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Fetal Membrane Inflammation Induces Preterm Birth Via Toll-Like Receptor 2 in Mice With Chronic Gingivitis

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Abstract

Inflammation is associated with preterm birth. We previously described a mouse model of chronic inflammation-induced preterm birth after dental *Porphyromonas gingivalis* infection. The aim of this study was to employ this model system to investigate the mechanisms through which enhanced uterine contractility induces preterm birth. Messenger RNA (mRNA) encoding contraction-associated proteins, such as oxytocin receptors, was measured at various gestational time points by real-time polymerase chain reaction (PCR). Spontaneous and oxytocin-induced uterine contractile activity at gestational day 18 was assessed using a tissue organ bath. The expression levels of Toll-like receptor 2 (TLR2), TLR4, cyclooxygenase (COX)-2, nuclear factor-kappa B (NF-κB) p65, and p38 mitogen-activated protein kinase (MAPK) on gestational day 18 were also determined by real-time PCR or Western blotting. Messenger RNA encoding contraction-associated proteins was increased at gestational day 18, and the spontaneous contractile activity (1.6-fold greater area under the contraction curve) and sensitivity to oxytocin (EC_{50}: 8.8 nM vs 2.2 nM) were enhanced in the *P gingivalis* group compared to those in the control group. In the *P gingivalis* group, COX-2 mRNA expression was not elevated in the placenta or myometrium but was upregulated 2.3-fold in the fetal membrane. The TLR2 mRNA levels in the fetal membrane were 2.7-fold higher in the *P gingivalis* group, whereas TLR4 levels were not elevated. Activation of the NF-κB p65 and p38 MAPK pathways was enhanced in the fetal membrane of the *P gingivalis* group. Thus, in mice with chronic dental *P gingivalis* infection, TLR2-induced inflammation in the fetal membrane leads to upregulation of uterine contractility, leading to preterm birth.

Keywords

inflammation-induced preterm birth, *Porphyromonas gingivalis*, toll-like receptor-2, uterine contractility

Introduction

Premature birth has a significant influence on neonatal morbidity and mortality.[1-3] The rate of preterm birth has increased in recent years,[4] despite significant efforts to prevent this. Two-thirds of preterm births occur after the spontaneous onset of labor, whereas the remainder is medically induced due to maternal or fetal complications such as preeclampsia or fetal growth restriction.[5] Thus, elucidation of the mechanisms underlying spontaneous preterm birth will facilitate the development of novel preventative and therapeutic strategies.

Enhanced uterine contraction, cervical ripening, and rupture of membranes often occur earlier in preterm birth than in term birth.[6] Preterm birth has also been reported to be associated with maternal inflammation.[7] In animal models, the injection of bacteria, lipopolysaccharide (LPS), or proinflammatory

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cytokines during pregnancy results in maternal acute inflammation and subsequent preterm birth.[8-11] For example, intraamniotic administration of interleukin (IL) 1β has been shown to enhance uterine contractions in pregnant rhesus monkeys.[12] However, no studies have examined whether enhanced uterine contraction is associated with maternal chronic inflammation.

Periodontal disease is associated with various systemic conditions such as cardiovascular disease, diabetes, nonalcoholic steatohepatitis,[13,14] and notably preterm birth and fetal growth restriction.[15-17] We have previously described a model of preterm birth using mice infected with *Porphyromonas gingivalis* , a common cause of periodontal disease.[18,19] Mice infected with *P gingivalis* before pregnancy have a shorter duration of pregnancy than healthy mice,[18] in addition to higher serum levels of pro-inflammatory cytokines, including IL-1β and tumor necrosis factor (TNF)-α both before and during pregnancy.[18,19] The major strengths of these models are that systemic inflammation persisted throughout the pregnancy period, and preterm delivery was observed, although there were no manipulations, such as LPS injection, for additional infectious conditions during pregnancy. Thus, we concluded that the *P gingivalis* mice will serve as a better model for the clinical condition in humans than the usual animal models. This indicates that a maternal chronic inflammatory condition might lead to preterm birth.

Myometrial activation is linked to the coordinated expression of contractile-associated proteins (CAPs) such as oxytocin receptors, prostaglandin F (FP) receptors, and connexin-43 (Cx43). Myometrial expression of CAPs is enhanced at the late stages of pregnancy, causing increased uterine contractility in both term and preterm delivery.[20] The *P gingivalis*-infected mice show elevated myometrial expression of messenger RNA (mRNA) encoding CAPs and Ca^{2+} channels (L-type Ca^{2+} channels and purinergic P2X7 receptors) at gestational day 18.[21,22] Thus, myometrial activation is a key component for the induction of preterm birth in our animal model.[19] However, serum levels of certain cytokines were not found to be associated with CAP expression, and the mRNA expression of IL-1β and TNF-α was not enhanced in the myometrium. Hence, the mediators leading to myometrial activation in this model remain unknown.

During pregnancy, prostaglandins are synthesized mainly in the myometrium and fetal membrane, stimulating myometrial contractions.[23] In our mouse model of preterm birth, the mRNA expression of IL-1β and TNF-α was enhanced in the fetal membrane,[19] suggesting that inflammation in the fetal membrane might activate the myometrium. Intrauterine bacterial infection, such as chorioamnionitis, is widely accepted as a common cause of preterm birth. Bacteria or LPS, 2 Toll-like receptor (TLR) ligands, activate downstream inflammatory responses such as nuclear factor-kappa B (NF-κB) and mitogen-activated protein kinase (MAPK) pathways.[24] In the uterus, this promotes the expression of proinflammatory cytokines and cyclooxygenase (COX)-2, leading to preterm birth. Interestingly, we have observed *P gingivalis* colonies within the placenta in our mouse model of preterm birth.[18]

The aim of this study was to identify the mechanisms underlying myometrial activation in our mouse model of chronic inflammation-induced preterm birth. We investigated the inflammatory pathways in the fetal membrane that are induced by TLRs, including NF-κB, MAPK, and COX-2, and evaluated myometrial activation by measuring gestational changes in CAPs and uterine contractility.

Materials and Methods

Animal Studies

Eight-week-old female C57BL/6J mice (Charles River Japan, Yokohama, Japan) were housed in a cage (12/12-hour light/dark cycle) at 22°C and fed a solid diet with access to water ad libitum. This study was performed in accordance with the guidelines of the Committee of Research Facilities for Laboratory Animal Science at Hiroshima University. Female mice were housed with male mice overnight, and pregnancy was confirmed by the appearance of a vaginal plug (defined as gestational day 0).

Chronic Inflammation-Induced Model of Preterm Birth

Dental infection with *P gingivalis* was performed as described previously.[25] Briefly, the roof of the pulp chamber of the upper jaw right and left first molars was removed using a #1/2 round bar. A small cotton swab of phosphate-buffered saline containing 10^7 *P gingivalis* cells (W83 strain) was inserted into the pulp chamber, which was sealed using Caviton (GC Corporation, Tokyo, Japan). After 6 weeks, a pathological periapical granuloma is observed in this model, and serum levels of IL-1β and TNF-α have been determined to increase 2-fold and 2.5-fold, respectively,[18] confirming chronic inflammation. Therefore, mating was started 6 weeks after *P gingivalis* infection. The average gestational periods for mice with (*P gingivalis* group) and without (control group) *P gingivalis* infection are 18.3 $\pm$ 0.9 days and 20.5 $\pm$ 0.5 days, respectively.[18]

Tissue Collection

Pregnant mice were killed under anesthesia on gestational days 15 to 18 and at the time of delivery (of one pup). The uterus was excised, and the myometrium and fetal membrane were collected separately for RNA and protein extraction. On gestational day 15, whole uterine tissues including the myometrium, fetal membrane, placenta, and pups were preserved for histologic examination, and a blood sample was collected from the heart or inferior vena cava.

RNA Extraction and Real-Time Reverse Transcriptase Polymerase Chain Reaction

Levels of mRNA encoding CAPs, TLR2, TLR4, COX-2, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were measured by real-time reverse transcriptase polymerase chain reaction. The relative gene expression levels were determined

as described previously.[19] The primer sequences for this analysis were as follows: mouse oxytocin receptor, forward: 5′-ATC CGC ACA GTG AAG ATG ACC T-3′ and reverse: 5′-GCA TGG CAA TGA TGA AGG CAG A-3′; mouse Cx43, forward: 5′-CCG AAC TCT CTT TTC CTT TGA CTC-3′ and reverse: 5′-ACC TTG TCC AGC AGC TTC CC-3′; mouse FP receptor, forward: 5′-CTG GCC ATA ATG TGC GTC TC-3′ and reverse: 5′-TGT CGT TTC ACA GGT CAC TGG-3′; mouse COX-2, forward: 5′-GAA TCA TTC ACC AGG CAA ATT G-3′ and reverse: 5′-TCT GTA CTG CGG GTG GAA CA-3′; mouse GAPDH, forward: 5′-GCA TCC TGG GCT ACA CTG AG-3′ and reverse: 5′-TCC ACC ACC CTG TTG CTG TA-3′. Gene expression was normalized to that of GAPDH. Each sample was run in duplicate, and output levels are reported as the average for 2 wells.

Measurement of Uterine Strip Contractility

Uterine strips ($\sim$5 mm wide and $\sim$7 mm long) at gestational day 18 were suspended vertically between 2 steel wires in an organ chamber containing 10 mL of modified Krebs solution (mmol/L: NaCl 122, KCl 4.7, $CaCl_2$ 2.5, $MgCl_2$ 1.2, KH_2PO_4 1.2 $NaHCO_3$ 15.4, D-glucose 11.5)[26] gassed with 5% CO_2 and 95% O_2 (pH 7.4 at 37°C). The resting tension was adjusted to 1 g. Spontaneous contractions were measured after stable responses to 100 mmol/L KCl had been achieved. Then, increasing concentrations of oxytocin were added (1 pmol/L to 300 nmol/L, at 20-minute intervals; Peptide Institute, Osaka, Japan). Following this, the tissue was washed and exposed to KCl (100 mmol/L) to elicit a tetanic response and confirm viability. Uterine contractions were recorded with a force transducer (UL2; Minebea Co Ltd, Osaka, Japan), and the data were digitized and analyzed using PowerLab and Lab Chart software (Bio Research, Nagoya, Japan). The number of contraction cycles, maximum intensity of each contraction, and duration (baseline to baseline) of each contraction were recorded. Total force was calculated as the area under the contraction curve (AUC) for the final 5 minutes at each drug concentration, and this value was normalized to the weight of the uterine strip.

Western Blotting

Western blot analysis of fetal membrane samples was performed to determine protein levels of NF-κB and MAPK. Protein extracts (1000 μg) were subjected to electrophoresis using 4% to 12% sodium dodecyl sulfate polyacrylamide electrophoresis gels, and the proteins were transferred to membranes using an iBlot 2 Dry Blotting System (Life Technologies, Carlsbad, California). Membranes were incubated overnight with primary antibodies as follows: anti-phosphorylated-NF-κB p65 (1:1000; Cell Signaling Technology [CST], Danvers, Massachusetts), anti-NF-κB p65 (1:3000; CST), anti-phosphorylated-p38 MAPK (1:3000; CST), anti-p38 MAPK (1:3000; CST), and anti-cyclophilin B (1:20,000; Abcam, Cambridge, United Kingdom). Cyclophilin B was used as a loading control.

Histologic Examination

Whole uterine tissues, including myometrium, fetal membrane, placenta, and pups, were fixed in 4% paraformaldehyde at gestational day 15 for immunolocalization of *P gingivalis* colonies as described previously.[19] Samples were incubated with primary antibody (rabbit antiserum against whole *P gingivalis*, 1:5000 dilution; kindly provided by Professor Kazuyuki Ishihara, Tokyo Dental College) for 1 hour at 4°C, followed by DAB substrate (Dako, Glostrup, Denmark) for 1 minute. The same tissues and ovaries were also stained with hematoxylin and eosin.

Measurement of Serum Progesterone

Blood samples were collected from the heart on gestational day 18. The serum was separated by centrifugation and stored at −80°C. Progesterone levels were measured by enzyme immunoassay, in accordance with the manufacturer's protocol (Cayman Chemical, Ann Arbor, Michigan), using a microtiter plate reader (at 405 nm).

Statistical Analysis

Statcel 3 software for Microsoft Excel (Microsoft, Redmond, Washington) was used for statistical analyses. Comparisons were made using the Student *t* test or the Mann-Whitney *U* test; $P < .05$ was considered statistically significant. KaleidaGraph 4.5 (Hulinks Inc, Tokyo, Japan) was used to construct graphs and fit dose–response curves using the least-squares method.

Results

Gestational Changes in the Expression of CAPs Associated With P gingivalis Infection

We first examined gestational changes in the myometrial expression of CAPs. In the control group, oxytocin receptor, Cx43, and FP receptor expression levels were low at gestational days 15 to 18 but were elevated 16.9-fold, 6.7-fold, and 3.1-fold, respectively, at delivery compared to levels at gestational day 15 (Figure 1A-C). The expression of CAPs at gestational days 15 to 17 was similar between the *P gingivalis* and control groups. However, compared to that in controls, the *P gingivalis* group showed elevated expression of CAPs at day 18 and at delivery (Figure 1A-C). Moreover, oxytocin receptor, Cx43, and FP receptor levels at gestational day 18 were increased 3.2-fold, 2.4-fold, and 2.4-fold, respectively, in the *P gingivalis* group compared to expression in the control group ($P < .05$, $n = 10$ per group).

Porphyromonas gingivalis Infection Induces Uterine Contractile Activity

Representative spontaneous and oxytocin-induced uterine oscillatory contractions are presented in Figure 2A. The mean

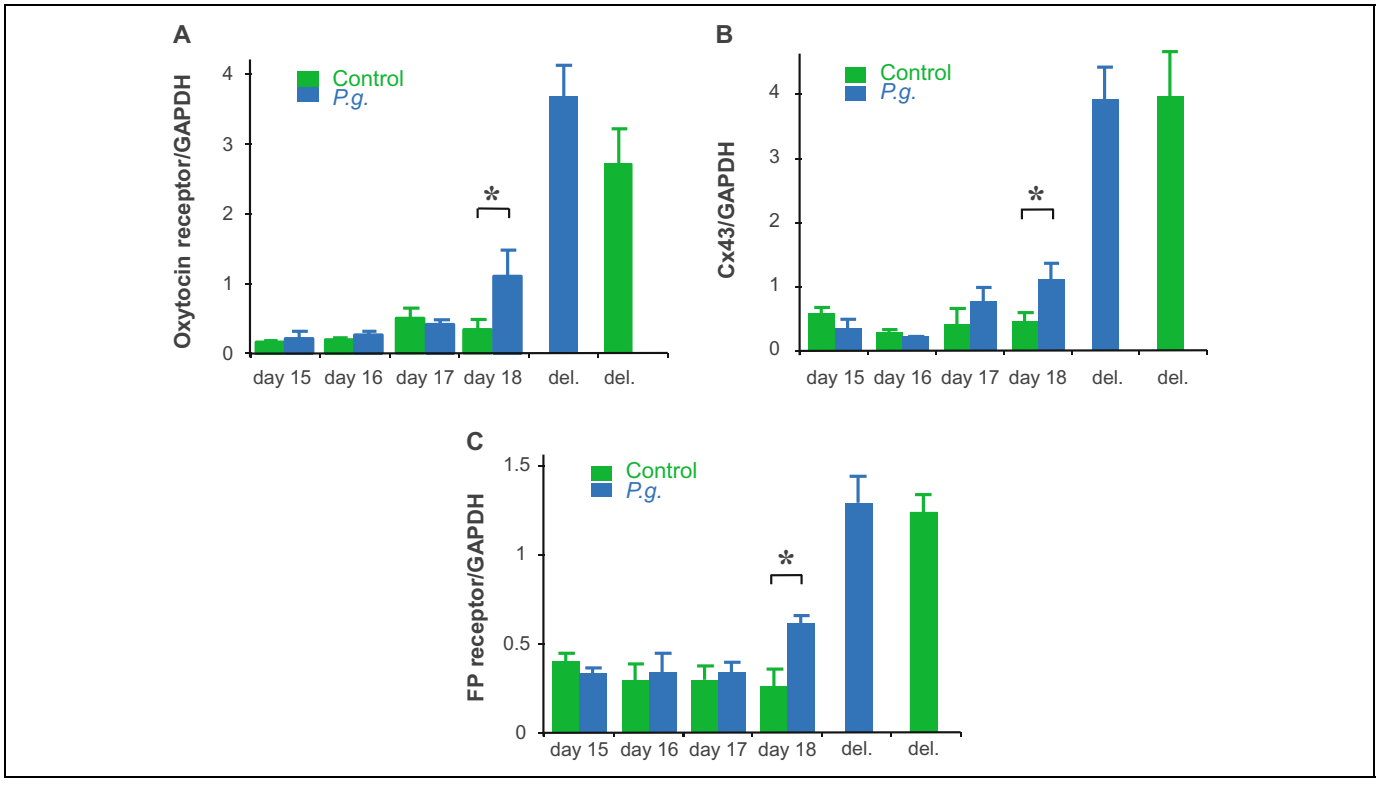

Figure 1. Gestational changes in the mRNA expression of CAPs in the myometrium with *Porphyromonas gingivalis* mRNA expression levels of CAPs in the myometrium at various time points were measured by real-time RT-PCR and normalized to those of GAPDH. A, Oxytocin receptor expression. B, Cx43 expression. C, FP receptor expression. Green bars: control group; blue bars: *P gingivalis* group. Gestational days 15, 16, and 17: n = 4 per group; gestational day 18: n = 10 per group; delivery (del.): n = 8 per group. *$P < .05$. mRNA expression levels of the oxytocin receptor, Cx43, and FP receptor in the myometrium at day 18 of gestation were elevated 3.2-fold ($P = .008$), 2.4-fold ($P = .02$), and 2.4-fold ($P = .001$), respectively, in the *P gingivalis* group compared to those in the control group. CAP indicates contractility-associated protein; Cx43, Connexin-43; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; mRNA, messenger RNA; PCR, polymerase chain reaction; FP, prostaglandin F; RT, reverse transcriptase.

numbers of spontaneous cyclic contractions per 5 minutes in the control and *P gingivalis* groups were 8.3 ± 0.7 and 8.4 ± 1.0, respectively (n = 6 per group). However, higher maximum intensity for each spontaneous contraction (0.15 ± 0.02 g vs 0.09 ± 0.01 g per 1 mg of uterine strip; $P = .01$) and longer durations of contractions (25.7 ± 1.1 s vs 21.7 ± 1.5 s; $P = .03$) were observed in the *P gingivalis* group compared to those in the control group. The mean AUC of spontaneous contractions over a 5-minute period was 1.6-fold higher in the *P gingivalis* group than in the control group ($P = .002$, n = 6 per group; Figure 2B), indicating that the *P gingivalis* group exhibited enhanced spontaneous uterine contractions.

Oxytocin increased the frequency and strength of uterine contractions in both the groups in a concentration-dependent manner (Figure 2A and C). However, AUC values were significantly higher in the *P gingivalis* group than in the control group for every oxytocin concentration, indicating an enhanced response to oxytocin in the *P gingivalis* group. In addition, the half-maximal effective concentration (EC_{50}) of oxytocin was 8.8 nmol/L in the control group and 2.2 nmol/L in the *P gingivalis* group.

Porphyromonas gingivalis *Infection-Induced Labor Is Not Associated With Progesterone Withdrawal*

Progesterone withdrawal leads to parturition in most mammals, including mice.[27,28] On gestational day 18, serum progesterone was not significantly different[15,16] between the control (41.9 ± 30.7 ng/mL) and the *P gingivalis* (24.0 ± 23.8 ng/mL) groups (n = 6 per group). The ovaries, specifically the corpus luteum, are the source of progesterone during pregnancy in mice. In hematoxylin–eosin-stained samples, we observed no significant regression of the corpus luteum at gestational day 18 (data not shown). These findings suggest that neither ovarian function nor serum progesterone is involved in the initiation of labor in our mouse model.

Porphyromonas gingivalis *Infection Upregulates COX-2 Expression in the Fetal Membrane*

The mRNA expression of COX-2 in the placenta and myometrium on gestational day 18 did not differ between the *P gingivalis* and the control groups (Figure 3A and B). However, COX-2 mRNA expression in the fetal membrane was

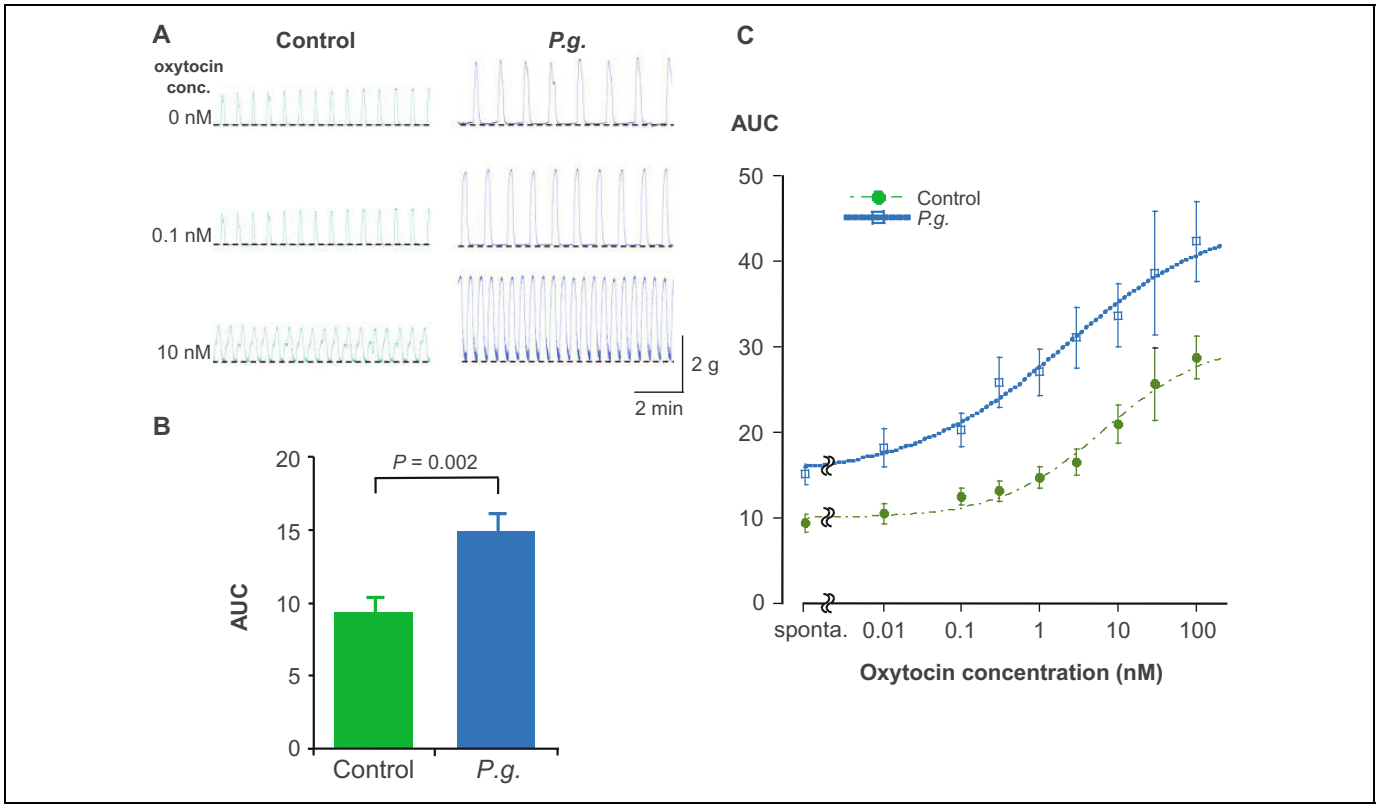

Figure 2. Differences in uterine contractility between mice in the control and *Porphyromonas gingivalis* infected groups. A, Representative traces showing uterine contractions in the presence of different concentrations of oxytocin (0, 0.1, and 10 nM). B, The mean AUC for spontaneous uterine contractile activity on day 18 of gestation was increased 1.6-fold in the *P gingivalis* group compared to that in the control group ($P = .002$, n = 6 per group). C, Mean AUC values for uterine contraction measured for spontaneous contractions (sponta.) and for contractions induced by various concentrations of oxytocin. At every oxytocin concentration, the AUC value for the *P gingivalis* group was higher than the corresponding value for the control group (n = 6 per group; $P < .05$). AUC indicates area under the contraction curve; *Porphyromonas gingivalis*.

2.3-fold higher in the *P gingivalis* group than in the control group ($P = .02$, n = 10 per group; Figure 3C).

Porphyromonas gingivalis *Is Found in the Fetal and Placental Membrane of Infected Mice*

We previously reported that in our mouse model of chronic inflammation-induced preterm birth, *P gingivalis* colonies can be observed in the placenta, and especially in villus cells on the embryo side, but not in the myometrium.[19] In the present study, immunolocalization of *P gingivalis* in intrauterine tissues was performed on gestational day 15 because all 3 layers of the fetal membrane can be clearly observed at this time. *Porphyromonas gingivalis* colonies were not detected in the control group. However, in the *P gingivalis* group, colonies were detected in the fetal membrane, mainly in the chorionic membrane and amniotic mesenchymal cells (Figure 4A). Interestingly, neutrophil infiltration and macrophage accumulation were slight in the fetal membrane or placenta hematoxylin–eosin-stained sections (Figure 4B and C). These findings suggest that *P gingivalis* might migrate to the placenta and fetal membrane through the blood to form colonies that induce local inflammation but not chorioamnionitis.

Inflammatory Pathways in the Fetal Membrane Are Activated by P gingivalis

The TLR2 and TLR4 are transmembrane proteins that recognize pathogen-associated molecular patterns in bacteria and activate inflammatory pathways. Messenger RNA levels of TLR2 and TLR4 in the placenta at gestational day 18 were similar between the *P gingivalis* and control groups (Figure 5A and B). Moreover, mRNA expression of TLR4 in the fetal membrane was also not elevated in the *P gingivalis* group (Figure 5C). However, the mRNA expression of TLR2 in the fetal membrane was 2.7-fold higher in the *P gingivalis* group than in the control group ($P = .003$, n = 10 per group; Figure 5D). Western blot analysis was performed to detect NF-κB and MAPK in the fetal membrane on gestational day 18. Compared to those in the control group, elevated levels of phosphorylated NF-κB p65 and p38 MAPK, but not total NF-κB p65 and p38 MAPK, were observed in the *P gingivalis*

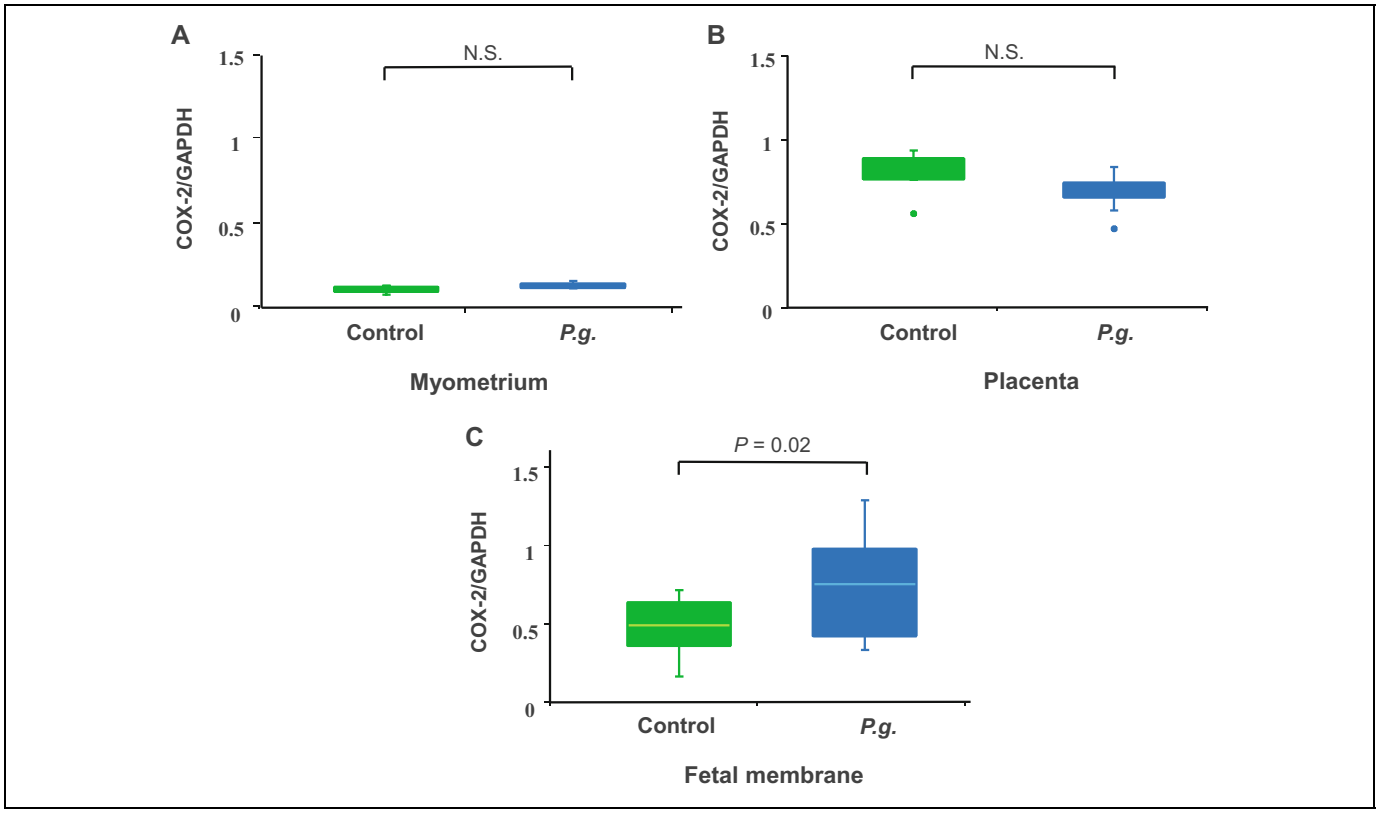

Figure 3. Expression of COX-2 mRNA in the placenta, fetal membrane, and myometrium at day 18 of gestation with *Porphyromonas gingivalis* infection. The mRNA expression of COX-2 was measured by real-time RT-PCR and normalized to that of GAPDH. A, Myometrium. B, Placenta. C, Fetal membrane. COX indicates cyclooxygenase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; mRNA, messenger RNA; NS, not significant; PCR, polymerase chain reaction; RT, reverse transcriptase.

group (Figure 6). These results suggest that TLR2-activated inflammatory pathways in the fetal membrane of *P gingivalis*-infected mice.

Discussion

Although many studies have indicated that maternal intrauterine infection or inflammation can cause preterm birth,[8-11] in most of these investigations, preterm birth was induced by acute and/or local infection at a late gestational stage. An important characteristic of our model system is that chronic inflammation is initiated before pregnancy onset and is maintained throughout the gestational period. Therefore, our model system has notable advantages over previous methodologies to investigate how chronic inflammation affects preterm birth.[18,19]

In the present study, we observed gestational changes in the expression of CAPs, which are necessary for the initiation and progression of uterine contractions. Messenger RNA expression of the oxytocin receptor, Cx43, and FP receptor was elevated at delivery in both groups. Interestingly, the mRNA expression of these CAPs began to increase at an earlier time (gestational day 18, Figure 1) in the *P gingivalis* group. Additionally, the *P gingivalis* group exhibited enhanced spontaneous uterine contractility and an increased sensitivity to oxytocin at this time (Figure 2). Thus, the upregulation of CAPs at an earlier time in gestation might initiate uterine contractility, thereby leading to preterm birth. Our findings are consistent with previous studies showing that LPS-induced acute maternal inflammation results in enhanced expression of CAPs[29-31] and increased uterine contractions.[32,33]

The expression levels of CAPs were similar to those of the control until day 17 but were significantly increased compared to those of the control at day 18. The switch of the myometrium from a quiescent state to a contractile state appears to occur in late period of gestation.[6] In this report, we cannot explain why the expression of CAPs significantly changed at day 18; however, we speculate that myometrial conditions such as some responses to cytokine signaling or sensitivity to progesterone were altered to facilitate the switch to a contractile state due to chronic inflammation. This subsequently led to the induction of myometrial activation at day 18. Further studies are needed to elucidate the detailed mechanisms.

We focused on inflammation as the mechanism underlying this earlier upregulation of CAPs in our model system, because there was no evidence of progesterone withdrawal in this report. High serum levels of IL-1 and TNF were observed before and during pregnancy in the *P gingivalis* group,[18,19] suggesting that systemic inflammation might affect the expression of CAPs. However, we also confirmed the presence of

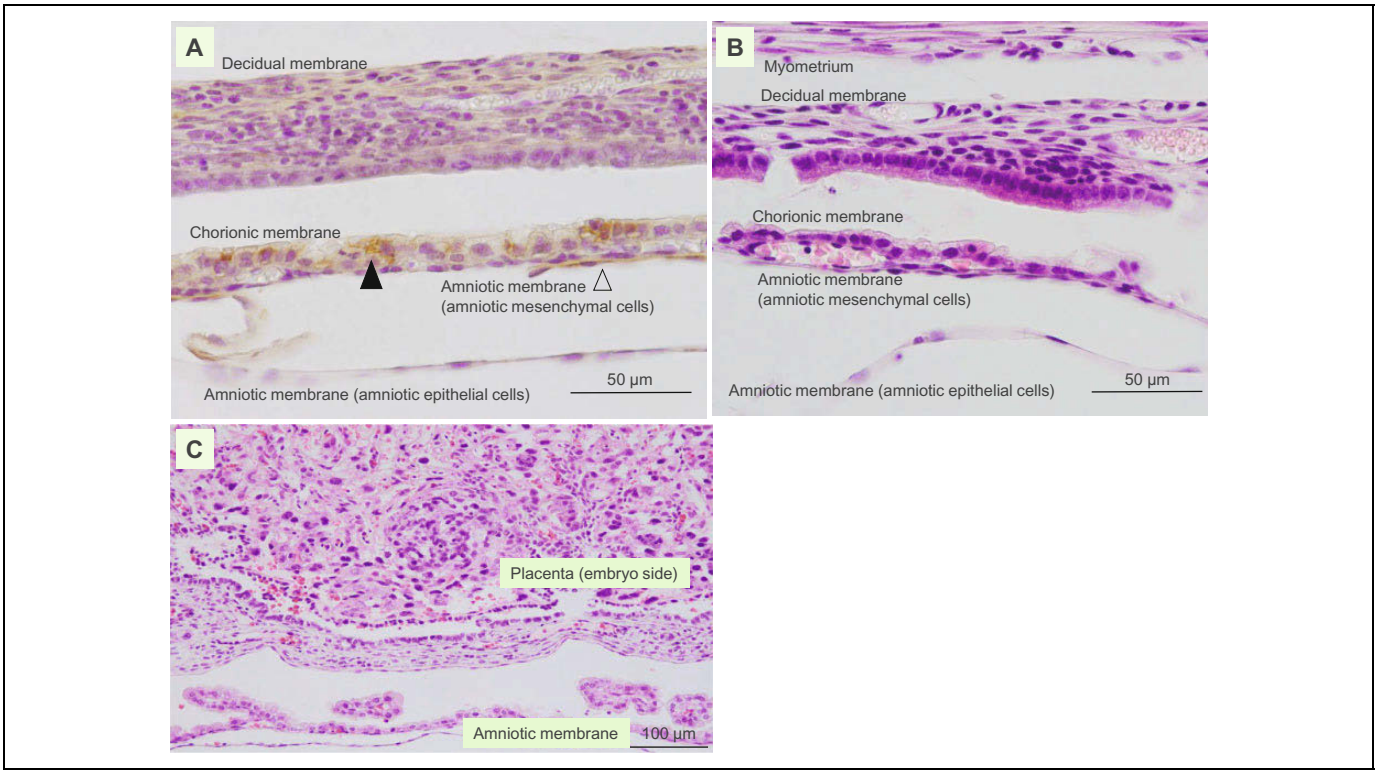

Figure 4. Detection of *Porphyromonas gingivalis* colonies in fetal membrane tissue by immunohistochemistry. A, *P gingivalis* colonies (stained brown) were observed in the fetal membrane, and mainly in the chorionic membrane (▲) and amniotic mesenchymal cells (Δ). B and C, Neutrophil infiltration and macrophage accumulation were not observed in the fetal membrane (B) or placenta (C). Magnification: ×600.

local intrauterine inflammation. As described previously,[19] IL-1 and TNF levels in the *P gingivalis* group were increased in the placenta, and especially in the fetal membrane, but not in the myometrium. The mRNA expression of COX-2 in the fetal membrane was also elevated in the *P gingivalis* group (Figure 3). Since the fetal membrane is recognized as a major source of prostaglandin, which is a potent mediator of uterine contraction,[23] we investigated inflammatory pathways in the fetal membrane.

Porphyromonas gingivalis colonies were detected in the fetal membrane (Figure 4). Therefore, we measured expression levels of TLR2 and TLR4. It is widely accepted that TLR4 recognizes LPS from Gram-negative bacteria such as *Escherichia coli* and activates downstream inflammatory responses. However, we found no difference in TLR4 expression between the control and the *P gingivalis* groups. Regarding the mRNA expression level of TLR2, there was a significant difference in the fetal membrane but not in the placenta. This is because the whole amount of fetal membrane, on the other hand, the part of the placenta was prepared for the tissue collection (Figure 5). We may have to improve the tissue collection, especially placenta. Notably, TLR2 plays an important role in the primary recognition of *P gingivalis* and *P gingivalis*-associated LPS.[34]

The NF-κB and MAPK pathways are involved in the regulation of many genes including those encoding COX-2 and several pro-inflammatory cytokines.[35,36] We observed that phosphorylated (ie, activated) NF-κB and MAPK levels were increased in the *P gingivalis* group (Figure 6). Taken together, our data indicate that NF-κB and MAPK pathways were activated via TLR2 in the fetal membrane of the *P gingivalis* - infected animals, leading to upregulation of CAPs at an earlier time in gestation. As the presence of *P gingivalis* colonies and *P gingivalis*-associated LPS promotes TLR2 expression and the induction of inflammatory cytokines in chorion-derived cells,[16] it is likely that TLR2 represents an important mechanism of preterm birth in this model. Indeed, a previous study reported that increased expression of TLR2 in the fetal membrane is associated with human spontaneous preterm birth, regardless of whether the membrane was intact or ruptured.[37]

Various systematic reviews and meta-analysis concur that treatment of periodontal disease with scaling and root planning during gestational period does not affect the outcome of pregnancy.[38-41] Because the presence and colonization of *P gingivalis* leads to persistent inflammation, more than scaling therapeutic strategies, such as TLR blockade, might be needed for the treatment of pregnant women with periodontal disease.[42]

The mechanism of *P gingivalis* colony formation in the fetal membranes and placental tissue remains unclear. It is likely that *P gingivalis* in the oral cavity would be migrated to the chorionic tissues, including the amniotic fluid, through the blood stream.[43] Indeed, we reported that *P gingivalis* colony was observed in aortic walls[44] and hepatocytes[25] in our *P gingivalis* mice. Hematoxylin–eosin staining of the fetal membrane revealed minor signs of chorioamnionitis. Cysteine

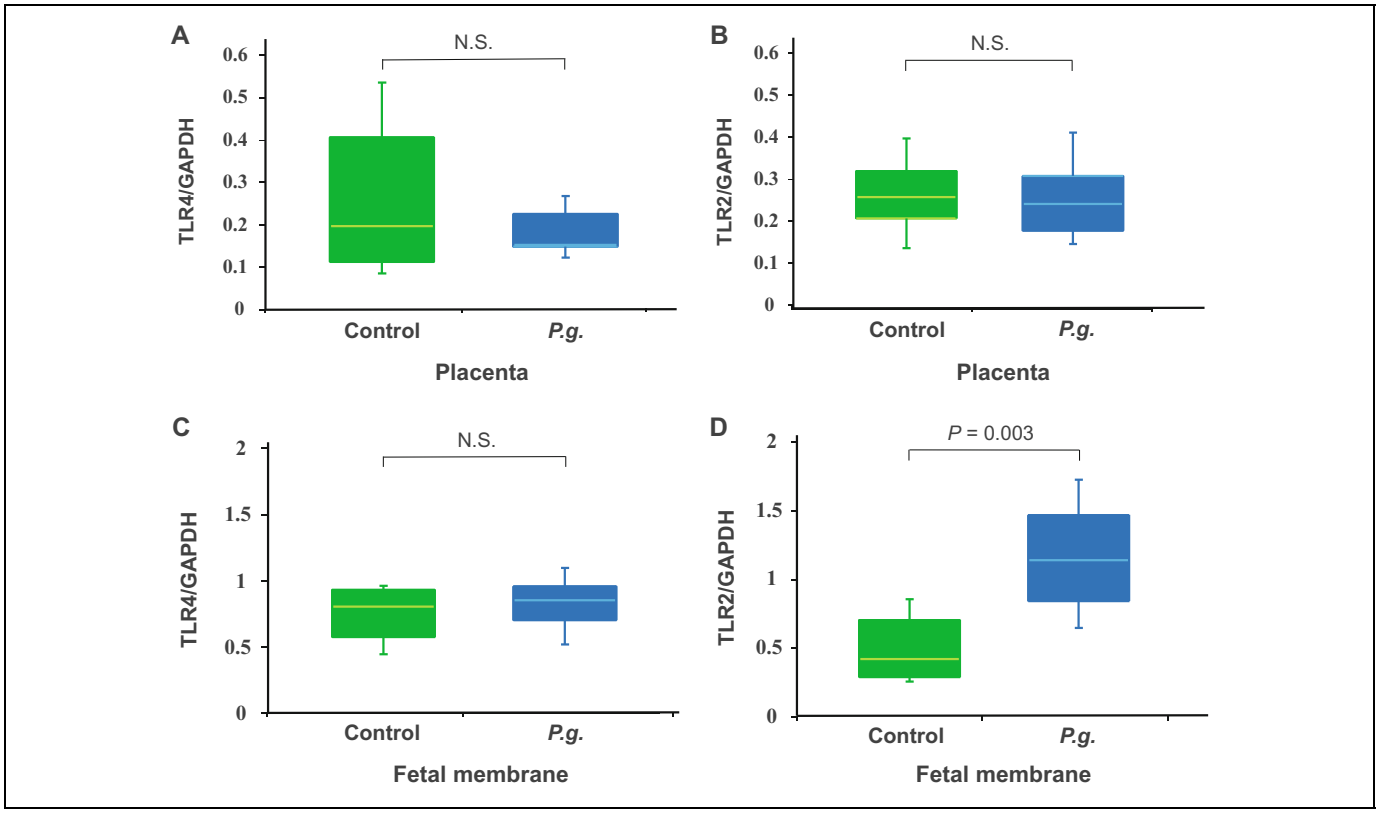

Figure 5. mRNA expression of TLR2 and TLR4 in the placenta and fetal membrane after *Porphyromonas gingivalis* infection. mRNA expression levels of TLR2 and TLR4 were measured by real-time RT-PCR and normalized to those of GAPDH. A, TLR4 mRNA expression in the placenta (n = 10 per group). B, TLR2 mRNA expression in the placenta (n = 10 per group). C, TLR4 mRNA expression in the fetal membrane (n = 10 per group). D, TLR2 mRNA expression in the fetal membrane (n = 10 per group). TLR2 mRNA levels were increased by 2.7-fold in the *P gingivalis* group when compared to those in the control group ($P = .003$). GAPDH indicates glyceraldehyde-3-phosphate dehydrogenase; mRNA, messenger RNA; NS, not significant; PCR, polymerase chain reaction; RT, reverse transcriptase; TLR2, toll-like receptor-2.

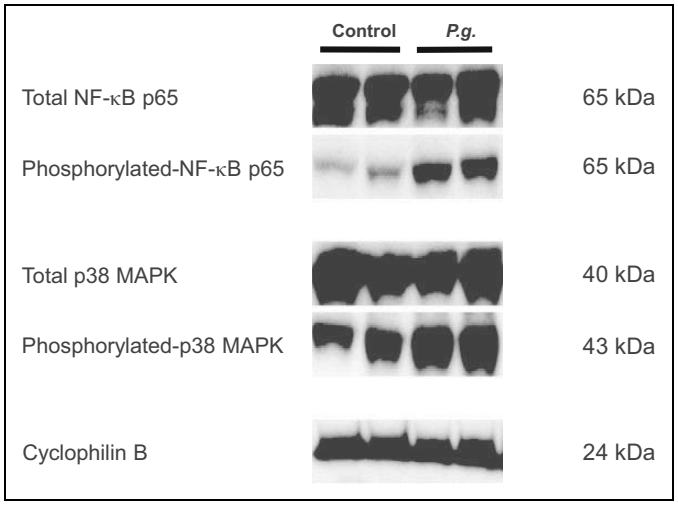

Figure 6. Protein expression of NF-κB and MAPK in the fetal membrane after *Porphyromonas gingivalis* infection. Levels of phosphorylated-NF-κB p65, total NF-κB p65, phosphorylated-p38 MAPK, and total p38 MAPK (n = 2 per group) were detected by Western blotting. Cyclophilin B expression was used as an internal control. MAPK indicates mitogen-activated protein kinase; NF-κB, nuclear factor-kappa B.

proteases (gingipains) from *P gingivalis* have been implicated in the pathogenesis of periodontal disease through mechanisms that include the inhibition of macrophage migration and the phagocytosis of primary apoptotic neutrophils.[45] Due to inefficient clearance of apoptotic cells (which is required for resolution of inflammation), chronic inflammatory conditions are maintained in periodontal lesions, leading to systemic inflammation. A similar mechanism might operate in the fetal membrane of our animal model.

In conclusion, we have shown that enhanced CAP expression at an earlier time in gestation is associated with increased uterine contractility, leading to preterm birth in our model of chronic inflammation-induced preterm birth. The mechanism underlying preterm birth might involve not only systemic inflammation but also activation of inflammatory pathways (via TLR2) in the fetal membrane. Additional studies are needed to further examine the role and ubiquitous expression of TLR2 and identify the cells that mediate the immune response within the fetal membrane.

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Declaration of Conflicting Interests

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