Prostaglandin E$_2$ promotes Pam3CSK4-induced inflammation in endometrial epithelial cells of cattle

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ABSTRACT

Bacterial contamination often impairs uterine function in cattle leading to uterine diseases such as endometritis. Inflammatory responses to bacterial infections in the uterus of cattle are generated through pattern recognition receptors, including Toll-like receptor 2 (TLR2), which is responsible for Pam3CSK4 recognition. This cellular response induces inflammatory responses through stimulation of mitogen-activated protein kinases (MAPKs) and nuclear factor (NF)-κB signaling activation, stimulating the expression of inflammatory mediators. Prostaglandin (PG) E$_2$ has important actions in bacterial endometritis, although details through which these mechanisms regulate Pam3CSK4-induced inflammatory responses in cattle endometrial epithelial cells (bEECs) remain unclear. In the present study there was examination of the actions of exogenous PGE$_2$ in Pam3CSK4-induced inflammatory responses. The bEECs pre-treated with exogenous PGE$_2$ prior to Pam3CSK4 treatment had an augmented Pam3CSK4-stimulated phosphorylation of protein kinase A (PKA), extracellular signal-regulated kinase (ERK), and IκB-α; stimulation of TLR2, cyclooxygenase-2, and interleukin-6 functions; and suppression of the activation of PGE$_2$ receptor 4. Thus, Pam3CSK4-induced inflammatory responses through TLR2 signaling in bEECs were enhanced by exogenous PGE$_2$ pre-treatment.

1. Introduction

Bacterial contamination often impairs uterine function in cattle, causing uterine diseases such as endometritis (Sheldon and Dobson, 2004). The endometrial epithelium has an important function in the initial immune response against bacterial invasion through recognition by Toll-like receptors (TLRs) (Davies et al., 2008). The TLRs are type I transmembrane glycoproteins consisting of an extracellular leucine-rich repeat domain, a transmembrane domain, and an intracellular Toll-interleukin-1 (IL-1) receptor domain (Akira et al., 2006; O'Neill and Bowie, 2007). Endometrial epithelia of cattle is able to detect and respond to bacterial lipopeptides with TLR2, -1, and -6 activation (Cronin et al., 2012; Turner et al., 2014). Triacylated lipopeptides are commonly found

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in gram-negative bacteria and bind TLR2, which heterodimerizes with TLR1 in mice. Diacylated lipopeptides are found in gram-positive bacteria or Mycoplasma and bind TLR2/TLR6 heterodimers (Takeuchi et al., 2001; Jin et al., 2007). The Pam3CSK4 is a commercially available synthetic structural analog of the Braun lipoprotein and a TLR2 agonist (Neilsen et al., 2001). For TLR2 responses, there is a MyD88-dependent signaling pathway activated that is associated with TLR1 or TLR6 to recognize the target ligands and initiate signaling, which induces inflammatory responses via MAPKs and NF-κB signaling. Activation of NF-κB and MAPK in the nucleus initiates the transcription of inflammatory cytokine genes such as IL-6 and IL-8 (Pietrocola et al., 2011).

Endometritis in cattle is a mucosal disease characterized by sustained neutrophil infiltration and increased IL-6 and IL-8 concentrations following postpartum gram-negative bacterial infection (Cronin et al., 2016). Gene transcripts for IL-6 and IL-8 are more abundant in the endometrium of cattle with a uterine disease than in animals that do not have this disease (Herath et al., 2009; Wathes et al., 2009). The IL-8 secretion products attract neutrophils from the peripheral circulation, whereas IL-6 has multiple functions including activation of neutrophils and stimulation of the acute-phase response (Bell et al., 2004).

Prostaglandins (PGs) are lipid mediators not stored by cells, but synthesized from arachidonic acid as a result of actions of cyclooxygenase (COX) enzymes, either constitutively or in response to cell specific trauma, stimuli, or signaling molecules (Berenbaum, 2000; Funk, 2001). The COX-2 protein is a key enzyme in the production of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and prostaglandin E_2 (PGE_2) (Fortier et al., 2008). When basal conditions prevail, $PGF_{2\alpha}$, the principal prostaglandin produced by epithelial cells, is produced through stimulation of transcription occurring as a result of the constitutively expressed COX-1 gene (Parent et al., 2003). The PGE_2 molecule functions as an endogenous hormone essential for normal physiological functions of various organs in the female reproductive, gastrointestinal, and cardiovascular systems; however, when pathological conditions are prevalent, PGE_2 also functions as an inflammatory mediator or, in some cases, an anti-inflammatory mediator (Takayama et al., 2002; Wanggren et al., 2006; Diaz-Munoz et al., 2012). The most abundant eicosanoid lipid in the inflammatory environment is PGE_2, and results of studies indicate this hormone signals through cyclic adenylyl monophosphate (cAMP)/PKA to suppress inflammation, although the specific mechanism is unclear (Xu et al., 2008; Wall et al., 2009). There is an important function of PGE_2 in the immune response as well as in endocrine function through actions on the E prostanoid (EP)-2 and EP4 receptors to control inflammation (Harizi and Gualde, 2006; Okano et al., 2006).

Taking into consideration these previous findings, the present study was conducted to determine whether exogenous PGE_2 is associated with Pam3CSK4-induced inflammatory responses in endometrial epithelial cells (bEECs) of cattle.

2. Materials and methods

2.1. Ethics statement

All animal studies were conducted in accordance with the experimental practices and standards approved by the animal welfare and research ethics committee of Inner Mongolia Agricultural University (Approval ID: 20160829-1), and all efforts were made to minimize animal suffering.

2.2. Culture of bEECs

Fresh specimens from healthy Holstein cattle (approximately 6 years old and not pregnant) were obtained from a local slaughterhouse. Primary bEECs were isolated from the uterine horn using enzymatic digestion and mechanical separation. The fresh uterine tissues were washed three times with phosphate-buffered saline (PBS) with 100 IU/mL penicillin and streptomycin (Gibco, Grand Island, NY, USA).Initially, each uterine horn (< 2 cm in length) was surgically isolated. To expose the endometrial tissue, the uterine horn was cut longitudinally and washed three times with Dulbecco's PBS supplemented with 100 IU/mL penicillin and streptomycin. The endometrial tissue was subsequently digested in 1% protease from *Streptomyces griseus* (Sigma Aldrich, St. Louis, MO, USA) overnight at 4 °C. For bEEC collection, the endometrial tissue was gently scraped, and the resulting cell slurry was washed three times with PBS supplemented with 100 IU/mL penicillin and streptomycin and centrifuged (300 × *g* for 5 min at 4 °C). The cells were seeded into cell culture flasks containing Dulbecco's modified Eagle's medium/F12 medium (Gibco) supplemented with 15% fetal bovine serum (ExCell Biolog, Shanghai, China) and 100 IU/mL penicillin and streptomycin and incubated at 37 °C with 5% CO_2. The culture medium was replaced every 48 h until cells reached confluence. The cells were then digested with trypsin (0.25%) supplemented with 0.1% ethylenediaminetetraacetic acid-2Na. Subsequently, the pure bEECs were isolated after three passages for further analysis. After three passages, the cellular morphology of bEECs was stable, and cells plated at a density of 1×10^6 cells/ml in six-well plates used for subsequent analyses, such as real-time polymerase chain reaction (PCR) analysis, western blotting, and enzyme-linked immunosorbent assays (ELISAs) (Gao et al., 2017).

2.3. Total RNA isolation and real-time PCR analysis

Total RNA was isolated from bEECs at 4, 8, and 12 h after pre-treatment with PGE_2 and/or $PGF_{2\alpha}$ (CAYMAN, Ann Arbor, MI, USA) and Pam3CSK4 (InvivoGen, San Diego, CA, USA) alone or in combination with TRIzol (AxyGen, Union City, CA, USA). Total RNA was treated with DNase I and reverse transcribed using a Revert Aid first-strand cDNA synthesis kit (Thermo Scientific, Schaumburg, IL, USA). Total cDNA was used as starting material for real-time PCR with FastStart Universal SYBR Green Master (Roche, Mannheim, Germany) on an iQ5 multicolor real-time PCR detection system (Bio-Rad, Hercules, CA, USA). The primers used for real-time reverse transcription (RT)-PCR are presented in Table 1. The glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) gene was used as the

Table 1
Primers used for *GAPDH*, TLR2, *COX-2*, *IL-8*, *IL-6*, *EP4* gene amplification, sequencing, and Accession No.

Gene symbol	Accession No.	Primer sequence
GAPDH	NM_001034034.2	Forward: 5′-CCTGCCAAGTATGATGAGAT-3′
TLR2	AC_000174.1	Reverse: 5′-AGTGTCGCTGTTGAAGTC-3′
COX-2	NM_174445.2	Forward: 5′-CGATGACTACCGCTGTGACTC-3′
IL-8	NM_173925.2	Reverse: 5′-CCTTCCTGGGCTTCCTCTT-3′
IL-6	NM_173923.2	Forward: 5′-GGTGCCTGGTCTGATGATGT-3′
EP4	NM_174589.2	Reverse: 5′-GATTAGCCTGCTTGTCTGGAAC-3′
		Forward: 5′-ACACATTCCACACCTTTCCA-3′
		Reverse: 5′-GGTTTAGGCAGACCTCGTTT-3′
		Forward: 5′-ATGCTTCCAATCTGGGTTC-3′
		Reverse: 5′-TGAGGATAATCTTTGCGTTC-3′
		Forward: 5′-TCCCAGTGAAACGCTGAAC-3′
		Reverse: 5′-CTCGTCTGTCTGCAAAGTGC-3′

internal control. The results were expressed as $2^{-\Delta\Delta Ct}$ (where $\Delta\Delta Ct = \Delta Ct - \Delta Ct_{\text{control}}$ and $\Delta Ct = Ct_{\text{target}} - Ct_{\text{GAPDH}}$).

2.4. Western blot analysis

The bEECs were treated with M-PER mammalian protein extraction reagent (Thermo Scientific) for total protein extraction at the indicated time points (15 min, 30 min, 60 min, and 18 h post-treatment). The concentrations of the protein samples were determined using a BCA assay kit (Thermo Scientific). For western blotting analysis, 10 μg total protein per lane was resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis on 12% and 15% gels and blotted onto polyvinylidene difluoride membranes. Rabbit anti-phospho-extracellular signal-regulated kinase (ERK), anti-ERK, anti-phospho-c-Jun N-terminal kinase (JNK), anti-JNK, anti-phospho-p38, anti-p38, anti-phospho-PKA, and anti-phospho-IκB-α monoclonal antibodies; mouse anti-IκB-α monoclonal antibodies (1:1000; Cell Signaling Technology, Beverly, MA, USA); rabbit anti-TLR2 polyclonal antibodies (1:250; Novus, CO, USA); mouse anti-IL-8 monoclonal antibodies (1:1000); rabbit anti-GAPDH monoclonal antibodies (1:10,000); rabbit anti-EP4 polyclonal antibodies (1:1000; Abcam, Cambridge, UK), and goat anti-COX-2 polyclonal antibodies (Acris, Germany) were used for protein detection. Proteins were visualized using secondary horseradish peroxidase-COnjugated goat anti-rabbit and goat anti-mouse antibodies (1:10,000; Cell Signaling Technology), donkey anti-goat antibodies (1:10,000; Abcam), and Pierce SuperSignal West Femto chemi-luminescent substrate (Thermo Fisher Scientific, IL, USA). Protein signal intensity was measured by densitometry analysis using ImageJ software (National Institutes of Health).

2.5. ELISA

The bEECs were pre-treated with PGE_2 (10^{-7} M) and $PGF_{2\alpha}$ (10^{-7} M) alone or in combination for 24 h. The concentrations of IL-6 in the supernatant secretions from cells with or without Pam3CSK4 (100 ng/mL) treatment at 24 h were measured using ELISA kits for IL-6 (Thermo Scientific) according to the manufacturer's instructions.

2.6. Data analyses

All data were analyzed using GraphPad Prism 6 (GraphPad InStat Software, San Diego, CA, USA) and expressed as the mean ± standard deviation (SD). Statistical significance was analyzed using the Student's t tests or one-way analysis of variance (ANOVA) followed by post-test analysis (Tukey's multiple comparison tests for control comparisons) when applicable. Differences with P values of 0.05 or less were considered statistically significant.

3. Results

3.1. Effects of PGE_2 and $PGF_{2\alpha}$ on relative abundance of TLR2 in Pam3CSK4-stimulated bEECs

The bEECs were pre-treated with PGE_2 (10^{-7} M) and $PGF_{2\alpha}$ (10^{-7} M) alone or in combination for 24 h. The relative abundance of TLR2 mRNA (4 h) and abundance of TLR2 protein (18 h) in cells with or without Pam3CSK4 (100 ng/mL) treatment at the indicated time points were determined using real-time RT-PCR and western blotting, respectively. As depicted in Fig. 1, cells pre-treated with PGE_2 had a greater relative abundance of TLR2 mRNA ($P < 0.01$) and abundance of protein ($P < 0.05$) compared with those in control cells. These results indicated that relative abundance of TLR2 mRNA and abundance of protein was greater in Pam3CSK4-stimulated cells compared with those in the control group ($P < 0.001$). In addition, relative abundance of mRNA with Pam3CSK4-induction of TLR2 was enhanced by treatment with PGE_2 and when combined with the $PGF_{2\alpha}$ treatment ($P < 0.001$). The $PGF_{2\alpha}$ pre-treatment alone had no effect on relative abundance of Pam3CSK4-induced TLR2 mRNA in bEECs.
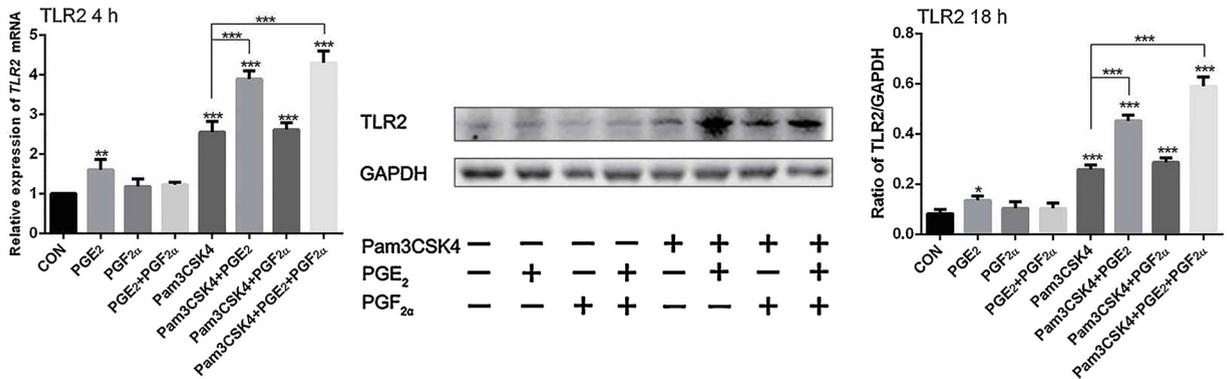
Fig. 1. Effects of PGE$_2$ and PGF$_{2\alpha}$ on TLR2 relative abundance of Pam3CSK4-stimulated bEECs. Relative abundance of TLR2 mRNA and abundance of TLR2 protein were analyzed in Pam3CSK4-induced bEECs pre-treated with PGE$_2$ (10^{-7} M) or PGF$_{2\alpha}$ (10^{-7} M) for 24 h; mRNA was extracted after treatment with Pam3CSK4 (100 ng/mL) for 4 h, whereas total proteins were extracted after Pam3CSK4 treatment for 18 h; Western blot and real-time RT-PCR analyses were used to determine abundance of protein and relative abundance of TLR2, respectively, are depicted. Target/GAPDH abundance are indicated in gray; Results are expressed as means $\pm$ SDs ($n = 3$); *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ relative to that of the control (Con).

3.2. Effects of PGE$_2$ and PGF$_{2\alpha}$ on Pam3CSK4-induced MAPKs and NF-kB signaling pathway activation in bEECs

The bEECs were pre-treated with PGE$_2$ (10^{-7} M) and PGF$_{2\alpha}$ (10^{-7} M) alone or in combination for 24 h. Phosphorylation of ERK, JNK, p38, and IκB-α after Pam3CSK4 (100 ng/mL) treatment was analyzed at the indicated time points (15, 30, and 60 min post-treatment) by western blotting (Fig. 2A). As depicted in Fig. 2B–E, PGE$_2$ and PGF$_{2\alpha}$ pre-treatment alone or in combination had no effect on the activation of MAPK and NF-kB signaling compared with that in the control cells. The Pam3CSK4-stimulated groups had greater ERK, JNK, p38, and IκB-α phosphorylation (Fig. 2B–E). In addition, cells pre-treated with PGE$_2$ had a greater Pam3CSK4-induced ERK ($P < 0.01$, Fig. 2B) and IκB-α ($P < 0.001$, Fig. 2E) phosphorylation at 15 min post-treatment, but there was no effect on Pam3CSK4-induced phosphorylation of JNK and p38 at 30 min post-treatment (Fig. 2C, D). Furthermore, cells pre-treated with PGE$_2$ combined with PGF$_{2\alpha}$ had greater Pam3CSK4-induced ERK phosphorylation ($P < 0.001$, Fig. 2B) at 15 min post-treatment, but there was no effect on Pam3CSK4-induced p38 and JNK phosphorylation at 30 min post-treatment and this resulted in a decrease in Pam3CSK4-induced IκB-α phosphorylation at 15 min post-treatment ($P < 0.05$, Fig. 2E).

3.3. Effects of PGE$_2$ and PGF$_{2\alpha}$ on cAMP/PKA signaling pathway activation in Pam3CSK4-stimulated bEECs

The bEECs were pre-treated with PGE$_2$ (10^{-7} M) and PGF$_{2\alpha}$ (10^{-7} M) alone or in combination for 24 h. Phosphorylation of cAMP/PKA after Pam3CSK4 (100 ng/mL) treatment was subsequently quantified at the indicated time points (15, 30, and 60 min post-treatment) by western blotting. Results indicated that PKA phosphorylation was greater in the Pam3CSK4-stimulated groups (Fig. 3). In addition, cells pre-treated with PGE$_2$ alone or in combination with PGF$_{2\alpha}$ had an increased PKA phosphorylation after Pam3CSK4 treatment compared with that in cells stimulated with Pam3CSK4 alone. The results of ERK phosphorylation were similar
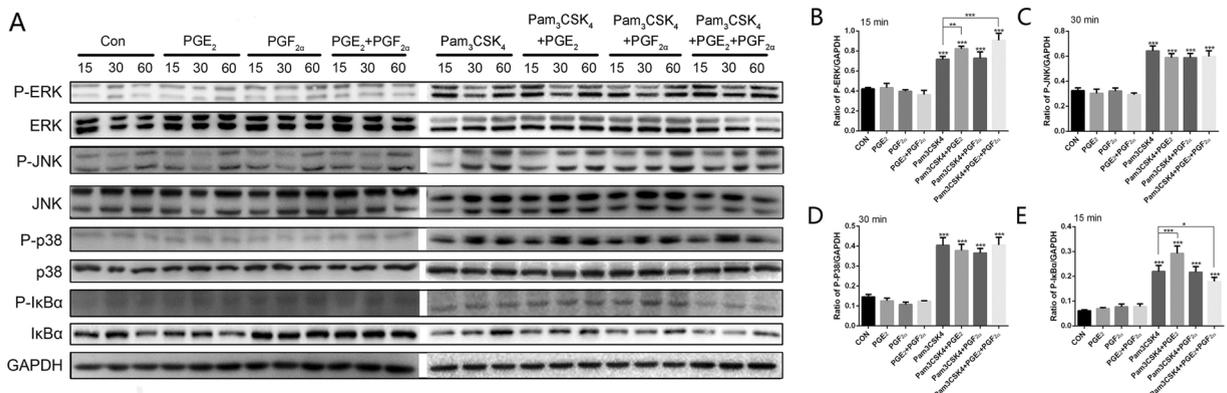

Fig. 2. Effects of treatments with PGE$_2$ and PGF$_{2\alpha}$ on Pam3CSK4-induced MAPKs and NF-kB signaling pathway activation in bEECs; bEECs were pre-treated with PGE$_2$ (10^{-7} M) and/or PGF$_{2\alpha}$ (10^{-7} M) for 24 h and then treated with 100 ng/mL Pam3CSK4 for 15, 30, and 60 min; Protein samples were analyzed by western blotting with specific antibodies, and the abundance of phosphorylated proteins were determined based on the abundance of phosphorylated protein relative to abundance of GAPDH; Results are expressed as the means $\pm$ SDs of three independent experiments; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with the control (Con).
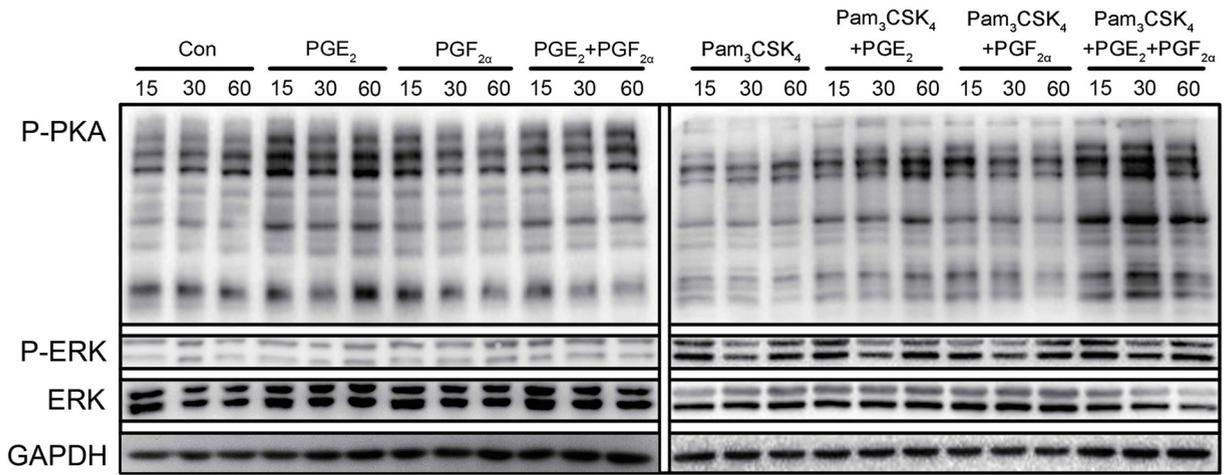
Fig. 3. Effects of PGE$_2$ and PGF$_{2\alpha}$ on cAMP/PKA signaling pathway activation in Pam3CSK4-stimulated bEECs. bEECs were pre-treated with PGE$_2$ (10^{-7} M) and/or PGF$_{2\alpha}$ (10^{-7} M) for 24 h; Cells were then treated with 100 ng/mL Pam3CSK4 for 15, 30, and 60 min; Protein samples were analyzed by western blotting using specific antibodies.

to those of PKA phosphorylation.

3.4. Effects of PGE$_2$ and PGF$_{2\alpha}$ on relative abundance of COX-2 in Pam3CSK4-stimulated bEECs

The bEECs were pre-treated with PGE$_2$ (10^{-7} M) and PGF$_{2\alpha}$ (10^{-7} M) alone or in combination for 24 h. The relative abundance of COX-2 mRNA (12 h) and abundance of protein (18 h) in cells with or without Pam3CSK4 (100 ng/mL) treatment were then measured at the indicated time points using real-time RT-PCR and western blotting, respectively. Results indicated that cells pre-treated with PGE$_2$ had greater relative abundances of COX-2 mRNA and abundance of protein compared with those in control cells ($P < 0.01$, Fig. 4). Furthermore, relative abundance of COX-2 mRNA and abundance of protein were greater in Pam3CSK4-stimulated cells after PGE$_2$ pre-treatment ($P < 0.05$, Fig. 4). Cells pre-treated with PGE$_2$ combined with PGF$_{2\alpha}$ had a greater relative abundance of COX-2 mRNA and abundance of protein after Pam3CSK4 treatment compared with those in cells stimulated with Pam3CSK4 alone ($P < 0.001$, Fig. 4), and PGF$_{2\alpha}$ pre-treatment alone had no effect on Pam3CSK4-induced COX-2 relative abundance in bEECs.

3.5. Effects of PGE$_2$ and PGF$_{2\alpha}$ on the secretion of inflammatory mediators in Pam3CSK4-stimulated bEECs

The bEECs were pre-treated with PGE$_2$ (10^{-7} M) and PGF$_{2\alpha}$ (10^{-7} M) alone or in combination for 24 h. The relative abundance of IL-8 mRNA (12 h) and abundance of protein (18 h) and relative abundance of IL-6 mRNA (12 h) in cells and IL-6 secretion (24 h) into the culture medium with or without Pam3CSK4 (100 ng/mL) treatment were then measured at the indicated time points using real-time RT-PCR, western blotting, and ELISA. The results indicated that cells pre-treated with PGE$_2$ had a greater relative abundance of in IL-8 mRNA and abundance of protein compared with those in the control group ($P < 0.001$, Fig. 5A). In addition, cells pre-treated
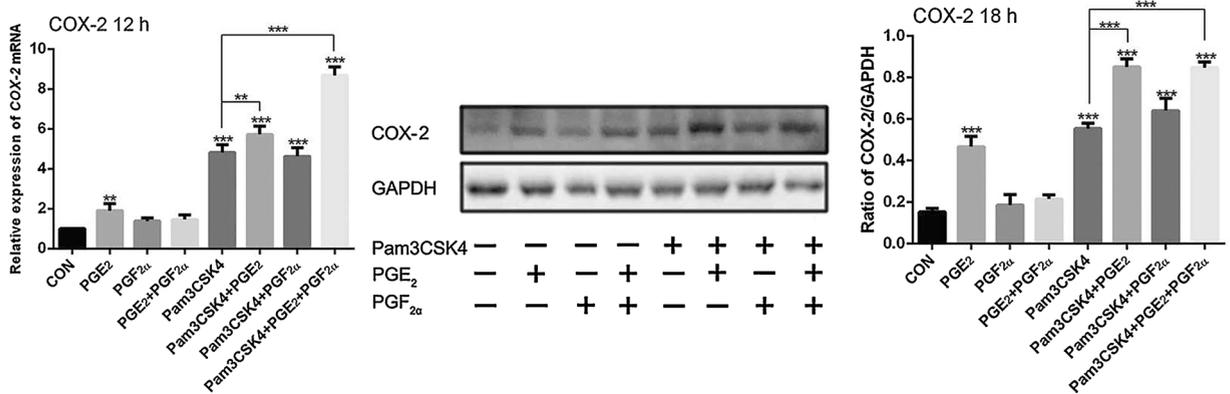

Fig. 4. Effects of PGE$_2$ and PGF$_{2\alpha}$ on relative abundance of COX-2 mRNA in Pam3CSK4-stimulated bEECs; Relative abundance of COX-2 mRNA was examined by western blotting and real-time PCR in Pam3CSK4-induced bEECs after PGE$_2$ (10^{-7} M) and/or PGF$_{2\alpha}$ (10^{-7} M) pre-treatment for 24 h and treatment with 100 ng/mL Pam3CSK4 for 12 or 18 h (for mRNA and protein, respectively); Target/GAPDH protein relative abundances are indicated in gray; Results are expressed as means ± SDs ($n = 3$); *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ relative to the control (Con).
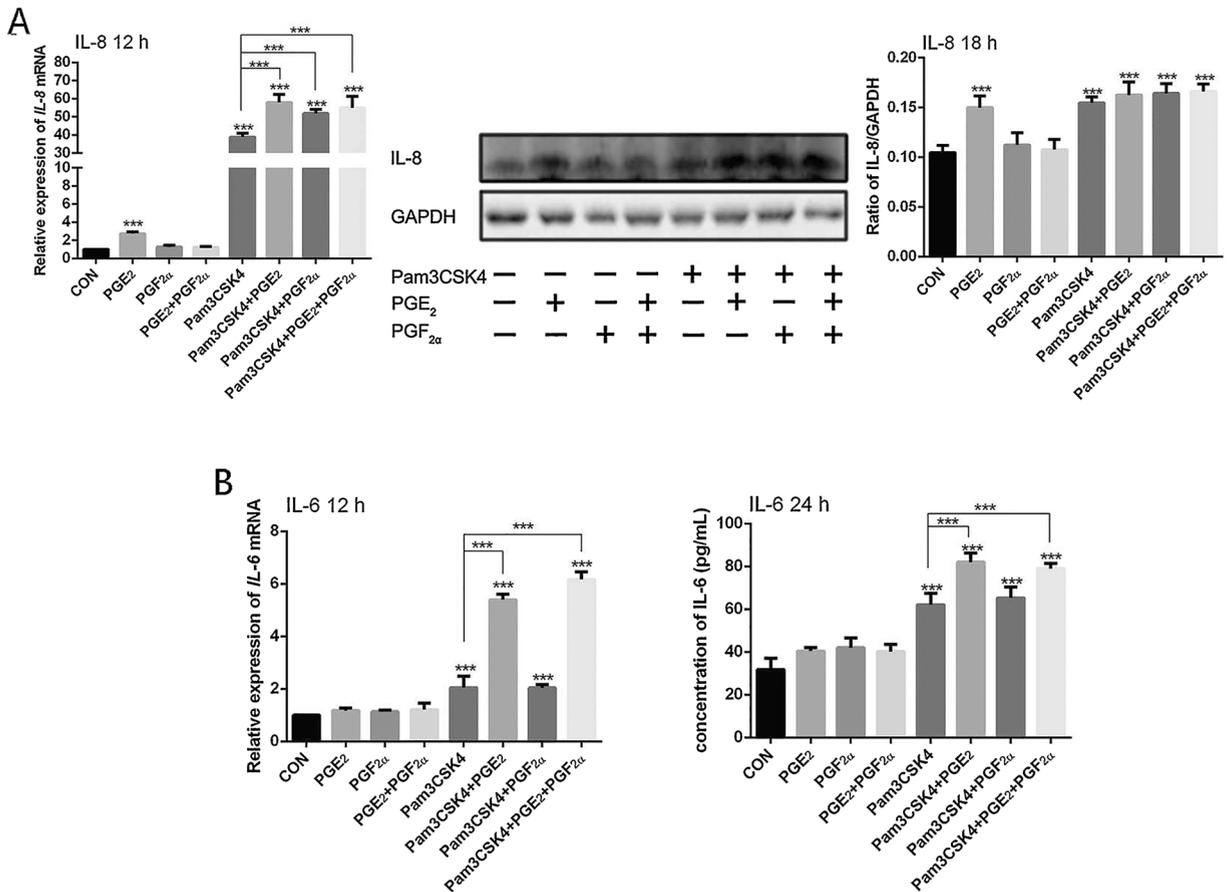
Fig. 5. Effects of PGE_2 and $PGF_{2\alpha}$ on the secretion of inflammatory mediators in Pam3CSK4-stimulated bEECs; Secretion of IL-8 (Panel A) and IL-6 (Panel B) in bEECs after PGE_2 (10^{-7} M) and/or $PGF_{2\alpha}$ (10^{-7} M) pre-treatment for 24 h; mRNA was extracted after treatment with 100 ng/mL Pam3CSK4 for 12 h whereas protein was extracted after treatment with 100 ng/mL Pam3CSK4 for 18 (IL-8) or 24 h (IL-6); Results are expressed as the means ± SDs ($n = 3$); *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with the control (Con).

with PGE_2 and $PGF_{2\alpha}$ alone or in combination had a greater relative abundance of IL-8 mRNA and there was no difference in abundance of IL-8 protein after Pam3CSK4 treatment compared with that in cells treated with Pam3CSK4 alone ($P < 0.001$, Fig. 5A). As depicted in Fig. 5B, PGE_2 and $PGF_{2\alpha}$ pre-treatment had no effect on relative abundance of IL-6 mRNA and abundance of protein in bEECs without Pam3CSK4 treatment. The relative abundance of Pam3CSK4-induced IL-6 mRNA and abundance of IL-6 protein were greater in the PGE_2 or combined PGE_2 and $PGF_{2\alpha}$ treatment groups ($P < 0.001$). The $PGF_{2\alpha}$ pre-treatment alone had no effect on Pam3CSK4-induced relative abundance of IL-6 in bEECs.

3.6. Effects of PGE_2 and $PGF_{2\alpha}$ on relative abundance of EP4 in Pam3CSK4-stimulated bEECs

The bEECs were pre-treated with PGE_2 (10^{-7} M) and $PGF_{2\alpha}$ (10^{-7} M) alone or in combination for 24 h. The relative abundance of EP4 mRNA (4 h) and abundance of protein (18 h) were then quantified in cells with or without Pam3CSK4 (100 ng/mL) treatment at the indicated time points using real-time RT-PCR and western blotting, respectively. The results indicated that PGE_2 and $PGF_{2\alpha}$ pre-treatment alone or in combination had no effect on the relative abundance of EP4 mRNA and abundance of protein compared with the control treatment (Fig. 6). The relative abundance of EP4 mRNA and abundance of protein was greater in Pam3CSK4-stimulated groups compared with those in the control group ($P < 0.001$, Fig. 6). In addition, relative abundance of EP4 after Pam3CSK4-treatment was less in PGE_2 and with the combination treatment with PGE_2 and $PGF_{2\alpha}$ ($P < 0.05$). The $PGF_{2\alpha}$ pre-treatment alone had no effect on relative abundance of EP4 after Pam3CSK4 treatment of bEECs.

4. Discussion

The innate immune responses to pathogens are very important for the removal of bacteria, the regulation of inflammation, and the maintenance of endometrial health (Hirata et al., 2005; Herath et al., 2006). Epithelial and stromal cells have a generalized function in innate immunity to initiate inflammatory responses against a wide range of bacteria that infect the endometrium by
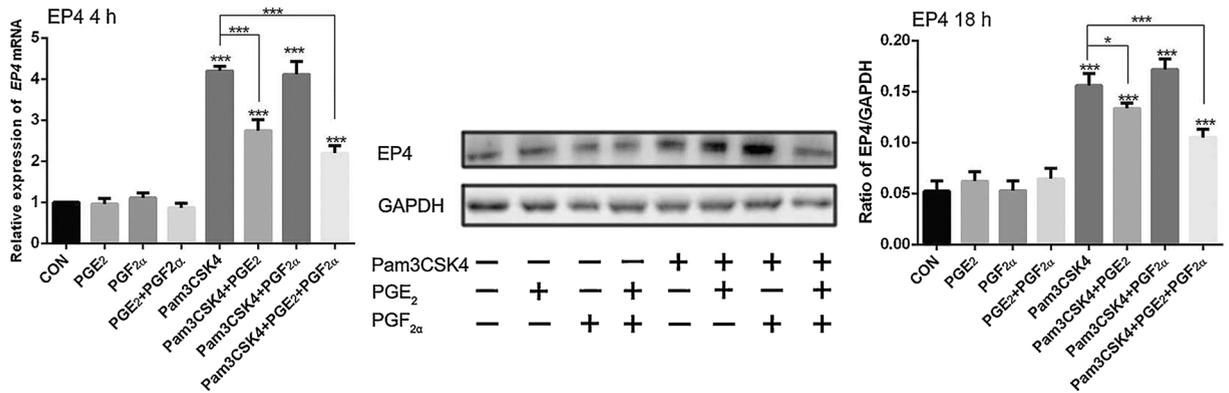
Fig. 6. Effects of PGE_2 and $PGF_{2\alpha}$ on relative abundance of EP4 in Pam3CSK4-stimulated bEECs; Relative of abundance of EP4 in Pam3CSK4-treated bEECs after PGE_2 (10^{-7} M) and/or $PGF_{2\alpha}$ (10^{-7} M) pre-treatment for 24 h was determined; mRNA was extracted after treatment with 100 ng/mL Pam3CSK4 for 4 h, and total proteins were extracted after treatment with 100 ng/mL Pam3CSK4 for 18 h; Relative abundance of EP4 mRNA was assessed by western blotting and real-time RT-PCR; Results are expressed as the means $\pm$ SDs ($n = 3$);. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with the control (Con).

sensing bacterial lipopeptides as a result of TLR2 in concert with TLR1 and TLR6 (Turner et al., 2014). Bacterial lipopeptides are important for innate immunity because of production by all bacteria, and thus TLR2 has generalized functions in detecting bacteria, usually involving monocytic immune cells (Aliprantis et al., 1999). The Pam3CSK4 is a commercially available synthetic triacylated lipopeptide and specific agonist of the TLR2/1 heterodimer complex (Neilsen et al., 2001; Stivers et al., 2017). The most abundant prostanoid in the mammalian body is PGE_2, which is synthesized in large amounts in response to cell specific trauma, stimuli, pathogen infection, or signaling molecules (Serhan and Levy, 2003; Park et al., 2006). In addition, PGE_2 can exert homeostatic, inflammatory, or anti-inflammatory effects (Davies et al., 1984; Sugimoto et al., 2000; Takayama et al., 2002). Inhibition of PGE_2 synthesis is considered an important anti-inflammatory reaction (Vane and Botting, 2003). In this study, Pam3CSK4-induced TLR2 gene expression was enhanced by exogenous PGE_2 pre-treatment, thereby implying that PGE_2 may have pro-inflammatory functions.

The TLR2 signaling has been thought to occur through MyD88 resulting in the activation of NF-κB and MAPKs and induction of pro-inflammatory cytokines (Akira et al., 2006). In the present study, the concentrations of phosphorylated ERK and IκB-α were increased by PGE_2 pre-treatment in bEECs following Pam3CSK4 treatment. According to previous studies, cAMP/PKA can activate ERK signaling through additional mechanisms, and PGE_2 has an important function in the modulation of immune cell processes through cAMP/PKA signaling. The primary intracellular second messenger of PGE_2 signaling is cAMP (Stork and Schmitt, 2002; Su et al., 2008). Thus, the effects of exogenous PGE_2 on PKA phosphorylation were assessed in Pam3CSK4-treated bEECs. The results indicated that phosphorylation of cAMP/PKA after Pam3CSK4 treatment at the indicated time points was enhanced by treatment with PGE_2. These results suggest that there is signaling between cAMP and MAPK pathways after Pam3CSK4 treatment of bEECs and this signaling was enhanced by PGE_2, resulting in increased ERK phosphorylation.

The primary product of COX-1 and COX-2 actions is PGE_2. The COX-1 gene is constitutively expressed, whereas the COX-2 gene is induced by various immune stimuli such as cytokines and various pathogen-associated molecular patterns (Dannenberg and Subbaramaiah, 2003; Rodriguez et al., 2014). Furthermore, COX-2 gene expression is enhanced in endometrial tissues of cattle during bacterial endometritis, implying that PGE_2 may contribute to this particular infection (Peter et al., 2015). It appears as though COX-2 is the primary COX controlling PGE_2 synthesis in response to inflammation (Park et al., 2006). In the present study, PGE_2 production increased with an increase in COX-2 gene expression in bEECs treated with or not treated with Pam3CSK4, supporting the line of thought that that exogenous PGE_2 could be involved in regulating the Pam3CSK4-induced inflammatory responses in bEECs.

The NF-κB and MAPK pathways regulate pro-inflammatory mediators (Ha et al., 2010; Lee et al., 2011). Once NF-κB is activated, subunit p65 dissociates from the inhibitory protein IκB-α and translocates from the cytoplasm to the nucleus, where it induces the transcription of specific target genes such as TNF-α, IL-1β, and IL-6 (Akira et al., 2006; Hennessy et al., 2010). Endometritis in cattle is characterized by greater IL-6 and IL-8 gene expression following postpartum gram-negative bacterial infection (Cronin et al., 2016). In cattle endometrial cultures, there is secretion of IL-1β, IL-6, and IL-8 in response to *Escherichia coli* and *Trueperella pyogenes* (Borges et al., 2012). An especially potent chemoattractant is IL-8 for neutrophils in the uterus (Zerbe et al., 2003). In cattle endometrium explants, IL-8 production increases *in vitro* in response to both gram-positive and gram-negative bacteria (Borges et al., 2012). The findings of the present study indicate that there was increased production of IL-8 as a result of PGE_2 pre-treatment in Pam3CSK4-unstimulated bEECs. In addition, after Pam3CSK4 treatment there was a greater relative abundance of IL-8 mRNA in bEECs and the abundance was greater after PGE_2 and $PGF_{2\alpha}$ pre-treatment alone or in combination, whereas PGE_2 and $PGF_{2\alpha}$ had no effect on abundance of IL-8 protein. A primary function of IL-8 is to stimulate angiogenic potential when it is secreted by the endothelium during menstruation than when it is secreted by the secretory endometrium *in vivo*, thereby suggesting that increased amounts of IL-8 coincide with endometrial repair (Maybin et al., 2011). Results of previous studies indicate that IL-8 secretion is induced by the activation of PGE_2/EP2 and $PGF_{2\alpha}$/$PGF_{2\alpha}$ receptor (FP) signaling and is involved in endometrial growth during estrous cycles and peri-implantation (Zhang et al., 2017a, b), consistent with the results of the present study. Furthermore, results of

the present study also indicate that in bEECs, PGE2 and PGF2α pre-treatment did not regulate the Pam3CSK4-induced expression of the IL-8 gene.

The primary cells that produce IL-6 are macrophages and monocytes (Bell et al., 2004) and IL-6 functions as an activator of acute-phase responses and as a lymphocyte stimulatory factor. Greater expression of the IL-6 gene occurs in bEECs stimulated with *E. coli* (Chapwanya et al., 2013). The IL-6 protein is promptly and transiently produced in response to infections and tissue injuries contributes to host defense through the induction of acute phase responses, hematopoiesis, and immune reactions (Tanaka et al., 2014). Results of the present study indicate that Pam3CSK4-induced IL-6 gene expression in bEECs was enhanced by treatment with PGE_2, which is consistent with the results of IκB-α phosphorylation, indicating that inflammatory responses in bEECs could be enhanced by treatment with PGE_2 as a result of IL-6 secretion.

The effects of PGE_2 often occur as a result of activation of four G protein-coupled receptors (EP1–4) (Konya et al., 2013). Among these receptors, EP2 and EP4 activate adenylate cyclase, induce intracellular cAMP production, and stimulate PKA signaling (Alfranca et al., 2006). The expression of the EP2 gene in the endometrium of cattle is greatest during the estrus, while EP4 is undetectable in the normal physiological state, which is consistent with the hypothesis that EP2 contributes to preventing necrosis and that EP4 is important in responding to pathogen infections (Arosh et al., 2003; Nishimura et al., 2013). The EP4 receptor activation contributes to the anti-inflammatory activity of PGE_2 in a range of disease relevant models (Birrell et al., 2015). Data from the present study also indicate that Pam3CSK4-induced EP4 gene expression in bEECs decreased following PGE_2 pre-treatment, indicating that PGE_2 may exert pro-inflammatory effects when there are Pam3CSK4-induced inflammatory responses in bEECs.

In conclusion, findings in the present study indicate that secretion of the pro-inflammatory cytokine IL-6 was enhanced in bEECs after treatment with the TLR2 agonist Pam3CSK4 and exogenous PGE_2 at a dose of 10^{-7} M, which is consistent with the results where there was IκB-α phosphorylation. In addition, Pam3CSK4-induced TLR2 gene expression was enhanced and EP4 gene expression was suppressed after treatment with PGE_2. Taken together, these findings indicated that PGE_2 enhances Pam3CSK4-induced inflammatory responses through activation of the TLR2/NF-κB signaling pathway in bEECs. In the present study, however, there was not an assessment of the specific functions of PGE_2 in the pathogenesis of bacterial endometritis. Furthermore, whether PGE_2 and its analogs could be used for the prevention and treatment of bacterial endometritis in cattle remains unknown. Thus, further studies are needed to address this potential use of PGE_2.

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Conflicts of interests

The authors declare that they have no conflicts of interests.

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References

Akira, S., Uematsu, S., Takeuchi, O., 2006. Pathogen recognition and innate immunity. Cell 124, 783–801.
Alfranca, A., Iniguez, M.A., Fresno, M., Redondo, J.M., 2006. Prostanoid signal transduction and gene expression in the endothelium: role in cardiovascular diseases. Cardiovasc. Res. 70, 446–456.
Aliprantis, A.O., Yang, R.B., Mark, M.R., Suggett, S., Devaux, B., Radolf, J.D., Klimpel, G.R., Godowski, P., Zychlinsky, A., 1999. Cell activation and apoptosis by bacterial lipoproteins through toll-like receptor-2. Science 285, 736–739.
Arosh, J.A., Banu, S.K., Chapdelaine, P., Emond, V., Kim, J.J., MacLaren, L.A., Fortier, M.A., 2003. Molecular cloning and characterization of bovine prostaglandin E-2 receptors EP2 and EP4: expression and regulation in endometrium and myometrium during the estrous cycle and early pregnancy. Endocrinology 144, 3076–3091.
Bell, M.J., Hallenbeck, J.M., Gallo, V., 2004. Determining the fetal inflammatory response in an experimental model of intrauterine inflammation in rats. Pediatr. Res. 56, 541–546.
Berenbaum, F., 2000. Proinflammatory cytokines, prostaglandins, and the chondrocyte: mechanisms of intracellular activation. Joint Bone Spine 67, 561–564.
Birrell, M.A., Maher, S.A., Dekkak, B., Jones, V., Wong, S., Brook, P., Belvisi, M.G., 2015. Anti-inflammatory effects of PGE(2) in the lung: role of the EP4 receptor subtype. Thorax 70, 740–747.
Borges, A.M., Healey, G.D., Sheldon, I.M., 2012. Explants of intact endometrium to model bovine innate immunity and inflammation ex vivo. Am. J. Reprod. Immunol. 67, 526–539.
Chapwanya, A., Meade, K.G., Doherty, M.L., Callanan, J.J., O'Farrelly, C., 2013. Endometrial epithelial cells are potent producers of tracheal antimicrobial peptide and serum amyloid A3 gene expression in response to E. Coli stimulation. Vet. Immunol. Immunop 151, 157–162.
Cronin, J.G., Kanamarlapudi, V., Thornton, C.A., Sheldon, I.M., 2016. Signal transducer and activator of transcription-3 licenses Toll-like receptor 4-dependent interleukin (IL)-6 and IL-8 production via IL-6 receptor-positive feedback in endometrial cells. Mucosal Immunol. 9, 1125–1136.
Cronin, J.G., Turner, M.L., Goetze, L., Bryant, C.E., Sheldon, I.M., 2012. Toll-like receptor 4 and YD88-dependent signaling mechanisms of the innate immune system are essential for the response to lipopolysaccharide by epithelial and stromal cells of the bovine endometrium. Biol. Reprod. 86.
Dannenberg, A.J., Subbaramaiah, K., 2003. Targeting cyclooxygenase-2 in human neoplasia: rationale and promise. Cancer Cell 4, 431–436.
Davies, D., Meade, K.G., Herath, S., Eckersall, P.D., Gonzalez, D., White, J.O., Conlan, R.S., O'Farrelly, C., Sheldon, I.M., 2008. Toll-like receptor and antimicrobial peptide expression in the bovine endometrium. Reprod. Biol. Endocrinol. 6.
Davies, P., Bailey, P.J., Goldenberg, M.M., Ford-Hutchinson, A.W., 1984. The role of arachidonic acid oxygenation products in pain and inflammation. Annu. Rev. Immunol. 2, 335–357.

Diaz-Munoz, M.D., Osma-Garcia, I.C., Fresno, M., Iniguez, M.A., 2012. Involvement of PGE(2) and the cAMP signalling pathway in the up-regulation of COX-2 and mPGES-1 expression in LPS-activated macrophages. Biochem. J. 443, 451–461.

Fortier, M.A., Krishnaswamy, K., Danyod, G., Boucher-Kovalik, S., Chapdelaine, J.A.P., 2008. A postgenomic integratred view of prostaglandins in reproduction: implications for, other body systems. J. Physiol. Pharmacol. 59, 65–89.

Funk, C.D., 2001. Prostaglandins and leukotrienes: advances in eicosanoid biology. Science 294, 1871–1875.

Gao, L., Liu, B., Mao, W., Gao, R., Zhang, S., Duritahala Fu, C., Shen, Y., Zhang, Y., Zhang, N., Wu, J., Deng, Y., Wu, X., Cao, J., 2017. PTGER2 activation induces PTGS-2 and growth factor gene expression in endometrial epithelial cells of cattle. Anim. Reprod. Sci. 187, 54–63.

Ha, Y.M., Chung, S.W., Kim, J.M., Kim, D.H., Kim, J.Y., Lee, E.K., Lee, J., Kim, Y.J., Yoo, M.A., Jeong, K.S., Chung, H.Y., 2010. Molecular activation of NF-kappa B, pro-inflammatory mediators, and signal pathways in gamma-irradiated mice. Biotechnol. Lett. 32, 373–378.

Harizi, H., Gualde, N., 2006. Pivotal role of PGE(2) and IL-10 in the cross-regulation of dendritic cell-derived inflammatory mediators. Cell. Mol. Immunol. 3, 271–277.

Hennessy, E.J., Parker, A.E., O'Neill, L.A.J., 2010. Targeting toll-like receptors: emerging therapeutics? Nat. Rev. Drug. Discov. 9, 293–307.

Herath, S., Fischer, D.P., Werling, D., Williams, E.J., Lilly, S.T., Dobson, H., Bryant, C.E., Sheldon, I.M., 2006. Expression and function of Toll-like receptor 4 in the endometrial cells of the uterus. Endocrinology 147, 562–570.

Herath, S., Lilly, S.T., Santos, N.R., Gilbert, R.O., Goetze, L., Bryant, C.E., White, J.O., Cronin, J., Sheldon, I.M., 2009. Expression of genes associated with immunity in the endometrium of cattle with disparate postpartum uterine disease and fertility. Reprod. Biol. Endocrinol. 7.

Hirata, T., Osuga, Y., Hirota, Y., Koga, K., Yoshino, O., Harada, M., Morimoto, C., Yano, T., Nishii, O., Tsutsumi, O., Taketani, Y., 2005. Evidence for the presence of Toll-like receptor 4 system in the human endometrium. J. Clin. Endocr. Metab. 90, 548–556.

Jin, M.S., Kim, S.E., Heo, J.Y., Lee, M.E., Kim, H.M., Paik, S.G., Lee, H.Y., Lee, J.O., 2007. Crystal structure of the TLR1-TLR2 heterodimer induced by binding of a tri-acylated lipopeptide. Cell 130, 1071–1082.

Konya, V., Marsche, G., Schuligoi, R., Heinemann, A., 2013. E-type prostanoid receptor 4 (EP4) in disease and therapy. Pharmacol. Therapeut. 138, 485–502.

Lee, S.-M., Kim, E.-J., Suk, K., Lee, W.-H., 2011. Stimulation of Fas (CD95) induces production of pro-inflammatory mediators through ERK/JNK-dependent activation of NF-kappa B in THP-1 cells. Cell. Immunol. 271, 157–162.

Maybin, J.A., Hirani, N., Jabbour, H.N., Critchley, H.O.D., 2011. Novel roles for hypoxia and prostaglandin E-2 in the regulation of IL-8 during endometrial repair. Am. J. Pathol. 178, 1245–1256.

Neilsen, P.O., Zimmerman, G.A., McIntyre, T.M., 2001. Escherichia coli Braun lipoprotein induces a lipopolysaccharide-like endotoxic response from primary human endothelial cells. J. Immunol. (Baltimore, Md. : 1950) 167, 5231–5239.

Nishimura, T., Zhao, X., Gan, H., Koyasu, S., Remold, H.G., 2013. The prostaglandin E-2 receptor EP4 is integral to a positive feedback loop for prostaglandin E-2 production in human macrophages infected with Mycobacterium tuberculosis. FASEB J. 27, 3827–3836.

O'Neill, L.A., Bowie, A.G., 2007. The family of five: TIR-domain-containing adaptors in Toll-like eceptor signalling. Nat. Rev. Immunol. 7, 353–364.

Okano, M., Sugata, Y., Fujiwara, T., Matsumoto, R., Nishibori, M., Shimizu, K., Maeda, M., Kimura, Y., Kariya, S., Hattori, H., Yokoyama, M., Kino, K., Nishizaki, K., 2006. E prostanoid 2 (EP2)/EP4-mediated suppression of antigen-specific human T-cell responses by prostaglandin E-2. Immunology 118, 343–352.

Parent, J., Villeneuve, C., Fortier, M.A., 2003. Evaluation of the contribution of cyclooxygenase 1 and cyclooxygenase 2 to the production of PGE(2) and PGF(2 alpha) in epithelial cells from bovine endometrium. Reproduction 126, 539–547.

Park, J.Y., Pillinger, M.H., Abramson, S.B., 2006. Prostaglandin E-2 synthesis and secretion: the role of PGE(2) synthases. Clin. Immunol. 119, 229–240.

Peter, S., Michel, G., Hahn, A., Ibrahim, M., Lubke-Becker, A., Jung, M., Einspanier, R., Gabler, C., 2015. Puerperal influence of bovine uterine health status on the mrna expression of pro-inflammatory factors. J. Physiol. Pharmacol. 66, 449–462.

Pietrocola, G., Arciola, C.R., Rindi, S., Di Poto, A., Missineo, A., Montanaro, L., Speziale, P., 2011. Toll-like receptors (TLRs) in innate immune defense against Staphylococcus aureus. Int. J. Artif. Organs 34, 799–810.

Rodriguez, M., Domingo, E., Municio, C., Alvarez, Y., Hugo, E., Fernandez, N., Crespo, M.S., 2014. Polarization of the innate immune response by prostaglandin E-2: a puzzle of receptors and signals. Mol. Pharmacol. 85, 187–197.

Serhan, C.N., Levy, B., 2003. Success of prostaglandin E-2 in structure-function is a challenge for structure-based therapeutics. Proc. Natl. Acad. Sci. U. S. A. 100, 8609–8611.

Sheldon, I.M., Dobson, H., 2004. Postpartum uterine health in cattle. Anim. Reprod. Sci. 295–306 82-3.

Stivers, N.S., Pelisch, N., Orem, B.C., Williams, J., Nally, J.M., Stirling, D.P., 2017. The toll-like eceptor 2 agonist Pam3CSK4 is neuroprotective after spinal cord injury. Exp. Neurol. 294, 1–11.

Stork, P.J., Schmitt, J.M., 2002. Crosstalk between cAMP and MAP kinase signaling in the regulation of cell proliferation. Trends Cell Biol. 12, 258–266.

Su, Y., Huang, X., Raskovalova, T., Zacharia, L., Lokshin, A., Jackson, E., Gorelik, E., 2008. Cooperation of adenosine and prostaglandin E2 (PGE2) in amplification of cAMP-PKA signaling and immunosuppression. Cancer Immunol. Immunother. 57, 1611–1623.

Sugimoto, Y., Narumiya, S., Ichikawa, A., 2000. Distribution and function of prostanoid receptors: studies from knockout mice. Prog. Lipid Res. 39, 289–314.

Takayama, K., Garcia-Cardena, G., Sukhova, G.K., Comander, J., Gimbrone, M.A., Libby, P., 2002. Prostaglandin E-2 suppresses chemokine production in human macrophages through the EP4 receptor. J. Biol. Chem. 277, 44147–44154.

Takeuchi, O., Kawai, T., Muhlradt, P.F., Morr, M., Radolf, J.D., Zychlinsky, A., Takeda, K., Akira, S., 2001. Discrimination of bacterial lipoproteins by Toll-like receptor 6. Int. Immunol. 13, 933–940.

Tanaka, T., Narazaki, M., Kishimoto, T., 2014. IL-6 in inflammation, immunity, and disease. CSH. Perspect. Biol. 6, a016295.

Turner, M.L., Cronin, J.G., Healey, G.D., Sheldon, I.M., 2014. Epithelial and stromal cells of bovine endometrium have roles in innate immunity and initiate in-flammatory responses to bacterial lipopeptides in vitro via toll-like receptors TLR2, TLR1, and TLR6. Endocrinology 155, 1453–1465.

Vane, J.R., Botting, R.M., 2003. The mechanism of action of aspirin. Thromb. Res. 110, 255–258.

Wall, E.A., Zavzavadjian, J.R., Chang, M.S., Randhawa, B., Zhu, X., Hsueh, R.C., Liu, J., Driver, A., Bao, X.R., Sternweis, P.C., Simon, M.I., Fraser, I.D.C., 2009. Suppression of LPS-Induced TNF-alpha production in macrophages by cAMP is mediated by PKA-AKAP95-p105. Sci. Signal. 2.

Wanggren, K., Lalitkumar, P.G., Stavreus-Evers, A., Stabi, B., Gemzell-Danielsson, K., 2006. Prostaglandin E-2 and F-2 alpha receptors in the human Fallopian tube before and after mifepristone treatment. Mol. Hum. Reprod. 12, 577–585.

Wathes, D.C., Cheng, Z., Chowdhury, W., Fenwick, M.A., Fitzpatrick, R., Morris, D.G., Patton, J., Murphy, J.J., 2009. Negative energy balance alters global gene expression and immune responses in the uterus of postpartum dairy cows. Physiol. Genomics 39, 1–13.

Xu, X.J., Reichner, J.S., Mastrofrancesco, B., Henry Jr, W.L., Albina, J.E., 2008. Prostaglandin E-2 suppresses lipopolysaccharide-stimulated IFN-beta production. J. Immunol. 180, 2125–2131.

Zerbe, H., Schuberth, H., Engelke, F., Frank, J., Klug, E., Leibold, W., 2003. Development and comparison of in vivo and in vitro models for endometritis in cows and mares. Theriogenology 60, 209–223.

Zhang, S., Liu, B., Gao, L., Mao, W., Fu, C., Duritahala, Zhang, N., Zhang, Y., Shen, Y., Cao, J., 2017a. Prostaglandin F2??PTGFR signalling activation, growth factor expression and cell proliferation in bovine endometrial explants. Reprod. Fert. Dev.

Zhang, S., Liu, B., Mao, W., Li, Q., Fu, C., Zhang, N., Zhang, Y., Gao, L., Shen, Y., Cao, J., 2017b. The effect of prostaglandin E2 receptor (PTGER2) activation on growth factor expression and cell proliferation in bovine endometrial explants. Prostaglandins Leukot. Essent. Fatty Acids 122, 16–23.