NOD1 and NOD2 Regulate Proinflammatory and Prolabor Mediators in Human Fetal Membranes and Myometrium via Nuclear Factor-Kappa B

Martha Lappas

Department of Obstetrics and Gynaecology, University of Melbourne, Melbourne, Victoria, Australia, and Mercy Perinatal Research Centre, Mercy Hospital for Women, Heidelberg, Victoria, Australia

ABSTRACT

Preterm birth remains one of the most important issues facing perinatal medicine today, with chronic inflammation and/or infection being the biggest etiological factor. The nucleotide oligomerization domain (NOD) intracellular molecules recognize a wide range of microbial products as well as other intracellular danger signals, thereby initiating inflammation through activation of nuclear factor KB (NFkB), a central regulator of the terminal processes of human labor and delivery. The aims of this study were to determine the effect of 1) human labor, proinflammatory cytokines, and bacterial endotoxin LPS on NOD1 and NOD2 expression and 2) NOD1 and NOD2 activation on the expression of prolabor mediators in human fetal membranes and myometrium. NOD1 and NOD2 expression was significantly higher in fetal membranes and myometrium after spontaneous labor when compared to nonlaboring tissues. Bacterial endotoxin LPS and the proinflammatory cytokines TNF and IL1B significantly increased NOD2, but not NOD1, expression. Furthermore, LPS-induced NOD2 expression was decreased by the NFkB inhibitor BAY 11–7082. In both fetal membranes and myometrium, the NOD1 ligand bacterial iE-DAP and the NOD2 ligand bacterial MDP significantly increased the expression and secretion of proinflammatory cytokines (IL6 and IL8), cyclooxygenase (PTG2) expression and subsequent release of prostaglandins PGE, and PG, and the expression and activity of MMP9. The effects of these NOD1 and NOD2 ligands were mediated via NFkB, as both iE-DAP and MDP significantly increased NFkB activation and secretion of proinflammatory cytokines (IL6 and IL8), cyclooxygenase (PTG2) expression and subsequent release of prostaglandins PGE, and PG, and the expression and activity of MMP9. The effect of NOD2 ligands were mediated via NFkB, as both iE-DAP and MDP significantly increased NFkB activation and secretion of proinflammatory cytokines (IL6 and IL8), cyclooxygenase (PTG2) expression and subsequent release of prostaglandins PGE, and PG, and the expression and activity of MMP9. The effect of NOD2 ligands were mediated via NFkB, as both iE-DAP and MDP significantly increased NFkB activation and secretion of proinflammatory cytokines (IL6 and IL8), cyclooxygenase (PTG2) expression and subsequent release of prostaglandins PGE, and PG, and the expression and activity of MMP9. The effect of NOD2 ligands were mediated via NFkB, as both iE-DAP and MDP significantly increased NFkB activation and secretion of proinflammatory cytokines (IL6 and IL8), cyclooxygenase (PTG2) expression and subsequent release of prostaglandins PGE, and PG, and the expression and activity of MMP9. The effect of NOD2 ligands were mediated via NFkB, as both iE-DAP and MDP significantly increased NFkB activation and secretion of proinflammatory cytokines (IL6 and IL8), cyclooxygenase (PTG2) expression and subsequent release of prostaglandins PGE, and PG, and the expression and activity of MMP9. The effect of NOD2 ligands were mediated via NFkB, as both iE-DAP and MDP significantly increased NFkB activation and secretion of proinflammatory cytokines (IL6 and IL8), cyclooxygenase (PTG2) expression and subsequent release of prostaglandins PGE, and PG, and the expression and activity of MMP9. The effect of NOD2 ligands were mediated via NFkB, as both iE-DAP and MDP significantly increased NFkB activation and secretion of proinflammatory cytokines (IL6 and IL8), cyclooxygenase (PTG2) expression and subsequent release of prostaglandins PGE, and PG, and the expression and activity of MMP9. The effect of NOD2 ligands were mediated via NFkB, as both iE-DAP and MDP significantly increased NFkB activation and secretion of proinflammatory cytokines (IL6 and IL8), cyclooxygenase (PTG2) expression and subsequent release of prostaglandins PGE, and PG, and the expression and activity of MMP9. The effect of NOD2 ligands were mediated via NFkB, as both iE-DAP and MDP significantly increased NFkB activation and secretion of proinflammatory cytokines (IL6 and IL8), cyclooxygenase (PTG2) expression and subsequent release of prostaglandins PGE, and PG, and the expression and activity of MMP9.

INTRODUCTION

Worldwide, preterm birth is one of the most significant healthcare issues facing perinatal medicine; it accounts for greater than 70% of perinatal mortality [1]. In the long term, the babies that survive being born early are often faced with adverse complications that continue into childhood [2, 3], levying a considerable cost burden to the health care system [4] and emotional and financial strain to the families affected [5]. Thus, it is vital to understand the cellular and molecular mechanisms that govern birth if we are to develop interventions capable of preventing the serious clinical sequelae associated with preterm birth.

Preterm prelabor rupture of the membranes (PPROM) and spontaneous (with intact membranes) preterm labor are the most common antecedents of preterm birth. Although there are many causes of preterm birth, intra-amniotic infection and chorioamnionitis are the most significant etiological factors [6]. Infection and inflammation trigger the activation of inflammatory cytokines, cyclooxygenase (PTG2) and prostaglandins, and extracellular matrix (ECM) remodeling enzymes (e.g., matrix metalloproteinase [MMP], which are involved in the processes leading to uterine contractions and membrane rupture [7–12]. Nuclear factor-kappa B (NFkB) is a key proinflammatory transcription factor involved in promoting the formation of these proinflammatory and prolabor mediators in human gestational tissues [13–15].

Nucleotide-binding oligomerization domain-containing 1 and 2 (NOD1 and NOD2) are intracellular pattern-recognition receptors (PRRs) involved in the sensing of numerous microbes or microbial components, which have gained access to the cell’s cytoplasm [16]. NOD1 and NOD2 detect distinct structures of bacterial peptidoglycan; NOD1 recognizes the bacterial peptidoglycan-derived peptides g-D-glutamyl-meso-diaminopimelic acid (iE-DAP) derived primarily from Gram-negative bacilli and particular Gram-positive bacteria [17], whereas NOD2 recognizes the minimal peptidoglycan muramyl dipeptide (MDP), the largest peptidoglycan motif common to all bacteria [18]. The activation of NOD1 or NOD2 promotes the activation of NFkB-mediated proinflammatory gene expression [18–25]. For example, stimulation of NOD1 or NOD2 results in the secretion of proinflammatory cytokines and chemokines [26–32], PTG2-prostaglandin pathway [32–34], and MMPs [35]. As such, there is mounting evidence that deregulation of NOD1 and NOD2 signaling causes or contributes to a variety of human diseases, including asthma, cancer, and inflammatory bowel disease [16, 21, 36–39].

NOD1 and NOD2 have recently been characterized in placental trophoblasts. Human first-trimester placental syncytiotrophoblast and cytotrophoblast cells and first-trimester isolated primary trophoblast cultures express both NOD1 and NOD2 [40], and bacterial endotoxin lipopolysaccharide (LPS) upregulates the gene expression of NOD1 and NOD2 in...
trophoblast cells [41]. Exposure to NOD1 and NOD2 agonists induces an inflammatory cytokine-chemokine response in first-trimester trophoblast primary cells [40, 41]. Furthermore, in term trophoblasts, NOD1 activation by iE-DAP induces the secretion of the proinflammatory cytokines IL6, GROa, and MCP-1. Although these cytokines were not upregulated in response to NOD2 stimulation with bacterial MDP, MDP did induce IL8 and MIP1 alpha [42]. NOD1 also contributes to the induction of IL1B secretion in human trophoblasts after sensing of *Chlamydia trachomatis* [43]. These data clearly establish a role for NOD1 in regulating proinflammatory cytokines in human placental trophoblast cells.

The relevance of NOD1 has also recently been established in pregnancy using a mouse model. The administration of high-dose bacterial iE-DAP to pregnant C57BL/6J mice on Embryonic Day 14.5 triggers preterm delivery within 20 h [42]. Maternal exposure to iE-DAP at lower doses does not induce preterm birth; however, it leads to an extensive inflammatory response in the fetus, evidenced by the upregulation of a wide array of cytokines and chemokines. However, the expression and regulation of NOD1 and NOD2 in human myometrium and fetal membranes, which play a role in uterine contractions and rupture of fetal membranes, has yet to be investigated. The aim of this study was, thus, to determine the effect of spontaneous labor on NOD1 and NOD2 gene expression in fetal membranes and myometrium. The effects of mediators of labor, namely LPS and proinflammatory cytokines, on NOD1 and NOD2 gene expression were also assessed in fetal membranes and myometrium. To determine if NOD1 and NOD2 regulate proinflammatory and prolabor mediators, the effect of the NOD1 ligand iE-DAP and the NOD2 ligand MDP were examined in fetal membranes and myometrium.

**MATERIALS AND METHODS**

**Tissue Collection**

Human placenta and attached fetal membranes and myometrium were obtained (with the Research Ethics Committee of Mercy Health approval) from consenting women who delivered singleton infants. All tissues were obtained within 15 min of delivery.

Fetal membranes were obtained from consenting women at the time of preterm delivery (<37-wk gestation). Fetal membranes were obtained from the following two groups: 1) preterm no labor (Cesarean section with no labor [n = 9 patients]) and 2) preterm labor (after spontaneous labor and normal vaginal delivery [n = 9 patients]). Clinical details of the patients have been described previously [44]. All placentas collected from preterm gestations were swabbed for microbiological culture investigations and histopathological examination, and patients with chorioamnionitis were excluded. Women with pre-eclampsia, pre-existing diabetes, asthma, multiple pregnancies, and fetuses with chromosomal abnormalities were also excluded. Indications for preterm delivery (in the absence of labor) were placenta prævia, placental abruption, antepartum hemorrhage or Rhesus isoimmunization. Fetal membranes were obtained 2 cm form the periplacental edge.

Myometrium was obtained from consenting women at the time of term Cesarean section (>37-wk gestation). Myometrial biopsies were collected from two groups of women: 1) pregnant women undergoing elective Cesarean section in the absence of labor (n = 6 patients) and 2) pregnant women who delivered during active labor (n = 9 patients). Women were excluded from the study if they had a multiple pregnancy or evidence of active infection. A myometrial biopsy was obtained from the upper margin of the lower uterine segment incision during the Cesarean section. Tissue samples were snap frozen in liquid nitrogen and immediately stored at −80°C.

For the explants studies, fetal membranes (obtained 2 cm from the periplacental edge) and myometrium (obtained from the upper margin of the incision made in the lower uterine segment) were obtained from women who delivered healthy, singleton infants at term (>37-wk gestation) from elective Cesarean section in the absence of labor. Indications for Cesarean section were breech presentation and/or previous Cesarean section. A sample of fetal membrane and myometrium was also fixed and paraffin embedded for immunohistochemical analysis.

**Immunohistochemistry**

To determine the localization of NOD1 and NOD2 in fetal membranes and myometrium, immunohistochemistry was performed as described previously [45] with minor modifications. Immunohistochemistry was performed using the IHC Select Immunoperoxidase Secondary Detection System (Millipore). All incubations were performed at room temperature in a humidity chamber. Sections were blocked with normal goat serum for 30 min, followed by primary antibody for 60 min. Mouse monoclonal anti-NOD1 (MAB7090; R&D Systems) and rabbit polyclonal anti-NOD2 (NB500-253; Novus Biologicals) were used at 2.5 µg/ml and 5 µg/ml, respectively. Negative control slides, where primary antibody was replaced with normal rabbit or mouse IgG serum, were also included. Slides were incubated with biotinylated secondary antibody for 10 min followed by incubation with Streptavidin-HRP solution for 10 min and DAB chromagen reaction for 5 min. Slides were counterstained with Harris hematoxylin and mounted.

**Tissue Explant Culture**

To determine the effect of LPS, TNF, and/or IL1B on NOD1 and NOD2 gene expression, tissue explants were performed. Fresh fetal membranes and myometrium (collected as detailed above) were placed in Dulbecco modified Eagle medium (DMEM) at 37°C in a humidified atmosphere of 8% O2 and 5% CO2 for 1 h. Tissues were blotted dry on sterile filter paper and transferred to 24-well tissue culture plates (100 mg wet weight/well for fetal membranes and 50 mg wet weight/well for myometrium). The explants were incubated in 1 ml DMEM containing 100 µg/ml penicillin G and 100 µg/ml streptomycin. Tissues were incubated in the absence or presence of 10 µg/ml LPS, 20 ng/ml TNF, and/or 10 ng/ml IL1B for 20 h. In order to determine if LPS regulates NOD1 and NOD2 gene expression via NFκB and MAPK, additional experiments were also performed whereby fetal membranes and myometrium were pretreated with 25 µM SB202190 (p38 MAPK inhibitor), 10 µM U0126 (ERK inhibitor), and 25 µM SP600125 (cJun inhibitor) and/or 50 µM BAY 11–7082 (NFκB inhibitor) for 60 min before the addition of 10 µg/ml LPS for 20 h. The concentration of these inhibitors is based on previous studies in human gestational tissues [46, 47]. NOD1 and NOD2 expression was assayed by quantitative RT-PCR (qRT-PCR), as detailed below. Experiments were performed on fetal membranes from six patients and myometrium from five patients.

Tissue explants were also performed to determine the effect of the NOD1 ligand iE-DAP and the NOD2 ligand MDP on prolabor mediators in fetal membranes and myometrium. C12-iE-DAP and L18-MDP were purchased from InvivoGen (San Diego, CA). Tissue explants (prepared as detailed above) were incubated for 20 h in the presence or absence of 1 µg/ml iE-DAP or 1 µg/ml MDP. The concentrations of iE-DAP and MDP were based on past studies [48, 49]. Tissue explants were also performed in the absence or presence of 10 ng/ml LPS, 20 ng/ml TNF, and/or 10 ng/ml IL1B for 20 h. In order to determine if LPS regulates NOD1 and NOD2 gene expression via NFκB and MAPK, additional experiments were also performed whereby tissues were pretreated with 50 µM BAY 11–7082 for 60 min before the addition of iE-DAP and MDP for 20 h. After final incubation, tissue and media were collected separately and stored at −80°C for further analysis, as detailed below. Experiments were performed on myometrium from five patients, and fetal membranes from six patients.

**Gene Silencing of NOD1 and NOD2 with siRNA in Primary Myometrial Cells**

Primary myometrial cells were used to investigate the effect of siRNA-mediated gene silencing of NOD1 and NOD2 on prolabor mediators. Myometrial cells were isolated as previously described [50], and siRNA transfections were performed as previously described [44, 51, 52]. Briefly, cells at approximately 50% confluence were transfected using SilenceMag reagent according to manufacturer guidelines (Oz Biosciences, France). Cells were transfected with 100 nM NOD1 and NOD2 or nonspecific (NS) siRNA (Ambion) in DMEM/F-12 (containing 10% heat-inactivated fetal calf serum (FCS)) for 72 h. The medium was then replaced with DMEM/F-12 (containing 0.5% BSA) with or without 1 µg/ml iE-DAP or 1 µg/ml MDP, and the cells incubated at 37°C for an additional 20 h. Cells were collected and stored at −80°C until assayed for mRNA expression by qRT-PCR, as detailed below. Media were collected and stored at −80°C until assayed for cytokine, prostaglandin, and MMP release, as detailed below. Experiments were performed on myometrial cells obtained from six patients.
RNA Extraction and qRT-PCR

Total RNA was extracted from cells and tissues using TRIzol according to manufacturer’s instructions (Bioline). RNA concentration and purity were measured using a NanoDrop ND1000 spectrophotometer (Thermo Scientific, Pittsburgh, PA). RNA quality and integrity was determined via the A260/280 ratio. RNA (1 μg for tissue and 0.25 μg for cells) was converted to cDNA using the SuperScript VILO cDNA synthesis kit (Invitrogen) according to the manufacturer’s instructions. The cDNA was diluted 50-fold, and 4 μl of this was used to perform RT-PCR using SensiFAST SYBR (Bioline) and 100 nM of predesigned and validated primers (QuantiTect primer assays; Qiagen). The RT-PCR was performed using a CFX384 Real-Time PCR detection system from Bio-Rad Laboratories. Average gene Ct values were normalized to the average GAPDH Ct values of the same cDNA sample. Fold differences were determined using the comparative Ct method and are shown as mean ± SEM of relative gene expression. For the studies in the cells, there was a large variability of baseline values, which is normal for cells derived from different patients. Data were normalized to the respective control for cell preparations derived from each patient. Thus, data are presented as fold change in expression relative to the expression level in the NS siRNA transfected cells, which was set at 1.

NFKB Transcriptional Activity

A luciferase assay was utilized to determine the effect of iE-DAP and MDP on NFKB transcriptional activity. Primary myometrial cells, prepared as described above, at approximately 70% confluence, were transfected using XtremeGene HP DNA transfection reagent according to manufacturer guidelines (Roche Applied Science). Cells were transfected with 0.25 μg pNFKB-luc (Qiagen) for 48 h. The medium was then replaced with DMEM/F-12 with 2% heat-inactivated FCS, with or without 1 μg/ml iE-DAP or 1 μg/ml MDP, and the cells incubated at 37°C for an additional 20 h. The cells were harvested in lysis buffer and luminescence activity was measured using the Luciferase reporter assay kit (BioVision) as instructed. NFKB reporter activity was normalized to Renilla luciferase activity using the Pierce Renilla luciferase flash assay kit (Thermo Scientific). The normalized results are expressed as a ratio of luciferase activity of NFKB-luc reporter, which was set at 1. Experiments were performed in myometrial cells obtained from five patients.

Cytokine and Prostaglandin Assays

The release of IL6 and IL8 was performed by sandwich ELISA according to the manufacturer’s instructions (Life Technologies). The release of PGE2 and PGF2α into the incubation medium was assayed using a commercially available competitive enzyme immunoassay kit according to the manufacturer’s specifications (Kokubunba Kits; Sapphire Bioscience). Results were normalized to total protein using bicinchoninic acid protein assay (Thermo Scientific) and data expressed as either picograms or nanograms per milligram protein, unless otherwise specified.

Gelatin Zymography

Assessment of enzymes of ECM weakening and rupture (MMP9) was performed by gelatin zymography as previously described [53] on conditioned media collected from primary amnion and myometrium cells. Proteolytic activity was visualized as clear zones of lysis on a blue background of undigested gelatin. Gels were scanned (ChemiDoc; Bio-Rad Laboratories), inverted, and densitometry performed using Quantity One image analysis software (Bio-Rad Laboratories). Fold change was calculated relative to basal, which was set at 1.

Statistical Analysis

Statistical analysis was performed on the normalized data unless otherwise specified. All statistical analyses were undertaken using GraphPad Prism (GraphPad Software, La Jolla, CA). Two sample comparisons, either a paired or unpaired Student t-test, were used to assess statistical significance between normally distributed data; otherwise, the nonparametric Mann-Whitney U-test (unpaired) or the Wilcoxon test (matched pairs) were used. For all other comparisons, the homogeneity of data was assessed by the Bartlett test and, when significant, the data were logarithmically transformed before further analysis using a one-way ANOVA (using least significant difference correction to discriminate among the means). Statistical significance was ascribed to a P value < 0.05. Data are expressed as mean ± SEM.

RESULTS

Localization of NOD1 and NOD2 in Human Fetal Membranes and Myometrium

Immunohistochemistry was used to determine the localization of NOD1 and NOD2 in human fetal membranes (Fig. 1A) and human pregnant myometrium (Fig. 1B) at term. In the fetal membranes, amnion epithelial cells, choricionic cytotrophoblasts and the decidual cells exhibited strong NOD1 protein expression. There was also some NOD1 staining in the fibroblast cells of the connective tissue layer, NOD2 staining was less intense in the fetal membranes. The protein was localized to the choricionic cytotrophoblasts, the decidual cells and the fibroblast cells of the connective tissue layer. No NOD2 expression was observed in the amnion epithelium. For the myometrium, there was strong cytoplasmic NOD1 staining and moderate cytoplasmic NOD2 staining in the myometrial smooth muscle cells (myocytes). No staining was observed in the negative controls for both fetal membranes and myometrium.

Effect of Human Labor on NOD1 and NOD2 Gene Expression in Fetal Membranes and Myometrium

The next aim was to determine the effect of human labor on NOD1 and NOD2 expression. Fetal membranes were obtained from preterm deliveries, whereas myometrium samples were obtained from term deliveries. In fetal membranes (Fig. 2, A and B) and myometrium (Fig. 2, C and D), NOD1 and NOD2 expression was significantly higher after spontaneous labor when compared to nonlaboring tissues.

Effect of LPS, TNF and/or IL1B on NOD1 and NOD2 Gene Expression

Given that infection/inflammation is the biggest factor for preterm birth, the next aim was to determine the effect of the endotoxin LPS and the proinflammatory cytokines TNF and/or IL1B on NOD1 and NOD2 gene expression in fetal membranes and myometrium. Tissues were incubated in the absence or presence of 10 μg/ml LPS, 20 ng/ml TNF, and/or 10 ng/ml IL1B for 20 h. For both fetal membranes and myometrium, there was no effect of LPS or proinflammatory cytokines on NOD1 expression (data not shown). In fetal membranes, LPS, TNF, and IL1B significantly increased NOD2 expression (Fig. 3A). Likewise, in myometrium, LPS and IL1B significantly increased the expression of NOD2 (Fig. 3B).

Previous studies in gestational tissues have shown that LPS activates both the NFKB and MAPK pathway [13, 14, 46, 54, 55]. Further to this, in nongestational tissues, NFKB and MAPK proteins regulate NOD expression [18–20, 56]. Thus, the next aim was to determine if LPS regulates NOD2 gene expression via NFKB or MAPK pathway in gestational tissues. To do this, fetal membranes and myometrium were incubated with LPS in the absence or presence of the NFKB inhibitor BAY 11–7082. Additionally, fetal membranes were also incuated with LPS in the absence or presence of the p38 MAPK inhibitor SB202190, ERK inhibitor U0126, and cJun inhibitor SP600125. We have previously demonstrated the efficacy of BAY 11–7082 in inhibiting NFKB activation in human gestational tissues [57]. Likewise, we have also previously reported the specificity of SB202190 in inhibiting p38 MAPK, U0126 in inhibiting ERK, and SP600125 in inhibiting cJun inhibitor in fetal membranes [46, 58]. As presented in Figure 3, BAY 11–7082 significantly attenuates LPS-induced NOD2 expression in both fetal membranes (Fig.

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3C) and myometrium (Fig. 3D). In addition, in fetal membranes, inhibitors of the MAPK pathway significantly attenuate LPS-induced NOD2 expression (Fig. 3E).

**Effect of NOD1 and NOD2 Activation and Inhibition on the Expression of Prolabor Mediators in Human Fetal Membranes and Myometrium**

Having shown that NOD1 and NOD2 expression were upregulated by labor, the next aim was to determine if NOD1 and NOD2 regulate proinflammatory and prolabor mediators in fetal membranes and myometrium. This was done by incubating tissues with the NOD1 agonist iE-DAP and the NOD2 agonist MDP for 20 h. Cytokine and prostaglandin release was measured by ELISA, secretory MMP9 activity was measured by gelatin zymography, and gene expression by qRT-PCR. The data for fetal membranes are presented in Figure 4, and the data for myometrium presented in Figure 5. The iE-DAP and MDP significantly increased *IL6* and *IL8* mRNA expression and cytokine release from human fetal membranes (Fig. 4, A–D) and myometrium (Fig. 5, A–D). Similarly, in myometrium, both iE-DAP and MDP significantly increased *PTGS2* expression, which was associated with an increase in PGE₂ and PGF₂α release (Fig. 5, E–G). For fetal membranes, PGE₂ and PGF₂α were undetectable. The gene expression of *MMP9* and enzyme activity of MMP9 was also significantly augmented by iE-DAP and MDP in fetal membranes (Fig. 4, E and F) and myometrium (Fig. 5, H and I).

The next objective was to confirm that iE-DAP mediates its effects via NOD1 and that MDP acts via NOD2. Thus, to validate the specificity of the ligands to induce prolabor mediators, NOD1 and NOD2 were knocked out using siRNA in myometrial cells and the response to iE-DAP and MDP was determined. The efficacy of transfection was analyzed by qRT-PCR, with a 50% knockdown observed for *NOD1* and a 70% knockdown for *NOD2*. As expected, iE-DAP and MDP increased cytokine expression and release (Fig. 6, A–D), *PTGS2* and resultant prostaglandin release (Fig. 6, E and F).
and MMP9 mRNA expression and MMP9 activity (Fig. 6, G and H). However, in NOD1 siRNA transfected cells, the response to iE-DAP was significantly attenuated. Likewise, in NOD2 siRNA transfected cells, MDP failed to induce cytokine expression and release (Fig. 6, A–D), PTGS2 and resultant prostaglandin release (Fig. 6, E and F), and MMP9 gene expression and MMP9 activity (Fig. 6, G and H). Of note, these experiments were not performed in primary amnion cells, as NOD2 was not present in these cells (Fig. 1A).

NOD1 and NOD2 Activate the NFKB Pathway

NFKB is a key transcription factor responsible for regulation of inflammation in human gestational tissues [13, 14]. In nongestational tissues, NOD1 and NOD2 ligand activation mediates inflammation via activation of NFKB [18–22]. Thus, the next aim was to determine the effect of iE-DAP and MDP on NFKB transcriptional activity. This was performed by transfecting human myometrial cells with NFKB-luc reporter. After 48-h transfection, cells were incubated in the absence (basal) or presence of iE-DAP and MDP for 20 h (n = 6 patients) and NFKB transcriptional activity was assessed by luminescence. As shown in Figure 7, in myometrial cells expressing the NFKB-luc reporter, treatment with iE-DAP or MDP significantly increased luciferase activity.

NOD1 and NOD2 Regulate Prolabor Mediators via NFKB

The final aim was to explore whether the NOD1 agonist iE-DAP and the NOD2 agonist MDP regulate prolabor mediators in human gestational tissues via NFKB. To address this, we conducted experiments in which fetal membranes (n = 6 patients) or myometrium (n = 5 patients) were preincubated for 60 min with the NFKB inhibitor BAY 11–7082 before stimulation with iE-DAP and MDP for 20 h. The data for fetal membranes are presented in Figure 8, and the data for myometrium presented in Figure 9. In fetal membranes, BAY 11–7082 significantly attenuated iE-DAP- and MDP-induced cytokine gene expression (Fig. 8, A and C) and release (Fig. 8, B and D), and MMP9 mRNA expression and activity (Fig. 8, E and F). In myometrium, BAY 11–7082 significantly attenuated iE-DAP- and MDP-induced release of cytokines (Fig. 9, B and D), prostaglandins (Fig. 9, F and G), and pro-MMP9 release (Fig. 9I). In addition, MDP-induced IL6 and IL8 gene expression was also reduced by BAY 11–7082 (Fig. 9, A and C). There was, however, no effect of BAY 11–7082 on iE-
DAP-induced gene expression of *IL6* (Fig. 9A) and *IL8* (Fig. 9C). BAY 11–7082 had no effect on iE-DAP- or MDP-induced PTGS2 gene expression (Fig. 9E).

**DISCUSSION**

The data presented in this study demonstrate, for the first time, the presence of functional NOD1 and NOD2 receptors in human fetal membranes and myometrium. In this study, we found that NOD1 and NOD2 are expressed in human fetal membranes and human pregnant myometrium. Preterm labor induced an increase in *NOD1* and *NOD2* expression in human fetal membranes. In addition, *NOD1* and *NOD2* expression was also increased in term myometrium at labor compared with nonlaboring tissues. Bacterial endotoxin LPS induced a significant increase in *NOD2* gene expression, which was under the control of NFKB and MAPK proteins. The NOD1 ligand iE-DAP and the NOD2 ligand MDP significantly increased proinflammatory and prolabor mediators in human fetal membranes and myometrium. These NOD1- and NOD2-induced effects appeared to be elicited through activation of NFKB, as 1) iE-DAP and MDP induced NFKB transcriptional activity and 2) the NFKB inhibitor BAY 11–7082 significantly attenuated iE-DAP- and MDP-induced prolabor effects.
There are many causes of preterm labor; however, infection/inflammation is responsible for the majority of early preterm births [59]. Toll-like receptors (TLRs) are PRRs in the early host defense against pathogen invasion that are expressed on plasma membranes and lysosomal and/or endosomal vesicles [60]. However, certain types of bacteria may have the ability to evade TLR recognition by gaining access to the cell’s cytoplasm. NOD1 and NOD2 are cytoplasmic-based PRRs that can detect such intracellular bacteria [16]. The mechanisms by which these fragments enter host cells to induce NOD1 and NOD2 activation are poorly understood. They may enter the cell’s cytosol via pore-forming toxins, endocytosis, pathogen secretion systems [16, 21], or from an invasive intracellular bacterium [61]. Previous studies have shown in other cell types that LPS upregulates NOD1 and NOD2 expression [62]. Similarly, in trophoblast cells, LPS upregulates NOD1 and NOD2 mRNA expression [41], and the intracellular bacterial pathogen Chlamydia trachomatis induces NOD1 expression [43]. In this study, we report that LPS increases the expression of NOD2 in fetal membranes and myometrium. There was, however, no effect of LPS on NOD1 expression in these tissues.

Having shown that LPS upregulates NOD2 expression in fetal membranes and myometrium, the next objective was to determine the molecular mechanism by which LPS was modulating NOD2 mRNA levels in fetal membranes and myometrium. In keeping with the NOD2 promoter containing NFKB binding sites [63], studies have shown that NFKB regulates NOD expression. In trophoblast cells, LPS-induced NOD1 and NOD2 mRNA expression is significantly reduced by the presence of an NFKB inhibitor [41]. NFKB regulation of NOD proteins has also been reported for other cells, including epithelial and myelomonocytic cells [62]. We have also previously shown that the MAPK pathway is activated in...
FIG. 8. NOD1 and NOD2 ligands increase proinflammatory and prolabor mediators via the NFKB pathway in fetal membranes. Fetal membranes were preincubated in the absence or presence of 50 μM BAY 11–7082 for 60 min and then treated with 1 μg/ml iE-DAP or 1 μg/ml MDP for 20 h (n = 6 patients). A, C, and E IL6, IL8, and MMP9 gene expression was analyzed by qRT-PCR. Gene expression was normalized to GAPDH mRNA expression and the fold change was calculated relative to basal. Data are displayed as mean ± SEM. *P < 0.05 vs. basal (one-way ANOVA); **P < 0.05 vs. iE-DAP (one-way ANOVA); #P < 0.05 vs. MDP (one-way ANOVA). B and D) The incubation medium was assayed for concentration of IL6 and IL8 by ELISA. Each bar represents the mean ± SEM. *P < 0.05 vs. basal (one-way ANOVA); **P < 0.05 vs. iE-DAP (one-way ANOVA); $P < 0.05 vs. MDP (one-way ANOVA). F) Representative zymography from one patient and quantitation for MMP9. MMP9 expression levels were confirmed by densitometry. The fold change was calculated relative to basal. Each bar represents the mean ± SEM. *P < 0.05 vs. basal (one-way ANOVA); **P < 0.05 vs. iE-DAP (one-way ANOVA); $P < 0.05 vs. MDP (one-way ANOVA).

response to LPS in human gestational tissues [46]. Thus, we sought to determine if NFKB and MAPK proteins regulate LPS-induced NOD2 expression. We found that LPS-induced NOD2 expression was significantly attenuated by treatment with the NFKB inhibitor BAY 11–7082 in both fetal membranes and myometrium. In addition, the ERK inhibitor U0126, the p38 MAPK inhibitor SB202190, and the JUN inhibitor SP600125 also significantly attenuated LPS-induced NOD2 expression in fetal membranes. Collectively, these data suggest that NOD2 transcription is under the control of NFKB and MAPK proteins.

TNF and IL1B are two key cytokines linked to the pathogenesis of preterm birth. Labor is associated with increased concentrations of TNF and IL1B in both gestational tissues and biological fluids [9], and intra-amniotic and/or systemic administration of TNF or IL1B to mice and monkeys induces preterm labor [64–66]. Previous studies in nongestational tissues have shown that TNF and IL1B upregulate NOD1 and/or NOD2 mRNA expression in various cell lineages [62, 63, 67]. Likewise, in this study, TNF and/or IL1B induced NOD2 gene expression in fetal membranes and myometrium.

Even though labor was associated with an increase in NOD1 expression, there was no effect of LPS, TNF, or IL1B on NOD1 expression in fetal membranes. These data suggest that the increase in NOD1 observed in fetal membranes after spontaneous preterm labor and delivery may be mediated via factors other than infection and/or inflammation. Indeed, there are many other causes of preterm labor. These include stress activation of the maternal-fetal hypothalamic-pituitary-adrenal axis, uteroplacental thrombosis associated with decidual hemorrhage, uterine overdistention via multifetal gestations, or polyhydramnios.

During labor, there is an influx of macrophages, neutrophils, and T cells into the myometrium [68, 69]. The activated leucocytes express proinflammatory cytokines, which can amplify or initiate the process of parturition. For example, IL1B can increase proinflammatory cytokines (e.g., IL6 and TNF), chemokines (e.g., IL8), PTGS2, prostaglandins, and ECM remodeling enzymes (e.g., MMP9), which initiate spontaneous premature uterine contractions [8–12, 70–72]. In this study, the NOD1 ligand bacterial iE-DAP and the NOD2 ligand bacterial MDP induced proinflammatory and prolabor mediators in human myometrium. These effects of iE-DAP and MDP were specific for NOD1 and NOD2, respectively. Specifically, iE-DAP failed to induce a proinflammatory effect in NOD1-deficient cells. Likewise, in myometrial cells where NOD2 was knocked out using siRNA, MDP failed to induce prolabor mediators.

Approximately one-third of all cases of preterm birth are due to the untimely rupture of the fetal membranes. In cases of PPROM, there is a significant increase in adverse clinical perinatal outcomes and health care costs [73]. PPROM arises from multiple pathologic pathways; however, most commonly, chronic inflammation and/or infection are the biggest factors associated with PPROM [6]. This results in the activation of inflammatory cytokines and the ECM-degrading enzyme MMP9, which are involved in the processes leading to membrane rupture [7–12]. The data presented in this study demonstrate that NOD1 and NOD2 are increased in fetal membranes after spontaneous preterm labor and delivery. Furthermore, the NOD1 and NOD2 ligands increased proinflammatory cytokine expression and MMP9 activity in fetal membranes. To my knowledge, this is the first study demonstrating the prolabor and proinflammatory actions of NOD1 and NOD2 in human fetal membranes and myometrium. However, the data presented in this paper confirm the studies by Abrahams’ group that have shown an important role for NOD1 in regulating proinflammatory cytokines in placental trophoblast cells [40–43]. Importantly, they are also in support of a recent study that demonstrated that administration of high-
FIG. 9. NOD1 and NOD2 ligands increase proinflammatory and prolabor mediators via the NFKB pathway in myometrium. Myometrium was incubated in the absence or presence of 1 μg/ml iE-DAP or 1 μg/ml MDP with or without 50 μM BAY 11–7082 for 20 h (n = 5 patients). A, C, E, and H IL6, IL8, PTGS2, and MMP9 gene expression was analyzed by qRT-PCR. Gene expression was normalized to GAPDH mRNA expression and the fold change was calculated relative to basal. Data is displayed as the mean ± SEM. *P < 0.05 vs. basal (one-way ANOVA); **P < 0.05 vs. iE-DAP (one-way ANOVA); #P < 0.05 vs. MDP (one-way ANOVA). B, D, F, and G The incubation medium was assayed for concentration of IL6, IL8, PGE2, and PGF2α by ELISA. Each bar represents the mean ± SEM. *P < 0.05 vs. basal (one-way ANOVA); **P < 0.05 vs. iE-DAP (one-way ANOVA); #P < 0.05 vs. MDP (one-way ANOVA). I Representative zymography (from one patient) and quantitation for MMP9. Expression was confirmed by densitometry. The fold change was calculated relative to basal. Data is displayed as the mean ± SEM. *P < 0.05 vs. basal (one-way ANOVA); **P < 0.05 vs. iE-DAP (one-way ANOVA); #P < 0.05 vs. MDP (one-way ANOVA).

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THE ROLE OF NOD1 AND NOD2 AND HUMAN LABOR

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ANALYSIS

To date, the NOD signaling cascade has been shown to be involved in the activation of the NFKB pathway that drives the transcription of numerous genes involved in inflammation [19, 49, 54, 67, 74–76]. Of note, although lower doses of iE-DAP did not induce preterm birth, they altered the cytokine profile at the maternal-fetal interface, and induced fetal inflammation [42]. The effect of NOD2 ligands, however, was not evaluated in this study. Like TLRs, stimulation of NOD1 or NOD2 results in the activation of NFKB, which drives the transcription of numerous genes involved in inflammation [19, 49, 54, 67, 74–76]. There is now much evidence to demonstrate that NFKB also regulate the key terminal processes of human labor and delivery by driving the expression of many prolabor genes [13–15]. Inhibition of NFKB activity in ex situ human gestational tissues, suppresses the formation of labor-mediating effectors, including proinflammatory cytokines, PTGS2-prostaglandin pathways, and ECM-remodeling enzymes [77–79]. Furthermore, labor and delivery are associated with changes in NFKB content in fetal membranes and myometrium and increased NFKB DNA binding activity in the fetal membranes [13–15]. The results of this study show that, in human gestational tissues, NOD1 and NOD2 elicit their prolaboratory and prolabor actions via NFKB. Specifically, the NOD1 and NOD2 ligands activated NFKB transcriptional activity, and the NFKB inhibitor BAY 11–7082 significantly abrogated the proinflammatory and prolabor effects of iE-DAP and MDP.

Following microbial sensing, NOD1 or NOD2 directly recruit the signaling effector protein, RIP-like interacting CAMP kinase (RICK) [19, 75]. RICK null cells of mice fail to activate NFKB signaling downstream of NOD1 and NOD2 ligand stimulation, indicating that RICK is required in vivo for signaling [74, 80, 81]. Studies are now in progress to determine if RICK is also required for NOD1 and NOD2 signaling in human gestational tissues.

Regardless of the cause of preterm labor, they all share many common pathways in the activation of common downstream cellular and molecular effectors. Thus, understanding the basis of molecular mechanisms that trigger the onset and process of rupture of membranes and uterine contractions may lead to therapeutic treatment and interventions that are vital to the future health and well-being of the infant. The results of this study indicate that the NOD system is functional within human fetal membranes and myometrium, and may contribute to the inflammatory response elicited by exposure to bacterial products. It is of note that administration of the NOD1 ligand iE-DAP to Day-14.5 pregnant mice increases proinflammatory cytokines in maternal and fetal tissues and importantly induces preterm birth [42]. Taken together, these studies demonstrate that NOD receptors may represent therapeutic targets for the treatment and/or management of infection-induced preterm birth.
REFERENCES


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