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Prokineticin 1 Induces Inflammatory Response in Human Myometrium

A Potential Role in Initiating Term and Preterm Parturition

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The infiltration of human myometrium and cervix
with leukocytes and the formation of a pro-inflam-
matory environment within the uterus have been
associated with the initiation of both term and pre-
term parturition. The mechanism regulating the on-
set of this pro-inflammatory cascade is not fully
elucidated. We demonstrate that prokineticin 1
(PROK1) is up-regulated in human myometrium
and placenta during labor. The expression of
PROK1 receptor remains unchanged during labor
and is abundantly expressed in the myometrium.
Gene array analysis identified 65 genes up-regu-
lated by PROK1 in human myometrium, mainly cy-
tokines and chemokines, including IL-1β, chemo-
kine C-C motif ligand 3, and colony-stimulating
factor 3. In addition, we demonstrate that PROK1
increases the expression of chemokine C-C motif
ligand 20, IL-6, IL-8, prostaglandin synthase 2, and
prostaglandin E$_2$ and F$_{2\alpha}$ secretion. The treatment
of myometrial explants with 100 ng/mL of lipopoly-
saccharide up-regulates the expression of PROK1,
PROK1 receptor, and inflammatory mediators. The
infection of myometrial explants with lentiviral mi-
croRNA targeting PROK1, preceding treatment with
lipopolysaccharide, reduces the expression of in-
flammatory genes. We propose that PROK1 is a
novel inflammatory mediator that can contribute to
the onset of human parturition at term and partially
mediate premature onset of inflammatory path-
ways during bacterial infection. *(Am J Pathol 2011,
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Human parturition is a pro-inflammatory event associated
with the influx of leukocytes, such as neutrophils and
macrophages, to the uterus.[1,2] Indeed, 40% to 60% of
genes up-regulated in the myometrium and cervix at la-
bor are involved in inflammation and chemotaxis.[3] Re-
cruited leukocytes secrete cytokines, including IL-1β,
IL-6, and IL-8,[4] and prostaglandins E$_2$ (PGE$_2$) and F$_{2\alpha}$
(PGF$_{2\alpha}$). These cytokines and prostaglandins can stimu-
late production of matrix metalloproteinases that are in-
volved in membrane rupture[5] and can also promote cer-
vical ripening.[6] However, during labor, the greatest influx
of leukocytes is observed in the myometrium.[1] Cytokines,
such as IL-1β, released by leukocytes, promote the syn-
thesis of prostaglandin synthase 2 (PTGS2)–dependent
prostaglandins (PGE$_2$ and PGF$_{2\alpha}$), which are important
regulators of myometrial contractility.[7,8] More important,
independently of prostaglandins, IL-1β also regulates
basal and store-operated calcium entry in myometrial
smooth muscle cells, which is important for contractions
of individual smooth muscle cells.[9]

The same pro-inflammatory events can be initiated
before 37 weeks of gestation and lead to premature onset
of parturition and preterm birth. Nearly 11% of all single-
ton deliveries are preterm, and this constitutes the big-
gest cause of neonatal morbidity and mortality.[10,11] Al-
though several factors, such as smoking, alcohol,
advanced maternal age, and structural abnormalities of
the cervix,[12,13] can contribute to preterm initiation of la-
bor, bacterial infection in the uteroplacental unit is be-
lieved to be a major cause. Such infections are detected
in 79% of births at 23 weeks and in 11% of births at 31 to
34 weeks.[14,15] In most cases of preterm birth, even in the

absence of infection, histological evidence of inflammation is apparent in the uteroplacental unit.[16,17] Intrauterine inflammation is associated with adverse perinatal outcome.[18] However, therapies for preterm birth have predominantly focused on inhibition of the onset of labor. Therefore, there is increasing recognition that effective therapies should target myometrial contractility and inhibit/reduce intrauterine inflammation.

Prokineticins 1 and 2 (PROK1 and PROK2, respectively) are multifunctional secreted proteins that signal via two G protein–coupled receptors termed PROK receptors 1 and 2 (PROKR1 and PROKR2, respectively). PROKs induce smooth muscle contractility of the gut,[19,20] which is mediated via increased calcium influx into smooth muscle cells.[19,20] PROKs also have potent effects in multiple other processes, including neurogenesis, angiogenesis, hematopoiesis, and nociception.[21] There is increasing evidence that PROK1, via PROKR1, can modulate immune responses. PROK1 can promote differentiation of bone marrow progenitors into macrophage-like adherent cells.[22] In addition, PROK1 induces the expression of chemokines, including chemokine C-C motif ligand 4 (CCL4), CXCL1, and IL-8 (CXCL8) in human monocytes, which constitutes an important regulatory mechanism for leukocyte recruitment to the site of inflammation.[23]

PROKs may be important regulators of female reproductive functions, including regulation of uterine receptivity[24] and placental function.[25–27] Considering their well-described roles in mediating inflammatory pathways and smooth muscle contractility, we hypothesized that PROK1, via PROKR1, may be important in regulating events leading to the onset of parturition. This study was designed to determine the expression and potential role of PROK1 and PROKR1 in the human myometrium during labor.

Materials and Methods

Patients and Tissue Collection

Full-thickness lower-segment biopsy specimens of human myometrium, placenta, and fetal membranes were collected from women undergoing elective caesarean section at term (>37 weeks) before the onset of labor (NL) and women in spontaneous labor at term (>37 weeks) who required emergency lower-segment caesarean section (L). Women with medical complications, such as diabetes or hypertension, or with symptoms of infection (determined by temperature >38°C) were excluded from the study. Patients were also excluded if the pregnancy was multiple or if they received either prostaglandin for induction or oxytocin for augmentation of labor. Written consent was obtained from each patient before recruitment, in accordance with approval from the Lothian Local Research Ethics Committee (REC reference no. 07/S1103/25). Immediately after collection, tissue was placed in RNAlater (Applied Biosystems, Warrington, UK) for RNA extraction, fixed in 4% neutral-buffered formalin, and wax embedded for immunohistochemistry (IHC).

Tissue Culture and Treatments

Myometrial tissue explants from women at term and not in labor were collected for *in vitro* studies. Tissue was routinely maintained in RPMI 1640 medium (GIBCO, Invitrogen, Paisley, UK) supplemented with 100 IU penicillin and 100 μg/mL streptomycin (Sigma, Poole, UK) at 37°C and 5% CO_2, as recommended. After collection, explants were finely chopped into equal portions with scissors and incubated overnight. Tissue was treated with vehicle (sterile double-distilled water), 100 ng/mL of lipopolysaccharide (LPS) from *Salmonella abortus equi* (Alexis Biochemicals, Axxora, Nottingham, UK), or 40 nmol/L PROK1 (PeproTech, London, UK) for 2, 4, 6, 8, and 24 hours. In previous studies, the PROK1 and LPS doses used in this study have given optimal responses.[28,29] Tissue and supernatants were collected at all time points. All experiments were performed in duplicate.

Gene Array and Data Analysis

Total RNA was extracted from myometrial explants treated with either vehicle or 40 nmol/L PROK1 for 6 and 24 hours ($n = 6$ for each time point). Sample preparation, array hybridization, and data analysis were performed by the Finnish Microarray and Sequencing Centre and the Turku Centre for Biotechnology (Turku, Finland). Briefly, total RNA was biotin labeled by a single *in vitro* transcription amplification step. Samples were hybridized to Illumina Human HT-12 v.3 Expression BeadChips (Illumina, San Diego, CA) and stained with streptavadin-Cy3. Signal intensities within data sets were normalized by applying quantile normalization. R package Limma[30] (Bioconductor, Open Source Software for Bioinformatics) was used for performing the statistical testing between the groups. Functional grouping of the gene list into statistically significant overrepresented ontologies was performed using the protein analysis through evolutionary relationships database analysis tools (PANTHER).[31]

TaqMan Quantitative Real-Time PCR

Tissue was homogenized in QIAzol (Qiagen, Crawley, UK), followed by RNA extraction with on-column DNase digestion using the RNeasy Mini Kit, according to the manufacturer's instructions (Qiagen). RNA was reverse transcribed using the VILO SuperScript VILO cDNA Synthesis Kit (Invitrogen). TaqMan quantitative real-time PCR was performed using an ABI Prism 7500 System (Applied Biosystems). The sequences of all primers and FAM-labeled probes are listed in Table 1. The expression of target genes was normalized to RNA loading, measured with primers and VIC-labeled probe for ribosomal 18S RNA. Relative gene expression was calculated by using the comparative C_T method. Results of *in vitro* experiments are presented relative to expression in vehicle-treated samples. A comparison of gene expression in tissues collected from women in labor versus not in labor is presented as relative to a calibrator endometrial sample included in all reactions.[28]

Table 1. Sequences of Primers and Probes Used for Analysis of Gene Expression by TaqMan Quantitative Real-Time PCR

Gene	Sequence or category number
PROK1	
Forward	5′-GTGCCACCCGGGCAG-3′
Reverse	5′-AGCAAGGACAGGTGTGGTGC-3′
FAM	5′-ACAAGGTCCCCTTGTTCAGGAAACGCA-3′
CCL20	
Forward	5′-TCCTGGCTGCTTTGATGTCA-3′
Reverse	5′-CCAAGACAGCAGTCAAAGTTGCT-3′
FAM	5′-TGCTGCTACTCCACCTCTGCGGC-3′
PROKR1	
Forward	5′-TCTTACAATGGCGGTAAGTCCA-3′
Reverse	5′-CTCTTCGGTGGCAGGCAT-3′
FAM	5′-TGCAGACCTGGACCTCAAGACAATTGG-3′
IL-1β	
Forward	5′-CGCATCCAGCTACGAAT-3′
Reverse	5′-CATGGCACAACAACTGA-3′
FAM	5′-CGACCACCACTACAGC-3′
IL-6	
Forward	5′-GCCGCCCCACACAGACA-3′
Reverse	5′-CCGTCGAGGATGTACGGAAT-3′
FAM	5′-CCACTCACCTCTTCAGAACGAATTGACAAAC-3′
IL-8	
Forward	5′-CTGGCCGTGGCTCTCTT-3′
Reverse	5′-TTAGCACTCCTTGGCAAAACTG-3′
FAM	5′-CCTTCCTGATTTCTGCAGCTCTGTGTGAA-3′
COX-2	
Forward	5′-CCTTCCTCCTGTGCCTGATG-3′
Reverse	5′-ACAATCTCATTTGAATCAGGAAGCT-3′
FAM	5′-TGCCCGACTCCCTTGGGTGTCA-3′
18S	
Forward	5′-CGGCTACCACATCCAAGGAA-3′
Reverse	5′-GCTGGAATTACCGCGGCT-3′
VIC	5′-TGCTGGCACCAGACTTGCCCTC-3′
CCL3	Hs00234142-m1
CSF3	Hs99999083-m1
VCAM1	Hs01003372-m1
TACC2	Hs00610617-m1
CCL2	Hs00234140-m1
CCL8	Hs00271615-m1

IHC Data

Paraffin-embedded sections were dewaxed in xylene and dehydrated in graded ethanol. Antigen retrieval was performed by boiling for 5 minutes in 0.01 mol/L citrate buffer (pH 6.0), followed by quenching the activity of endogenous peroxidase with 30% H_2O_2 in methanol.

Sections were blocked in a solution of 20% normal goat serum and 5% bovine serum albumin at room temperature, followed by incubation with rabbit anti-human PROK1 at 1:500 dilution (Phoenix Pharmaceuticals, Belmont, CA) or PROKR1 at 1:250 dilution (Life Span Biosciences, Atlanta, GA) overnight at 4°C; negative controls were incubated with rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA). Subsequently, sections were incubated with biotinylated goat anti-rabbit antibody (Vector Laboratories, Peterborough, UK) (1:500), followed by incubation with streptavidin–horseradish peroxidase (GE Healthcare UK, Little Chalfont) (1:1000). Staining was detected with 3,3′-diaminobenzidine (Vector Laboratories, Peterborough, UK) as the chromogen.

Lentivirus miRNA Gene Silencing

Lentivirus microRNA (miRNA) targeting PROK1 mRNA was used to knock down PROK1 expression, as previously described.[28,32] Briefly, myometrial explants ($n = 8$) were finely chopped and subsequently infected with lentivirus containing scrambled sequence (plenti6/V5-EmGFP-miR-neg) as a negative control or PROK1 miRNA (pLenti6/V5-EmGFP-hum-PROK1-72-287) chained constructs for 72 hours. Subsequently, explants were incubated in the presence or absence of 100 ng/mL of LPS for 24 hours. Tissue and supernatants were then harvested, and RNA was extracted from tissues for PCR analysis. Results are presented relative to expression in vehicle-treated samples.

## Measurement of Prostaglandins PGE_2 and $PGF_{2\alpha}$

Myometrium explants ($n = 5$) were incubated in the presence or absence of 40 nmol/L PROK1 for 24 hours. Medium was collected and assayed by enzyme-linked immunosorbent assay for PGE_2 and $PGF_{2\alpha}$, as previously described.[33] Results are presented as pg/mg tissue.

Measurement of IL-1β, CCL3, and CCL20

Myometrial explants were infected with lentiviral constructs containing scrambled or PROK1 miRNA and subsequently treated with 100 ng/mL of LPS for 24 hours, as previously described. Conditioned media were collected
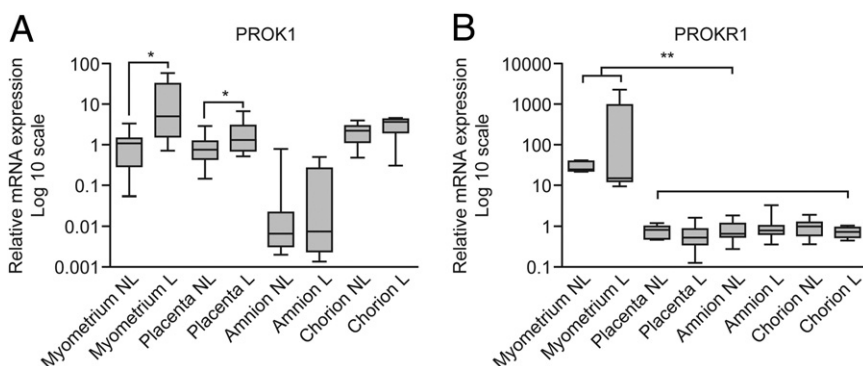
Figure 1. Expression of PROK1 and PROKR1 mRNA in human uteroplacental tissues at term. PROK1 mRNA expression is elevated in myometrium and placenta during labor at term compared with the tissues collected at term before the onset of labor (**A**). Levels of PROKR1 mRNA do not vary with the onset of labor; however, notably, PROKR1 levels are highest in myometrium compared with other uteroplacental tissues (**B**). Results are presented as mean ± SEM. *$P < 0.05$, **$P < 0.0001$ ($n = 10$ to 12).
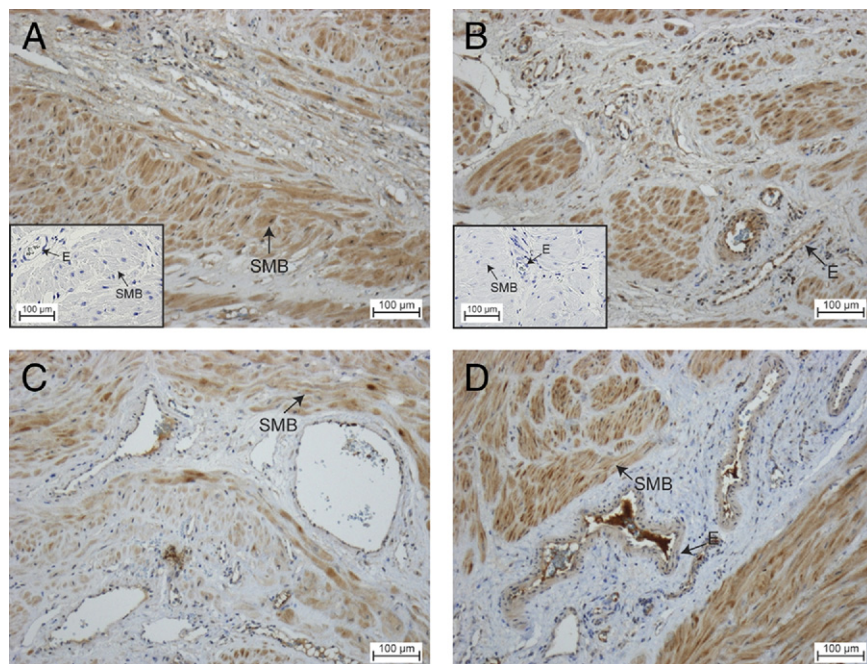
Figure 2. Immunolocalization of PROK1 and PROKR1 in myometrium collected from women at term before the onset of labor or during labor. PROK1 is localized to the smooth muscle bundles (**A**, not in labor; **B**, in labor). PROKR1 was also immunolocalized to the smooth muscle bundles in myometrial tissue collected from women not in labor (**C**) and in labor (**D**). During labor, PROKR1 expression is also localized to vasculature. Control sections were negative for immunoreactivity (**insets**). E, endothelium; SMB, smooth muscle bundles ($n = 16$: $n = 8$ for myometrium collected from women not in labor, and $n = 8$ for myometrium collected from women in labor).

and assayed for IL-1β, CCL3, and CCL20 by using appropriate DuoSet ELISA Development Systems, according to the manufacturer's protocols (R&D Systems, Abingdon, UK). Briefly, 96-well microplates were coated overnight with 100 μL of working concentration of appropriate Capture Antibody diluted in PBS (1:180). The following day, the plates were blocked with 300 μL of 1% bovine serum albumin in PBS for 1 hour at room temperature. Subsequently, 100 μL of samples and appropriate standards were added for 2 hours, followed by incubation with Detection Antibody diluted (1:180) for 2 hours and then incubation with streptavidin–horseradish peroxidase (1:200) for 20 minutes. After the addition of TMB Substrate Solution, optical density was determined at 450 nm using a microplate reader. Results are presented relative to expression in vehicle-treated samples.
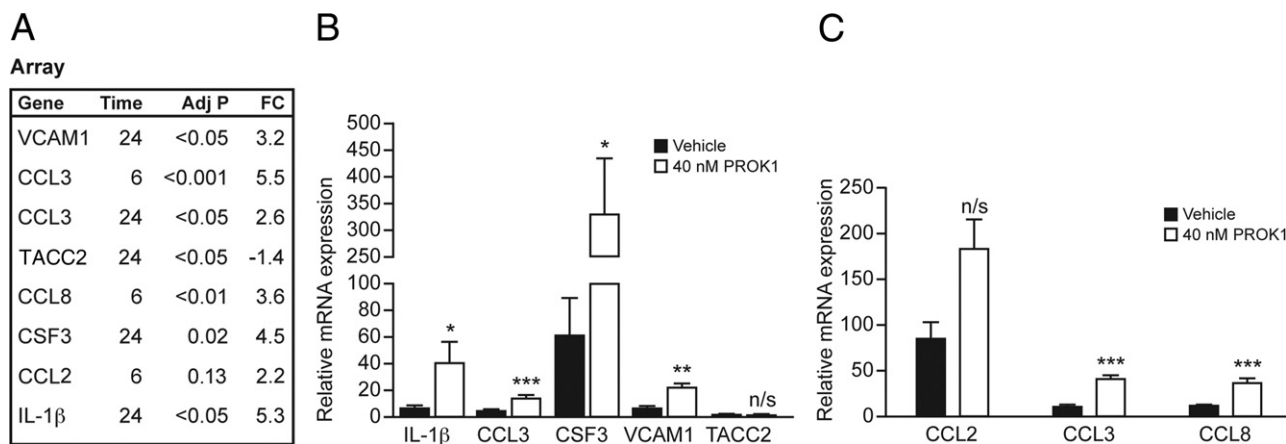
Statistical Analysis

Data were analyzed using an unpaired *t*-test with Welch's correction or a paired *t*-test (GraphPad Prism 5.0; GraphPad Software, La Jolla, CA). The *n* values represent the number of independent experiments. Unless otherwise indicated, data are given as mean ± SEM.

Results

Expression of PROK1 Increases in Uteroplacental Tissues at Labor

PROK1 mRNA expression is significantly elevated during labor (L) versus not labor (NL) in the myometrium (16.78 ±

A

Array

Gene	Time	Adj P	FC
VCAM1	24	<0.05	3.2
CCL3	6	<0.001	5.5
CCL3	24	<0.05	2.6
TACC2	24	<0.05	-1.4
CCL8	6	<0.01	3.6
CSF3	24	0.02	4.5
CCL2	6	0.13	2.2
IL-1β	24	<0.05	5.3

Figure 3. Validation of gene array analysis by quantitative real-time PCR. **A:** List of genes selected to verify the array analysis, including genes representing high- and low-intensity signals and genes that were up- or down-regulated at either the 6- or 24-hour time point. Adj, adjusted; FC, fold change. **B:** Treatment of myometrial explants with PROK1 for 24 hours up-regulates expression of IL-1β, CCL3, CSF3, and VCAM1, whereas levels of transforming acidic coiled-coil–containing protein 2 remained unaffected. **C:** Treatment of myometrial explants with 40 nmol/L PROK1 for 6 hours elevated expression of CCL3 and CCL8, whereas there was no difference in the expression of CCL2. *$P < 0.05$, **$P < 0.005$, and ***$P < 0.0001$. n/s, not significant ($n = 6$). Results are presented as mean ± SEM.

7.0-fold versus 1.15 ± 0.37-fold; $P < 0.05$) and placenta (1.97 ± 0.56-fold versus 0.9 ± 0.17-fold; $P < 0.05$) but not in fetal membranes (Figure 1A). PROKR1 expression does not change at term with the onset of labor in any of the uteroplacental tissues. However, expression is highest in the myometrium compared with other uteroplacental tissues ($P < 0.0001$), suggesting that myometrium may be the main target tissue for PROK1 signaling during labor (Figure 1B).

We subsequently focused on the role of PROK1 and PROKR1 in human myometrium. During IHC, PROK1 was localized in the smooth muscle bundles in the myometrium of women at term before the onset of labor (Figure 2A) and during labor (Figure 2B). Similarly, PROKR1 was immunolocalized to the smooth muscle bundles in myometrial tissue collected from women at term before the onset of labor (Figure 2C) and during labor (Figure 2D). During labor, PROKR1 expression is additionally localized to the vasculature (Figure 2D).

PROK1/PROKR1 Regulates Inflammatory Mediators in Human Myometrium

After having established that PROK1 expression is elevated in the myometrium during labor and that PROKR1 transcript levels are highest in myometrial tissue, we sought to identify the downstream targets of PROK1/PROKR1 signaling in the myometrium. Gene array analysis was used to identify PROK1-regulated transcripts in myometrial explants at term (in six independent samples), treated with 40 nmol/L PROK1 for 6 or 24 hours and compared with vehicle treatment for each time point. The array analysis identified both up- and down-regulated transcripts at both time points (see Supplemental Figure S1, A and B, at *http://ajp.amjpathol.org*; *http://www.ncbi.nlm.nih.gov/geo*, Accession no. GSE28272), with an increase in the number of regulated transcripts from 6 to 24 hours (see Supplemental Figure S1C at *http://ajp.amjpathol.org*). The analysis shown there was a distinct response at 6 hours after PROK1 treatment compared with the response at 24 hours, although approximately 30% of the genes at 6 hours were common to 24-hour treatment, indicating a sustained regulation of a subset of genes. Genes were selected to verify the array analysis by real-time PCR. These genes were selected to represent high- and low-intensity signals and genes that were up- or down-regulated at either time point (Figure 3A). PCR verification confirmed the increased expression of CCL3 and CCL8 at 6 hours (Figure 3C) and IL-1β, CCL3, colony-stimulating factor 3 (CSF3), and vascular cell adhesion molecule 1 (VCAM1) at the 24-hour time point (Figure 3B). However, PCR did not validate regulation of transforming acidic coiled-coil–containing protein 2 and CCL2. The PCR validation permitted cutoff values to be established to generate a reliable list of 65 PROK1 regulated genes (Table 2). The gene list indicated that the response to PROK1 at 6 hours was mainly constituted of chemokine genes (7 of the 11 genes). Also, chemokines (ie, CCL3, CCL3L1, and CCL3L3) represented the genes that were regulated at both 6 and 24 hours after

Table 2. Differential Gene Expression of 65 Genes after PROK1 Treatment of Term Myometrial Explants

Gene	Mean fold change	Adjusted P value	Time (hours)
ADAMDEC1	1.6	0.0741	24
AMPD3	1.6	0.0861	24
BDKRB1	1.9	0.0506	24
C15orf48	3.2	0.0327	24
C8orf4	1.6	0.0506	24
CCL20	3.9	0.0709	24
CCL3	5.5	0.0001	6
CCL3	2.6	0.0296	24
CCL3L1	3.6	0.0017	6
CCL3L1	2.5	0.0327	24
CCL3L3	4.9	0.0011	6
CCL3L3	3.2	0.0249	24
CCL4L1	2.6	0.0118	6
CCL4L2	6.9	0.0000	6
CCL8	3.6	0.0071	6
CCR7	2.0	0.0969	24
CD82	1.8	0.0420	24
CSF3	4.5	0.0249	24
CX3CL1	3.0	0.0100	6
CXCL1	2.6	0.0249	24
CXCL2	3.1	0.0474	24
CXCL5	5.0	0.0000	24
CXCL6	5.3	0.0062	24
CYP27B1	3.0	0.0709	24
ELL2	1.6	0.0085	24
FBP1	1.8	0.0709	24
FCGR2A	1.6	0.0900	24
G0S2	1.9	0.0841	24
HCK	1.8	0.0792	6
IL1B	5.3	0.0249	24
IL1RN	2.3	0.0861	24
IL24	3.2	0.0581	24
IL32	1.7	0.0280	24
IL7R	2.5	0.0177	24
IRAK2	1.8	0.0474	24
LILRB4	1.6	0.0177	24
LTB	2.7	0.0249	24
MEOX1	2.6	0.0018	6
MME	1.6	0.0769	24
MMP9	3.5	0.0313	24
MT1H	2.1	0.0275	24
MUCL1	2.0	0.0799	24
NAMPT	1.7	0.0249	24
NFKB2	1.5	0.0747	24
NFKBIZ	1.9	0.0707	24
NRCAM	1.5	0.0615	24
OLR1	1.9	0.0387	24
OSM	1.9	0.0474	24
PDE4B	1.8	0.0327	24
PPL	−1.6	0.0709	24
PSME2	1.5	0.0480	24
RCSD1	1.5	0.0383	24
RND1	1.9	0.0172	6
SELE	6.0	0.0496	6
SEMA4A	1.7	0.0085	24
SERPINB2	4.0	0.0085	24
SERPINB7	3.5	0.0870	24
SLC11A2	1.5	0.0730	24
SLC39A14	1.6	0.0280	24
SOCS1	1.5	0.0348	24
SRGN	1.7	0.0724	24
TNFAIP8	1.6	0.0861	24
TNFRSF6B	1.7	0.0056	24
TNIP1	1.6	0.0249	24
UBD	2.3	0.0709	24
VCAM1	3.2	0.0474	24
WNT5A	1.6	0.0841	24
ZC3H12A	1.8	0.0673	24

The cutoff was established by quantitative PCR verification, a fold change >1.5, and an adjusted P value <0.1.

Table 3.   Over-represented PANTHER Ontologies

Biological process	No. of genes in the category	No. of genes in the PROK1 list	No. of genes expected	P value
Immune response	756	23	2.39	4.54×10^{-17}
Immune system process	2628	36	8.32	2.63×10^{-16}
Response to stimulus	1798	29	5.69	1.78×10^{-14}
Cell-cell signaling	1331	25	4.21	8.34×10^{-14}
Cell surface receptor–linked signal transduction	2235	30	7.07	6.25×10^{-13}
Signal transduction	4191	39	13.26	2.58×10^{-12}
Cell communication	4365	39	13.81	9.76×10^{-12}
Macrophage activation	305	10	0.97	4.33×10^{-8}
Cellular process	6258	40	19.80	1.67×10^{-7}
Cellular defense response	457	10	1.45	1.71×10^{-6}
Intracellular signaling cascade	1568	16	4.96	2.21×10^{-5}
Response to interferon-γ	105	5	0.33	2.22×10^{-5}
Angiogenesis	408	8	1.29	4.41×10^{-5}
B-cell–mediated immunity	314	7	0.99	6.19×10^{-5}
Mesoderm development	1528	14	4.83	2.50×10^{-4}
Response to external stimulus	325	6	1.03	5.80×10^{-4}
Blood coagulation	325	6	1.03	5.80×10^{-4}
Natural killer cell activation	121	4	0.38	6.10×10^{-4}
Cell motion	964	10	3.05	8.55×10^{-4}
Apoptosis	966	9	3.06	3.27×10^{-3}
Cell-cell adhesion	799	8	2.53	3.63×10^{-3}
System development	2031	14	6.43	3.93×10^{-3}
Cell adhesion	1333	10	4.22	8.77×10^{-3}

PANTHER, protein analysis through evolutionary relationships.

PROK1 treatment. The response to PROK1 treatment after 24 hours consisted mainly of chemokines and cytokines (ie, 22 of the 57 genes). A functional grouping of the gene list into statistically significant overrepresented ontologies revealed the immune response and related pathways to predominate, including macrophage activation and response to interferon-γ (Table 3).

Selected PROK1 up-regulated genes identified from the gene array list were analyzed for expression in term myometrium in L versus NL. The analysis demonstrated that the following cytokines are up-regulated during labor: IL-1β (L versus NL, 16.78 ± 7.0-fold versus 1.16 ± 0.37-fold; $P < 0.05$), CCL3 (L versus NL, 12.79 ± 5.23-fold versus 1.3 ± 0.33-fold; $P < 0.05$), and CSF3 (L versus NL, 66.7 ± 57.28-fold versus 0.06 ± 0.33-fold; $P < 0.0001$). There was no change in expression of VCAM1 (L versus NL, 10.36 ± 2.4-fold versus 13.04 ± 4.91-fold) or CCL8 (NL versus L, 13.45 ± 4.9-fold versus 27.82 ± 12.02-fold) (Figure 4). An elevation in expression of PROK1 at labor is included in the figure for comparison.

There were several genes demonstrated by the gene array to be up-regulated in response to PROK1, such as PTGS2, IL-6, IL-8, and CCL20; however, they were less than cutoff values. Nevertheless, because they play an important role in the physiological characteristics of labor and have been previously demonstrated to be up-regulated in the myometrium during labor,[3] we sought to confirm whether they are regulated by PROK1. We demonstrate that treatment with PROK1 resulted in up-regulation of mRNA levels for the following variables: PTGS2 (3.89 ± 1.38-fold over vehicle, $P < 0.05$), IL-6 (3.2 ± 0.76-fold over vehicle, $P < 0.05$), IL-8 (4.6 ± 1.4-fold over vehicle, $P < 0.05$), and CCL20 (6.27 ± 2.39-fold over vehicle, $P < 0.05$) (Figure 5A). We also demonstrate that

the increase in PTGS2 transcript levels is reflected by an increase in the secretion of PTGS2-dependent prostaglandins: PGE_2 (Figure 5B) [70.7 ± 17.1 versus 12.14 ± 2.28 pg/mL/mg tissue for PROK1 versus vehicle treatment; $P < 0.05$] and $PGF_2\alpha$ (Figure 5C) (32.54 ± 10.9 versus 11.84 ± 4.22 pg/mL/mg tissue for PROK1 versus vehicle treatment; $P < 0.05$).

LPS Increases Expression of PROK1 and PROKR1 in Term Myometrium

Subsequently, we examined whether treatment with a mimetic of infection, such as LPS, can regulate the expression of PROK1 and PROKR1. Treatment of term myometrial explants with 100 ng/mL LPS resulted in an increase in PROK1 mRNA at 2 hours (58.29 ± 30.25-fold
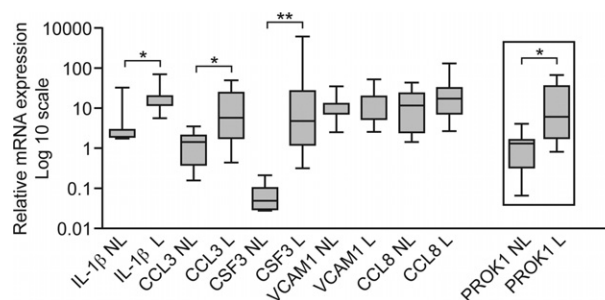
Figure 4. PROK1-regulated genes are elevated in myometrium during labor. The expression of genes that were demonstrated by gene array to be up-regulated in response to PROK1 was subsequently examined in term myometrium NL versus term myometrium L. Quantitative real-time PCR analysis demonstrated that IL-1β, CCL3, and CSF3 are up-regulated at labor, similar to PROK1, whereas there was no change in the level of VCAM1 and CCL8. *$P < 0.05$, **$P < 0.0001$ ($n = 10$). Results are presented as mean ± SEM.
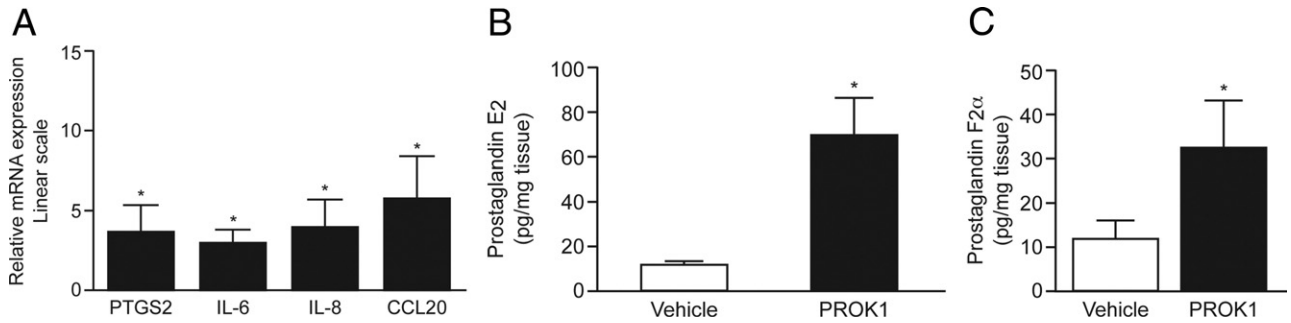
Figure 5. PROK1 up-regulates the expression of inflammatory mediators involved in term and preterm onset of parturition. Treatment of myometrial explants with 40 nmol/L PROK1 for 24 hours increased mRNA levels for PTGS2, IL-6, IL-8, and CCL20 ($n = 8$) (**A**). Treatment with 40 nmol/L PROK1 promoted secretion of PTGS2-dependent prostaglandins, PGE_2 (**B**) and $PGF_{2\alpha}$ (**C**), at 24 hours ($n = 5$). *$P < 0.05$. Results are presented as mean ± SEM.

over vehicle; $P < 0.05$) and PROKR1 mRNA at 6 hours (3.84 ± 1.6-fold over vehicle; $P < 0.05$) (Figure 6).

PROK1 Mediates LPS-Induced Inflammation in Term Myometrium

Several inflammatory mediators that are elevated during labor are also induced by bacterial infection.[34,35] After having demonstrated up-regulation of PROK1 and PROKR1 in response to LPS and PROK1-mediated regulation of inflammatory factors, we aimed to determine whether the pro-inflammatory effect of LPS is mediated via PROK1. Myometrial tissue was infected with lentiviral constructs containing a scrambled control miRNA sequence or a PROK1 chained miRNA sequence and subsequently treated with vehicle or 100 ng/mL of LPS. The expression of PROK1 was efficiently suppressed in myometrial tissue in response to infection with PROK1 miRNA versus scrambled miRNA (0.22 ± 0.07-fold versus $1.2 \pm 0.39$-fold; $P < 0.05$) (see Supplemental Figure S2 at *http://ajp.amjpathol.org*). The infection of myometrial tissue with PROK1 miRNA decreased the response to LPS: IL-1β (57.21 ± 17.87-fold after infection with scrambled miRNA versus 9.53 ± 4.23-fold after infection with PROK1 miRNA), CCL3 (16.6 ± 3.65-fold after infection with scrambled miRNA verus 5.9 ± 2.7-fold after infection with PROK1 miRNA), CSF3 (25.52 ± 18.81-fold after infection with scrambled miRNA versus 1.68 ± 0.53-fold after infection with PROK1 miRNA), and CCL20 (50.1 ± 3.65-fold after infection with scrambled miRNA versus 5.9 ± 2.4-fold after infection with PROK1 miRNA) ($P <$

0.05 for all genes) (Figure 7, A–D). However, there was no effect of PROK1 suppression on the level of PTGS2 (2.9 ± 0.74-fold after infection with scrambled miRNA versus 3.3 ± 1.84-fold after infection with PROK1 miRNA) (Figure 7E). As a negative control, we demonstrated that there was no effect of LPS on PTGS1 expression or when PROK1 was suppressed (Figure 7F). These data suggest that LPS-induced inflammation in term myometrium may be partially mediated via expression of PROK1.

We subsequently investigated the effect of PROK1 suppression on secreted proteins in the supernatants of myometrial explant cultures. The treatment of myometrial explants with PROK1 miRNA reduced secretion of IL-β (13.92 ± 3.86-fold after infection with scrambled miRNA versus 0.58 ± 0.17-fold after infection with PROK1 miRNA), CCL3 (69.6 ± 26.23-fold after infection with scrambled miRNA versus 5.34 ± 1.76-fold after infection with PROK1 miRNA), and CCL20 (39.5 ± 22.25-fold after infection with scrambled miRNA versus 1.63 ± 0.97-fold after infection with PROK1 miRNA ($P < 0.05$ for all proteins) (Figure 8, A–C).

Discussion

Human parturition is well described as a pro-inflammatory event associated with recruitment of leukocytes, mainly neutrophils and macrophages, to the myometrium and cervix.[1] Data indicate that 40% to 60% of genes up-regulated in these tissues at labor are involved in inflammation and chemotaxis.[3] Recruited leukocytes secrete cytokines, such as IL-1β, IL-6, and IL-8, and pros-
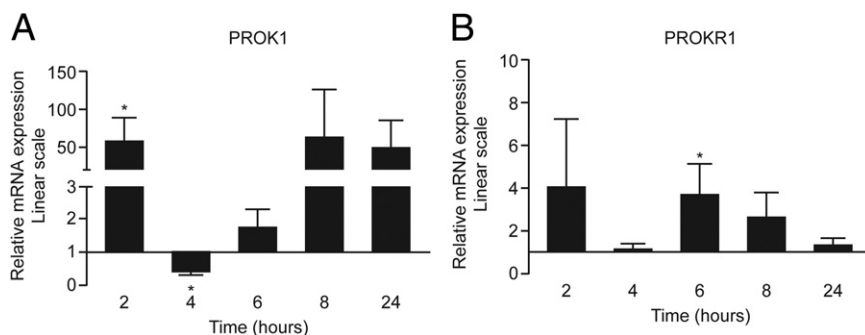
Figure 6. LPS promotes expression of PROK1 and PROKR1 in term myometrium. Myometrial explants collected from women at term, not in labor, were treated with 100 ng/mL LPS for 24 hours. Treatment with LPS up-regulated the mRNA level for PROK1 (**A**) and PROKR1 (**B**). *$P < 0.05$ ($n = 8$). Results are presented as mean ± SEM.
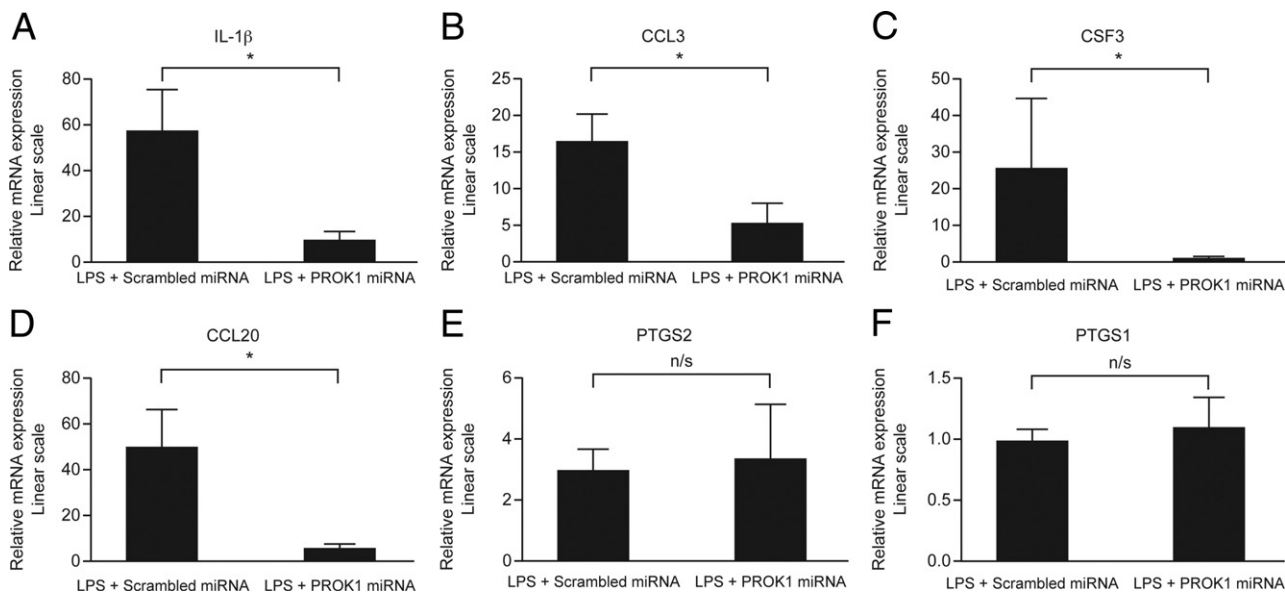
Figure 7. PROK1 mediates LPS-induced expression of inflammatory mediators in myometrium. Term myometrium was infected with lentivirus containing miRNA constructs targeting PROK1 or scrambled sequence. Infected explants were subsequently treated with 100 ng/mL of LPS for 24 hours. The expression of IL-1β (**A**), CCL3 (**B**), CSF3 (**C**), and CCL20 (**D**) in response to 100 ng/mL of LPS was significantly decreased by PROK1 miRNA constructs compared with the scrambled sequence. However, PROK1 suppression did not reduce the effect of LPS on PTGS2 expression (**E**). LPS treatment had no effect on PTGS1 expression, and this remained unaffected when PROK1 was suppressed (**F**). *$P < 0.05$. n/s, not significant ($n = 8$). Results are presented as mean $\pm$ SEM.

taglandins PGE_2 and $PGF_{2\alpha}$. These cytokines can stimulate production of matrix metalloproteinases that are involved in membrane rupture[5] and can also promote cervical ripening.[4] Although inflammatory events during parturition are well characterized, the underlying mechanism remains unknown.

Increasing evidence suggests that PROK1 can modulate immune responses. The expression of PROK1 and PROKR1 is increased at sites where inflammation occurs, and they are involved in mediating inflammatory pain.[36,37] PROK1 promoted differentiation of bone marrow progenitors into macrophage-like adherent cells in a mouse model and in human progenitor cells.[22] PROK1 also induces expression of CCL4, CXCL1, and IL-8 in human monocytes, which is a part of an important regulatory mechanism for leukocyte recruitment to the site of inflammation.[23] We have previously demonstrated that the expression of PROK1 and PROKR1 plays an important role during preg-

nancy.[24] However, the expression of PROK1 and PROKR1 is also elevated during the third trimester in the placenta, where it regulates the expression of inflammatory mediators with established roles in the initiation of labor: IL-8 and PTGS2.[25] This finding suggests that PROK1 may contribute to the induction of processes leading to the onset of parturition.

We demonstrate that the expression of PROK1 is significantly increased in term myometrium and placenta at labor compared with nonlaboring tissues. However, although not significantly affected by labor, the expression of PROKR1 is abundantly expressed in myometrium, suggesting that, within the uteroplacental unit, the myometrium is a major target tissue for PROK1 action.

Immunolocalization of PROKR1 demonstrated expression in term myometrium, predominantly in smooth muscle cells, confirming what we previously observed in nonpregnant myometrium.[38] Because PROK1 was a
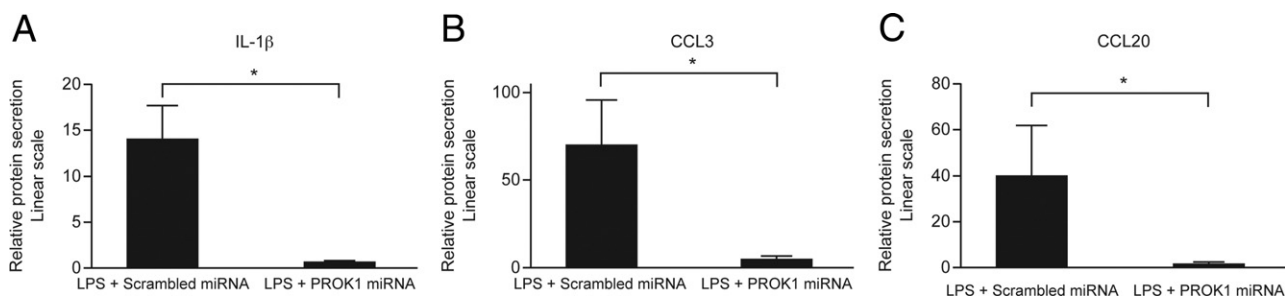
Figure 8. Targeting the *PROK1* gene with miRNA reduces the secretion of inflammatory proteins in response to LPS. Term myometrium was infected with lentivirus containing miRNA constructs targeting PROK1 or a scrambled sequence. Infected explants were subsequently treated with either vehicle or 100 ng/mL of LPS for 24 hours. Results are presented relative to expression in vehicle-treated samples. IL-1β (**A**), CCL3 (**B**), and CCL20 (**C**) protein levels in culture supernatants after treatment with LPS were reduced in tissues infected with PROK1 miRNA compared with tissues infected with a scrambled sequence. *$P < 0.05$ ($n = 5$ to 7). Results are presented as mean $\pm$ SEM.

potent inducer of contractility of guinea pig ileum,[20] our results indicate that elevated expression of PROK1 in the myometrium, with the onset of labor, may regulate contractions of smooth muscle cells, resulting in uterine contractions during labor. This is further supported by the regulatory effect of PROK1 on expression of IL-1β demonstrated in this study. IL-1β plays a pivotal role in uterine contractility because it promotes an influx of calcium into uterine smooth muscle cells, enhancing contraction.[9] In addition, IL-1β, via induction of PTGS2, stimulates production of prostaglandins that are well described to induce contractions of the uterus.[7,8] More important, our results demonstrate that PROK1 up-regulates expression of PTGS2 and secretion of PGE$_2$ and PGF$_{2\alpha}$. It is possible that this occurs directly via PROK1 regulation of PTGS2 expression[24] and/or indirectly via prior induction of IL-1β. These data warrant further research to confirm a role of PROK1/PROKR1 in myometrial contractility.

By using microarray gene expression analysis, we examined the effect of PROK1 on gene expression in myometrial tissue before the onset of labor at term. We identified 65 genes to be regulated by PROK1 in the myometrium, most of which are cytokines and chemokines. A functional grouping of these genes revealed that immune response and related pathways are predominantly regulated by PROK1, including IL-1β, CCL3, CSF3, CCL20, IL-6, IL-8, and PTGS2, which have been previously up-regulated in human myometrium and cervix during labor.[3] Such a pronounced effect of PROK1 on immune pathways suggests that PROK1 may regulate recruitment of leukocytes to the myometrium at term and their activation and, thus, contributes to the initiation of parturition.

IL-1β, IL-8, and IL-6 may constitute an initial pro-inflammatory stimulus that then further establishes an inflammatory microenvironment in the uterus by activating local cells to secrete chemoattractants or effector molecules, such as prostaglandins or matrix metalloproteinases.[39,40] IL-8, which in gestational tissues is mainly stimulated by IL-1β, is a potent chemoattractant and activating factor, mainly for neutrophils.[41,42] Its concentrations correlate with the number of neutrophils and macrophages in the lower segment of the myometrium and cervix.[2,43] The main role of IL-8 during parturition may be to maintain neutrophil numbers in gestational tissues and disseminate a pro-inflammatory state.[40] As labor progresses, the initial neutrophil influx may be followed by monocyte recruitment, which is believed to be regulated by IL-6.[3,44] Monocytes further promote inflammation by producing chemokines, such as CCL2, CCL3, CCL8, or CCL20, that are involved in further recruitment of leukocytes.[40]

During parturition, the absolute and percentage numbers of circulating neutrophils and monocytes are increased, indicating that proliferation of these cells is enhanced during labor.[45] Our results suggest that PROK1 may also regulate this process. In addition to regulation of IL-8 and IL-6, we demonstrate that PROK1 up-regulates the expression of CSF3. The main function of CSF3 is to stimulate bone marrow to produce and mobilize

neutrophils to the bloodstream and initiate their differentiation.[46,47] This is vital for the physiological features of labor, which is well associated with an influx of neutrophils to the myometrium and cervix.

In preterm birth, the pro-inflammatory environment is initiated prematurely. Although several factors, such as smoking, alcohol, advanced maternal age, and structural abnormalities of the cervix,[12,13] can contribute to preterm initiation of labor; bacterial infections account for nearly 25% of all incidents of preterm deliveries and they are detected in 79% of births at 23 weeks and in 11% of births at 31 to 34 weeks.[14,15] More important, antimicrobial therapies with antibiotics have had little or no benefit.[15] In most cases, even though intrauterine infections are polymicrobial, *Escherichia coli* and LPS demonstrate the most robust effect on concentration of prostaglandins PGE$_2$ and PGF$_{2\alpha}$ and cytokines, such as IL-1β, IL-6, and IL-8.[48] In most cases, evidence of intrauterine infection is not apparent or underlying infection is asymptomatic; nevertheless, histological evidence of inflammation is present in the decidua, fetal membranes, and umbilical cord.[16,17,49,50] Therefore, targeting downstream inflammation could constitute a novel and beneficial solution to prevent bacterially induced preterm births.

In this study, we demonstrate that several genes regulated by PROK1 increase during parturition both term and preterm and are associated with intrauterine bacterial infection.[34,35,51] Considering the induction of PROK1 and PROKR1 by LPS, we suggest that the role of PROK1 in inducing a pro-inflammatory response may contribute to the activation of inflammatory events and preterm birth associated with bacterial infections.

Further evidence to support a role of PROK1 in mediating an inflammatory response was shown using a mimetic of infection, LPS. We selected three genes that were demonstrated by the gene array to be the most responsive to PROK1 (IL-1β, CCL3, and CFS) and also included CCL20 and PTGS2. The study demonstrated that LPS induction of pro-inflammatory genes CCL20, CCL3, CFS, and IL-1β is decreased by inhibiting PROK1 gene expression. LPS may regulate the expression of these genes in the human myometrium via toll-like receptor 2, which is similar to PROK1 and localizes to smooth muscle bundles.[52]

Our study demonstrates a novel role for PROK1 in regulating a wide range of inflammatory mediators in human myometrial tissue, which plays a pivotal role in the physiological characteristics of labor. We propose that PROK1 regulates inflammatory processes, in particular those involved in the recruitment of leukocytes to the myometrium, which, in turn, contribute to the onset of parturition at term. In addition, PROK1 may mediate the inflammatory cascade in myometrium during infection, resulting in premature initiation of labor. Therefore, targeting PROK1 signaling could provide novel and efficient therapy by reducing the inflammatory response during premature initiation of labor and, hence, improve neonatal survival.

Acknowledgments

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