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) F2α 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 PGF2α 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 PGF2α and PGE2 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.

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 infection 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 (E2) concentrations and perturbation of hypothalamic and pituitary function (Sheldon et al. 2009b). LPS was found in the follicular fluid of such animals, likely additionally contributing to the delay in ovulation (Sheldon et al. 2009b). 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 (P4) secretion by the bovine corpus luteum (CL) and increased plasma...
concentrations of prostaglandin (PG) F₂alpha 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 (Lüttenau 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 luprostriol (PGF₂alpha analog, Prosolvin; Virbac AG, Glattbrugg, Switzerland) 7 days later and, finally, 10 µg buserelin 60 hours after PGF₂alpha (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 P₄**

Blood samples were collected from the coccygeal blood vessels into evacuated tubes containing EDTA as anti-coagulant (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 ºC until analyses were performed.

Concentrations of P₄ were measured using a radio-immunoassay (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 Reproduction (2016) 151 391–399
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 (ΔΔCT 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, Atlı et al. 2012).
Table 1 Accession numbers and sequences of PCR primers for assayed genes from bovine CL cells, and length of PCR products.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Accession numbers</th>
<th>Primer sequences</th>
<th>Product length</th>
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<tr>
<td>TLR2</td>
<td>NM_174197</td>
<td>Forward: 5'-TCACAGGACTGTGGTACAGTA-3' Revert: 5'-ACAGGAAGCCTGCTGATAGCA-3' TaqMan probe: 5'-CCAGGAGGCTTCGCGG-3'</td>
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<td>TLR4</td>
<td>NM_174198</td>
<td>Forward: 5'-AAGACTCTGGTGCGAATGAAGC-3' Revert: 5'-CCTTACCGTTTGTGAGAAAAC-3' TaqMan probe: 5'-TGGCATCGGCCCGCTG-3'</td>
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<td>STAR</td>
<td>NM_174189</td>
<td>Forward: 5'-AAGTCCCTAAGGAGAACAAACTC-3' Revert: 5'-TCCGAGAGGCACTGTTG-3' TaqMan probe: 5'-ACCTACAGGATGTCGCTCGGAA-3'</td>
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<td>HSD3B</td>
<td>NM_174343</td>
<td>Forward: 5'-CACACAGGCTCTGGTACAGT-3' Revert: 5'-GTACGCTGGAGCTGACCA-3' TaqMan probe: 5'-TCCCGCAGACCTACGATTCA-3'</td>
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<tr>
<td>COX2</td>
<td>NM_174445</td>
<td>Forward: 5'-CCACACAGGCTCTGGTACAGT-3' Revert: 5'-AGGCAAGCTGTTACCATCTG-3' TaqMan probe: 5'-TTGGCCTCCACCGAGCCATCA-3'</td>
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<td>mPTGES</td>
<td>NM_174443</td>
<td>Forward: 5'-CAAGTTAGGTCCGGAGAA-3' Revert: 5'-AGGGCAGCGTTCCACATCG-3' TaqMan probe: 5'-TCCCGCAGACCTACGATTCA-3'</td>
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<td>HSD20A</td>
<td>NM_00102519</td>
<td>Forward: 5'-ACCTGAGACTCTACCCATCTCA-3' Revert: 5'-TCTTACGCAATGGGAAAGAATG-3' TaqMan probe: 5'-CCCACAGGGCTCTGGGGA-3'</td>
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<td>PTGFR</td>
<td>NM_181025</td>
<td>Forward: 5'-GCCACTTGGAAGAGACCTTCT-3' Revert: 5'-CTCTGTATCCTGCTAGGATGAC-3' TaqMan probe: 5'-CTGTGGGGAATCTTATCGAACG-3'</td>
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<td>SDHA</td>
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<tr>
<td>GAPDH</td>
<td>NM_001034034</td>
<td>Forward: 5'-GGCATCATCTACTTCCTACTCTTGCA-3' Revert: 5'-TCGTAACGAGAAATGACCTTGAC-3' TaqMan probe: 5'-ACCTGAGACTCTACCCATCTCA-3'</td>
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<td>ACTB</td>
<td>NM_173979.3</td>
<td>Applied Biosystems, prod. nr.: B03279175_g1</td>
<td>144</td>
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**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, PGE2 synthase (mPTGES), PGF2α synthase (HSD20A/PGFS), PGF2α 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<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...
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 (Lüttenau 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 (Ailia & 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,
with luteal expression of several factors that were investigated in the present study (differences in the general approach of the recent and the present study). 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 P₄ concentrations reported by Herzog et al. (2012). Both STAR and HSD3B catalyze key steps of steroidogenesis (Couët et al. 1990, Stocco & Clark 1996) and are inhibited by PGF₂α (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 PGE₂ after LPS treatment (Herzog et al. 2012). It is known that pulsatile release of PGF₂α from the uterus and administration of PGF₂α increase luteal PGF₂α synthesis (Stocco et al. 2007, Shirasuna et al. 2010). The increase in luteal expression of both luteolytic PGE₂ and luteotropic PGE₃ 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 PGF₂α concentrations, because PTGFR mRNA was also reduced by PGF₂α 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.

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**Figure 5** Relative levels (X±SD) of luteal mRNA expression of IL1A (A), IL1B (B), IL1R1 (C), TNFR1 (D), TNFR2 (E) and TNFR2 (F) on Day 10 of the estrous cycle (Day 1 = ovulation) at 12 h after intravenous treatment with LPS (Escherichia coli O55:5; 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≤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.
and the acute phase response (Bannerman et al. 2004). Furthermore, IL1A, IL1B and TNFA are potent stimulators of luteal prostaglandins including PGF2α and PGE2 (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 (Korzewka 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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References
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