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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-inflammatory environment within the uterus have been associated with the initiation of both term and preterm parturition. The mechanism regulating the onset 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-regulated by PROK1 in human myometrium, mainly cytokines and chemokines, including IL-1β, chemokine 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 E2 and F2α secretion. The treatment of myometrial explants with 100 ng/mL of lipopolysaccharide up-regulates the expression of PROK1, PROK1 receptor, and inflammatory mediators. The infection of myometrial explants with lentiviral microRNA targeting PROK1, preceding treatment with lipopolysaccharide, reduces the expression of inflammatory 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 pathways during bacterial infection. (Am J Pathol 2011, 179:2709–2719; DOI: 10.1016/j.ajpath.2011.08.029)

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 labor are involved in inflammation and chemotaxis.3 Recruited leukocytes secrete cytokines, including IL-1β, IL-6, and IL-8, and prostaglandins E2 (PGE2) and F2α (PGF2α). These cytokines and prostaglandins can stimulate production of matrix metalloproteinases that are involved in membrane rupture and can also promote cervical ripening.4 However, during labor, the greatest influx of leukocytes is observed in the myometrium.5 Cytokines, such as IL-1β, released by leukocytes, promote the synthesis of prostaglandin synthase 2 (PTGS2)–dependent prostaglandins (PGE2 and PGF2α), which are important regulators of myometrial contractility.6,7 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 singleton deliveries are preterm, and this constitutes the biggest cause of neonatal morbidity and mortality.10,11 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 infection in the uteroplacental unit is believed 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. Intraterine inflammation is associated with adverse perinatal outcome. 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 intraterine 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, which is mediated via increased calcium influx into smooth muscle cells. PROKs also have potent effects in multiple other processes, including neurogenesis, angiogenesis, hematopoiesis, and nociception. 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. 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.

PROKs may be important regulators of female reproductive functions, including regulation of uterine receptivity and placental function. 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% CO2, 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. 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 streptavidin-Cy3. Signal intensities within data sets were normalized by applying quantile normalization. R package Limma (Biocductor, 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).

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 Ct 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.
endogenous peroxidase with 30% H2O2 in methanol. Buffer (pH 6.0), followed by quenching the activity performed by boiling for 5 minutes in 0.01 mol/L citrate and dehydrated in graded ethanol. Antigen retrieval was Paraffin-embedded sections were dewaxed in xylene.

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% H2O2 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. Briefly, myometrial explants (n = 8) were finely chopped and subsequently infected with lentivirus containing scrambled sequence (pLenti6/V5-EmGFPMiR-neg) as a negative control or PROK1 miRNA (pLenti6/V5-EmGFPHum-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 PGE2 and PGF2α

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 PGE2 and PGF2α, as previously described. Results are presented as pg/mg tissue.

Measurement of IL-1β, CCL3, and CCL20

Myometrial explants were incubated 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.

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′-CTGGCACCACGGCGCAAG-3′   |
| Reverse    | 5′-ACCAAGAGCACAGTGTGGTC-3′ |
| FAM        | 5′-ACAGGCTCTCTTTGTTCCAGA-3′|
| CCL20      |                            |
| Forward    | 5′-TCCTCTGTTCCTTTGGTCA-3′  |
| Reverse    | 5′-CCCTCTGTGGGAGCCAGGT-3′  |
| FAM        | 5′-TGCCAGCCTTCGACCTAGA-3′  |
| IL-1β      |                            |
| Forward    | 5′-GCCCTCCACCTACGAACT-3′   |
| Reverse    | 5′-CCGCTGAGGAGTACCGAAAT-3′ |
| FAM        | 5′-CCATCAACTTCTGGAAGAATGAC-3′|
| IL-8       |                            |
| Forward    | 5′-CTGGCCGCTGCTCTTCC-3′    |
| Reverse    | 5′-TTAGACCCTCTTTGCGGAAACATG-3′|
| FAM        | 5′-CCCTCTGTTCCTTGACCTGGAAG-3′|
| COX-2      |                            |
| Forward    | 5′-CCCTCTCTTGCCGTCTAGT-3′  |
| Reverse    | 5′-ACAATTCTATGATGAACTGG-3′ |
| FAM        | 5′-TGCCCGACTCTCTTGTGGTCA-3′|
| 18S        |                            |
| Forward    | 5′-CCGTACCCACCTACGAAAG-3′  |
| Reverse    | 5′-GCTGGATTACCGAGGCT-3′    |
| VIC        | 5′-TGCTGCGACCCAGACTTGCCTC-3′|
| CCL3       | Hs00234142-m1              |
| CSF3       | Hs00110983-m1              |
| VCAM1      | Hs01033372-m1              |
| TACC2      | Hs00610167-m1              |
| CCL2       | Hs00234140-m1              |
| CCL8       | Hs00271615-m1              |

![Figure 1](https://via.placeholder.com/150)

Figure 1. Expression of PROK1 and PROKR1 mRNA in human uterine 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 PROK1 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).
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 ±
PROK1/PROKR1 Regulates Inflammatory Mediators in Human Myometrium

After having established that PROK1 expression is elevated in the myometrium during labor and that PROK1 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 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). PROK1 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. During IHC, PROK1 was localized to the vasculature (Figure 2D). During labor, PROK1 expression is additionally localized 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). 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).

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           |
| BDGRB1      | 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           | 24           |
| CCL3        | 2.6              | 0.0296           | 24           |
| CCL3L1      | 3.6              | 0.0017           | 6            |
| CCL3L1      | 2.5              | 0.0327           | 24           |
| CCL3L3      | 4.9              | 0.0111           | 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           |
| CDS2        | 1.8              | 0.0420           | 24           |
| CSF3        | 4.5              | 0.0249           | 24           |
| CXC3CL1     | 3.0              | 0.0100           | 6            |
| CXC1L1      | 2.6              | 0.0249           | 24           |
| CXC1L2      | 3.1              | 0.0474           | 24           |
| CXC1L5      | 5.0              | 0.0000           | 24           |
| CXC1L6      | 5.3              | 0.0062           | 24           |
| CYP27B1     | 3.0              | 0.0709           | 24           |
| ELL2        | 1.6              | 0.0085           | 24           |
| FB1p        | 1.8              | 0.0709           | 24           |
| FCGR2A      | 1.6              | 0.0000           | 24           |
| GOS2        | 1.9              | 0.0841           | 24           |
| HCK         | 1.8              | 0.0792           | 6            |
| IL1β        | 5.3              | 0.0249           | 24           |
| IL1R        | 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           |
| IL1RB4      | 1.6              | 0.0177           | 24           |
| LTβ         | 2.7              | 0.0249           | 24           |
| MEOX1       | 2.6              | 0.0018           | 6            |
| MME         | 1.6              | 0.0769           | 24           |
| MMP9        | 3.5              | 0.0031           | 24           |
| MT1H        | 2.1              | 0.0275           | 24           |
| MUC1        | 2.0              | 0.0799           | 24           |
| NAMPT       | 1.7              | 0.0249           | 24           |
| NFKB2       | 1.5              | 0.0747           | 24           |
| NFKB1Z      | 1.9              | 0.0707           | 24           |
| NRCAM       | 1.5              | 0.0615           | 24           |
| OLR1        | 1.9              | 0.0387           | 24           |
| OSM         | 1.9              | 0.0474           | 24           |
| PDE4A6      | 1.8              | 0.0327           | 24           |
| PPI         | 1.6              | 0.0709           | 24           |
| PSME2       | 1.5              | 0.0480           | 24           |
| RCSD1       | 1.5              | 0.0383           | 24           |
| RGD1        | 1.9              | 0.0172           | 6            |
| SELC        | 6.0              | 0.0496           | 24           |
| SEMA4A      | 1.7              | 0.0085           | 24           |
| SERPINA2    | 4.0              | 0.0085           | 24           |
| SERPINA7    | 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           |
| TNFAIP6     | 1.6              | 0.0861           | 24           |
| TNFRSF6B    | 1.7              | 0.0506           | 24           |
| TNF1        | 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.
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 cytokotins 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 demonstrated 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 demonstrated that the increase in PTGS2 transcript levels is reflected by an increase in the secretion of PTGS2-dependent prostaglandins: PGE2 (Figure 5B) (70.7 ± 17.1 versus 12.14 ± 2.28 pg/mL/mg tissue for PROK1 versus vehicle treatment; P < 0.05) and PGF2α (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...
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 ± 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 versus 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-1β (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-
taglands PGE₂ and PGF₂₀α. These cytokines can stimulate production of matrix metalloproteinases that are involved in membrane rupture⁵ 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.⁶,⁷ 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 pregnancy.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
potent inducer of contractility of guinea pig ileum, 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. In addition, IL-1β, via induction of PTGS2, stimulates production of prostaglandins that are well described to induce contractions of the uterus. 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 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. 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 metalloproteinas. IL-8, which in gestational tissues is mainly secreted by local cells to secrete chemoattractants or effector molecules, such as prostaglandins PGE$_2$ and PGF$_{2\alpha}$ and cytokines, such as IL-1β, IL-6, and IL-8. 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. 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. 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 FF) and also included CCL20 and PTGS2. The study demonstrated that LPS induction of pro-inflammatory genes CCL20, CCL3, and FF 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.

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.
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