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LPS-mediated effects and spatio-temporal expression of TLR2 and TLR4 in the bovine corpus luteum

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Abstract

When given intravenously (iv), lipopolysaccharide (LPS) transiently suppresses the structure and function of the bovine corpus luteum (CL). This is associated with increased release of prostaglandin (PG) $F_{2\alpha}$ metabolite. The underlying regulatory mechanisms of this process remain, however, obscure. Therefore, the aims of this study were: i) to investigate the expression of the LPS receptor toll-like receptor 4 (*TLR4*) and 2 (*TLR2*) in the bovine CL during early, mid- and late luteal phases; and ii) to further dissect the mechanisms of LPS-mediated suppression of luteal function. As revealed by semi-quantitative qPCR and immunohistochemistry, both receptors were detectable throughout the luteal lifespan. Their mRNA levels increased from the early toward the mid-luteal phase; no further changes were observed thereafter. The TLR4 protein seemed more highly represented than TLR2. The cellular localization of TLRs was in blood vessels; weaker signals were observed in luteal cells. Additionally, cows were treated either with LPS (iv, 0.5 μg/kg BW) or with saline on Day 10 after ovulation. Samples were collected 1200 h after treatment and on Day 10 of the respective subsequent (untreated) cycle. The mRNA expression of several possible regulatory factors was investigated, revealing the suppression of $PGF_{2\alpha}$ receptor (*PTGFR*), *STAR* protein and 3β-hydroxysteroid dehydrogenase, compared with controls and subsequent cycles. The expression of *TLR2* and *TLR4*, interleukin 1α (*IL1A*) and *1β* (*IL1B*) and of $PGF_{2\alpha}$ and PGE_2 synthases (*HSD20A* and *mPTGES* respectively) was increased. The results demonstrate the presence of TLR2 and TLR4 in the bovine CL, and implicate their possible involvement in the deleterious effects of LPS on its function.
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Introduction

Inflammatory diseases, especially of the uterus and the mammary gland, reduce fertility in dairy cows (Hansen *et al*. 2004, Sheldon *et al*. 2009a). Infertility or subfertility in cows with uterine inflammation might be exclusively due to local effects in some cases, whereas a systemic inflammatory response is required to decrease fertility in cows with inflammation outside the genital tract, e.g. mastitis (Hansen *et al*. 2004). A systemic inflammatory response due to bovine metritis and mastitis is often associated with the presence of lipopolysaccharide (LPS), the endotoxin of gram-negative bacteria (Bannerman *et al*. 2004, Williams *et al*. 2005). There is increasing evidence that, at least in part, *Escherichia coli* LPS causes infertility by interfering with ovarian function (Suzuki *et al*. 2001, Herath *et al*. 2007, Lavon *et al*. 2008).

Ovarian dysfunction due to LPS in cows with inflammatory diseases might be due to impairment of the hypothalamic-pituitary axis and interference with ovarian follicular and luteal function (Hansen *et al*. 2004). Thus, cows with uterine infections after parturition were less likely to ovulate, probably because of slower growth of dominant follicles, lower peripheral estradiol (E_2) concentrations and perturbation of hypothalamic and pituitary function (Sheldon *et al*. 2009*b*). LPS was found in the follicular fluid of such animals, likely additionally contributing to the delay in ovulation (Sheldon *et al*. 2009*b*). Similar effects associated with a delayed luteinizing hormone surge and the resulting postponed ovulation were observed after intravenous or intramammary administration of LPS in cows (Lavon *et al*. 2008). Furthermore, intravenous treatment with LPS transiently suppressed progesterone (P_4) secretion by the bovine corpus luteum (CL) and increased plasma

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concentrations of prostaglandin (PG) $F_{2\alpha}$ metabolites (PGFM) (Herzog *et al*. 2012). Cows with uterine infections after parturition had increased concentrations of LPS not only in the uterine fluid but also in plasma and follicular fluid (Mateus *et al*. 2003, Williams *et al*. 2007, Magata *et al*. 2015). Thus, it may be assumed that LPS directly reaches the CL. Indeed, since LPS induced apoptosis in luteal cell cultures (Grant *et al*. 2007, Mishra & Dhali 2007), the possibility of a direct effect of LPS on the bovine CL should be considered. The direct effects of LPS on target organs depend, however, on the local presence of its specific receptor, toll-like receptor 4 (TLR4) (Gerold *et al*. 2007, Kannaki *et al*. 2011), the mRNA expression of which has recently been shown in the bovine mid-cycle CL (Lüttgenau *et al*. 2016).

Whereas toll-like receptor 2 (TLR2) recognizes bacterial lipids such as lipoteichoic acid and peptidoglycan from gram-positive bacteria, TLR4 in a complex with its co-receptors cluster of differentiation 14 (CD14) and myeloid differentiation factor 2 binds LPS, leading to signal transduction and activation of the innate immune system (Beutler 2004). However, expression of both *TLR2* and *TLR4* mRNA increased in bovine mammary (Ibeagha-Awemu *et al*. 2008, Ma *et al*. 2011) and endometrial epithelial cells (Fu *et al*. 2013) stimulated with LPS. Furthermore, these studies indicate that TLR4 is present on cells other than leukocytes. In cows, *TLR4* mRNA and protein were detected in endometrial stromal and epithelial cells by RT–PCR and flow cytometry (Herath *et al*. 2006). Activation of TLRs by LPS was observed in endometrial (Fu *et al*. 2013) as well as in mammary epithelial cells (Ibeagha-Awemu *et al*. 2008), thus inducing the downstream signaling cascade that culminates in the secretion of proinflammatory cytokines. In bovine granulosa cells, TLR4 was detected and an inflammatory response to LPS was observed and linked to reduced fertility, due to reduced follicular steroidogenesis (Herath *et al*. 2007). Since murine granulosa cells increased the expression of *TLR4* in response to LPS challenge (Shimada *et al*. 2006), granulosa cells seem to have immune capabilities (Herath *et al*. 2007). However, to the best of our knowledge, there are no reports available describing the spatio-temporal expression of TLR2 and TLR4 on the protein level in the bovine CL.

Consequently, the aims of the present study were: i) to provide evidence for the expression of *TLR2* and *TLR4* in the bovine CL throughout the luteal phase, and to determine their cellular localization; and ii) using samples derived from our previous study (Herzog *et al*. 2012) to further characterize the underlying mechanisms of transient suppression of luteal function after intravenous LPS treatment by analyzing the mRNA expression of cytokines and factors associated with prostaglandin synthesis and steroidogenesis. Expression analyses in the subsequent cycles (after the treated cycles) were aimed at detecting possible carryover effects of LPS.

Materials and methods

Study 1: Expression of TLR2 and TLR4 in bovine CL throughout the luteal phase

CL collection

Ovaries with CL were harvested from the carcasses of 14 clinically healthy cows (*Bos taurus*) including Red Holstein ($n=12$), Holstein Friesian ($n=1$) and Red Holstein×Limousin crossbred ($n=1$), that were slaughtered at a commercial abattoir. Before slaughter, cows were housed in a tie stall barn, and a modified ovulation synchronization (Ovsynch) protocol was started (at different times) after normal cyclic activity had been ultrasonographically confirmed in each cow. The protocol consisted of 10 µg buserelin (GNRH analog, Receptal; MSD Animal Health GmbH, Luzern, Switzerland), 15 mg luprostiol ($PGF_{2\alpha}$ analog, Prosolvin; Virbac AG, Glattbrugg, Switzerland) 7 days later and, finally, 10 µg buserel 60 hours after $PGF_{2\alpha}$ (all treatments were given intramuscularly). Ovulation occurred in all cows within 36 hours after the second GNRH treatment. Starting 17 days after ovulation, transrectal B-mode ultrasonography was performed in all cows at 2-day intervals to detect the time of ovulation (Day 1) and, subsequently, every 2–3 days to monitor normal development of the CL. Blood sampling and ultrasonography (B-mode and Power mode) of the CL were performed within 6 hours before the cows were slaughtered (at different cycle stages), and ovaries with CL were collected from the carcasses. For each cow, the time points during the luteal phase were randomly selected and allotted to the following groups according to Miyamoto *et al*. (2000): early (Days 5–7; $n=4$), mid- (Days 8–12; $n=5$) or late (Days 13–18; $n=5$) luteal phase.

Collection of blood and analysis of P_4

Blood samples were collected from the coccygeal blood vessels into evacuated tubes containing EDTA as anticoagulant (Vacuette 9 ml K3EDTA; Greiner Bio One, Kremsmünster, Austria). Tubes were immediately placed in ice, and plasma was separated by centrifugation (3000 ***g***, 15 min) and frozen at −20 °C until analyses were performed.

Concentrations of P_4 were measured using a radioimmunoassay (RIA Kit IM1188; Beckman Coulter GmbH, Krefeld, Germany). The range of standard concentrations for this test was 0.03–53 ng/ml, intra- and inter-assay coefficients of variation were ≤8.5 and ≤8.7% respectively and 50% of relative binding was reached at 1.6 ng/ml.

Ultrasonography

Transrectal ultrasonographic examinations of the uterus and ovaries were performed using a portable ultrasound device (GE LOGIQ e Premium BT11; General Electric

Medical System, Solingen, Germany), equipped with a 4.0–12.0 MHz linear-array transducer. B-mode and Power mode ultrasonography and a computer-assisted image analysis software (PixelFlux Version 1.0; Chameleon Software, Leipzig, Germany) were applied to determine luteal tissue area (LTA), luteal blood flow (LBF) and relative LBF (rLBF; LBF divided by LTA). A detailed description of the methodology was provided in previous studies (Lüttgenau *et al.* 2011*a*,*b*). In cows showing ovulation of two dominant follicles between Days 0 and 1 (double ovulation), and subsequent development of two CLs, their sum was applied for luteal measurements, as suggested by Bollwein *et al.* (2002).

Preparation of luteal tissue

Immediately after collection, the CL was removed from the ovary, trimmed of connective tissue, incubated for 24 h in RNA later (Ambion Biotechnologie GmbH, Wiesbaden, Germany) at 4 °C and, finally, stored at −80 °C until analysis. For immunohistochemistry (IHC), tissue samples were fixed for 24 hours in 10% neutral phosphate-buffered formalin, washed with PBS, dehydrated in a graded ethanol series and embedded in paraffin-equivalent Histo-Comp (Vogel Medizintechnik, Giessen, Germany).

Expression analysis

Luteal mRNA expression was determined for *TLR2*, *TLR4*, steroidogenic acute regulatory protein (*STAR*) and 3β-hydroxysteroid dehydrogenase (*HSD3B*). Accordingly, total RNA from luteal tissue samples was isolated using TRIzol reagent following the manufacturer's protocol (Invitrogen) and as described in Kowalewski *et al.* (2013). Semi-quantitative real-time (TaqMan) PCR was carried out in an automated fluorometer ABI PRISM 7500 Sequence Detection System (Applied Biosystems by Thermo Fisher) according to a previously described protocol (Kowalewski *et al.* 2006).

Samples were run in duplicates with Fast Start Universal Probe Master (ROX) from Roche Diagnostics. Autoclaved water instead of RNA and the so-called RT minus control were used as negative controls. Integrity of RNA and the assay procedure were tested by amplification of different independent endogenous references (*SDHA*, *GAPDH* and *ACTB*). The assays were set up to ensure approximately 100% efficiency of the PCRs. Relative gene expression was calculated using the comparative CT method (ΔΔ*C*T method) according to the manufacturer's protocols for the ABI PRISM 7500 Sequence Detection System (Applied Biosystems) and as described previously by Kowalewski *et al.* (2006, 2011).

Specificity of the selected PCR products was confirmed by sequencing (Microsynth, Galbach, Switzerland). A detailed description of the RT–qPCR method is provided in Kowalewski *et al.* (2006, 2011, 2013). The primers used to amplify specific fragments referring to selected genes purchased either from Microsynth or Applied Biosystems are presented in Table 1.

Immunohistochemistry

Methods of indirect immunoperoxidase IHC were described in detail by Kowalewski *et al.* (2006, 2011, 2013). In brief, luteal cross sections (2–3 µm thick) were cut and mounted on Super-Frost-Plus microscope slides. Antigen retrieval was done using citrate buffer (pH 6.0). Non-specific binding sites were blocked with normal 10% goat serum. The following antibodies were used: affinity purified polyclonal rabbit anti-TLR2 (Biorbyt, Cambridge, UK) and affinity purified polyclonal rabbit anti-TLR4 (Abbiotec, San Diego, CA, USA). The concentration of both primary antibodies was 1:200. Rabbit IgG (Vector Laboratories, Ltd., Burlingame, CA, USA) was used at the same protein concentration as the isotype-specific negative control. The secondary antibody was biotinylated goat anti-rabbit IgG (BA-1000) from Vector Laboratories, Ltd. (dilution 1:100). Peroxidase activity was visualized using the DAB Substrate Kit (Dako Schweiz AG, Baar, Switzerland), and slides were counterstained with hematoxylin.

Study 2: LPS-induced alterations in the expression of TLR2, TLR4 and other luteal factors

CL biopsies

CL biopsies from a previous experiment (Herzog *et al.* 2012) were used. In that study, transrectal ultrasonography was performed in each of seven clinically healthy, non-lactating German Holstein cows (*Bos taurus*) at 12, 24 and 36 hours after the second GNRH treatment of a modified Ovsynch protocol to detect the time of ovulation (defined as Day 1 of the estrous cycle). On Day 10, cows were treated with 10 ml saline (NaCl 0.9%; intravenously (iv), during 1 min), and luteal tissue was collected for biopsy 12 h after treatment and additionally on Day 10 of the subsequent (untreated) cycle. Then, the Ovsynch protocol was repeated, and cows were treated with 0.5 µg/kg body weight *E. coli* LPS (O55:B5; Sigma-Aldrich; diluted in 10 ml sterile water; iv, during 1 min) on Day 10 of the estrous cycle. Again, the collection of luteal tissue was performed at 12 h after the treatment and on Day 10 of the subsequent (untreated) cycle.

The collection of biopsy samples (~15×1×1 mm each) from the maximum diameter (including cells from the periphery and the center) of the CL was performed using a semi-automatic, high-speed biopsy needle (TEMNO Evolution; Fa. Walter, Baruth/Mark, Germany), as described previously (Herzog *et al.* 2012).

This method allowed repeated biopsy sampling from a single CL without impairing its subsequent function (Tsai *et al.* 2001, Atli *et al.* 2012).

Table 1 Accession numbers and sequences of PCR primers for assayed genes from bovine CL cells, and length of PCR products.

Primer	Accession numbers	Primer sequences	Product length
TLR2	NM_174197	Forward: 5′-TCCACGGACTGTGGTACATGA-3′ Reverse: 5′-ACACGAAGGCGTCGTAGCA-3′ TaqMan probe: 5′-CCAGGAAGGCTCCCCGCAGG-3′	102
TLR4	NM_174198	Forward: 5′-AAGACTGGGTGCGGAATGAAC-3′ Reverse: 5′-CCTTACGGCTTTTGTGGAAACC-3′ TaqMan probe: 5′-TGGCCATCGCCGCCAATATCATC-3′	144
STAR	NM_174189	Forward: 5′-AAGTCCCTCAAGGACCAAACTC-3′ Reverse: 5′-TGCGAGAGGACCTGGTTGAT-3′ TaqMan probe: 5′-ACCTCAAGGGATGGCTGCCGAAGA-3′	90
HSD3B	NM_174343	Forward: 5′-CACACCGCCTCTGTCATTGA-3′ Reverse: 5′-GTACGCTGGCCTGGACACA-3′ TaqMan probe: 5′-TGCTGTCCCGCGAGACCATCA-3′	112
COX2 (PTGS2)	NM_174445	Forward: 5′-GCACAAATCTGATGTTTGCATTC-3′ Reverse: 5′-GGTCCTCGTTCAAAATCTGTCT-3′ TaqMan probe: 5′-TTGCCCAGCACTTCACCCATCAATT-3′	76
mPTGES (PGES)	NM_174443	Forward: 5′-CAAGTGAGGCTGCGGAAGA-3′ Reverse: AGGCAGCGTTCCACATCTG-3′ TaqMan probe: 5′-TTTGCCAACCCCGAGGACGCTC-3′	101
HSD20A/PGFS (AKR1B5)	NM_001012519	Forward: 5′-ACCTGGACCTCTACCTCATCCA-3′ Reverse: 5′-TCCTCATCCAATGGGAAGAAGT-3′ TaqMan probe: 5′-CCCACAGGCTTCAAGCCTGGGA-3′	73
PTGFR (FP)	NM_181025	Forward: 5′-GCCAACTGGAAGAAGACCTTTC-′3 Reverse: 5′-CTGGTATGCCTTCATGAGGATAGC-3′ TaqMan probe: 5′-CAGTGGGAATCTTATCGAACAGCCTGGC-3′	101
FGF1	NM_174055.2	Applied Biosystems, prod. nr.: Bt03212662_m1	67
FGF2	NM_174056.3	Applied Biosystems, prod. nr.: Bt03259205_m1	100
IL1A	NM_174092	Applied Biosystems, prod. nr.: Bt03212739_m1	94
IL1B	NM_174093	Applied Biosystems, prod. nr.: Bt03212745_m1	129
IL1R1	NM_001206735	Applied Biosystems, prod. nr.: Bt04300521_m1	94
TNFA	NM_173966.3	Applied Biosystems, prod. nr.: Bt03259154_m1	84
TNFR1	NM_174674.2	Forward: 5′-GTTATGTCCAACCCGACCTTCA-3′ Reverse: 5′-GGCAAAGCCCGAAGACAAT-3′ TaqMan probe: 5′-AAGACTCTCAGGACCCAGG-3′	92
TNFR2	NM_001040490.2	Forward: 5′-GTCACCGCATGCTTTAGCTGTA-3′ Reverse: 5′-TGGCTTGCAGGTGCAGATG-3′ TaqMan probe: 5′-AACTCAAGCCTGCACAAC-3′	99
SDHA	NM_174178	Forward: 5′-ATGGAAGGTCTCTGCGCTAT-3′ Reverse: 5′-ATGGACCCGTTCTTCTATGC-3′ TaqMan probe: 5′-ACAGAGCGATCACACCGCGG-3′	119
GAPDH	NM_001034034	Forward: 5′-GCGATACTCACTCTTCTACCTTCGA-3′ Reverse: 5′-TCGTACCAGGAAATGAGCTTGAC-3′ TaqMan probe: 5′-CTGGCATTGCCCTCAACGACCACTT-3′	82
ACTB	NM_173979.3	Applied Biosystems, prod. nr.: Bt03279175_g1	144

Expression analysis

Semi-quantitative real-time PCR was applied for investigating the mRNA expression levels of target genes: *TLR2*, *TLR4*, *STAR*, *HSD3B*, cyclooxygenase 2 (*COX2*, *PTGS2*), PGE_2 synthase (*mPTGES*), $PGF_{2\alpha}$ synthase (*HSD20A*/*PGFS*), $PGF_{2\alpha}$ receptor (*PTGFR*, *FP*), fibroblast growth factor 1 (*FGF1*) and 2 (*FGF2*), interleukin 1α *(IL1A)* and 1β (*IL1B*), interleukin 1 receptor type 1 (*IL1R1*), tumor necrosis factor α (*TNFA*) and TNF receptor 1 (*TNFR1*) and 2 (*TNFR2*). The methodological approach was as presented above for Study 1. The primers and TaqMan probes sequences were purchased from Microsynth and are presented cumulatively in Table 1. The commercially available TaqMan systems for FGF1, FGF2, IL1A, IL1B, IL1R1, TNFA, and ACTB were purchased from Applied Biosystems.

Statistical analysis

Due to the uneven distribution of data obtained by semi-quantitative real-time PCR, logarithmic transformation was performed to normalize the data, and the geometric means (Xg) ± deviation factors (DF) were calculated for the analysis of target gene expression. The effects of observational group on target gene expression were then calculated by one-way ANOVA, followed by the Tukey–Kramer multiple comparison test. Since ultrasonography data were normally distributed, one-way ANOVA was performed for comparisons between early, mid- and late luteal phases.

All data were analyzed using the statistical analysis system V9.1 (SAS Institute, Inc., Cary, NC, USA) and the statistical software GraphPad3 (GraphPad Software, Inc., San Diego, CA, USA) respectively. Differences were considered significant at $P \leq 0.05$.

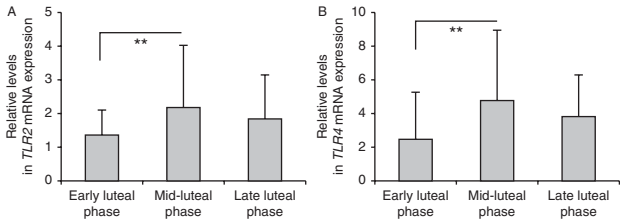

Figure 1 Relative levels (Xg±DF) of luteal mRNA expression of *TLR2* (A) and *TLR4* (B) during the early (Days 5–7; $n=4$), mid- (Days 8–12; $n=5$) and late (Days 13–18; $n=5$) luteal phases (Day 1 = ovulation). Parametric ANOVA ($P\leq0.05$) was applied, followed by the Tukey–Kramer multiple comparisons test; (**) indicates $P<0.05$.

Results

Study 1: Expression of TLR2 and TLR4 in bovine CL throughout the luteal phase

Luteal mRNA expression of *TLR2* and *TLR4* was detectable in all samples investigated at selected time points during the luteal phase, revealing a significant effect of time (ANOVA $P<0.05$ and $P=0.02$ for *TLR2* and *TLR4* respectively). Both receptors showed a similar expression pattern with significantly ($P<0.05$) increasing mRNA levels from the early to mid-luteal phase; no further changes were observed toward the late luteal phase (Fig. 1A and B). IHC localized the expression of TLR2 and TLR4 predominantly to steroidogenic cells and luteal blood vessels (Fig. 2). In general, especially in luteal cells, TLR4 seemed to be more strongly expressed than TLR2, as indicated by more intense staining of TLR4 which could already be observed during the early luteal phase. During the mid-luteal phase, the signal intensity appeared stronger with both receptors staining intensively, especially in large luteal cells. Within blood vessels, the *tunica intima* and *media* stained strongly for both receptors. In particular, vascular endothelial cells clearly revealed positive signals throughout the luteal phase.

Additionally, the luteal mRNA expression of *STAR* and *HSD3B* was investigated. Their expression was time dependent (ANOVA $P=0.002$ and $P=0.01$ for *STAR* and *HSD3B* respectively). It increased ($P<0.01$ and $P<0.05$ respectively) from the early to mid-luteal phase and decreased (each $P<0.05$) thereafter to initial values in the late luteal phase (Supplementary Fig. 1A and B, see section on supplementary data given at the end of this article).

Mean LTA was numerically increased ($P>0.05$) in the mid-luteal phase compared with the early and late luteal phase (supplementary Table 1, see section on supplementary data given at the end of this article). There was no significant difference in absolute and relative LBF as well as plasma P_4 concentrations between the early, mid- and late luteal phase.

Study 2: LPS-induced alterations in the expression of TLR2, TLR4 and other luteal factors

Expression of *TLR2* mRNA was higher ($P<0.05$) after intravenous LPS challenge compared with the control cycle (Fig. 3A). However, mRNA abundance of *TLR2* in the estrous cycles that followed the LPS challenge cycle and the control cycle did not differ from mRNA abundance in the LPS challenge cycle or from that in the control cycle. Luteal mRNA expression of *TLR4* was increased ($P<0.05$) after LPS challenge compared with the control and subsequent cycles after the LPS challenge and control cycles (Fig. 3B). There was no difference in mRNA abundance of *TLR4* between the control cycle and the subsequent (untreated) cycles. Luteal mRNA expression of the steroidogenic factors *STAR* and *HSD3B* was decreased ($P<0.001$) after LPS challenge compared to the control cycle as well as compared to the subsequent cycles after the LPS challenge and control cycles (Fig. 3C and D).

Within prostaglandin-related factors, LPS challenge significantly increased mRNA abundance for *COX2*, *mPTGES* and *HSD20A/PGFS*, but decreased mRNA for *PTGFR* (Fig. 4A, B, C and D). Specifically, *COX2* mRNA expression was higher ($P<0.01$) in the LPS challenge cycle compared to the subsequent cycles after the LPS challenge and control cycles but did not differ significantly between the LPS challenge cycle and control cycle (Fig. 4A). Luteal expression of *mPTGES* and *HSD20A/PGFS* mRNA was higher ($P<0.001$) in the LPS challenge cycle compared to the control cycle and the subsequent cycle after the control cycle (Fig. 4B and C). However, mRNA abundance of *mPTGES* was also higher ($P<0.001$) in the LPS challenge cycle compared to the subsequent cycle, whereas mRNA

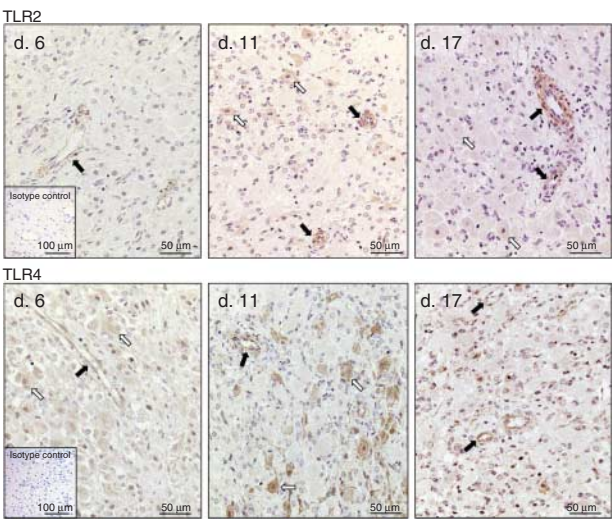

Figure 2 Immunohistochemistry localization of TLR2 and TLR4. Representative photographs are shown at Day 6 (early luteal phase), Day 11 (mid-luteal phase) and at Day 17 (late luteal phase). Open arrows = luteal cells; solid arrows = luteal vessels.

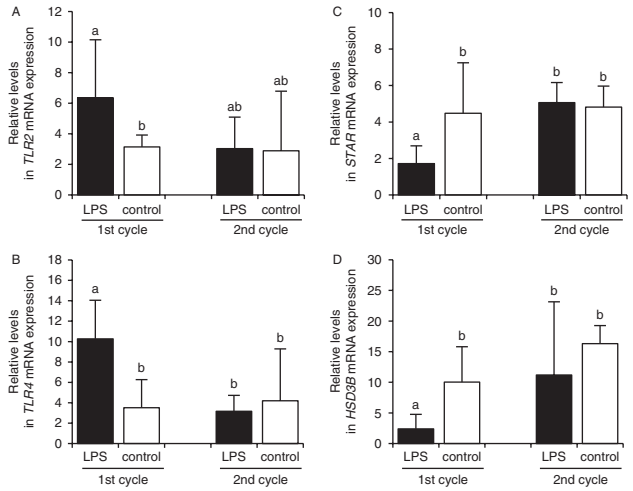

Figure 3 Relative levels (Xg±DF) of luteal mRNA expression of *TLR2* (A), *TLR4* (B), *STAR* (C) and *HSD3B* (D) on Day 10 of the estrous cycle (Day 1=ovulation) at 12 hours after intravenous treatment with LPS (*Escherichia coli* O55:B5; 0.5 µg/kg BW) or saline (control), and on Day 10 of the subsequent (2nd) cycle after the LPS challenge and control cycles. Parametric ANOVA ($P \leq 0.03$) was applied, followed by the Tukey–Kramer multiple comparisons test; [a,b]values with different superscripts differ ($P < 0.05$) between the indicated cycles.

abundance of *HSD20A/PGFS* in the subsequent cycle after the LPS challenge cycle did not differ significantly from the LPS challenge cycle or the control cycle. Luteal mRNA expression of *PTGFR* decreased ($P < 0.001$) after LPS challenge compared to the control and subsequent cycles (Fig. 4D). Within angiogenic factors, expression of *FGF1* mRNA also decreased ($P < 0.05$) in the LPS challenge cycle compared to the control and subsequent cycles, whereas mRNA abundance of *FGF2* increased ($P < 0.05$) after LPS challenge compared to the other cycles (Fig. 4E and F).

Expression levels of *IL1A* and *IL1B* mRNA were increased ($P < 0.001$) after LPS challenge compared to the control and subsequent cycles (Fig. 5A and B). Luteal mRNA abundance of *IL1R1* was also highest after LPS challenge, but it did not differ from values observed in the control cycle (Fig. 5C). However, *IL1R1* mRNA was more abundant ($P < 0.01$) in the LPS cycle compared with the untreated cycle that followed the control cycle. Luteal expression of *TNF* mRNA increased ($P < 0.01$) after LPS challenge compared to the control and subsequent cycles (Fig. 5D). The mRNA abundance of *TNFR1* did not differ between cycles, whereas mRNA for *TNFR2* was increased ($P < 0.001$) in the LPS cycle compared with the other cycles (Fig. 5E and F).

Discussion

The results of the present study establish, for the first time, expression patterns of TLR2 and TLR4 in the bovine CL during the luteal phase. On the mRNA level, the expression of *TLR2* and *TLR4* has recently been shown in the mid-cycle CL of isolated perfused bovine ovaries *in vitro* (Lüttgenau *et al.* 2016), which could be confirmed in the present *ex vivo* study. The cellular localization of both receptors was in steroidogenic cells and luteal vessels, indicating an immune capability of those cells directed toward components of gram-positive as well as gram-negative bacteria. Therefore, effects of LPS on the bovine CL do not seem to be restricted to TLR-bearing immune cells that are normally present in luteal tissue as reported in a review by Walusimbi & Pate (2013). The localization of TLR4 in luteal cells was consistent with previous reports that established the presence of TLR4 in granulosa (Herath *et al.* 2007, Bromfield & Sheldon 2011, Price & Sheldon 2013) and theca cells (Magata *et al.* 2014) from ovarian follicles, which are the progenitors of the large and small luteal cells respectively (Alila & Hansel 1984).

The mRNA levels of *TLR2* and *TLR4* increased from the early toward the mid-luteal phase and remained unchanged afterwards, i.e. during the late luteal phase. The expression patterns of *STAR* and *HSD3B* mRNA were determined in order to validate the allocation of CLs to their respective experimental groups (early,

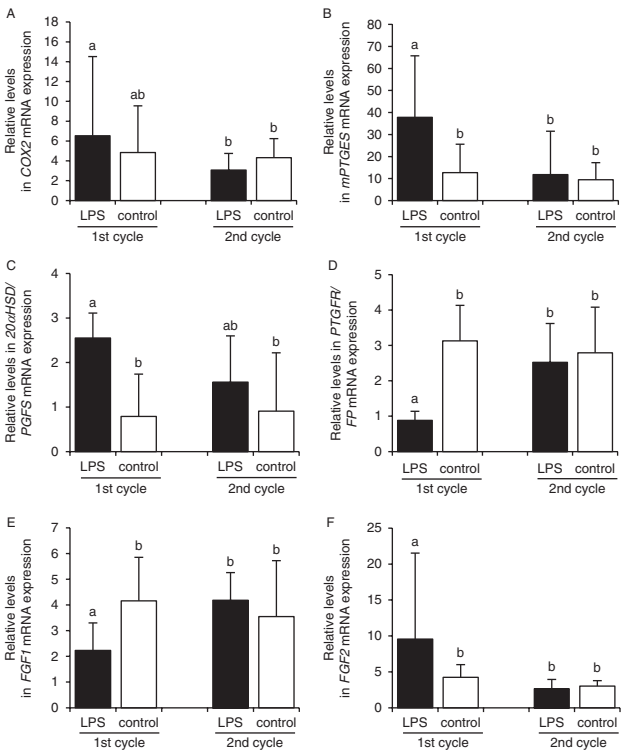

Figure 4 Relative levels (Xg±DF) in luteal mRNA expression of *COX2* (A), *mPTGES* (B), *HSD20A/PGFS* (C), *PTGFR* (D), *FGF1* (E) and *FGF2* (F) on Day 10 of the estrous cycle (Day 1=ovulation) 12 hours after intravenous treatment with LPS (*Escherichia coli* O55:B5; 0.5 µg/kg BW) or saline (control), and on Day 10 of the subsequent (2nd) cycle after the LPS challenge and control cycles. Parametric ANOVA ($P \leq 0.02$) was applied, followed by the Tukey–Kramer multiple comparisons test; [a,b]values with different superscripts differ ($P < 0.05$) between the indicated cycles.

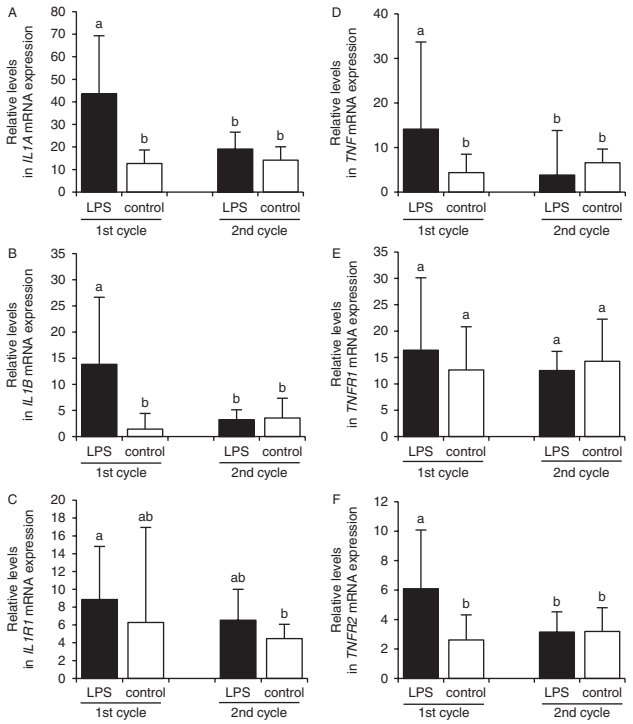

Figure 5 Relative levels (Xg±DF) of luteal mRNA expression of *IL1A* (A), *IL1B* (B), *IL1R1* (C), *TNF* (D), *TNFR1* (E) and *TNFR2* (F) on Day 10 of the estrous cycle (Day 1 = ovulation) at 12 h after intravenous treatment with LPS (*Escherichia coli* O55:B5; 0.5 µg/kg BW) or saline (control), and on Day 10 of the subsequent (2nd) cycle after the LPS challenge and control cycles. Parametric ANOVA ($P \leq 0.006$; except for *TNFR1* with $P > 0.05$) was applied, followed by the Tukey–Kramer multiple comparisons test; ^{a,b}values with different superscripts differ ($P < 0.01$) between the indicated cycles.

mid- or late luteal phase). Increased expression of *STAR* and *HSD3B* between the early and mid-luteal phase, and decreased expression between the mid- and late luteal phase were consistent with changes in steroidogenic capacity of the CL throughout its lifespan (Herzog *et al.* 2010). Determination of plasma P_4 concentrations, luteal size and blood flow further confirmed that only tissue derived from functional CLs was used.

Escherichia coli LPS given intravenously increased luteal mRNA expression of *TLR2* and *TLR4*. Because TLR4 is the receptor for the gram-negative endotoxin LPS (Poltorak *et al.* 1998), the increase in *TLR4* mRNA might indicate an auto-amplification pathway that supports increased binding of LPS. Binding and activation of TLR4 initiates the production of proinflammatory cytokines and leads to the recruitment of leukocytes (Sheldon & Bromfield 2011). However, in the recent study (Lüttgenau *et al.* 2016) that used isolated perfused ovaries, luteal mRNA expression of *TLR4* did not differ between LPS-treated and control ovaries during the first 3 hours after treatment. Apart from the differences in the general approach of the recent and the present study (*in vitro* vs *in vivo*), the different outcome in luteal expression of several factors that were investigated in both studies might be due to the different sampling time. Whereas biopsies in the present study were performed at 12 h after treatment, luteal tissue from isolated perfused ovaries was collected during the first 3 hours after challenge because viability of the ovary could not be guaranteed for a longer time. In both studies, LPS increased TLR2, which typically recognizes bacterial lipids from gram-positive bacteria (Takeda & Akira 2005). Increased expression of *TLR2* mRNA was also observed in mammary glands challenged with LPS (Ibeagha-Awemu *et al.* 2008) as well as *E. coli* (Yang *et al.* 2008). Although the reason for increased *TLR2* mRNA after LPS treatment is not known, studies in mice (Matsumura *et al.* 2000) and humans (Davanian *et al.* 2012) suggest that LPS-induced TNF increases *TLR2* mRNA and protein expression. In accordance with this, in the present study, *TNF* mRNA was also increased after LPS challenge.

The decrease in luteal mRNA expression of steroidogenic factors *STAR* and *HSD3B* after LPS challenge was consistent with the transient decrease in plasma P_4 concentrations reported by Herzog *et al.* (2012). Both STAR and *HSD3B* catalyze key steps of steroidogenesis (Couët *et al.* 1990, Stocco & Clark 1996) and were inhibited by $PGF_{2\alpha}$ (Stocco *et al.* 2007). The increase in mRNA expression of factors related to prostaglandin synthesis (*COX2*, *mPTGES* and *HSD20A/PGFS*) was in accordance with increased plasma concentrations of PGFM and PGE_2 after LPS treatment (Herzog *et al.* 2012). It is known that pulsatile release of $PGF_{2\alpha}$ from the uterus and administration of $PGF_{2\alpha}$ increase luteal $PGF_{2\alpha}$ synthesis (Stocco *et al.* 2007, Shirasuna *et al.* 2010). The increase in luteal expression of both luteolytic $PGF_{2\alpha}$ and luteotropic PGE_2 might explain the absence of complete premature luteolysis reported previously (Herzog *et al.* 2012). In contrast, luteal mRNA expression of *PTGFR* was decreased after LPS challenge, possibly due to increased $PGF_{2\alpha}$ concentrations, because *PTGFR* mRNA was also reduced by $PGF_{2\alpha}$ administration (Shirasuna *et al.* 2010).

Luteal mRNA expression of *FGF1* decreased whereas that of *FGF2* increased after LPS challenge. FGFs are potent mitogens for endothelial cells and other cell types, including luteal cells, and are therefore strong luteotropic factors within the CL (Yamashita *et al.* 2008, Shirasuna *et al.* 2010). The mechanisms that decrease FGF1 but increase FGF2 remain unknown; however, different expression levels of these luteotropic factors might have contributed to the incomplete luteolysis observed by Herzog *et al.* (2012).

In the present study, LPS increased luteal mRNA expression of the proinflammatory cytokines *IL1A*, *IL1B* and *TNF*. Increased concentrations of TNFA (in milk and plasma) were also observed after LPS-induced mastitis (Hoeben *et al.* 2000). The interleukins and TNF mediate the inflammatory response at both the local and systemic levels by promoting neutrophil transendothelial migration to the site of infection and by inducing fever

and the acute phase response (Bannerman *et al*. 2004). Furthermore, IL1A, IL1B and TNFA are potent stimulators of luteal prostaglandins including $PGF_{2\alpha}$ and PGE_2 (Nishimura *et al*. 2004, Sakumoto & Okuda 2004). Consistent with this, luteal mRNA expression of *HSD20A/PGFS* and *mPTGES* was increased after LPS challenge in the present study. Both TNFA receptors, TNFR1 and TNFR2, are expressed in the bovine CL (Korzekwa *et al*. 2008), but only the mRNA abundance of *TNFR2* was increased after LPS challenge.

In the estrous cycles that followed the LPS challenge and control cycles, luteal expression of none of the investigated parameters differed from that observed in the control cycles, indicating the absence of any carryover effect of LPS on the CL in the subsequent cycle.

In conclusion, the expression of TLR2 and TLR4 was predominantly localized to luteal cells and blood vessels and increased during the mid- and late luteal phase. It seems possible that luteal TLR2 and TLR4 are involved in the immune response of luteal tissue to an intravenous application of *E. coli* LPS *in vivo,* which is associated with the production of proinflammatory cytokines and reduced ovarian steroidogenesis in cows.

Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1530/REP-15-0520.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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