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RESEARCH ARTICLE

Oestrogen Receptors ERα, ERβ and GPER Mediate the Activation of AMPK and the Inhibition of the Inflammatory Signaling Pathway TLR4/NFκB in Neutrophils of Dairy Cow

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ABSTRACT

Different inflammatory diseases in dairy cows are related to the impairment of neutrophils function, which is affected by blood oestrogen level. In this study, the molecular mechanism of 17β-oestradiol (E2) receptors mediated by the basal physiological concentration of E2 in the immune activity and energy status of circulating cow neutrophils was elucidated *in vitro* based on 9 blood samples collected from 3 adult Simmental cows at 2-weeks intervals. Western blotting and qRT–PCR were used to investigate E2 nuclear receptors (ERα and ERβ) and G-protein coupled receptor (GPER) expression in resting and lipopolysaccharide (LPS)-activated neutrophils at the basal physiological level of E2 (20pg/mL) *in vitro* and the effect on the inflammatory signaling pathway Toll-like receptor 4 (TLR4)/nuclear factor kappa B (NFκB) and the AMP-activated protein kinase (AMPK), the regulators of energy metabolism. Results showed that E2 upregulated GPER protein expression, and decreased TLR4 mRNA and protein expression, myeloid differentiation Factor 88 (MyD88) mRNA expression and NFκB (p65) phosphorylation, but ERα and ERβ mRNA and protein expression were not changed. However, ERα mRNA and protein expression and GPER protein expression were decreased, and ERβ mRNA and protein expression was increased in activated neutrophils. TLR4 mRNA and protein expression, MyD88 mRNA expression and NFκB (p65) phosphorylation were upregulated, while AMPK phosphorylation was inhibited in activated neutrophils, with an opposite effect on oestrogen-treated neutrophils. In conclusion, basal physiological E2 concentration can mediate ERα, ERβ and GPER to slow down the activation of the TLR4/NFκB signaling pathway in LPS-activated neutrophils and to promote cell energy metabolism in cows.

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INTRODUCTION

Increased susceptibility of dairy cows to different inflammatory diseases of the mammary glands, uterus and other body organs is associated with decreased neutrophils function. Neutrophils are an important component of innate immune defense, and their function is mediated by cell-surface receptors, such as Toll-like receptors (TLRs) and is accompanied by changes in intracellular energy metabolism. Neutrophils function is known to be affected by oestrogen, the well-known sex steroid hormone, through the oestrogen receptors (ER), including nuclear receptors (ERα and ERβ) mediating slow genomic effects and membrane receptor G-protein coupled receptor (GPER/GPR30) mediating rapid non-genomic effects. In healthy cows, the basal physiological blood level of oestrogen (17β-oestradiol, E2) is approximately 20pg/mL.

The effects of E2 on neutrophils play an important role in innate immune defense. The classical mechanism of E2 action is that nuclear ERs act as transcription factors to stimulate gene expression and mediate the slow genomic pathway. In tissues and cell types in which ER is clearly expressed, ER expression levels are correlated with endogenous E2 levels. Previous studies have revealed that

ERα and ERβ are present in bovine neutrophils (Lamote *et al.*, 2006) and bovine neutrophils express ERα and ERβ mRNA and ERβ proteins, but not ERα proteins.

A non-classical mechanism of E2 action is through GPER, which acts in tandem with the traditional ER to regulate the physiological response of the cell. GPER is structurally independent of ERα or ERβ, widely distributed in the brain and surrounding tissues and plays a role in the blood transport system; it binds to E2 and mediates non-genomic effects. The expressed GPER in human neutrophils and nHL-60 cells can activate a variety of signal transduction pathways that ultimately lead to phosphorylation of substrate proteins, including some transcription factors (Flores *et al.*, 2016); therefore, ERα is a transcription factor downstream of GPER signaling pathways.

The diversity of neutrophils activity and function is mediated by a large number of receptors that recognize multiple exogenous and endogenous ligands and initiate immune responses (Lim *et al.*, 2017). Neutrophils are also directly detected by TLRs and activated by pathogen-associated molecular patterns (PAMPs), such as Toll-like receptor 1 (TLR1), TLR2, TLR4, TLR6, TLR7 and TLR10. As a major pattern recognition receptor for pathogenic microorganisms, TLR4 in cow neutrophils can respond to lipopolysaccharide (LPS) and play a key role in various inflammatory responses (Bassel and Caswell, 2018). LPS is one of the most potent inflammatory factors; it regulates and activates neutrophils, affecting cell motility or morphology, and also regulates the synthesis and release of inflammatory mediators and cytokines (Cheng *et al.*, 2018). TLR4 is the primary LPS receptor which mediates myeloid differentiation Factor 88 (MyD88)-dependent signaling (MyD88 is the major adaptor protein in the TLR signaling pathway that induces NFκB activation), which leads to downstream inflammatory responses. Nuclear factor kappa-B (NFκB) serves as a pluripotent regulator of several proinflammatory cytokines, and is activated by a variety of stimuli. In response to TLR4 receptor stimulation, intracellular MyD88-dependent signal transduction pathways activate cytosolic-associated cytokines, leading to NFκB activation. Then NFκB enters the nucleus and activates the related genes, transduces the corresponding mRNA, and synthesizes and releases interleukin (IL)-1, IL-6, IL-12, tumor necrosis factor (TNF)-α and other cytokines, causing a series of inflammatory reactions. Multilevel crosstalk between ER and TLR4/NFκB signals has been well described (Zhang *et al.*, 2018). ERα and ERβ inhibit NFκB activity in an oestrogen-dependent manner in multiple cell types. Meanwhile, alleviation of ischemic injury by GPER through inhibiting TLR4-mediated microglial inflammation was also reported (Zhang *et al.*, 2018).

In the cellular energy metabolism signaling network of neutrophils activity, the key cellular energy sensor AMPK (AMP-activated protein kinase) regulates cellular energy homeostasis and metabolic stress, and its activation promotes catabolism and inhibits anabolism of cellular energy. AMPK has several direct phosphorylation targets, but it indirectly inhibits NFκB signaling through its downstream mediators, like peroxisome-proliferator-activated receptor γ coactivator 1α (PGC1α) and silent information regulator 1 (SIRT1), which in turn inhibit the expression of inflammatory factors (Cantó and Auwerx, 2010). AMPK was also found to reduce LPS-mediated proinflammatory activation of neutrophils in mice and to inhibit IκBα degradation and NFκB activation (Zmijewski *et al.*, 2008). Thus, AMPK activation may help to inhibit neutrophils activation and the chemotaxis associated with bacterial eradication under inflammatory conditions.

However, there is little information about the role of basal physiological concentration of E2 in regulating the TLR4/NFκB signaling cascade that is mediated by ER in cow neutrophils. Moreover, whether GPER exists in cow neutrophils and how it coordinates with ERα and ERβ, especially under the basal level of E2, needs to be further investigated. Similarly, it is not clear whether E2 regulates the immune response of cow neutrophils by activating AMPK. Therefore, this study was planned to evaluate the possible effects of basal physiological level of oestrogen-mediated ER (ERα, ERβ and GPER) on the TLR4/NFκB signaling pathway and AMPK activity in cow neutrophils. Attempts were also made to further elucidate the effects and signaling mechanisms of ER-mediated cellular energy metabolism in cow neutrophils under basal level of 17β-oestradiol.

MATERIALS AND METHODS

Animals: The protocol of this study was duly approved by the Animal Use and Care Ethics Committee of Inner Mongolia Minzu University, China. The study was conducted from May to August 2019 in a family ranch in Tongliao City, China. Three clinically healthy Simmental cows (2-3 years old) were selected and their ovaries were surgically removed, as described previously (McLean *et al.*, 2016). The feeding and management of these cows was strengthened in strict accordance with postoperative nursing requirements.

Isolation and culture of the neutrophils: In this study, three blood samples were collected at 2-weeks interval from the three ovariectomized cows. About 100 mL jugular blood was collected from each cow and placed in heparin sodium tubes. Neutrophils were isolated from collected blood, as described previously (Wang *et al.*, 2021). After isolation, neutrophils were washed with PBS, resuspended (2.0×10^6 cells/mL) in RPMI 1640 medium and incubated for 30 min at 37°C under 5% CO_2. After 30 min, the neutrophils were seeded in 24-well plates and cultured in RPMI 1640 medium in four different treatment groups (three replicates for each sample) for 0, 1, 2, 4 and 8hrs: These groups included the 17β-oestradiol group (20pg/mL E2), E2+Fulvestrant (ICI: 10^{-6} M) group, LPS (100g/mL) group and E2+LPS group. The E2 and LPS treatment groups had induced neutrophils with E2 and LPS, respectively; the E2+ICI group had both E2 and ICI-182780 induced neutrophils; in the E2+LPS group neutrophils were induced by E2 for 2hrs, then LPS was added. Then the cells were collected at the corresponding time points for subsequent tests. In this study, 20pg/mL E2 was taken as basal physiological level, as has been reported earlier (Robertson, 1974).

Quantitative real-time PCR analysis: The extraction of RNA was carried out by using RNAiso plus (Takara, China), following the protocol of Wu *et al.* (2019).

Table 1: Primers and their sequences used for qRT–PCR analysis

Name of cDNA	Genes	Sequences of primers (5′→3′)	Length (bp)
ERα	AY238475.2	For: CAGCATCCCTTTCTCAACAGC Rev: CAGACCCTTGGCATCTATTCC	111
ERβ	NM_001216634.2	For: AAGTGATGGGAAATGACCTGGGAT Rev: TTTTTGGAAACTCCTTCTCTGTGGG	189
NFκB	DQ355511.1	For: GGGTGAATCGGAACTCTGG Rev: AGCCTGGTCCCGTGAAATA	105
MyD88	NM_001014382.2	For: TAGACAGCAGCATAACTCGGATAAA Rev: GCAGACCTCGTTTCCATTG	186
TLR4	NM_174198.6	For: GTTGCTGTTCTCACACTGATTTTG Rev: GGTGTTCTAGTTGCTCTAAGCCCAT	114
β-actin	AY141970.1	For: GTGACAGCAGTCGGTTGGAT Rev: CTTAGAGAGAAGCGGGGTGG	166

The obtained RNA was reverse transcribed using a primescript reverse transcriptase (Takara). qRT–PCR was performed based on the step one plus PCR system (Applied Biosystems, USA), using the SYBR Green-detection system (Roche, USA). The ERα, ERβ, NFκB, MyD88, TLR4 and β-actin were designed with primer express software (Applied Biosystems, USA). The reaction system was performed in 20μL volumes. The experimental conditions were as follows: one cycle of pre-denatured at 95°C for 60s, followed by 40 cycles of denatured at 95°C for 15s and extension at 60°C for 60s. The specificity of the PCR product was verified in the dissociation stage. The qRT–PCR primer sequences are shown in Table 1. The relative expression levels of each target gene were determined by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001), with β-actin was used as the internal reference.

Western blotting analysis: Total protein was extracted from neutrophils with lysate buffer (Solarbio, China). Protein concentrations were measured with Bicinchoninic Acid (BCA) Protein Assay kit (Applygen, China). Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) was used for the separation of proteins. Then samples were transferred to polyvinylidene fluoride (PVDF) membranes (Immobilon, USA) following Wang *et al.* (2022). Then the membranes were blocked with 5% bovine serum albumin for 2 hrs and imprinted with 1:1000 dilution of primary antibodies against ERα (Abcam, USA), ERβ (Abcam), GPER (Abcam), TLR4 (Abcam), MyD88 (Abcam), NFκB (p65) and p-NFκB (p65) (Abcam), AMPK (Abcam), p-AMPK (Abcam) and β-actin (Absin, China) at 4°C overnight. Then, the membrane was washed with Tris Buffered Saline Tween (TBST) and the secondary antibodies (Cell Signaling Technology, USA) conjugated to horseradish peroxidase were incubated for 1 hr at room temperature. The bands were visualized with an enhanced chemiluminescence system.

Statistical analysis: Results are presented as mean values (±SEM) for various parameters. The data were analyzed through ANOVA under completely randomized design, using SPSS 19.0 software (SPSS, Inc., Chicago, IL). Differences among group means were compared following LSD multiple comparison test.

RESULTS

Expression of ERα, ERβ and GPER in resting neutrophils: The results regarding the expression of ERα, ERβ and GPER in resting neutrophils showed that the protein expression of ERα and ERβ did not change significantly (Fig. 1A-C), while the expression of GPER was significantly enhanced ($P<0.05$) during 1-8 hrs of culture compared to 0 hr (Fig. 1A, D). The qRT–PCR also revealed that the mRNA relative expression of ERα and ERβ did not change significantly during1-8 hrs of culture (Fig. 1E, F).

Effect of GPER on TLR4/NFκB signaling in resting neutrophils: To further study the effect of GPER on the activity of resting neutrophils in cows, oestrogen nuclear receptor antagonist ICI-182780 was used to detect the TLR4/NFκB signaling in neutrophils. The results showed that ICI-182780 significantly downregulated the protein and mRNA expression of ERα and ERβ and upregulated ($P<0.05$) the protein expression of GPER (Fig. 2A-F) during 1-8 hrs of culture. Moreover, the protein and mRNA expression of TLR4, the mRNA expression of MyD88 and the phosphorylation of NFκB (p65) were significantly downregulated ($P<0.05$) in a time-dependent manner from 1-8 hrs in the E2-treated alone groups, as well as in E2+ICI groups (Fig. 2G-L).

Effect of oestrogen on TLR4/NFκB signaling pathway in LPS-activated neutrophils: In LPS-activated neutrophils treated with E2, the ERα protein, mRNA expression and GPER protein expression were decreased, and the protein and mRNA expression of ERβ was increased significantly ($P<0.05$) from 1-8 hrs of culture (Fig. 3A-F). To further elucidate the role of E2 in LPS activated neutrophils, the role of TLR4/NFκB signaling pathway in the inflammatory response was evaluated. The results showed that LPS treatment strongly upregulated ($P<0.05$) NFκB phosphorylation, TLR4 protein and mRNA expression and MyD88 mRNA expression. Compared with the LPS-treated group, the E2+LPS-treated group showed significantly inhibited ($P<0.05$) NFκB (p65) phosphorylation, TLR4 protein and mRNA expression and MyD88 mRNA expression (Fig. 3G-L). However, the E2+LPS-treated group showed significantly upregulated ($P<0.05$) expression of TLR4, NFκB (p65) phosphorylation and MyD88 mRNA expression from 1-8 hrs, with higher upregulation than in the E2-treated group (Fig. 3G-L).

Effect of E2 on the phosphorylation of AMPK in neutrophils: The results also showed that LPS treatment inhibited AMPK phosphorylation in neutrophils, while E2 significantly upregulated ($P<0.05$) AMPK phosphorylation induced by LPS from 1-8 hrs of culture in a time-dependent manner, with lower upregulation ($P<0.05$) than that with E2 treatment alone (Fig. 4A, B).

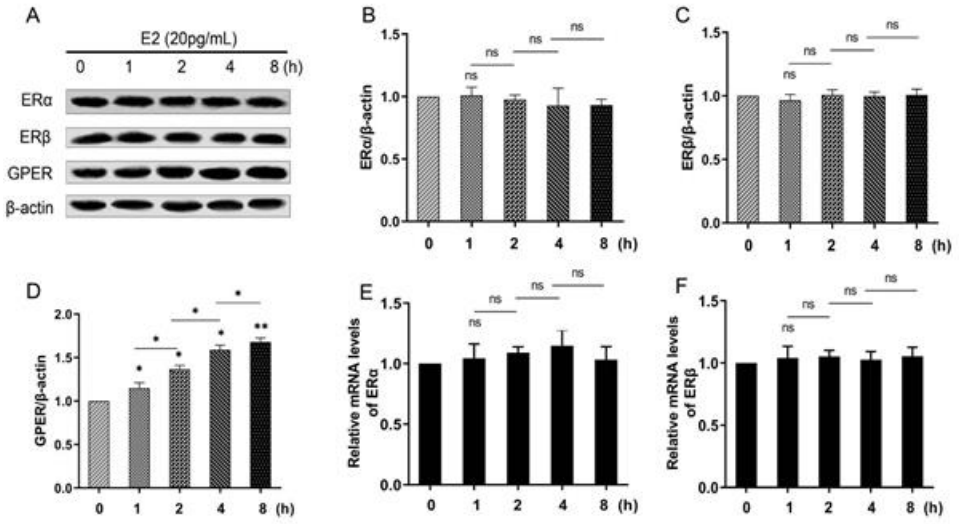
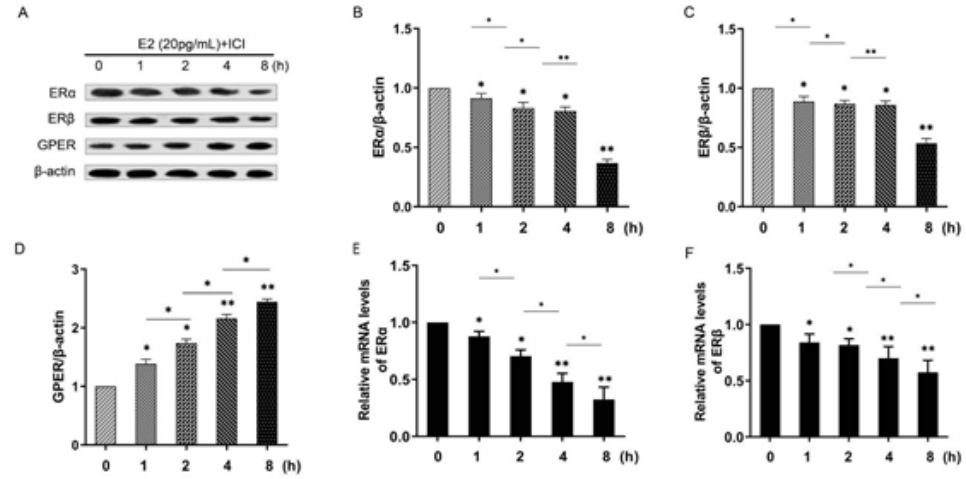


Fig. 1: Showing theeffects of E2 on the expression of ERα, ERβ and GPER in resting neutrophils of cows. (A): Results of Western blotting; (B, C, D): Protein expression levels of ERα, ERβ, and GPER; (E, F): mRNA expression levels of ERα and ERβ by qRT–PCR. *significant (P<0.05); ns non-significant.



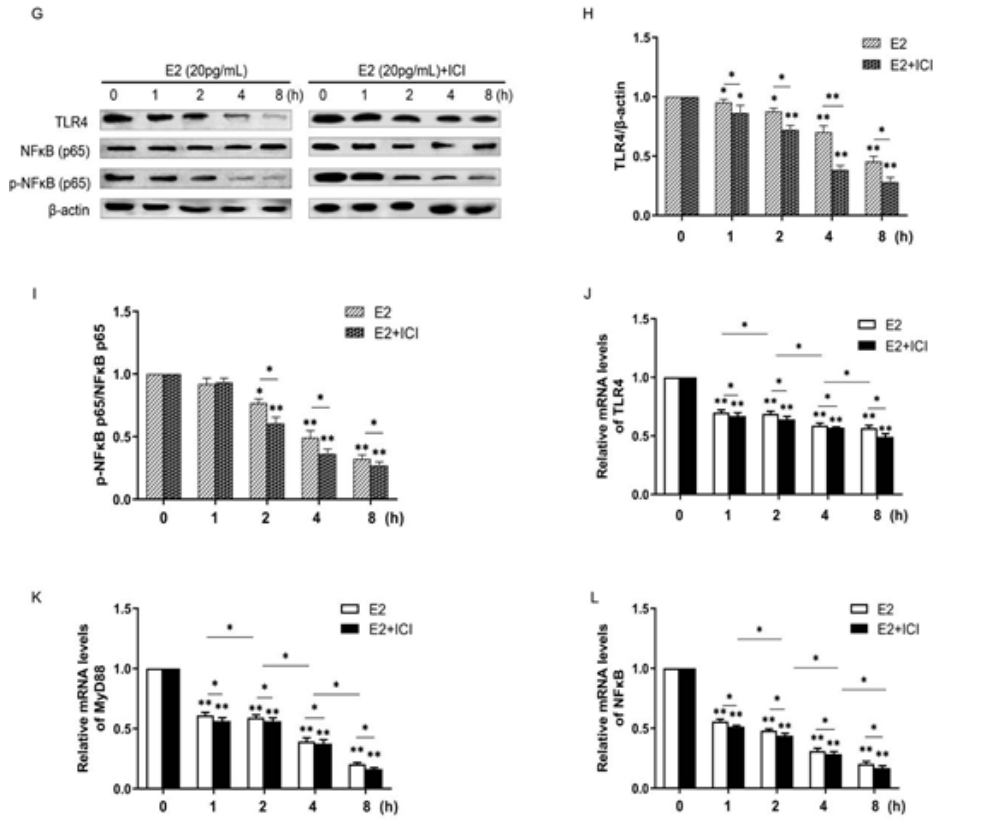


Fig. 2: Showing that E2 mediated GPER inhibits TLR4/NFκB signaling in resting neutrophils of cows. Neutrophils were treated with E2+ICI and E2 for 0, 1, 2, 4, and 8 hrs. (A, B, C, D, G, H, I): Protein expression levels of ERα, ERβ, GPER, TLR4, and p-NFκB (p65)/NFκB (p65) for the E2+ICI and E2 groups; (E, F, J, K, L): mRNA expression levels of ERα and ERβ, TLR4, MyD88 and NFκB by qRT–PCR for the E2+ICI and E2 groups. *significant (P<0.05); **significant (P<0.01).

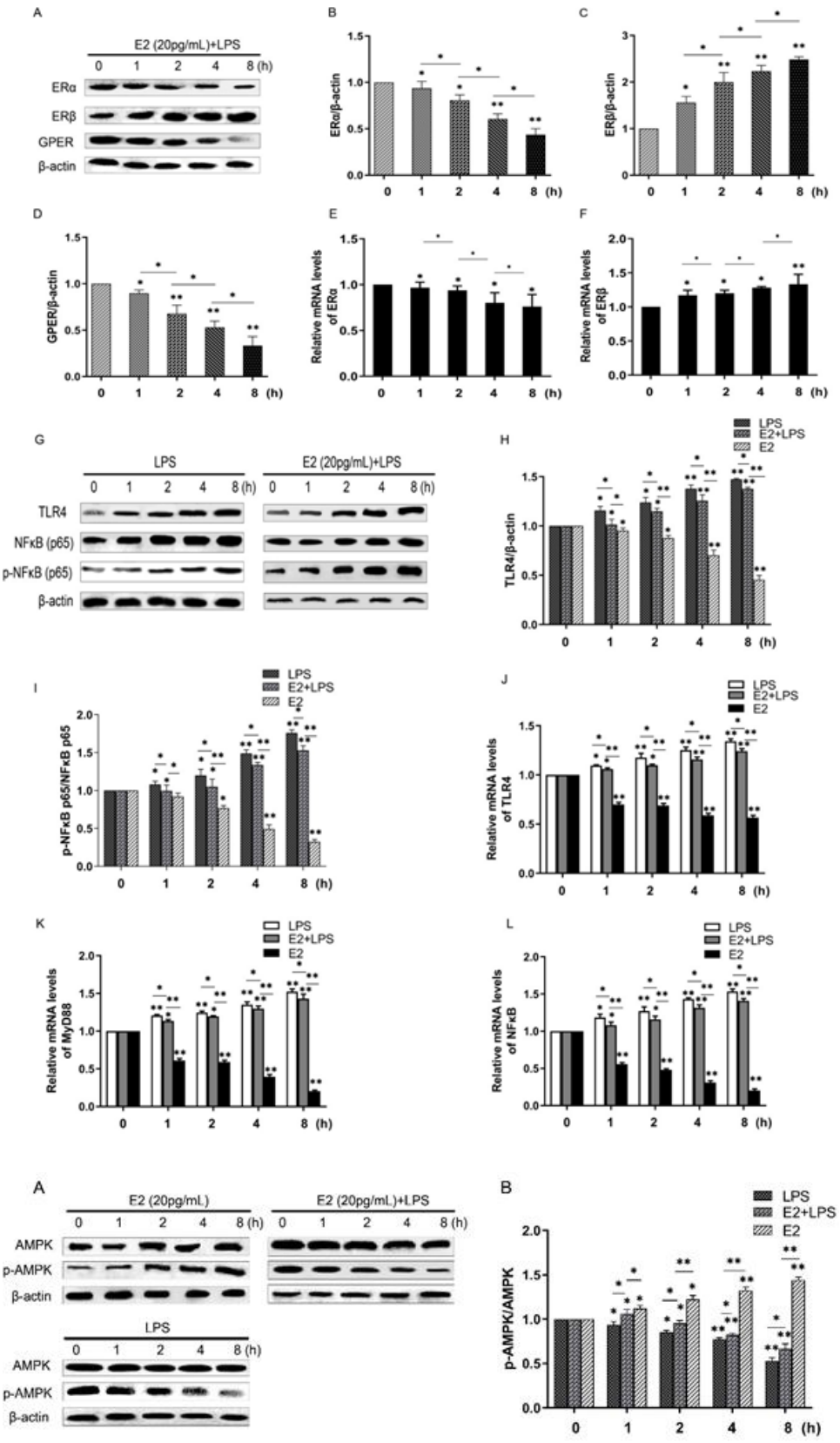


Fig. 3: Showing that E2 inhibits the TLR4/NFκB signaling pathway in LPS-activated neutrophils by upregulating ERβ. Neutrophils were treated with LPS and E2+LPS for 0, 1, 2, 4, and 8 hrs. (A, B, C, D): Protein expression levels of ERα, ERβ, and GPER by Western blotting for the E2+LPS group; (E, F): mRNA expression levels of ERα, ERβ, and GPER by qRT–PCR for the E2+LPS group; (G, H, I): Protein expression levels of TLR4 and p-NFκB (p65)/NFκB (p65) for the LPS, E2+LPS and E2 groups; (J, K, L): mRNA expression levels of TLR4, MyD88 and NFκB by qRT–PCR for the LPS, E2+LPS and E2 groups. *significant (P<0.05).

Fig. 4: Showing that E2 enhances the phosphorylation of AMPK in cow neutrophils. Neutrophils were treated with E2, LPS and E2+LPS for 0, 1, 2, 4, or 8 hrs. (A, B): Protein expression levels of AMPK for the E2, E2+LPS and LPS groups. *significant (P<0.05).

DISCUSSION

Physiological effects of E2 are largely attributable to E2 nuclear receptors (ERα and ERβ), which function as ligand-activated transcription factors. Lamote *et al.* (2006) found the expression of ERα and ERβ in neutrophils of cows during late pregnancy and early lactation by using flow cytometry and confocal microscopy, while mRNA levels of ERα and ERβ were detected by using qRT–PCR. In our study, Western blotting and qRT–PCR were used to confirm the presence of ERα and ERβ mRNA and protein in cow neutrophils *in vitro* at the basal level of oestrogen. Similarly, Molero *et al.* (2002) detected ERα and ERβ protein expression by Western blotting and found that both were enhanced in female neutrophils incubated with 17β-oestradiol (10mol/L) for 6 hrs. In addition, after the

addition of 17β-oestradiol (10pg/mL) to bovine oviduct epithelial cells *in vitro*, qRT–PCR results showed that from 1 to 6 hrs the expression of ERα mRNA was significantly decreased, but ERβ mRNA expression was not changed significantly, and ERα mRNA was 10 times higher than ERβ mRNA (Ulbrich *et al.,* 2003). However, our study showed that the protein and mRNA expression of ERα and ERβ did not change in cow neutrophils cultured with 20 pg/mL 17β-oestradiol for 0-8 hrs *in vitro*. Thus, the expression of ERα and ERβ may differ due to species and cell type and may be related to the concentration of 17β-oestradiol. Our study also showed that ERα and ERβ did not mediate the slow genomic effects of basal levels of E2 in resting neutrophils of cows during 0-8 hrs of culture. Rapid non-genomic signaling via GPER (Alexander *et al.*, 2017) is essential for oestrogenic activity, and our study detected GPER protein, which was upregulated in a time-dependent manner from 1 to 8 hrs, while the expression of ERα and ERβ did not change. These results suggest that the basal level of E2 mediated the rapid non-genomic effects through GPER in resting neutrophils of cows.

To further explore the role of GPER in oestrogen-mediated cellular effector and molecular signaling pathways in cow neutrophils, a GPER agonist/classical E2 receptor antagonist, ICI-182780, was used to block genomic signaling. A dose of 1 μm ICI-182780 has been shown to activate GPER in various tissues and cells, such as rhesus monkey neurons and human breast cancer cells (Noel *et al.,* 2009), and to inhibit ERα and ERβ (Chen *et al.,* 2014). We found that 10^{-6} M ICI-182780 also inhibited the expression of ERα and ERβ and enhanced the expression of GPER protein in cow neutrophils. According to Stefano *et al.* (2000), physiological doses of 17β-oestradiol (10^{-13} to 10^{-7} mol/L) could downregulate human neutrophils activity and reduce the risk of atherosclerosis in premenopausal women by activating ER and strongly stimulating NO release.

TLR4/NFκB, as the key signaling pathway in innate immunity experiments, has been used to verify that exposure of murine macrophages to physiological concentrations of 10^{-8} M 17β-oestradiol resulted in a significant reduction in cell-surface TLR4 levels within 10 min by mediating GPER (Rettew *et al.*, 2010). Our results showed that the physiological dose of 17β-oestradiol (20 pg/mL) inhibited the TLR4/NFκB signaling pathway and downregulated resting neutrophils immune activity within 1-8 hrs; the addition of ICI-182780 inhibited TLR4/NFκB signaling, while enhanced GPER. These results suggest that GPER rapidly downregulates TLR4/NFκB signaling in resting neutrophils in response to basal physiological doses of E2.

In the present study, oestrogen-mediated ER expression in LPS-activated neutrophils of cows was evaluated *in vitro.* The results showed that basal physiological doses of E2 inhibited ERα and GPER expression and enhanced ERβ expression in LPS-activated neutrophils. Differential expression of ER has been found in most studies, and ERβ shows a concentration-dependent antagonism to ERα-mediated transcription when ERα and ERβ are expressed simultaneously (Ascenzi *et al.,* 2006). Therefore, ERβ has a strong regulatory effect on LPS-activated neutrophils in cows.

The effects of E2 on TLR4/NFκB signaling in LPS-activated neutrophils of cows were also investigated *in vitro*. LPS has been shown to activate NFκB by binding TLR4 through the TLR4/MyD88 pathway, and the excessive and sustained activation of TLR4/NFκB leads to inflammatory responses. Imai *et al.* (2008) have found that oxidized phospholipids produced by overactivation of neutrophils can induce IL-6 overexpression through activation of the TLR4 signaling pathway. Our study has also indicated that LPS activates TLR4/NFκB signaling in cow neutrophils and induces inflammatory responses. Notably, E2 has been shown to be an inhibitor of inflammation, leading to reduced cytokine and chemokine production, primarily by reducing TLR4/NFκB signaling. According to Calippe *et al.* (2008), short-term *in vitro* treatment with physiological doses of E2 inhibited NFκB and attenuated mouse macrophage responses to LPS. Similarly, our study showed an inhibitory effect of basal physiological doses of E2 on the LPS-induced enhancement of the TLR4/NFκB signaling pathway in neutrophils. However, the basal level of E2 was not sufficient to resist LPS-induced enhancement of TLR4/NFκB signaling in cells compared with E2 treatment alone. These results suggest that basal physiological doses of E2 may attenuate LPS-induced inflammation by inhibiting the TLR4/NFκB signaling pathway in cow neutrophils.

Neutrophils function is closely related to cellular energy status. AMPK is a key kinase involved in the regulation of cellular energy metabolism, and its activation is mediated by inhibition of NFκB-induced inflammatory response systems in different cell types. In this study, AMPK phosphorylation levels were detected for further understanding the regulation of AMPK in the TLR4/NFκB signaling pathway in cow neutrophils. LPS has been shown to inhibit AMPK activation and increase inflammation and tissue damage in murine neutrophils (Zmijewski *et al.*, 2008). Park *et al.* (2013) recorded the time-dependent decrease in AMPK phosphorylation in LPS-induced neutrophils of mice. According to Zmijewski *et al.* (2008), metformin-induced activation of AMPK inhibited the degradation of IκBα, and NFκB activation reduced LPS-mediated proinflammatory activation of mouse neutrophils. Oestrogen has also been shown to improve E2 deficiency-induced cardiac contractile function by activating AMPK, enhancing cell activity to protect chondrocytes (Mei *et al.,* 2021), regulating AMPK/NFκB signaling pathway, reducing proinflammatory cytokines (IL-1β, IL-6 and TNFα), and demonstrating neuroprotective effects (Zhang *et al.,* 2020). Similarly, in the present study, LPS induced a time-dependent decrease in AMPK phosphorylation and NFκB activation in cow neutrophils, whereas E2 activated AMPK and inhibited NFκB activation. Park *et al.* (2013) have also shown that AMPK activation increased cell chemotaxis by inhibiting TLR4-related signaling pathways and enhanced bacterial uptake and killing by murine neutrophils *in vitro.* These findings support our results, which showed that LPS inhibited AMPK activation, whereas basal doses of E2 increased AMPK phosphorylation and slowed the inflammatory response by inhibiting TLR4/NFκB signaling.

Conclusions: In this study, we found that basal physiological concentrations of E2 could mediate ER (ERα, ERβ and GPER) expression, attenuate LPS-induced inhibition of energy metabolism through activation of AMPK and downregulate immune activity through inhibition of the TLR4/NFκB signaling pathway in cow neutrophils. This study further revealed the relationship between the basal level of E2 and cow neutrophils activity. These results will be helpful in understanding the regulatory mechanism of E2 on neutrophils function in different physiological stages of cows. However, due to the limited data, our study does not fully represent the real clinical situation, but it can be used as a reference to provide a theoretical basis for further understanding the immune activity and energy status of cow neutrophils at the basal physiological level of E2.

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Authors contribution: XW and LD conceived and designed the study. YZ, JM and YL executed the experimentS and detected the clinical samples. QD, MT, ZJ and XZ analyzed the data. All authors interpreted the data, critically reviewed the manuscript for important intellectual contents and approved the final version.

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