

Effect of TLR4 antagonist on immune responses of bovine endometrial cells

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Abstract

Bovine endometritis is one of the most common reproductive diseases and causes important economic losses. Lipopolysaccharide (LPS) from Gram-negative bacteria is recognized by Toll-like receptor 4 (TLR4). Binding of LPS and TLR4 mediates the expression of proinflammatory cytokines and chemokines. This study aimed to access the effect of TLR4 antagonist (RsLPS) on the immune responses of bovine endometrial cells. Primary culture of bovine endometrial cells were challenged with LPS (100 ng/ml) after treated with and without RsLPS (5000 ng/ml). Endometrial cells without RsLPS and LPS treatment served as control. The mRNA expression of *TLR4*, *CD14*, *TNF*, *IL1B* and *IL8* were investigated by using qPCR. The secretory protein of IL8 was measured by using ELISA. As expected, LPS up-regulated the mRNA expression of *TNF*, *IL1B*, *IL8* and the secretion of IL8 ($p < 0.01$). Meanwhile, endometrial cells pre-treated with RsLPS, after challenging with LPS showed an increased mRNA expression of all candidate genes ($p < 0.05$) except only *CD14* compared to control group. Interestingly, the secretion of IL8 was significantly decreased in LPS challenged endometrial cells pre-treated with RsLPS ($p < 0.01$) compared to LPS treatment group. Our findings suggested that RsLPS may act as antagonist for TLR4 in bovine endometrial cells. Nevertheless, other signaling pathways may involve with the link between innate immune responses and TLR4 antagonist. Therefore, the signaling mechanisms of RsLPS that related to the expression of proinflammatory mediators should be further investigated.

Keywords: Toll-like receptor 4, lipopolysaccharide, inflammation, endometrium

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Introduction

Uterine infection and the associated inflammatory immune responses compromise animal welfare and causing infertility. Cattle are remarkable among domestic animals as bacterial contamination of the uterus is ubiquitous postpartum (Sheldon et al., 2002; Williams et al., 2008). As the cervix dilates to allow passage of the calf at parturition, the anatomical barrier to bacterial contamination is breached, allowing microorganisms from skin, feces and surrounding environment to enter the uterus. Bovine endometritis may also occur after artificial insemination besides normal or abnormal parturition which associated with lower conception rates, increased intervals from calving to first service or conception and more culls for failure to conceive (William et al., 2008; Zhao et al., 2011). *Escherichia coli* (*E. coli*) is the most commonly isolated pathogen from the postpartum uterus (Hussain et al., 1990; Huszenicza et al., 1999; Galvão et al., 2012).

The innate immune mechanism of the uterus has developed to eradicate infection whereas maintaining the capability to adjust specialized physiological functions such as pregnancy and parturition. The key mediator of the innate immune system is pattern recognition receptors (PRRs) (Wira et al., 2005; Horne et al., 2008). Toll-like receptors (TLRs) are the major family of PRRs (Aflatoonian and Fazeli, 2008). TLRs recognized conserved pathogen-associated molecular patterns (PAMPs) produced by pathogens. Toll-like receptor 4 (TLR4) is the first mammalian TLR identified and it is, consequently, the best described of the family (Rock et al., 1998; Takeda et al., 2003; Linde et al., 2007).

TLR4 is the signaling receptor for LPS, the endotoxic component of Gram-negative bacteria (Poltorak et al., 1998; Hoshino et al., 1999; Yu et al., 2010). LPS derived from Gram-negative bacteria may directly cause endometrial damage. It is the role of TLR4 to detect LPS, although signaling through TLR4 also requires accessory molecules such as CD14, MD2 and LPS binding protein (LBP) (Beutler, 2004; William et al., 2008). The signaling pathway of TLR4 leading to the production of various proinflammatory cytokines (TNF α , IL1 β) and chemokines (IL8) that related to the inflammatory responses (Sheldon et al., 2014). Previously, bovine endometrial cells have been shown to increase expression of a number of genes in the presence of bacterial LPS, including TLR4, CD14, TNF, IL1 and IL8 (Herath et al., 2006; Davies et al., 2008; Lu et al., 2008; Bryant et al., 2010; Cronin et al., 2012; Swangchan-Uthai et al., 2012). However, if the host response is too strong, the excessive inflammatory reaction may occur and leading to the tissue destruction (Si-Tahar et al., 2009). Thus, treatment regimen tailored to disrupting the actions of LPS from *E. coli* which is detected by TLR4 in endometrium may diminish the detrimental effects of bovine uterine infection and inflammation due to this pathogen.

Clinically, the therapeutic potential of TLR4 antagonist as an effective treatment for endotoxemia and sepsis was first highlighted in human (Lynn et al., 2003; Raja and Dreyfus, 2007). In human blood

monocytes, TLR4 antagonist has a remarkable LPS inhibitory activity manifested via down-regulation of the intracellular generation of proinflammatory cytokines TNF α and IL6 (Czeslick et al., 2006). In rhesus monkeys, TLR4 antagonist pretreatment inhibited LPS-induced uterine activity and was associated with significantly lower TNF α , PGF $_{2\alpha}$ and PGE $_2$. And TLR4 antagonists, together with antibiotics may prevent infection-associated preterm birth (Waldorf et al., 2008). Inhibition of TLR4 with TLR4 antagonist significantly reduces markers of inflammatory response in an in situ murine model (Shimamoto et al., 2006). In rats, the studies by selectively inhibiting TLR4 expression using TLR4 antagonist observing that TLR4 inhibition protected the kidney from inflammatory cascades in renal ischemia-reperfusion injury (Liu et al., 2010).

Nowadays, the extensive using of antibiotics in animal husbandry has made the resistance to bacterial infection. The development of new therapeutic drug, TLR4 antagonists or inhibitors of TLR responses may be effective for treatment of inflammatory diseases as well as bovine endometritis. Accordingly, the study of the effect of *Rhodobacter sphaeroides* LPS (RsLPS) as TLR4 antagonist on bovine endometrium may lead to the novel therapeutic development of bovine endometritis.

Materials and Methods

Primary bovine endometrial cells culture: Bovine primary mixed epithelial and stromal cells were cultured using protocol described previously (Swangchan-Uthai et al., 2012). Briefly, fresh bovine uteri in early luteal phase were collected from nonpregnant cows at the slaughter house and transported to the cell culture laboratory in a plastic bag on ice. The stage of the estrous cycle was determined based on appearance of the ovaries and genital tracts (Ireland et al., 1980). After arriving at the laboratory, the outside of uterus was washed in 70% ethanol before opening under sterile conditions in a laminar flow hood. Endometrial tissues were separated by cutting into strips and transferred to Dulbecco's Modified Eagle Medium (DMEM/F-12, Gibco, Thermo Fisher Scientific). The endometrial tissues were chopped into 1 mm³ pieces and transferred to Hank's Balanced Salt Solution (HBSS; Gibco, Thermo Fisher Scientific). Tissue samples were then digested 150 ml of digestive solution made by dissolving 50 mg Trypsin (Worthington Biochemical Corporation), 50 mg Collagenase A (Roche), 100 mg Bovine Serum Albumin (BSA; Sigma) and 10 mg Deoxyribonuclease (DNase) I (Roche) per 100 ml of HBSS. After incubation for 120 min at 37°C in a shaking water bath, the cell suspension was filtered through cell strainer 70 μ m (Falcon, Fisher Scientific). The filtered cell suspension was resuspended with HBSS containing 10% Fetal Bovine Serum (FBS; Sigma) and centrifuged at 100 $\times$ g for 10 min before the washing procedure was repeated twice. After the cell integrity and number were checked by trypan blue staining under light microscope at 100 $\times$ magnification, the washed pellet was resuspended with DMEM/F-12 medium containing 100 U of penicillin, 100 μ g

streptomycin, 0.25 µg amphotericin B (Gibco, Thermo Fischer Scientific) and 10% FBS to 7×10^5 to 9×10^5 cells/ml. And then 1 ml of cell suspension was added per well into 24-well plate (Nunc, Thermo Scientific), followed by another 1 ml of culture medium per well. Cell cultures were incubated in a humid atmosphere at 37°C with 5% carbon dioxide (CO₂). Culture medium was changed every 48 hours for 7 to 10 days until the cells reached confluence.

Cytotoxicity assays: TLR 4 antagonist that used in this study was ultrapure lipopolysaccharide from *Rhodobacter sphaeroides* or RsLPS (Catalog # tlr1-prslps, Invivogen, San Diego, Ca, USA,) that act as TLR4-specific inhibition by blocking LPS-dependent activation of TLR4. The main mechanism is the direct competition between LPS for the same binding site (Teghanemt et al., 2005). Prior to the experimental study of gene expression, mixed epithelial and stromal endometrial cells cultures were grown to confluence in 96-well microplates (Nunc) to test the dose toxicity of RsLPS.

Sets of 6 well-replicates were exposed to RsLPS at concentrations of 0, 10, 100, 1000, 5000 ng/ml for 10 h. The number of viable cell in proliferation or cytotoxicity assay was determined by a colorimetric method, CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA). The absorbance (optical density, OD) of samples was recorded at 450 nm using a 96-well plate reader. The concentration of RsLPS that did not affect cell proliferation (5000 ng/ml) was selected for treatment group in the following experiment.

Experimental design: In 24-well plate containing confluent mixed bovine endometrial cells, ratio of approximately 1:9 epithelial to stromal cells respectively (Swangchan-Uthai et al., 2012), were used in our experiment. One-third of the wells were challenged with 100 ng/ml LPS for 6 h. Another one-third of the wells were pre-treated with RsLPS 5000 ng/ml for 4 h and then challenged with 100 ng/ml LPS for 6 h. The remaining wells without LPS and RsLPS served as control. Treatments were replicated four times in each group. Cells from each well were collected separately to evaluated mRNA expression of *TLR4*, *CD14*, *TNF*, *IL1B* and *IL8* by qPCR. Media samples from each well were collected separately and stored at -80°C for analysis of concentration of IL8. Experiments were conducted on three separate occasions.

RNA isolation and reverse transcription (RT): The procedures of RNA isolation, RT and qPCR were performed as described previously (Swangchan-Uthai et al., 2012). Briefly, total RNA was extracted from endometrial cells directly from the culture plate, using a column method (RNeasy mini kit; QIAGEN) in accordance with the guidelines supplied by the manufacturer. RNA concentration and purify of each sample were evaluated using NanoDrop® spectrophotometer (NanoDrop Technologies Inc.). RNA integrity was confirmed with clearly visible bands of the 18S and 28S ribosomal RNA in denaturing formaldehyde agarose gel electrophoresis

visualization. In the following stage, precisely 1 µg of total RNA was treated to remove genomic DNA carryover with an RNase-free DNase kit (Promega) according to guideline supplied by the manufacturer. Subsequently, the DNase -treated RNA was transcribed into complementary DNA (cDNA) using an RT system kit (Promega) with random hexamer primers following the protocol from the supplier. A master mixture of RT reagents was prepared once to minimize potential variation.

Real-time qPCR assay (qPCR): Primer sequences were obtained from the previous study (Swangchan-Uthai et al., 2012). For optimization of qPCR, primers were tested by conventional PCR amplification using GoTaq® Green Master Mix (Promega). The presence of single band of DNA by electrophoresis on a 1% (w/v) agarose gel confirmed specificity of PCR products without primer dimers. ACTB and RN18S1 were selected as reference genes. To minimized variation, all cDNA samples included in each analysis were derived from the same batch. A master mix of qPCR reactions (KAPA SYBR® FAST qPCR kit; KAPA Biosystems) was prepared once for each assay to avoid pipetting error. Standards for qPCR were prepared from purified PCR products by using QIAquick PCR purification kit (Qiagen). qPCR assays for both standards and samples were run in duplicate. A no-template control (NTC) with nuclease-free water was included in every assay. Data from samples that expressed below quantification cycle (C_q) of the NTC were excluded. Absolute concentrations of the PCR product were calculated by comparing C_q values of the unknown samples to that of a standard curve, using Applied Biosystems 7300 Real-time PCR software (Applied Biosystems Inc.).

Measurement of secreted protein using Enzyme-linked immunosorbent assay (ELISA) for IL8: Samples of medium were used to determine IL8 levels in duplicate by using Human CXCL8/IL-8 DuoSet ELISA (R&D Systems, Inc.) in accordance with the guidelines supplied by the manufacturer. This IL8 kit has been proved for measuring bovine IL8 with an acceptable cross-reactivity (Cronin et al., 2015). All samples were measured on the same occasion. The intra-assay coefficient of variation (CV) was 5.08%, and the detection limit was 6.44 pg/ml.

Statistical analysis: Data were derived from a total of 9-12 replications, obtained from three independent occasions. Data were analysed using IBM SPSS Statistics for Windows, Version 22.0 (Armonk, NY: IBM Corp.). For the cytotoxicity test, one-way ANOVA (analysis of variance) was used to analyse the effect of dose cytotoxicity of RsLPS, followed by Bonferroni post hoc tests for significant effects. For the qPCR and ELISA, data were tested for homogeneity of variance by using a Levene test and log transformed if appropriate. Univariate analysis of variance was used to evaluate the effect of LPS and RsLPS, in which batch was included as a random effect. Bonferroni post hoc tests were carried out to identify sources of differences. Differences with $p < 0.05$ were regarded as statistically significant, $p < 0.01$ as highly statistically significant.

Results

Comparison of endometrial cells from primary cultures challenged with RsLPS at concentrations of 0, 10, 100, 1000, or 5000 ng/ml (n=6 replicates of endometrial cells per dose) revealed no significant differences in absorbance values representing the number of viable cells (Fig 1). Subsequent experiments were therefore carried out with the concentration of RsLPS at 5000 ng/ml.

In this study, the mRNA expression of *TLR4*, *CD14*, *TNF*, *IL1B*, *IL8* and reference genes (*ACTB* and *RN18S1*) were determined in bovine endometrial cells. All treatments did not alter *ACTB* and *RN18S1* mRNA expression. Thereby, the data of mRNA expression of the measure genes were normalized with the corresponding value of *ACTB* and *RN18S1*. The data were presented as relative gene expression.

Not surprisingly, the mRNA expression of *TNF*, *IL1B*, *IL8* and the secretion of *IL8* were significantly increased in bovine endometrial cells challenged with LPS ($p < 0.01$) compared to control group. Meanwhile, endometrial cells treated with RsLPS, after challenging with LPS showed an increased mRNA expression of all candidate genes ($p < 0.05$) except only *CD14* compared to control group. In addition, there was no significant difference of mRNA expression of all candidate genes between endometrial cells treated with RsLPS before challenging with LPS and the LPS treated group (Fig 2). Interestingly, the secretion of *IL-8* was significantly decreased in LPS challenged bovine endometrial cells pre-treated with RsLPS ($p < 0.01$) compared to LPS treatment group (Fig 3).

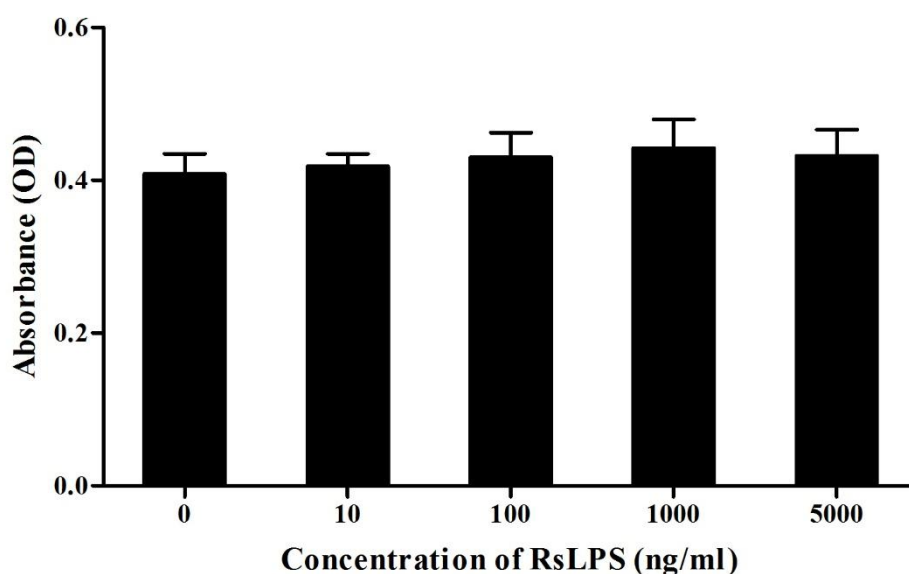
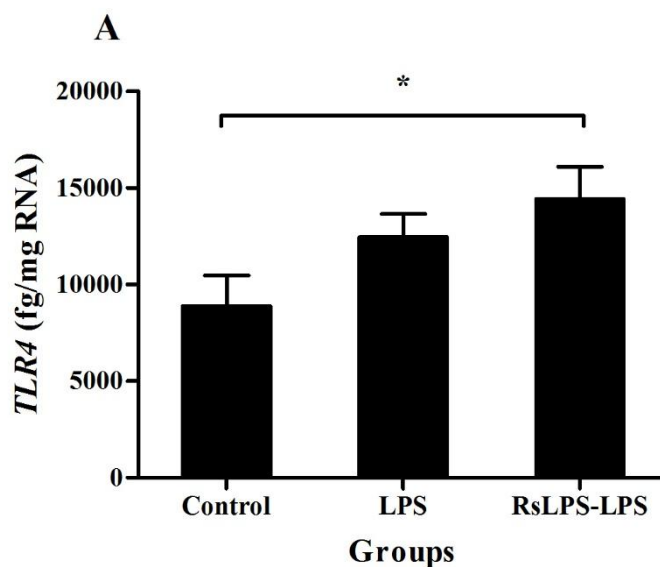
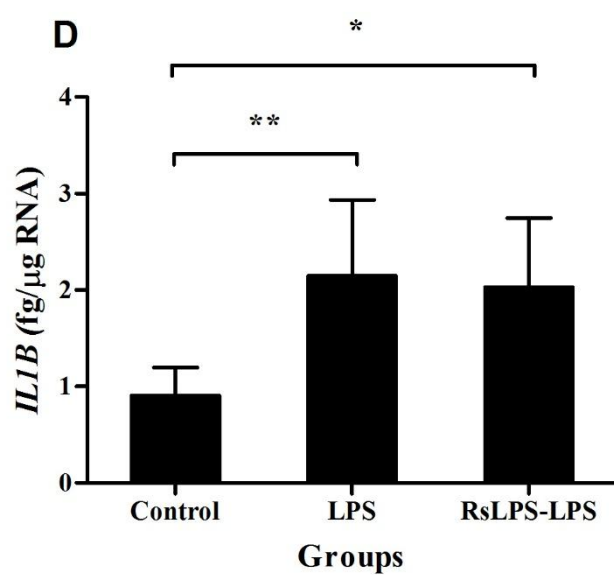
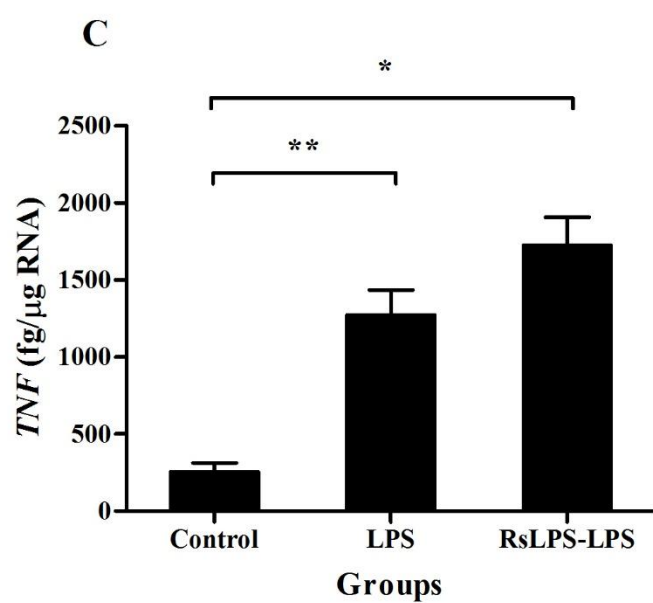
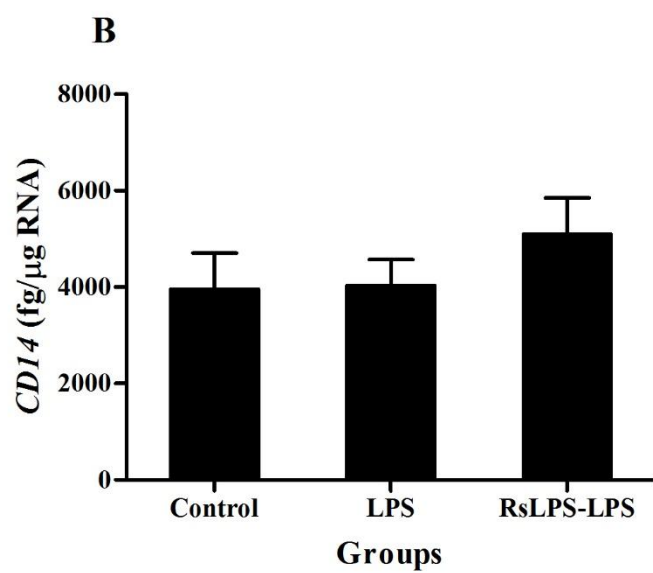


Figure 1 Cytotoxicity test at different concentrations of RsLPS (ng/ml) were determined in bovine mixed endometrial cell culture. Values are means $\pm$ SEM, n=6 replicates.





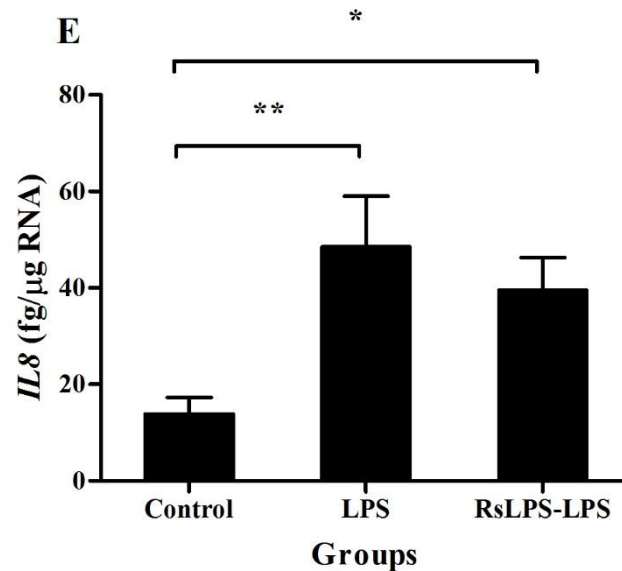


Figure 2 mRNA expression levels for *TLR4* (A), *CD14* (B), *TNF* (C), *IL1B* (D) and *IL8* (E) genes were determined by qPCR analysis in different groups (control, LPS, RsLPS-LPS). Values are means $\pm$ SEM. Differences between control, LPS and RsLPS-LPS treatments are indicated by * $p < 0.05$ and ** $p < 0.01$.

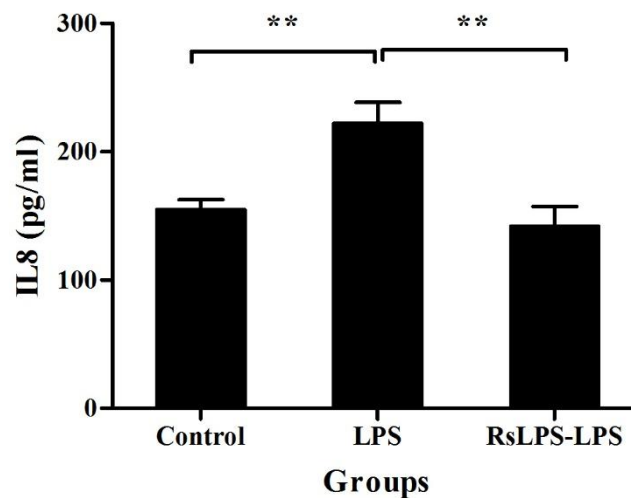


Figure 3 The concentrations of IL8 were measured, using the human IL8 ELISA in different groups (control, LPS, RsLPS-LPS). Values are means $\pm$ SEM. Differences between control, LPS and RsLPS-LPS treatments are indicated by ** $p < 0.01$.

Discussion

Many lines of evidences have shown that the innate immune responses were initially initiated by TLR4 in bovine endometrium (Herath et al., 2006; Devies et al., 2008; Chapwanya et al., 2009; Herath et al., 2009; Martins et al., 2011; Cronin et al., 2012; Silva et al., 2012; Swangchan-Uthai et al., 2012; Pinedo et al., 2013). TLR4 is the important pattern recognition receptor that recognized the LPS of Gram-negative bacteria in complex with CD14 and MD2 (Takeuchi and Akira, 2010; Sheldon et al., 2014). TLR4 receptor complex on the endometrial cells is important to detect LPS and then activate the inflammatory responses via NF κ B pathway. This signaling pathway is related to the expression of proinflammatory cytokines and chemokines such as TNF α , IL1B and IL8 (also known as CXCL8) (Sheldon et al., 2014). However, the excessive inflammatory responses may cause a

detrimental effect to the host. The systemic inflammatory responses to LPS from Gram-negative bacteria can cause sepsis and endotoxemia in both human and animals (Bryant et al., 2007; Lohmann et al., 2007).

In our study, we determined the proinflammatory cytokine and chemokine responses in bovine endometrial cells after incubated with LPS. As expected, LPS up-regulated the mRNA expression of all proinflammatory cytokines and chemokine and the secretion of IL8. In accordance with the previous studies, *IL1B* and *IL8* mRNA expression were markedly increased in bovine endometrial cells cultured with LPS (Cronin et al., 2012; Swangchan-Uthai et al., 2012). In addition, the study of time course of proinflammatory cytokines responses to LPS showed a rapid response by *TNF* at 1 h and the *IL1B* response later at 6 h (Swangchan-Uthai et al., 2012).

A natural, non-toxic molecule of LPS from *Rhodobacter sphaeroides* contains penta-acylated lipid A as opposed to LPS from *E. coli* that contains hexa-acylated lipid A. A structure in toxic LPS called lipid A could activate the inflammatory responses (Bryant et al., 2007; Rallabhandi et al., 2012). The antagonistic mechanism of RsLPS thought to be competitive with toxic LPS for the same binding site on MD2. The targets for competition may include CD14, TLR4 or other receptor components (Lohmann et al., 2003). Therefore, the use of LPS analogs or LPS antagonist for inhibits LPS-receptor complex has been widely studied for the development of novel therapeutic drugs (Rallabhandi et al., 2012; Lu et al., 2013; Anwar et al., 2015). From this study, endometrial cells treated with RsLPS, after challenging with LPS showed an increased mRNA expression of all candidate genes except CD14 compared to control group. The possibility is that RsLPS contains lipopeptide contaminants which may also stimulate Toll-like receptor 2 (TLR2) according to the manufacturer description. Even though, RsLPS ultrapure only has extremely weak TLR2 activity, but RsLPS alone could promote the proinflammatory cytokines from TLR2 signaling pathway. However, there was no significant difference of mRNA expression of all candidate genes between endometrial cells treated with RsLPS before challenging with LPS and the LPS treated group. In human and mice, LPS analogs that act antagonists such as RsLPS and diphosphoryl lipid A from *Rhodobacter sphaeroides* inhibited cellular binding of LPS from pathogenic bacteria and also inhibited proinflammatory cytokine release (Lien et al., 2000; Bryant et al., 2007; Lohmann et al., 2007). Whereas, the proinflammatory cytokines was not significantly decreased in equine cells pre-treated with RsLPS. These differences may be related to the species-specific characteristic (Lohmann et al., 2003).

IL8 is a potent chemotactic factor recruiting polymorphonuclear cells (PMNs) to the site of inflammation (Zerbe et al., 2003; Christoffersen et al., 2010). In bovine, IL8 appears particularly important during uterine disease in cattle since the signs of endometritis can be replicated by infusing recombinant bovine IL8 into the uterus. The expression levels of IL8 have shown to be the important biomarker that reflects the infection status of the cow when inflammation is established (Ghasemi et al., 2012; Cronin et al., 2015). Interestingly, the secretion of IL8 was significantly decreased in LPS challenged bovine endometrial cells pre-treated with RsLPS compared to LPS treatment group in our study. Analyzing of chemokine IL8 may be sufficient to monitor uterine inflammation. Since, the PMNs from the activation of IL8 may also express other proinflammatory cytokines via different signaling pathways (Wada et al., 2010; Ghasemi et al., 2012). Consequently, our findings suggested that RsLPS may act as antagonist for TLR4 in bovine endometrial cells. Nevertheless, other signaling pathways may involve with the link between innate immune responses and TLR4 antagonist. The signaling mechanisms of RsLPS that related to the expression of proinflammatory mediators should be further investigated.

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บทคัดย่อ

ผลของตัวรับทอลไลค์ 4 แอนทาโกนิสต์ต่อการตอบสนองทางภูมิคุ้มกัน ของเซลล์เยื่อบุผนังมดลูก

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การอักเสบของเยื่อบุผนังมดลูก เป็นโรคทางระบบสืบพันธุ์ที่พบส่วนใหญ่และทำให้เกิดปัญหาทางเศรษฐกิจที่สำคัญ โลโบโพลีแซคคาไรด์จากแบคทีเรียแกรมลบจะถูกรับรู้โดยตัวรับทอลไลค์ 4 การจับกันของโลโบโพลีแซคคาไรด์และตัวรับทอลไลค์ 4 จะกระตุ้นให้มีการแสดงออกของไซโตไคน์และคีโมไคน์ที่เหนี่ยวนำให้เกิดการอักเสบ ในการศึกษาครั้งนี้มีวัตถุประสงค์เพื่อศึกษาผลของตัวรับทอลไลค์ 4 แอนทาโกนิสต์ (RsLPS) ต่อการตอบสนองทางภูมิคุ้มกันของเซลล์เยื่อบุผนังมดลูก ทำการเลี้ยงเซลล์เยื่อบุผนังมดลูกและให้สาร RsLP (5000 นก./มล.) จากนั้นจึงให้สารโลโบโพลีแซคคาไรด์ (100 นก./มล.) ในขณะที่อีกกลุ่มให้สารโลโบโพลีแซคคาไรด์เพียงอย่างเดียว ส่วนเซลล์เยื่อบุผนังมดลูกที่ไม่ให้สาร RsLPS และสารโลโบโพลีแซคคาไรด์ จัดเป็นกลุ่มควบคุม ทำการตรวจปริมาณ mRNA ของยีน *TLR4*, *CD14*, *TNF*, *IL1B*, *IL8* โดยวิธี qPCR และตรวจปริมาณโปรตีน IL8 โดยวิธี ELISA ผลการศึกษาพบว่าสารโลโบโพลีแซคคาไรด์จะไปกระตุ้นให้มีการเพิ่มปริมาณ mRNA ของยีน *TNF*, *IL1B*, *IL8* และปริมาณโปรตีน IL8 ($p < 0.01$) ในขณะที่เซลล์เยื่อบุผนังมดลูกที่ได้รับสาร RsLPS และสารโลโบโพลีแซคคาไรด์ มีปริมาณ mRNA ของยีนทุกตัวที่ทำการศึกษาเพิ่มขึ้น ($p < 0.05$) ยกเว้น *CD14* เทียบกับกลุ่มควบคุม สิ่งที่น่าสนใจพบว่าปริมาณโปรตีน IL8 ลดลงอย่างมีนัยสำคัญทางสถิติ ($p < 0.01$) ในเซลล์เยื่อบุผนังมดลูกที่ได้รับสาร RsLPS และสารโลโบโพลีแซคคาไรด์ เทียบกับกลุ่มที่ได้รับสารโลโบโพลีแซคคาไรด์เพียงอย่างเดียว ผลการศึกษาแสดงให้เห็นว่า RsLPS อาจจะทำหน้าที่เป็นตัวรับทอลไลค์ 4 แอนทาโกนิสต์ในเยื่อบุผนังมดลูก อย่างไรก็ตามการส่งสัญญาณในส่วนอื่นๆ อาจเกี่ยวข้องกับการเชื่อมโยงระหว่างการตอบสนองของภูมิคุ้มกันอินเนตและตัวรับทอลไลค์ 4 แอนทาโกนิสต์ ดังนั้นกลไกการส่งสัญญาณของ RsLPS ซึ่งเกี่ยวข้องกับการแสดงออกของสารสื่ออักเสบ ควรที่จะมีการศึกษาต่อไป

คำสำคัญ: ตัวรับทอลไลค์ 4 โลโบโพลีแซคคาไรด์ การอักเสบ เยื่อบุผนังมดลูก

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