed during LPS-induced PTL (12-24 hours after the infusion) or 24 hours after a saline injection.

2.3.2 | RU486-induced PTL

On GD15, two groups of mice were subcutaneously injected with either mifepristone (RU486, 17ß- hydroxy-11α-[4-dimethylaminophenyl]-17-[1-propynyl]-estra-4,10-dien-3-ne; Biomol International, 150 μg in 100 μL corn oil containing 10% EtOH, RU486 group) or vehicle (100 μL corn oil containing 10% EtOH, vehicle group) (n = 4 per group). PTL occurred in 24 ± 2 hours post-RU486 treatment. Myometrial samples were collected from RU486 group during PTL after delivery of at least one pup, and 24 hours after vehicle injection from the control group.

2.4 | Myometrial tissue collection

2.4.1 | Human tissues

Healthy pregnant women with a singleton pregnancy undergoing elective caesarean delivery at term (gestational age ≥37 weeks) were recruited as ‘term not in labour’ (TNIL, n = 5). Caesarean delivery of ‘term in labour’ (TL, n = 5) women was performed after the onset of labour (with regular uterine contractions at 10-minutes interval and cervical dilatation >3 cm) for indications of foetal distress. Myometrial biopsy samples of approximately 1 cm³ were excised from the upper margin of the lower uterine segment post-delivery and washed in ice-cold PBS. For protein analysis, a small part of the biopsy was snap-frozen in liquid nitrogen and stored at −80°C; the rest was immediately transferred to 10% neutral-buffered formalin (NBF, Harleco) or 4% paraformaldehyde (PFA, Electron Microscopy Sciences), for 24 hours at 4°C, washed in PBS, dehydrated in increasing grades of ethanol and embedded in paraffin wax.

2.4.2 | Murine tissues

Mice were killed by carbon dioxide inhalation at specific gestational days and during TL or PTL. The part of uterine horn close to cervix from the horn from which a foetus was expelled (post-partum tissue) was removed; the remainder was collected for analysis. For biochemical analyses, uterine horns were bisected longitudinally in ice-cold PBS. The decidua basalis and decidua parietalis was removed as described previously. Myometrial samples were snap-frozen in liquid nitrogen and stored at −80°C for protein analysis. For immunohistochemistry analyses (IHC), one whole uterine horn was fixed in 10% NBF or 4% PFA and processed as described in (A) above.

2.5 | Cell lines and cell culture

The human telomerase immortalized myometrial cells (hTERT-HM) obtained from Dr Jennifer Condon were maintained in DMEM/F12, and human embryonic kidney cell line HEK293T, purchased from ATCC (cat # CRL-3216™), was maintained in DMEM. Media was supplemented with 10% FBS, 100 IU/mL penicillin and 100 μg/mL streptomycin. All the reagents for cell culture were purchased from Invitrogen Canada Inc.

2.5.1 | In vitro treatments

LPS purified from E coli (serotype O55:B5, Cat# L2880), 12-O-tetradecanoylphorbol-13-acetate (TPA, Cat # P1585) and Progesterone (P4, Cat # P8783) were purchased from Sigma-Aldrich. The NF-κB inhibitor: JSH (cat #ab144824) was obtained from Abcam, and AP-1 inhibitor: T5224 (Cat #22904) from Cayman Chemicals. LPS was reconstituted with ultrapure water, P4 with 70% ethanol, while TPA, JSH and T5224 were reconstituted with dimethyl sulphoxide (DMSO) as per manufacturer’s instructions.

2.5.2 | Assessment of AKR1C1 mRNA and protein

The hTERT-HM cells were serum-starved in FBS-free medium supplemented with 1% insulin selenium transferase (ITS), for overnight and then treated with 1) control (C, water) or LPS (1 μg/mL), 2) vehicle (V, 0.1% DMSO) or TPA (20 ng/mL), 3) serum-free medium (SF) or LPS-conditioned medium (CM) for 4 hours for RNA extraction or 24 hours for protein collection. The CM was prepared by treating the cells with LPS for 4 hours, and then, the LPS medium was removed, cells were washed with PBS−/− (without calcium and magnesium addition) and then incubated in serum-free medium (supplemented with 1% ITS) for 16 hours (overnight). The culture medium from cells was collected and named ‘CM’, enriched with secreted factors (cytokines and chemokines) from the LPS-treated cells.

2.5.3 | Activation and Inhibition of NF-κB

hTERT-HM cells were serum-starved as above and then treated with LPS (1 μg/mL) or control. Cell lysates for protein extraction were collected at 0, 15, 30 and 60 minutes to examine phosphorylation of IKBα. To validate the activity of NF-κB inhibitor, the cells were
serum-starved and pretreated with vehicle (0.1% DMSO) or JSH (20 µM) for 30 minutes and then control or LPS was added to cells. The cells were fixed at 2 hours post-LPS treatment, and NF-κB nuclear translocation was examined via immunofluorescence.

2.5.4 | Activation and Inhibition of AP-1

Cells were serum-starved and pretreated with vehicle (0.1% DMSO) or AP-1 inhibitor; T5224 (10 or 20 µM) for 30 minutes and then with TPA (20 or 50 ng/mL) for additional 30 minutes. Phosphorylation of cFOS was assessed via immunoblotting to validate the activation of AP-1 by TPA and inhibition by T5224.

2.5.5 | Progesterone ELISA

hTERT-HM cells were cultured in serum-free medium supplemented with 1% ITS and treated with 1 or 10 nM of P4, or its vehicle with control or LPS (1 µg/mL). Medium was then collected 18 hours post-treatment, and P4 was measured using a P4 ELISA kit according to the manufacturer’s protocol (Cayman Chemicals, Cat # 582601).

2.6 | Protein extraction and immunoblotting

Myometrial tissues were crushed on dry ice, homogenized in lysis buffer [0.08 M Tris/HCl (pH 6.8), 2% SDS, 10% Glycerol] with freshly added protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific Inc) using TissueLyser II (Qiagen). hTERT-HM cells were lysed on ice in lysis buffer (same as above), vortexed for 15 seconds twice and incubated on ice for 10 minutes in between. Homogenates from tissues and cell lysates were then sonicated on ice for 10 seconds and centrifuged at 20,000 × g for 25 minutes at 4°C, and supernatant was collected and stored at −20°C till further processing. Protein concentration was determined by BCA (Thermo Fisher Scientific Inc). Immunoblotting was performed as described earlier. Briefly, equal amount of protein was separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Trans-blot Turbo Midi PVDF, Bio-Rad) using Turbo Trans-Blot system (Bio-Rad). After blocking for an hour with 5% milk prepared in TBS-T, the membranes were incubated with primary antibody at 4°C for overnight. The membranes were subsequently incubated with horseradish peroxidase (HRP)–conjugated secondary antibody at room temperature for 1 hour. Signals were detected using Luminata HRP substrate (Millipore) or SuperSignal™ West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific), and imaging was performed with ChemiDoc imaging system (Bio-Rad). Antibodies used for immunoblotting are listed in Table 1. Immunoblotting for ERK2 was used as loading control. Densitometric analysis was performed using Image Lab system (Bio-Rad).

2.7 | Immunofluorescence

Paraffin-embedded human myometrium tissues were sectioned at 5 µm thickness, and slides were baked at 60°C overnight. The

| Antibody | Catalogue # | Dilution | Source |
|----------|-------------|----------|--------|
| 20α-HSD/AKR1C1 | GTX105620 | WB - 1:1000 | GeneTex |
| SMA | M0851 | 1:100 | DAKO |
| ERK2 | sc-154 | 1:2000 | Santa Cruz |
| p-cFOS | 5348 | 1:500 | Cell Signaling |
| cFOS | sc-52 | 1:200 | Santa Cruz |
| Phospho-IkBα (Ser32/36) | 9246 | 1:500 | Cell Signaling |
| IkBα | 4812 | 1:500 | Cell Signaling |
| NF-κB | ab16502 | 1:100 | Abcam |
| Cx43 | ab1728 | 1:1000 | Millipore |
| Goat anti-rabbit IgG-HRP | sc-2004 | 1:5000-1:10 000 | Santa Cruz |
| Goat anti-mouse IgG-HRP | sc-2005 | 1:5000-1:10 000 | Santa Cruz |
| Horse Anti-Rabbit IgG Antibody (H + L), Biotinylated | BA-1100-1.5 | 1:200 | Vector Labs |
| Streptavidin, Alexa Fluor™ 594 conjugate | S11227 | 1:1000 | Thermo Fisher |
| Donkey anti-Mouse IgG (H + L) Secondary Antibody, Alexa Fluor 488 | A21202 | 1:200 | Thermo Fisher |
| Donkey anti-Rabbit IgG (H + L) Secondary Antibody, Alexa Fluor 488 | A21206 | 1:300 | Thermo Fisher |

Abbreviations: WB, Western blotting; IF, immunofluorescence.
slides were de-waxed in xylene; 3 changes for 10, 5 and 5 minutes, respectively, and then rehydrated in the descending grades of ethanol (90%, 80%, 70%, 50% for 2 minutes each). Microwave heat treatment was performed for antigen retrieval using DAKO target antigen retrieval solution (Cat #S2367), followed by treatment with 0.01% Triton X-100 for 3 minutes. Auto-fluorescence was quenched using 1% Sudan black solution in 70% ethanol for 1 minute followed by 3 washes in PBS (10 minutes each). Slides were then treated with blocking solution (Dako protein block Cat # S3022) for one hour at room temperature. Incubation with primary antibodies (20α-HSD and smooth muscle actin (SMA), listed in Table 1) diluted in DAKO Antibody Diluent with Background Reducing Components (Cat # S3022) was done overnight at 4°C. The next day slides were washed 3 times with PBS (10 minutes each) and secondary antibodies, Alexa Fluor 488 donkey anti-mouse for SMA and biotinylated horse anti-rabbit for 20α-HSD, were applied together for one hour. After another set of PBS washes Streptavidin-Alexa Fluor™ 594 conjugate (for 20α-HSD) was applied to the slides. DAPI (1:1000; Sigma-Aldrich, Cat # D9542) was used to stain nuclei and slides were mounted with antifade mounting medium Vectashield, (Vector Labs). Rabbit and mouse IgGs (matched concentration as primary antibodies) were used as negative control.

NF-κB immunofluorescence staining in cells was performed utilizing a method described earlier. The hTERT-HM cells (treated as described above for 2 hours) in chamber slides (Cat # 154534PK, Thermo Fisher Scientific) were fixed with ice-cold (−20°C) methanol:acetone (1:1) for 3 minutes, washed with PBS thrice, permeabilized with 0.2% Triton X-100 for 5 minutes, washed with PBS thrice and blocked with 1% bovine serum albumin (BSA) solution prepared in PBS for 1 hour at room temperature. NF-κB antibody was applied to the cells for overnight at 4°C. Next day, cells were washed three times with PBS (10 minutes on rocking platform) and incubated with Alexa Fluor 488-conjugated donkey anti-rabbit IgG (H + L) secondary antibody for 30 minutes at room temperature. Cells were washed with PBS and mounted with antifade mounting medium.

**TABLE 2**  Sequences of primers used for RT-PCR

| Gene Symbol | Gene Symbol | Primer Sequence (5′ to 3′) | NCBI Ref. Sequence | Amplicon Size (bp) |
|-------------|-------------|----------------------------|--------------------|-------------------|
| AKR1C1      | Forward     | CAGCCAGGCTAGTGACAGAA       | NM_001353.6        | 145*              |
|             | Reverse     | ATTGCCAATTITGAGGCG       |                    |                   |
| GJA1        | Forward     | ATGACGACTGCTGCACTTTTG    | NM_000165.5        | 249               |
|             | Reverse     | TCTGCTTTAAGTGACATGTC    |                    |                   |
| GAPDH       | Forward     | AGATCATCACGCAATGCTCC     | NM_002046.7        | 92                |
|             | Reverse     | CATGACTCTCTTCCAGATAC     |                    |                   |
| YWHAZ       | Forward     | CGGCAGGGAAAAACGATAT      | NM_003406.4        | 94                |
|             | Reverse     | ACTTTTTGTACATTGTTGGCTC   |                    |                   |
| TBP         | Forward     | CCACAGCTTTTCCACTACA      | NM_003194.5        | 138               |
|             | Reverse     | CTGCCGTACAAATCCAGAAC     |                    |                   |

*Reference9.

**TABLE 3**  Sequences of primers used for mutagenesis of 20α-HSD promoter

| Primer name | Primer sequence (5′ to 3′) | Deletion |
|-------------|----------------------------|----------|
| NF-κB-distal-sense | 5′-CAGGACTGCACTTTGATCAGAGTTCATTACCAAGGC TAAATAAG-3′ | −586 to −572 |
| NF-κB-distal-antisense | 5′-CTTATTAGCCTTGAAGATCCCTGATCAAGGTCATGCA GTCCG-3′ | −324 to −312 |
| NF-κB-proximal-sense | 5′-GCCGCTAGAGGTTTCTGTATTCTTAAATTTTGTTCTA CAATCCTTTTGA-3′ | −464 to −456 |
| NF-κB-proximal-antisense | 5′-TCAGAAGTATTGTAGACCACAATTTAAGATACAG AAAACCTCAGCGGC-3′ | −417 to −409 |
Fluorescent microscopy was performed using Leica DM IL LED-Inverted fluorescence microscope with micropublisher 5.0 RTV Q imaging system or Leica Spinning Disc Confocal Microscope under different magnifications.

### 2.8 | RT-PCR

Total RNA was extracted from cells using RNeasy Mini Isolation Kit (Qiagen, Cat # 74134), according to manufacturer’s instructions. 500 ng of total RNA was reverse transcribed using iScript Reverse Transcription Supermix (Bio-Rad, Mississauga, Cat # 1725035), according to manufacturers’ instructions. Amplification was carried out in a volume of 25 μL containing PCR Mix, (Thermo Fisher Scientific) and 10 pmol each of forward and reverse primers (synthesized by Eurofin Genomics, Toronto, Ontario, Canada). Primer sequences for 20-α-HSD (AKR1C1) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; internal control) are provided in Table 2. Amplification protocol: 95°C for 30 seconds, 60°C for 45 seconds and 72°C for 30 seconds. For all PCRs, an initial step to activate HotStar Taq at 95°C for 3 minutes and a final extension of 5 minutes at 72°C were also performed. PCR products were visualized on a 1.5-2% agarose gel containing SYBR Safe (Thermo Fisher Scientific). The mRNA levels of GJA1 was determined by quantitative RT-PCR using SYBR green detection chemistry (Sigma). Expression of GJA1 was normalized to the geometric mean of three housekeeping genes: tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (YWHAZ), GAPDH, and TATA box binding protein (TBP) using CFX manager 3.1 software (Bio-Rad Laboratories Ltd.).

### 2.9 | Identification of putative NF-κB and AP-1 consensus binding sites in human AKR1C1 promoter

We used transcription factor binding site (TFBS) search 2.0 which utilizes LASAGNA (Length-Aware Site Alignment Guided by Nucleotide Association) algorithm (https://biogrid-lasagna.engr.uconn.edu/lasagna_search/index.php) to identify the putative NF-κB and AP-1 binding sites in the 5′ flanking region of human AKR1C1 promoter (~886 to +43). Two NF-κB consensus binding sites were identified at positions −586 to −572 and −324 to −312 and two of AP-1 sites at −464 to −456 and −417 to −409.

#### 2.9.1 | Construction of deletion vectors by site-directed mutagenesis

A plasmid vector, plightSwitch-Prom-AKR1C1 (referred as pWT), containing the human AKR1C1 (encodes 20α-HSD) promoter (~860 to +43 bases relative to the transcriptional start site) upstream of the open reading frame encoding firefly luciferase reporter was obtained from Switch Gear Genomics (Product ID: S711489). Deletion vectors for NF-κB (pan-κB) and AP-1 (pΔAP-1) consensus sites (identified above) were created (Figure 5A) using a site-directed mutagenesis kit (Agilent Technologies). Primer sequences for introducing desired mutations are provided in Table 3.

#### 2.9.2 | Transient transfection and luciferase reporter assay

Transient transfection was performed using Lipofectamine 3000 transfection reagent (Thermo Fisher Scientific), following manufacturer’s protocol. HEK293T cells were transiently co-transfected with
pWT and pRSVβgal vector. Cells were allowed to recover 5 hours after transfection by replacement of transfection medium with serum-free DMEM/F12 supplemented with 1% ITS and treated with P4 or vehicle for 24 hours and then harvested in passive lysis buffer (Promega). Luciferase activity was determined using LightSwitch luciferase assay kit (Active Motif, Switch Gear Genomics) as per manufacturer’s instructions. Transfection efficiency was normalized with β-galactosidase activity. Experiments were performed with at least triplicates for each experimental condition and replicated thrice as independent experiments.

2.10 Statistical analysis

The Student t test was used to determine the differences between two groups (non-labouring versus labouring samples or control versus treatment). Differences among several groups were determined by one-way analysis of variance (ANOVA), followed by Dunnet’s multiple comparison test using Prism software (GraphPad Prism). Two-way ANOVA and Tukey's or Sidak's post-tests were used to compare different variables. Differences were considered significant only where P values were less than .05.

3 RESULTS

3.1 20α-HSD is up-regulated in myometrium during labour

The onset of labour at term was associated with increased abundance of 20α-HSD protein in human myometrium as assessed by immunoblotting (P = .03, n = 7, Figure 1A) and immunofluorescence (Figure 1B). In mice, 20α-HSD protein abundance in myometrium assessed by immunoblotting was low during early gestation (GD8) and increased with the advancing gestation (GD15) peaking at GD19 (term not in labour, TNIL) and during TL (Figure 2A). Myometrial tissue collected during PTB induced by LPS or RU486 also had higher levels of 20α-HSD protein compared with sham/vehicle controls (LPS: P = .04, Figure 2B; RU486: P = .04, Figure 2C).
3.2 | Pro-inflammatory stimuli induce human 20α-HSD expression and activity in vitro

LPS and TPA increased the abundance of 20α-HSD mRNA (Figure 3A) and protein (Figure 3B) in hTERT-HM cells. To determine the contribution of secreted factors as a potential mechanism of LPS action, the effect of medium conditioned by myometrial cells treated with LPS (LPS-CM) on AKR1C1 expression was examined. LPS-CM increased AKR1C1 mRNA and 20α-HSD in hTERT-HM cells (Figure 3A,B). LPS decreased the recoverable amount of exogenous P4 in hTERT-HM cell media (Figure 3C). Furthermore, LPS increased GJA1 mRNA (encodes CX43) (Figure 3D) and GJA1 protein in hTERT-HM cells (Figure 3E).

3.3 | Pro-inflammatory stimuli induce human AKR1C1 promoter activity in vitro, NF-κB mediates the effect of LPS while AP-1 transcription factors mediate the effect of TPA on human AKR1C1 promoter activity

To assess the transcriptional regulation of human 20α-HSD by the pro-inflammatory stimuli, the human 20α-HSD promoter (~886 to +43) with a luciferase reporter (pWT) was transfected into human HEK293T cells and treated with LPS or TPA. We found that LPS (0.25-10 µg/mL) and TPA at ≥20 ng/mL were able to induce 20α-HSD promoter activity above the basal transcription levels (P < 0.05; Figure 4A). We determined that LPS treatment of hTERT-HM cells induced phosphorylation of NF-κB inhibitor alpha (IκBα) protein (Figure 4B) and subsequent nuclear translocation of NF-κB, which was blocked by the JSH (NF-κB inhibitor) (Figure 4C). TPA treatment induced AKR1C1 promoter activity in vitro, NF-κB mediates the effect of LPS while AP-1 transcription factors mediate the effect of TPA on human AKR1C1 promoter activity.
**DISCUSSION**

Our previous study\(^2\) and that of Williams et al\(^1\) suggest that parturition in women is, at least in part, triggered by localized \(P4\) withdrawal. Data in this study support the hypothesis that inflammatory mediators associated with term and preterm labour induce localized \(P4\) withdrawal in myometrial cells by increasing expression of \(AKR1C1\) and abundance and activity of its protein product \(20\alpha\)-HSD that metabolizes \(P4\) to an inactive form. Consistent with previous results,\(^1,2\) we found up-regulation of myometrial \(20\alpha\)-HSD during labour in...
human and mouse myometrium (Figures 1 and 2) and in mouse models of preterm labour (LPS- and RU486-model), which suggest that local P4 metabolism may be critical for myometrial activation and labour onset. Unfortunately, we were unable to obtain myometrial tissues from women in preterm labour to examine 20α-HSD expression, which could have strengthened the conclusions drawn from the murine models of PTB.

Our in vitro analyses using human myometrial cells (hTERT-HM) treated with inflammatory stimuli (mimicking infectious or non-infectious conditions) confirm the positive association of 20α-HSD levels with inflammation. Notably, the effect caused by LPS treatment itself was similar to the effect of LPS-CM in the induction of 20α-HSD levels. LPS-induced signal transduction leads to secretion of multiple pro- and anti-inflammatory cytokines/chemokines by the treated cells, which can impart their effects in an autocrine/paracrine manner. Our previous study showed that static mechanical stretch also induces secretion of numerous cytokines and chemokines (eg IL-6, CXCL8, CXCL1, MIF, VEGF, G-CSF, IL-12, bFGF and PDGF-bb) from the hTERT-HM cells, which suggests that uterine stretch may also contribute to 20α-HSD-mediated local P4 metabolism.

The consequence of 20α-HSD up-regulation is increased metabolism of P4 and reduction in the levels of biologically active P4. In this study, we provide evidence of P4 depletion in the culture medium of human myometrial cells treated with LPS. Since steroids freely move across the cell membrane down their concentration gradient, depletion of P4 in the culture medium of LPS-treated cells suggests increased metabolism of intracellular P4 within the myometrial cells. These data are consistent with increased 20α-HSD in response to inflammatory stimuli.

It is known that infection/inflammation regulate the activity of AP-1 and NF-κB. LPS differentially activates AP-1 and NF-κB depending upon its serotype. In this study, we treated hTERT-HM cells with LPS-O55:B5 serotype to mimic infection, which resulted in phosphorylation of NF-κB inhibitor, IκBα, and nuclear translocation/activation of NF-κB, while treatment with TPA to mimic non-infectious inflammation caused phospho-activation of AP-1 protein (Figure 4B-D). It has been reported that LPS acts through Toll-like receptor 4 to activate downstream signalling in which MyD88/TNF-associated factor 6 (TRAF6) phosphorylates IκBα, which results in its dissociation from and subsequent nuclear translocation/activation of NF-κB. TPA has been used as a non-infectious

**FIGURE 5** LPS mediates its effects on AKR1C1 promoter activity via NF-κB consensus sites, while TPA activates it through AP-1 binding sites. A, Diagram of human 20α-HSD/AKR1C1 promoter-luciferase reporter construct (pWT) showing number and position of AP-1 and NF-κB consensus binding sites in 5′ flanking region of human AKR1C1 gene (−860 to +43), and the mutant vectors generated via site-directed mutagenesis with deletion of AP-1 sites (pΔAP-1) or NF-κB sites (pΔκB). B, HEK293T cells were transiently co-transfected with pWT or pΔκB or pΔAP-1 and pRSVβgal, treated with control or LPS (1 µg/mL) or vehicle or TPA (50 ng/mL) for 24 h. The relative luciferase to β-galactosidase activity is represented by mean ± SEM, as fold induction compared to cells treated with vehicle/control. Data are pooled from n = 3 independent experiments performed in quadruplicate. Statistical significance was determined by one-way ANOVA followed by Tukey’s multiple comparisons test. Significance between control (C) and LPS is represented by ‘*’ and between pWT and mutant vectors is shown by ‘α’. ‘**’ denotes statistical significance at P < .05, ‘***’ and ‘ααα’ at P < .01 and ‘αααα’ at P < .001. RLU = Relative Luminescence Unit.
model of inflammation and is known to induce activity of AP-1 TFs and GJA1 expression through the protein kinase C pathway.\textsuperscript{22,33–35} Whether it is normal term labour (physiologic inflammation) or infection-induced PTL, myometrial activation of AP-1 and/or NF-κB is observed,\textsuperscript{21,36} and current study suggests that both of these TFs can induce AKR1C1 promoter activity and therefore might contribute to local P4 withdrawal.

Regulation of 20α-HSD in reproductive tissues is multi-faceted. While we determined that NF-κB and AP-1 affect the transcription of the AKR1C1 in the myometrium, studies conducted in other reproductive tissues have shown the involvement of other factors. For example, one of the molecular pathway attributed to the regulation of myometrial expression of AKR1C1 is the miR200-STAT5b axis. STAT5b is a transcriptional repressor of AKR1C1.\textsuperscript{9} The targeted degradation of STAT5b by miR200 results in de-repression of AKR1C1 transcription. However, this negative regulatory axis is under the influence of P4, which up-regulates TF: ZEB1, to keep the expression of miR200 family in check.\textsuperscript{13}

Prolactin is reported to suppress the ovarian expression of Akr1c18 (rat homologue of AKR1C1) during early pregnancy, leading to higher levels of P4.\textsuperscript{37,38} PGF\textsubscript{2α} mediates activation and up-regulation of AP-1 (JUND) and NUR77 TF in the corpus luteum of rats to induce Akr1c18 and participate in P4 withdrawal and labour onset.\textsuperscript{39} In mouse ovarian cells, Akr1c18/AKR1C1 transcription is shown to be regulated by Sp-1 and Sp-3 TFs\textsuperscript{40} and in human ovaries by NF-Y/CEBP.\textsuperscript{41}

The AP-1 family of TFs were previously implicated in regulation of 20α-HSD gene promoter activity in the monkey.\textsuperscript{42} In this study, we determined that the 5' flanking region of AKR1C1 (−860 to +43) is responsive...
to LPS and TPA. Moreover, blocking NF-κB or AP-1 activity, using specific inhibitors, attenuated AKR1C1 promoter activity suggesting that both TFs regulate human AKR1C1 transcription. The targeted deletion of NF-κB and AP-1 consensus sites revealed that NF-κB mediates infection (LPS)-induced AKR1C1 transcription, while the AP-1 pathway is critical for inflammation (TPA)-induced AKR1C1 transcription. Given that both AP-1 and NF-κB are activated in myometrium prior to labour, we suggest that these TFs are pivotal in the up-regulation of 20α-HSD and consequent localized P4 withdrawal in myometrial cells. We have previously found that the AP-1 specific dimer composition differentially regulates gene expression and that the activity of nuclear AP-1 heterodimers (FRA1/JUNB and FRA2/JUND) is associated with human labour. Elucidation of the specific role of different AP-1 dimers in the regulation of 20α-HSD transcription will be important to delineate the molecular regulation of AKR1C1 expression.

Taken together, our findings provide a molecular mechanism linking uterine inflammation and myometrial P4 withdrawal, which precedes labour onset (summarized in Figure 6). We propose that up-regulation of myometrial AKR1C1/20α-HSD during labour is conserved in term (mice and human) and preterm parturition (mice). We found that inflammatory signals, driven by mimics that are widely accepted as representing infection and inflammation, induce AKR1C1 expression through the activation of AP-1 or NF-κB TFs. The resultant increase in 20α-HSD may cause local P4 withdrawal that remove inhibition of CAP gene expression, especially GJA1, that cause the contraction of labour by increasing myometrial cell contractility and excitability. We suggest that present data on local metabolism of P4 in myometrium explain the conflicting results on the effectiveness of clinical P4 treatment aimed at preventing PTB. It may be possible, therefore, to clinically inhibit labour and prevent PTB using anti-inflammatory therapeutics that inhibit the activation of AKR1C1 expression and 20α-HSD activity in myometrial cells, thus preserving the relaxatory and pro-gestational actions of P4.

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CONFLICT OF INTERESTS

The authors have no conflicts of interest.

AUTHOR CONTRIBUTIONS

Lubna Nadeem: Conceptualization (lead); Data curation (lead); Formal analysis (lead); Funding acquisition (supporting); Investigation (lead); Methodology (lead); Project administration (lead); Supervision (lead); Validation (equal); Visualization (equal); Writing-original draft (equal).

Ratheesh Balendran: Data curation (supporting); Formal analysis (supporting); Investigation (supporting); Methodology (supporting); Validation (supporting); Visualization (supporting).

Anna Dorogin: Data curation (supporting); Investigation (supporting); Methodology (supporting); Validation (supporting); Visualization (supporting).

Sam Mesiano: Conceptualization (supporting); Funding acquisition (supporting); Writing-review & editing (equal).

Oksana Shynlova: Conceptualization (lead); Funding acquisition (equal); Investigation (supporting); Methodology (supporting); Project administration (equal); Supervision (equal); Validation (supporting); Visualization (supporting); Writing-review & editing (lead).

Stephen J Lye: Conceptualization (lead); Funding acquisition (lead); Project administration (lead); Resources (lead); Supervision (lead); Visualization (equal); Writing-review & editing (equal).

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

REFERENCES

1. 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. 2012;109:7529-7534.

2. 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.

3. Jez JM, Bennett MJ, Schlegel BP, Lewis M, Penning TM. Comparative anatomy of the aldol-keto reductase superfamily. Biochim J. 1997;326(Pt 3):625-636.

4. Penning TM. Molecular endocrinology of hydroxysteroid dehydrogenases. Endocr Rev. 1997;18:281-305.

5. Runnebaum B, Zander J. Progesterone and 20α-hydroxyprogesterone in human myometrium during pregnancy. Acta Endocrinol [Suppl]. 1971;150:3-45.

6. Wiest WG, Kidwell WR, Balogh K Jr. Progesterone catabolism in the rat ovary: a regulatory mechanism for prostaglandin potency during pregnancy. Endocrinol. 1968;82:844-859.

7. Albarracin CT, Parmer TG, Duan WR, Nelson SE, Gibori G. Identification of a major prolactin-regulated protein as 20α-hydroxysteroid dehydrogenase: coordinate regulation of its activity, protein content, and messenger ribonucleic acid expression. Endocrinol. 1994;134:2453-2460.

8. Teglund S, McKay C, Schuetz E, et al. Stat5a and Stat5b proteins have essential and nonessential, or redundant, roles in cytokine responses. Cell. 1998;93:841-850.

9. Piekorz RP, Gingras S, Hoffmeyer A, Ihle JN, Weinstein Y. Regulation of progesterone levels during pregnancy and parturition by signal transducer and activator of transcription 5 and 20α-hydroxysteroid dehydrogenase: Mol Endocrinol. 2007;19:431-440.

10. Ishida M, Choi JH, Hirabayashi K, et al. Reproductive phenotypes in mice with targeted disruption of the 20α-hydroxysteroid dehydrogenase gene. J Reprod Dev. 2007;53:499-508.

11. Poeltz G, Rao B, Latreille MT, Mignot TM, Cedard L. Progesterone levels in the circulating blood of the ovarian and uterine veins during gestation in the mouse. Biol Reprod. 1981;24:801-805.

12. Braden CP, Gregory PA, Kolesnikoff N, et al. A double-negative feedback loop between ZEB1-SIP1 and the microRNA-200 family regulates epithelial-mesenchymal transition. Cancer Res. 2008;68:7846-7854.

13. Renthal NE, Chen CC, Williams KC, Gerard RD, Prange-Kiel J, Mendelson CR. miR-200 family and targets, ZEB1 and ZEB2, modulate uterine quiescence and contractility during pregnancy and labor. Proc Natl Acad Sci. 2010;107:20828-20833.

14. Shynlova O, Lee YH, Srikhajon K, Lye SJ. Physiologic uterine inflammation and labor onset: integration of endocrine and mechanical signals. Reprod Sci. 2013;20:154-167.
15. Manabe Y, Sakaguchi M, Nakajima A. Initiation of uterine contractions by purely mechanical stretching of the uterus at midpregnancy. *Int J Biol Res Pregnancy*. 1981;2:63-69.

16. Condon JC, Jeyasuria P, Faust JM, Mendelson CR. Surfactant protein secreted by the maturing mouse fetal lung acts as a hormone that signals the initiation of parturition. *Proc Natl Acad Sci*. 2004;101:4978-4983.

17. Gao L, Rabitt EH, Condon JC, et al. Steroid receptor coactivators 1 and 2 mediate fetal-to-maternal signaling that initiates parturition. *J Clin Invest*. 2015;125:2808-2824.

18. Reini EL, England SK. Fetal-to-maternal signaling to initiate parturition. *J Clin Invest*. 2015;125:2569-2571.

19. Osman I, Young A, Ledingham MA, et al. Leukocyte density and pro-inflammatory cytokine expression in human fetal membranes, decidua, cervix and myometrium before and during labour at term. *Mol Hum Reprod*. 2003;9:41-45.

20. Shynlova O, Nadeem L, Zhang J, Dunk C, Lye S. Myometrial activation: Novel concepts underlying labor. *Placenta*. 2020;92:28-36.

21. MacIntyre DA, Lee YS, Migale R, et al. Activator protein 1 is a key terminal mediator of inflammation-induced preterm labor in mice. *FASEB J*. 2017;31:2358-2368.

22. Nadeem L, Farine T, Dorogin A, Matsyiak-Zablocki E, Shynlova O, Lye S. Differential expression of myometrial AP-1 proteins during gestation and labour. *J Cell Mol Med*. 2018;22:452-471.

23. Wang H, Stjernholm YV. Plasma membrane receptor mediated MAPK signaling pathways are activated in human uterine cervix at parturition. *Reprod Biol Endocrinol*. 2007;5:3.

24. Renthal NE, Williams KC, Montalbano AP, Chen C-C, Gao LU, Mendelson CR. Molecular regulation of parturition: a myometrial perspective. *Cold Spring Harb Perspect Med*. 2015;5(11):a023069.

25. Piersanti M, Lye SJ. Increase in messenger ribonucleic acid encoding the myometrial gap junction protein, connexin-43, requires protein synthesis and is associated with increased expression of the activator protein-1, c-fos. *Endocrinol*. 1995;136:3571-3578.

26. Condon J, Yin S, Mayhew B, et al. Telomerase immortalization of human myometrial cells. *Biol Reprod*. 2002;67:506-514.

27. Nadeem L, Shynlova O, Mesiano S, Lye S. Progesterone via its type-A receptor promotes myometrial gap junction coupling. *Sci Rep*. 2017;7:13357.

28. Lee C, Huang CH. LASAGNA: a novel algorithm for transcription factor binding site alignment. *BMC Bioinform*. 2013;14:108.

29. Li W, Yang S, Kim SO, Reid G, Challis JR, Bocking AD. Lipopolysaccharide-induced profiles of cytokine, chemokine, and growth factors produced by human decidual cells are altered by lactobacillus rhamnosus GR-1 supernatant. *Reprod Sci*. 2014;21:939-947.

30. Lee YH, Shynlova O, Lye SJ. Stretch-induced human myometrial cytokines enhance immune cell recruitment via endothelial activation. *Cell Mol Immunol*. 2015;12:231-242.

31. Migale R, Herbert BR, Lee YS, et al. Specific lipopolysaccharide serotypes induce differential maternal and neonatal inflammatory responses in a murine model of preterm labor. *Am J Path*. 2015;185:2390-2401.

32. Sakai J, Cammarota E, Wright JA, et al. Lipopolysaccharide-induced NF-kB nuclear translocation is primarily dependent on MyD88, but TNFα expression requires TRIF and MyD88. *Sci Rep*. 2017;7:1428.

33. Geimonen E, Jiang W, Ali M, Fishman GI, Garfield RE, Andersen J. Activation of protein kinase C in human uterine smooth muscle induces connexin-43 gene transcription through an AP-1 site in the promoter sequence. *J Biol Chem*. 1996;271:23667-23674.

34. Karin M, Liu Z, Zandi E. AP-1 function and regulation. *Curr Opin Cell Biol*. 1997;9:240-246.

35. Mitchell JA, Lye SJ. Regulation of connexin43 expression by c-fos and c-jun in myometrial cells. *Cell Commun Adhes*. 2001;8:299-302.

36. Condon JC, Hardy DB, Kovicar K, Mendelson CR. Up-regulation of the progesterone receptor (PR)-C isoform in laboring myometrium by activation of nuclear factor-kappaB may contribute to the onset of labor through inhibition of PR function. *Mol Endocrinol*. 2006;20:764-775.

37. Albarracin CT, Gibori G. Prolactin action on luteal protein expression in the corpus luteum. *Endocrinol*. 1991;129:1821-1830.

38. El-Kabbani O, Dhaqat U, Hara A. Inhibitors of human 20α-hydroxysteroid dehydrogenase (AKR1C1). *J Steroid Biochem Mol Biol*. 2011;125:105-111.

39. Stocco CO, Lau LF, Gibori G. A calcium/calmodulin-dependent activation of ERK1/2 mediates JunD phosphorylation and induction of nur77 and 20alpha-hsd genes by prostaglandin F2alpha in ovarian cells. *J Biol Chem*. 2002;277:3293-3302.

40. Hirabayashi K, Ishida M, Suzuki M, Yamanouchi K, Nishihara M. Characterization and functional analysis of the 5′-flanking region of the mouse 20alpha-hydroxysteroid dehydrogenase gene. *Biochem J*. 2004;382:975-980.

41. Pallai R, Simpkins H, Chen J, Parekh HK. The CCAAT box binding transcription factor, nuclear factor-Y (NF-Y) regulates transcription of human aldo-keto reductase 1C1 (AKR1C1) gene. *Gene*. 2010;459:11-23.

42. Nanjidurens T, Min K-S. The transcription factor Ap-1 regulates monkey 20α-hydroxysteroid dehydrogenase promoter activity in CHO cells. *BMC Biotechnol*. 2014;14:71.

43. Khanjani S, Kandola MK, Lindstrom TM, et al. NF-kappaB regulates a cassette of immune/inflammatory genes in human pregnant myometrium at term. *J Cell Mol Med*. 2011;15:809-824.

44. Lim S, MacIntyre DA, Lee YS, et al. Nuclear factor kappa B activation occurs in the amnion prior to labour onset and modulates the expression of numerous labour associated genes. *PLoS One*. 2012;7:e34707.

45. Meis PJ, Klebanoff M, Thom E, et al. Prevention of recurrent preterm delivery by 17 alpha-hydroxyprogesterone caproate. *N Engl J Med*. 2003;348:2379-2385.

46. Nelson DB, McIntire DD, McDonald J, Gard J, Turriichi P, Leveno KJ. 17-alpha Hydroxyprogesterone caproate did not reduce the rate of recurrent preterm birth in a prospective cohort study. *Am J Obstet Gynecol*. 2017;216:e1-600.e9.

47. Blackwell SC, G Yamfi-Bannerman C, Biggio JR Jr, et al. 17-OHPC to prevent recurrent preterm birth in singleton gestations (PROLONG Study): a Multicenter, International, randomized double-blind trial. *Am J Perinatol*. 2020;37:127-136.

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