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Cortisol inhibits NF-κB and MAPK pathways in LPS activated bovine endometrial epithelial cells

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ABSTRACT

The bovine uterus is subject to infection after calving, which may lead to endometritis. Elevated cortisol levels have been observed in postpartum cattle. However, the role of cortisol in the inflammatory response of the uterus has not been reported. The aim of this study was to investigate the anti-inflammatory effects of cortisol on lipopolysaccharide (LPS)-induced primary bovine endometrial epithelial cells (BEECs). BEECs were treated with various concentrations of cortisol (5, 15 and 30 ng/mL) in the presence of LPS. The mRNA expression of TLR4 and proinflammatory cytokines was measured with qPCR. The activation of NF-κB and MAPK signalling pathways was detected with Western blotting and immunofluorescence. Cortisol induced the down-regulation of the mRNA expression of toll-like receptor 4 (TLR4) and proinflammatory cytokines, including interleukin (IL)-1β, IL-6, IL-8, tumour necrosis factor–α (TNF-α), cyclooxygenase-2 (COX-2) and inducible NO synthase (iNOS). Cortisol inhibited the activity of nuclear factor-κB (NF-κB) via blocking the phosphorylation and degradation of IκB. Cortisol suppressed the phosphorylation of mitogen-activated protein kinase (MAPK), including extracellular signal-regulated kinase (ERK1/2), p38MAPK and c-Jun N-terminal kinase/stress-activated protein kinase (JNK). These results demonstrated that cortisol may exert its anti-inflammatory actions by regulating NF-κB activation and MAPK phosphorylation.

1. Introduction

After calving, most cattle (80%–90%) uteri are contaminated with genital tract bacteria, most of which are *Escherichia coli (E. coli)*, *Fusobacterium necrophorum*, *Arcanobacterium pyogenes*, *Prevotella* spp. and *Fusobacterium nucleatum* [1,2]. Endometritis, including clinical and subclinical endometritis, refers to the inflammation of the endometrium. On average, 20% of cattle develop clinical endometritis, which is characterized by purulent uterine secreta in the vagina 3 weeks after calving. Approximately 30% of cattle experience subclinical endometritis, leading to subfertility and infertility in the absence of systemic symptoms [3–5].

The bovine endometrial epithelium cells (BEECs) are the first line of defence to resist the infection caused by various invading agents [6]. Because the epithelium is partially destroyed after calving, the damaged uterus is exposed to a large number of microbes. *E. coli* is the most common pathogenic bacterium contributing to uterine infection via the production of the endotoxin lipopolysaccharide (LPS) [5,7]. Toll-like receptor 4 (TLR4) is the pattern recognition receptor that can recognize LPS binding to myeloid differentiation factor 2 (MD2) on the cell surface [1,8]. The binding of TLR4-LPS leads to the activation of nuclear factor κB (NF-κB) and mitogen-activated protein kinase (MAPK). The NF-κB pathway activates downstream inflammatory mediators, including cytokines (IL-1β, IL-6, TNF-α, COX-2 and iNOS) and a chemokine (IL-8) [9,10]. The MAPK pathways, including extracellular signal-regulated kinase 1/2 (ERK1/2), p38, and c-Jun NH2-terminal kinase (JNK), have been reported to regulate the LPS-induced expression of TNF-α [11].

Natural and synthetic glucocorticoids are widely used as anti-inflammatory and immunosuppressive therapies. They are effective at inhibiting the inflammatory response to microbe invasion and avoiding excessive body inflammation [12–14]. Endogenic cortisol reaches high concentrations due to multiple stress stimulations, such as pregnancy stress, labour stress, and lactation stress in the bovine perinatal period [15]. However, the effect of cortisol on the LPS-induced inflammatory response of BEECs has not been clarified. The purpose of this study was to investigate the anti-inflammatory effects of cortisol on LPS-induced inflammation in BEECs and to clarify a possible mechanism.

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Table 1
The list of primer sequences used for amplification of qPCR.

Gene	Forward primers	Reverse primer	Accession number	Product size (bp)
β-Actin	CATCACCATCGGCAATGAGC	AGCACCGTGTTGGCGTAGAG	NM_173979.3	156
TLR4	GCTCTGCCTTCACTACAGGGACT	CTGGGACACCACGACAATAACC	NM_174198.6	106
IL-8	TTCCTCAGTAAAGATGCCAATG	TGACAACCCTACACCAGACCCA	NM_173925.2	86
IL-6	TGAAAGCAGCAAGGAGACACT	TGATTGAACCCAGATTGGAAGC	NM_173923.2	90
IL-1β	TGATGACCCTAAACAGATGAAGAGC	CCACGATGACCGACACCACCT	NM_174093.1	134
TNF-α	GGGCTTTACCTCATCTACTCACAG	GATGGCAGACAGGATGTTGACC	NM_173966.3	132
iNOS	GAGTGACTTTCCAAGACACGC	TGAAGGAGCCGTAATACTGGT	NM_001076799.1	186
COX-2	CCAGAGCTCTTCCTCCTGTG	AAGCTGGTCCTCGTTCAAAA	NM_174445.2	213

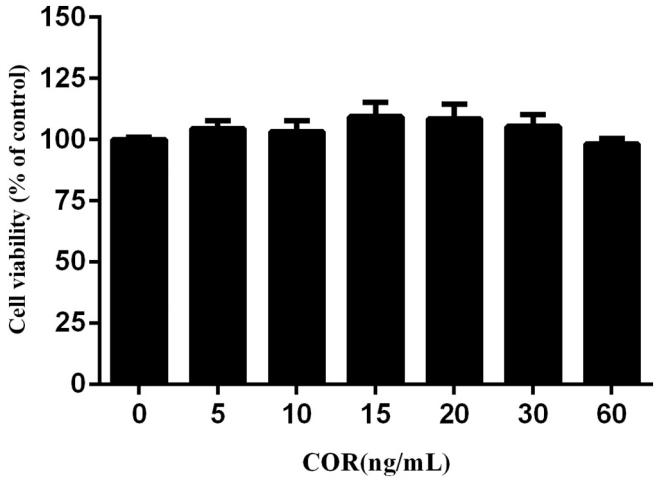

Fig. 1. The effects of different concentrations (5–60 ng/mL) of cortisol on bovine endometrial epithelial cell viability were measured by CCK-8 assay. The data are means ± SEM (n = 6). *$p < .05$ vs the control group.

2. Materials and methods

2.1. Culture of endometrial epithelial cells

Bovine uteri were collected at an abattoir from cattle without any evidence of genital disease or microbial infection, based on visual inspection, and the uteri were kept on ice until further processing at the laboratory. The uteri of postpartum cattle were abandoned in this study due to common bacterial contamination of the uterus, existing endometrial inflammation and damage to the epithelium after calving. Moreover, uteri at ovarian stage I (day 1 to 4 of the oestrous cycle) were selected for cell culture because peripheral plasma progesterone concentrations were similar to that of postpartum cattle [16]. In brief, the uterine horn was dissected and cut into 3–4 cm long pieces. Uterine tissue was digested with 0.1% protease from *Streptomyces griseus* (P5147, Sigma, USA) diluted in DMEM-F12 (D8900, Sigma, USA). After 18 h incubation at 4 °C, the uterine horn was removed from the digestive solution under aseptic conditions and incised longitudinally. The endometrium was scraped using a sterile surgical blade and ophthalmic forceps, and the scraped materials were washed in PBS (pH values from 7.2 to 7.4). Then, the cell suspension was collected. The cell suspension was centrifuged at 100 ×*g* for 5 min and followed twice further washes with PBS. Then, cells were cultured in Dulbecco's modified Eagle's medium/nutrient mixture F-12 ham containing 15% foetal bovine serum (FBS, Gibco, USA) and 50 U/mL of penicillin/streptomycin. The cells were seeded into a 25 cm^2 flasks and cultured at 37 °C with a 5% CO_2 and 95% sterile air. The purification of BEECs was confirmed to be above 99% by detection of CK-18 using immunohistochemistry. The medium was changed every 1–2 days until the cells reached approximately 90% confluence.

2.2. Cell viability assay

The effect of cortisol on cell viability was measured using the Cell Counting Kit-8 (CCK-8). The cells were plated on 96-well plates at a density of 1×10^4 cells per well and grown to 80% confluence in a 37 °C, 5% CO_2 incubator. Then, the cells were treated with various concentrations (5, 10, 15, 20, 30, 60 ng/mL) of cortisol (H0888, Sigma, USA) for 24 h. After stimulation, CCK-8 was added to each well, and the plate was incubated at 37 °C for 2 h. The optical density was then read at 450 nm using a microplate reader (Tecan, Austria). The CCK-8 was obtained from Dojindo Molecular Technologies, Inc. (Kumamoto, Japan).

2.3. RNA extraction and quantitative PCR (qPCR)

At the end of each experiment, endometrial epithelial cells were washed with 1 mL of PBS, and the RNA of the cells was extracted according to the manufacturer's instructions using TRIzol reagent (ET111, TRAN, China). The extracted RNA was quantified using a Nanodrop 2000 spectrophotometer (Thermo, USA), and the A260/280 ratio of each sample was between 1.8 and 2.1. Then, RNA was converted to cDNA using a reverse transcriptase synthesis kit (DRR047A, Takara, Japan). qPCR was performed using a CFX 96 Real-Time PCR Detection System (BIO-RAD, US). Amplification mixtures contained 12.5 μL of SYBR Green PCR mix, 1 μL of each primer, and 1 μL of cDNA template in a final volume of 25 μL per reaction (RR820A, Takara, Japan), and the following cycling conditions were performed: 95 °C for 30 s, 40 cycles of 95 °C for 5 s, 60 °C for 30 s. The $2^{-\triangle\triangle Ct}$ method was used to calculate the relative gene expression (target gene expression normalized to the expression of the endogenous control gene) [17]. The PCR analyses were performed in triplicate. The sequences of the primers are presented in Table 1.

2.4. Western blot analysis

The endometrial epithelial cells were stimulated with LPS (L2630, Sigma, USA) alone or together with cortisol. The total proteins were extracted and the protein concentrations were determined with a bicinchoninic acid (BCA) protein assay kit (P0010, Beyotime, China). Total protein (20–30 μg) was separated on 10% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Germany). The membranes were incubated with tris-buffered saline containing 0.05% Tween 20 and 5% nonfat milk to block nonspecific binding. Membranes were incubated with primary antibodies specific for p-p65, p65, p-IκBα, IκBα, p-ERK1/2, ERK1/2, p-p38, p38, p-JNK, JNK, and β-actin (all at 1:1000 dilution in 5% BSA) at 4 °C overnight and then incubated with the HRP-conjugated secondary antibodies (all at 1:2000 dilution in 5% nonfat milk) at room temperature for 1 h. Proteins were detected using a chemiluminescence (ECL) assay according to the manufacturer's instructions. The following antibodies were used: p-p65, p65, p-IκBα, IκBα, p-ERK1/2, ERK1/2, p-p38, p38, p-JNK, JNK, and β-actin (#3033, #8242, #2859, #4812, #4370, #4695, #4511, #8690, #4668, #9258, #4970; Cell Signalling Technology,