Intramammary lipopolysaccharide infusion alters gene expression but does not induce lysis of the bovine corpus luteum

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ABSTRACT

Data from various studies indicate that the ovarian function in dairy cows can be compromised during intramammary infections. Therefore, in this study, we investigated if an experimentally induced mastitis has an effect on corpus luteum (CL) function in 14 lactating cows. On d 9 of the estrous cycle (d 1 = ovulation), cows received a single dose of 200 µg of *Escherichia coli* lipopolysaccharide (LPS; dissolved in 10 mL of NaCl; n = 8) or 10 mL of saline (control; n = 6) into one quarter of the mammary gland. Measurements included plasma cortisol, haptoglobin, and progesterone (P₄) concentrations, as well as luteal size (LTA) and relative luteal blood flow (rLBF). Sampling was performed on d 1, 4, and 8. On d 9, the main examination day, sampling was performed immediately before (0 h), every 1 h (or at 3-h intervals for LTA and rLBF) until 9 h, as well as 12 and 24 h after treatment. Thereafter, measurements were taken on d 12, 15, 18, and then every 2 d until ovulation. Luteal tissue was collected for biopsy 24 h before and 6 h after treatment. Quantitative real-time PCR was applied to assess mRNA expression of steroidogenic factors (*STAR*, *HSD3B*), caspase 3, toll-like receptors (*TLR2*, *-4*), tumor necrosis factor α (*TNFA*), and prostaglandin-related factors (*PGES*, *PGFS*, *PTGFR*). Intramammary LPS infusion caused considerable inflammatory responses in the treated udder quarters. No decrease in plasma P₄ concentrations was noted after LPS-challenge, and P₄ levels did not differ between LPS-treated and control cows. Furthermore, LTA and rLBF values were not decreased after LPS challenge compared with the values obtained immediately before treatment. However, LPS infusion increased plasma levels of cortisol and haptoglobin compared with the control group. In the CL, mRNA

abundance of *TLR2* and *TNFA* was increased in cows after LPS-challenge (but not in control cows), whereas *TLR4*, steroidogenic, and prostaglandin-related factors remained similar to the mRNA abundance before treatment. In conclusion, intramammary LPS challenge induces systemic inflammatory reactions which alter the luteal mRNA abundance of *TLR2* and *TNFA* but does not induce lysis of the CL.

Key words: corpus luteum, mastitis, inflammation, endotoxin, cow

INTRODUCTION

For over half a century pregnancy rates in dairy cattle have been decreasing, mainly due to increased embryonic loss (Walsh et al., 2011). The causes of embryonic death are multifarious and the underlying mechanisms are only partly understood. One hypothesis was that inflammatory processes that occur even outside the reproductive tract interfere with embryonic survival and can reduce the fertility of dairy cows.

Mastitis, an inflammation of the mammary gland that often results from bacterial infection, is regarded as the most prevalent disease in dairy cattle (Lavon et al., 2008). Apart from local inflammation of the mammary tissue, mastitis exerts varied systemic effects (Bannerman et al., 2004). Moreover, deterioration of reproductive performance is observed in cows with clinical as well as subclinical mastitis (Pinedo et al., 2009; Lavon et al., 2011a; Roth et al., 2013).

Gram-negative bacteria are responsible for approximately 40% of all clinical cases of bovine mastitis (Schukken et al., 2012). Clinical mastitis due to gram-negative bacteria has a more detrimental effect on the probability of conception compared with infections caused by other pathogens (Hertl et al., 2010). Among the gram-negative bacteria, *Escherichia coli* is the most common cause of bovine clinical mastitis (Wilson et al., 1997). This mastitis can be simulated by the intramammary administration of the *E. coli* endotoxin LPS, which exhibits a dose-dependent inflammatory

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response in the treated quarters of cows (Bruckmaier et al., 1993; Wellnitz et al., 2011, 2015).

Although the exact mechanisms by which intramammary LPS reduces fertility are not clear, LPS-induced cytokines probably play a crucial role. Their effects can be exerted at the level of hypothalamic-pituitary axis, ovary, reproductive tract, or embryo (Hansen et al., 2004). The cytokine IFN- α is able to inhibit the release of the LH from the pituitary (Barros et al., 1992), which leads to anovulation or delayed ovulation in cows. Consistently, the intravenous or intramammary administration of LPS delays ovulation due to decreased LH surges (Lavon et al., 2008). Other cytokines that are enhanced due to mastitis can exert direct effects on the ovary. For instance, IL-6 inhibits estradiol secretion from granulosa cells of the bovine follicle (Alpizar and Spicer, 1994), and tumor necrosis factor (TNF)- α and IFN- γ have a toxic effect on bovine luteal cells in vitro (Petroff et al., 2001). Negative effects of mastitis on corpus luteum (CL) function may also be mediated by increased endometrial synthesis of luteolytic PGF $_{2\alpha}$ due to elevated blood levels of TNF- α (Skarzynski et al., 2000).

As maintenance of morphological and functional integrity of the CL in cows with inflammatory diseases is an essential precondition for embryonic survival, a recent study (Herzog et al., 2012) investigated the effects of an intravenous treatment with *E. coli* LPS on luteal structure and function using ultrasonography, hormone and gene expression analyses. Both structure and function of the bovine CL were transiently suppressed after LPS challenge. Only a small number of studies have examined the direct effects of inflammation of the mammary gland on the bovine CL. The current study investigates the possible alterations in luteal size, secretory function, and gene expression following LPS-induced clinical mastitis.

MATERIALS AND METHODS

Cattle

Sixteen lactating Red Holstein ($n = 10$), Holstein Friesian ($n = 3$), and Swiss Fleckvieh ($n = 3$) cows were enrolled for the purposes of this study. These cows were (mean $\pm$ SEM) 5.1 ± 0.5 yr old, with a BW of 657.6 ± 18.5 kg, and a BCS of 3.0 (median; scale, 1–5). The cows were in mo 2 to 7 (4.2 ± 0.4) of lactation 1 to 7 (3.3 ± 0.5), and were milked twice daily with a bucket milking unit. All cows were clinically healthy and none of the udders showed clinical signs of mastitis. During the experiments, the cows were housed in a tiestall barn and fed high-quality hay, grass, and concentrates with ad libitum access to water. The experimental proce-

dures complied with the Swiss Federal Law on Animal Protection and were approved by the Committee of Animal Experiments of the Canton Fribourg, Switzerland.

Study Design

A modified ovulation synchronization protocol (Ovsynch) was initiated after normal cyclic activity had been confirmed by ultrasonography in all cows. The protocol consisted of 10 μ g of buserelin (GnRH analog; Receptal, MSD Animal Health GmbH, Luzern, Switzerland), 15 mg of luproliol (PGF $_{2\alpha}$ analog; Pro-solvin, Virbac AG, Glattbrugg, Switzerland) 7 d later, and finally 10 μ g of buserelin 60 h after PGF $_{2\alpha}$ (all treatments given i.m.). The time of the second GnRH application was defined as estrus (d 0). Ovulation, which occurred in all cows within 36 h after the second GnRH treatment, was defined as d 1 h 0.

On d 0, 1, 4, and 8, clinical examination (data not shown), blood sampling, and ultrasonography were performed to verify health and luteal development in cows. Clinical examination included measurements of rectal temperature, respiratory and cardiac frequencies, palpation of the udder, as well as visual examination of conjunctiva and episcleral vessels. An intravenous catheter was inserted into one jugular vein on d 8 (removed after 48 h) to facilitate blood sampling during the main examination day (d 9).

After the morning milking on d 9, cows were randomly allocated to receive either a single intramammary treatment with 200 μ g of *E. coli* LPS (serotype O26:B6; Sigma Aldrich, Buchs, Switzerland), diluted in 10 mL of saline solution, or with 10 mL saline only (control). Within LPS-treated cows, another quarter was infused with 10 mL of saline (control quarter). Immediately before treatments, the teat tips were carefully disinfected with 70% ethanol. The treated quarters of the mammary gland were randomly chosen (front or rear, left or right) and equally distributed. Clinical examination and blood sampling was performed immediately before (0 h), hourly until 9 h (11 h for clinical examination), and 12 and 24 h after the administration of LPS or saline. Ultrasonographic measurements were performed 0, 3, 6, 9, 12, and 24 h after treatment, and luteal tissue was collected for biopsy 24 h before and 6 h after treatment. Within LPS-treated cows, foremilk samples (approximately 10 mL) were collected by hand from LPS-challenged and control quarters hourly between 0 and 2 h, and every 15 min between 2 and 5 h after treatment. Milk SCC was measured using a cell counter (DCC; DeLaval, Tumba, Sweden) immediately after collection. Starting on d 12, clinical examination, blood sampling, and ultrasonography were performed every 3

d, and from d 18 onwards, every 2 d until ovulation had occurred (or until d 30 if no ovulation was observed).

Collection of Blood and CL Biopsy Samples

Blood samples were collected from the jugular vein (d 8 to 10) or from the coccygeal blood vessels (all other times) into evacuated tubes containing EDTA as anticoagulant (Vacuette 9 mL K3EDTA; Greiner Bio-One, Kremsmünster, Austria). Tubes were immediately stored on ice and plasma was separated by centrifugation ($3,000 \times g$ for 15 min at 4°C), and frozen at -20°C until the analyses.

Prior to the collection of CL biopsy samples, caudal epidural anesthesia was provided to the cows by incorporating 50 mg of lidocaine hydrochloride (Lidocain 2% Streuli; Streuli Pharma AG, Uznach, Switzerland) to reduce rectal contractions. Two samples (approximately $15 \times 1 \times 1$ mm each) were obtained from the maximum diameter of the CL (including cells from the periphery and the center), using a semiautomatic, high-speed biopsy needle (TEMNO Evolution; Fa. Walter, Baruth/Mark, Germany) that was rendered free of RNase (RNase-ExitusPlus; AppliChem, Darmstadt, Germany). For visual control, a portable ultrasound device (GE Logiq e Premium BT11; General Electric Medical System, Solingen, Germany), equipped with a 7.5-MHz convex transducer was used. The biopsy needle and the ultrasound transducer were guided transvaginally using a bearing system (type Hannover) and the CL was placed in front of the vaginal fornix by transrectal manual assistance. Tissue samples were immediately placed in sterile DNase- and RNase-free cryo tubes (Fa. Brand, Wertheim, Germany), frozen in liquid nitrogen, and stored at -80°C until expression analysis. This method allowed repeated biopsy sampling from a single CL without impairing its subsequent function (Tsai et al., 2001; Lüttgenau et al., 2011b; Atli et al., 2012).

Ultrasonography

Transrectal ultrasonographic examinations of the ovaries were performed using a portable ultrasound device (GE Logiq e Premium BT11), equipped with a 4.0- to 12.0-MHz, linear-array transducer. An exhaustive description of this approach was given recently (Lüttgenau et al., 2011a,b). In brief, 3 cross-sectional images with maximal areas were frozen and recorded for every detectable CL (using B-mode ultrasonography). The total area of the CL was measured using computer-assisted image analysis software (PixelFlux Version 1.0; Chameleon Software, Leipzig, Germany).

When a cavity was present within a CL, the cavity area was assessed separately and subtracted from the total area of the CL. The difference between total area of the CL and its cavity was defined as area of luteal tissue (**LTA**). For each CL, mean values of the measurements of the 3 recorded images were used for data analysis.

The second part of the ultrasonographic examination used Power Mode ultrasonography to assess luteal blood flow (**LBF**). The transducer was positioned at the maximal diameter of the CL, and small adjustments in location (relative to the ovary) were made to achieve a maximal number of color pixels in the luteal parenchyma before freezing and recording the image. The number of pixels with color was determined (PixelFlux software) as a semiquantitative assessment of LBF. For each CL, 3 images were recorded, and the average number of pixels was used for further analysis. To calculate the relative LBF (**rLBF**), LBF was divided by LTA.

All ultrasonographic examinations (except from those before and during the Ovsynch protocol) were performed by the same investigator, and the intraclass correlation coefficients of LTA and LBF measurements were 1.00 and 0.99, respectively.

Plasma Concentration Measurements

Plasma concentrations of cortisol and haptoglobin were determined with a RIA according to Thun et al. (1981) and with a commercial ELISA (E-10HPT Bovine Haptoglobin ELISA kit; Immunology Consultants Laboratory, Portland, OR), respectively. Intra- and interassay coefficients of variation were 8.5 and 9.1%, respectively, for cortisol, and $<10\%$ for both assays for haptoglobin.

An RIA (RIA kit IM1188; Beckman Coulter GmbH, Krefeld, Germany) was used to measure plasma progesterone (**P₄**) concentrations, and intra- and interassay coefficients of variation were ≤ 6.5 and $\leq 7.2\%$, respectively.

Plasma concentrations of prostaglandin E_2 (**PGE₂**) and PGF_{2 α} metabolites (**PGFM**; 13,14-dihydro-15-keto-PGF_{2 α}) were determined in blood samples collected immediately before treatment, as well as 1 and 2 h (only for PGFM) and 3, 6, 9, 12, and 24 h after treatment with a commercial enzyme-immunoassay (PGE₂ High Sensitivity EIA kit; Enzo Life Sciences AG, Lausen, Switzerland) and with an enzyme-immunoassay according to Mishra et al. (2003), respectively. Commercial PGFM (Sigma Aldrich) was used for the standard curve, and PGFM-horseradish peroxidase conjugate and antiserum were kindly provided by the Department of Physiology in Weihenstephan (Technical University Munich, Germany). Intra- and interassay

say coefficients of variation were ≤ 9.8 and $\leq 12.6\%$ for PGE₂, and ≤ 7.5 and $\leq 12.7\%$ for PGFM, respectively.

RNA Extraction and Quantitative Real-Time PCR

Luteal mRNA expression was determined for steroidogenic acute regulatory protein (*STAR*), 3 β -hydroxysteroid dehydrogenase (*HSD3B*), toll-like receptor (*TLR*)-2 and -4, *TNFA*, caspase 3 (*CASP3*), PGE (*PGES/PTGES*) and PGF synthases (*PGFS/AKR1B1*), and PGF receptor (*PTGFR/FP*). Total RNA from luteal tissue samples was extracted using the miRNeasy Mini Kit (Qiagen, Hilden, Germany). Homogenization of the tissues was achieved with the Qiagen TissueLyser II and 5-mm stainless steel beads (Qiagen). The RNA concentration and integrity were quantified using the NanoDrop 2000 (peqLab, Erlangen, Germany) and the Bioanalyzer 2100 (Agilent Technologies, Waldbronn, Germany), respectively. The RNA integrity numbers ranged from 6.7 to 10 (average 8.3). Five hundred nanograms of RNA were reverse transcribed using the M-MLV Reverse Transcriptase, RNase H Minus, Point Mutant (Promega, Madison, WI), as recently described (Pistek et al., 2013).

Luteal mRNA expression was determined in a 2-step quantitative real-time PCR (qPCR) using the CFX384 Real-Time PCR Detection System (Bio-Rad, Munich, Germany) and the SsoFast EvaGreen Supermix (Bio-Rad). The qPCR was performed in a reaction volume of 10 μ L, consisting of 5 μ L of EvaGreen Mix, 0.4 μ L of each primer (10 μ M), 0.07 μ L of VisiBlue (TATAA Bio-center, Göteborg, Sweden), 3.13 μ L of water, and 1 μ L of cDNA. The primers used to amplify specific fragments referring to selected regulated genes are shown on Table 1. The annealing temperature and the melting points are outlined. The cycle number (C_q) required to achieve a definite SYBR Green fluorescence signal was calculated by the regression method (Bio-Rad CFX Manager 3.1). The C_q was inversely correlated with the logarithm of the initial template concentration. The C_q determined for the target genes were normalized against the geometrical mean of the 5 reference genes *YWHAZ*, *H3F3A*, *CNOT11*, *SUZ12*, and *TBP* (ΔC_q). The ΔC_q was normalized to its mean for all cows on the day before treatment ($\Delta \Delta C_q$). This was done to reduce the intersubject variance due to different baseline expression, which is a major confounding factor in qPCR experiments (Tichopad et al., 2009). To avoid negative digits, while allowing the estimation of a comparison between 2 genes, data were presented as means $\pm$ standard error of the mean added to the arbitrary value 20 ($\Delta \Delta C_q$). Thus, a high $\Delta \Delta C_q$ proportionally resembled high transcript abundance (Livak and Schmittgen, 2001).

Statistical Analyses

Two cows (both from the control group) that ovulated or had mastitis before treatment, were excluded from the study; thus, data from 14 cows were eligible for analyses. One of the 14 included cows (also from the control group) showed mastitis of an untreated quarter on d 21 (3 d before the next ovulation); the measurements of this cow taken between recognition of mastitis and ovulation were omitted from the analysis.

Time between induced (d 1) and subsequent ovulations varied among cows. To facilitate comparability, data were normalized to an average cycle length. Therefore, d 0 to 15 of the cycle were maintained, and d 4, 2, and 0 before the subsequent ovulation were used to characterize the remaining cycle. Examination days between d 15 and 4 before the subsequent ovulation were excluded from the analysis. In 3 cows without an ovulation until d 30 (all from the LPS group), the day of ovulation was defined as 2 d after first occurrence of plasma P₄ ≤ 1 ng/mL (based on the time interval between P₄ ≤ 1 ng/mL and ovulation that was measured for the remaining cows of the study).

Analysis of PGFM had a lower detection limit of 25 pg/mL. For measurements below this limit, 24 pg/mL was used as an arbitrary value to facilitate statistical analysis. Values of prostaglandin analyses were normalized to their mean on the day before treatment.

Statistical analysis was conducted using SAS V9.3 (SAS Institute Inc., Cary, NC). The distribution of the data was tested for normality visually (PROC CHART) and by means of the Shapiro-Wilk-test (PROC UNIVARIATE). For non-normal data of quantitative variables a Cochran–Mantel–Haenszel test based on rank scores was applied as equivalent to Friedman's 2-way ANOVA for repeated measures (PROC FREQ). Significant results were further evaluated with Wilcoxon's 2-sample test (PROC NPAR1WAY) for independent pairwise comparisons (between groups), and with Wilcoxon's signed rank test (PROC UNIVARIATE) for dependent pairwise comparisons (within group). For normal data of quantitative variables a repeated measures ANOVA (PROC GLM) was performed. Significant results were further evaluated using a 2-sample *t*-test (PROC TTEST) or a Student's *t*-test (PROC UNIVARIATE) for pairwise comparisons between independent and dependent groups, respectively. Differences in categorical variables were evaluated using Fisher's exact test (PROC FREQ). Intraclass correlations for intrarater reliability were calculated by means of the INTRACC macro of SAS (PROC GLM). Data are presented as mean $\pm$ standard error of the mean or median, depending on the distribution of the data, and differences were considered significant at $P \leq 0.05$.

Table 1. Sequences and accession numbers of PCR primers for assayed genes from bovine corpus luteum cells, and length, annealing (AT) and melting point (MP) temperatures of PCR products

Gene	Gene symbol	Reference (accession no.)	Forward primer (5'-3')	Reverse primer (5'-3')	PCR product (bp)	AT (°C)	MP (°C)
Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta	<i>YWHAZ</i>	NM_174814.2	AGGCTGAGCGATATGATGAC	GACCCTCCAAGATGACCTAC	141	60	81
Histone	<i>H3F3A</i>	NM_001014389.2	ACTGGCTACAAAAGCCGCTC	ACTTGCCTCCTGCAAAGCAC	233	60	85
CCR4-NOT transcription complex, subunit 11	<i>CNOT11</i>	XM_582695.6	TCAGTGGACCAAAGCCACCTA	CTCCACACCGGTGCTGTTCT	170	60	87
Suppressor of zeste 12 homolog (Drosophila)	<i>SUZ12</i>	NM_001205587.1	CATCCAAAAGGTGCTAGGATAGATG	TGGGCCTGCACACAAGAATG	160	60	82
TATA box binding protein	<i>TBP</i>	NM_001075742.1	CAGAGAGCTCCGGGATCGT	CACCATCTTCCCAGAACTGAATAT	194	60	83
Steroidogenic acute regulatory protein	<i>STAR</i>	NM_174189.2	GGATTAACCAGGTTCCGGCG	CTCTCCTTCTTCCAGCCCTC	157	60	89
3-β-hydroxysteroid dehydrogenase	<i>HSD3B</i>	NM_174343.3	TACCCAGCTGCTGTTGGA	ATGCCGTTGTTATTCAAGGC	322	60	87
Caspase 3	<i>CASP3</i>	NM_001077840.1	AACCTCCGTGGATTCAAAATC	TTCAGGRTAATCCATTTTGTAAC ¹	114	60	80
Toll-like receptor 2	<i>TLR2</i>	NM_174197.2	CCATGTGGAGAGGGTGTT	GGGGACACAAAACAGCACTT	138	60	81
Toll-like receptor 4	<i>TLR4</i>	NM_174198.6	GACCCTTGCGTACAGGTTGT	GGTCCAGCATCTTGGTTGAT	103	60	80
Tumor necrosis factor α	<i>TNFA</i>	NM_173966.3	CCACGTTGTAGCCGACATC	ACCACCAGCTGGTTGTCTTC	108	60	86
Prostaglandin E synthase	<i>PGES</i> (<i>PTGES</i>)	NM_174443.2	TCCTGGTCTTCTTCCTGGG	CCCAGACAATCTGCAGGG	132	60	88
Prostaglandin F synthase	<i>PGFS</i> (<i>AKR1B1</i>)	NM_001012519.1	ATACAAGCCGGCGGTTAAC	TGTCTGCAATCGCTTTGATC	188	60	86
Prostaglandin F receptor	<i>PTGFR</i> (<i>FP</i>)	NM_181025.3	AGCCTTGCCATTGCTATCC	TAGTTCCATTGATGAGGTGCC	127	60	80

¹Degenerate multispecies primer, R = A or G.

RESULTS

Cows

Breed, age, BW and BCS, lactation number, month of lactation, and estrous cycle length did not differ between LPS ($n = 8$) and control groups ($n = 6$). The proportions of Red Holstein, Holstein Friesian, and Swiss Fleckvieh cows were 50.0, 25.0, and 25.0%, and 66.7, 16.7, and 16.7% in LPS and control groups, respectively. The mean ages were 4.6 ± 0.9 and 5.1 ± 0.7 yr, the BW were 618.8 ± 27.8 and 703.5 ± 20.8 kg, and the median BCS were 2.9 and 3.1 in LPS and control groups, respectively. The cows of the LPS and the control group had mean lactation numbers of 2.8 ± 0.8 and 3.2 ± 0.7 , were in mo 4.8 ± 0.8 and 3.7 ± 0.4 of lactation, and had an estrous cycle length of 21.8 ± 0.5 ($n = 5$) and 22.0 ± 0.9 d ($n = 6$), respectively. Three cows from the LPS group did not ovulate until d 30, although P_4 concentrations ≤ 1 ng/mL were observed between d 18 and 26.

Mastitis

Two hours after the intramammary infusion with LPS, cows seemed to perceive pain of the treated quarter and kicked with the hind legs toward the udder. The treated quarter was warm, slightly red, and massively swollen.

Somatic cell counts in milk from LPS-challenged quarters increased ($P = 0.005$) from $77 \pm 24 \times 1,000$ cells/mL at 0 h to $390 \pm 116 \times 1,000$ cells/mL at 2 h 45 min after treatment, and increased further until the end of the examination period ($1,149 \pm 191 \times 1,000$ cells/mL). No significant changes of SCC were observed in control quarters. Exhaustive data for SCC and a detailed description of further changes during mastitis in cows of the present study have been reported recently (Wellnitz et al., 2015).

Vital Parameters and Clinical Findings

Before LPS challenge, rectal temperature and cardiac and respiratory frequencies were in physiological ranges and did not differ between cows of the LPS and the control group (Figure 1). After LPS treatment, rectal temperature increased significantly in all cows at 3 h and peaked at 6 h (range = 41.1 – 42.3°C) before it decreased to initial values (Figure 1A). Cardiac and respiratory frequencies increased significantly to peak values 7 h after LPS challenge and returned thereafter to initial values (Figure 1B and C). Rectal temperature and cardiac and respiratory frequencies were higher ($P \leq 0.05$) at 2 to 9, 4 to 8, and 6 to 9 h after treatment in cows of the LPS compared with the control group.

Figure 1. Changes (means $\pm$ SEM) in rectal temperature (A), cardiac frequency (B), and respiratory frequency (C) of cows during the first 24 h after intramammary treatment with either LPS ($n = 8$) or saline (control; $n = 6$). An asterisk (*) represents the difference between the 2 groups ($P \leq 0.05$) at times indicated.

Two hours after intramammary treatment with LPS, cows stopped eating, and during transrectal examinations the rumen appeared to be distended compared

Figure 2. Changes (means $\pm$ SEM) in plasma concentrations of cortisol (A, B) and haptoglobin (C, D) of cows during the whole estrous cycle (A, C) and during the first 24 h (B, D, enlargements of shaded areas in A, C) after intramammary treatment with either LPS ($n = 8$) or saline (control; $n = 6$). An asterisk (*) represents the difference between the 2 groups ($P \leq 0.05$) at times indicated. Day 1 and Ov = ovulation.

with that of control cows. From 3 to 12 h after LPS challenge, conjunctiva was reddened in 6 out of 8 cows and episcleral vessels were injected (but not blurred) in 3 out of 8 cows, whereas conjunctiva and episcleral vessels remained unchanged in all cows of the control group.

Plasma Concentrations of Cortisol and Haptoglobin

Plasma cortisol and haptoglobin concentrations did not differ between cows of the LPS and the control group before treatment (Figure 2A and C). After LPS challenge and in comparison to the values before infusion, cortisol levels increased significantly between 1 and 4 h and returned to initial values at 24 h (Figure 2A and B). Haptoglobin levels increased significantly from 2 to 72 h after LPS treatment and returned to

initial values thereafter (Figure 2C and D). Cortisol and haptoglobin concentrations were higher ($P \leq 0.02$) in LPS-treated compared with control cows between 2 and 12 h and 2 and 72 h after treatment, respectively.

Plasma Concentrations of Progesterone and Luteal Size and Blood Flow

Progesterone concentrations did not differ between LPS and control groups during the whole estrous cycle (Figure 3A). However, within the LPS-treated cows a significant increase was noted in plasma P_4 levels between 2 and 4 h after the challenge, and a significant decrease between 4 and 6 h (Figure 3B).

The LTA in the LPS-treated cows was smaller 24 h before ($P = 0.05$) and 12 h after ($P = 0.03$) the challenge compared with the LTA of control cows (Figure

Figure 3. Changes (means $\pm$ SEM) in plasma progesterone concentrations (A, B), the area of luteal tissue (C, D), and the relative area of colored pixels expressing luteal blood flow (E, F) of cows during the whole estrous cycle (A, C, E) and during the first 24 h (B, D, F, enlargements of shaded areas in A, C, E) after intramammary treatment with either LPS ($n = 8$) or saline (control; $n = 6$). An asterisk (*) represents the difference between the 2 groups ($P \leq 0.05$) at times indicated. Day 1 and Ov = ovulation.

3C and D). During the first 24 h after treatment, the LTA remained constant within the LPS group (Figure 3D).

Relative LBF did not differ between the LPS and the control group before treatment (Figure 3E), but was higher ($P = 0.05$) in LPS-treated cows 6 h after

challenge (Figure 3F). Within the LPS group, rLBF increased significantly from 0 to 6 h and returned thereafter to initial values.

Plasma Concentrations of Prostaglandin E_2 and Prostaglandin $F_{2\alpha}$ Metabolites

Plasma concentrations of PGE_2 and PGFM did not differ between LPS and control groups during the first 24 h after treatment (Figure 4A, B). In this period, PGE_2 and PGFM concentrations remained very stable in the LPS group. However, they decreased during the first 6 h after treatment and increased during the subsequent 6 h in control cows, returning to initial values at 24 h.

Luteal mRNA Expression

Changes in mRNA expression of all investigated parameters are shown in Figure 5. No difference regarding the luteal mRNA expression of steroidogenic factors *STAR* and *HSD3B* was noted between the 2 groups 24 h before and 6 h after treatment, nor within each group and between the 2 times of analysis (Figure 5A). Expression of apoptotic factor *CASP3* was higher 6 h after treatment compared with 24 h before treatment within both the LPS ($P = 0.004$) and the control ($P = 0.03$) group, but did not differ between groups at any respective time (Figure 5A).

Expression of *TLR2* mRNA was higher ($P = 0.005$) in the LPS group 6 h after compared with 24 h before treatment and revealed a higher ($P < 0.0001$) mRNA abundance after treatment in the LPS compared with the control group (Figure 5B). In contrast, expression of *TLR4* neither differed between groups at any time nor changed after treatment within groups (Figure 5B). Lipopolysaccharide treatment increased ($P = 0.0005$) proinflammatory cytokine *TNFA*, but no difference was observed between groups at any respective time (Figure 5B).

Luteal mRNA expression of *PGES* and *PGFS* differed neither between groups nor within groups and successive time points (Figure 5C). Although there was no difference in the expressions of *PTGFR* mRNA between 24 h before and 6 h after treatment in either LPS or control groups, the mRNA abundance was higher ($P = 0.03$) in the LPS compared with the control group 6 h after treatment (Figure 5C).

DISCUSSION

To the best of our knowledge, this is the first detailed description regarding the effects of an experimentally induced mastitis via *E. coli* LPS on the development

Figure 4. Changes (means $\pm$ SEM) in plasma concentrations of prostaglandin E_2 (PGE_2 ; A) and $PGF_{2\alpha}$ metabolites (PGFM; B) of cows during the first 24 h after intramammary treatment with either LPS ($n = 8$) or saline (control; $n = 6$). No significant difference between the 2 groups was noted at the times indicated.

and function of the bovine CL, including analyses of luteal blood flow and gene expression.

In all cows intramammary LPS challenge induced a clinical mastitis with obvious clinical symptoms within 2 h after treatment. Furthermore, the significant increase in milk SCC at 2 h 45 min indicated the presence of the innate mammary immune response (Wellnitz and Bruckmaier, 2012; Wellnitz et al., 2015). The considerable elevation of SCC was expected because we used a relatively high dosage of LPS (Baumert et al., 2009). The increase in SCC roughly coincided with the increase in rectal temperature, suggesting an association between the local infiltration of PMNL and the release of pyrogenic cytokines.

Plasma P_4 concentrations increased significantly between 2 and 4 h after intramammary LPS challenge, showing consistency with the elevated P_4 levels noticed

A) Steroidogenic factors and apoptosis-related enzyme

B) Receptors for LPS and proinflammatory cytokine

C) Prostaglandin-related factors

Figure 5. Luteal mRNA expressions (means $\pm$ SEM) of steroidogenic acute regulatory protein (*STAR*), 3 β -hydroxysteroid dehydrogenase (*HSD3B*), caspase3 (*CASP3*), toll-like receptor (*TLR*)2, *TLR*4, tumor necrosis factor α (*TNFA*), prostaglandinE synthase (*PGES*), *PGFS*, and prostaglandinF receptor (*PTGFR*) at 24h before and 6h after an intramammary treatment of cows with either LPS (black columns; n = 8) or saline (control; white columns; n = 6). Different letters (a,b) represent a difference between times ($P \leq 0.05$) within groups indicated; an asterisk (*) represents a difference between groups ($P \leq 0.05$) at times indicated. $\Delta\Delta Cq$ = mRNA expression (PCR cycle of quantification) normalized against reference genes and the baseline expression before the treatment.

within 0.5 (Herzog et al., 2012) and 4 h (Gilbert et al., 1990) after intravenous LPS treatment. We suggest that the transient increase in P_4 was of adrenal origin because a similar P_4 pattern was induced by LPS in ovariectomized heifers (Kujjo et al., 1995). Peak P_4 and cortisol concentrations were coincident 4 h after LPS challenge in the present study. The temporary increase in both P_4 and cortisol concentrations might be a result of a central activation of the neuroendocrine stress axis induced by the LPS (Suzuki et al., 2001). However, the reduced size of the CL 12 h after infusion in the LPS-treated compared with the control cows was probably not induced by the endotoxin because luteal size was already smaller before treatment. The different sizes of the CL between groups were possibly due to different times of ovulation (within the 36 h between the second GnRH treatment and the defined time of ovulation). In contrast, the size-corrected blood flow of the CL did not differ between LPS and control groups before treatment, but increased within 6 h after LPS challenge. The peak value of rLBF in the LPS group was temporally coincident with the peak values of cardiac frequencies, consistent with organ perfusion depending on cardiac frequency and output (Bose et al., 2015).

In previous studies, the transient increase in plasma P_4 concentrations after intravenous (Herzog et al., 2012) or intrauterine (Williams et al., 2008) treatments with LPS was followed by a decrease in plasma P_4 due to reduced luteal P_4 secretion. Progesterone concentration is correlated with LTA and LBF (Herzog et al., 2010; Lüttgenau et al., 2011b). Thus, consistency exists between the study of Herzog et al. (2012) reporting a depression in P_4 , LTA, and LBF after intravenous LPS treatment, and our study reporting no depression of any of these parameters. Maintenance of luteal size and function after intramammary LPS treatment compared with the partial luteolysis noticed after intravenous treatment might be due to the LPS being limited to the mammary gland, thus inducing lower concentrations of luteolytic $PGF_{2\alpha}$. Our assumption was confirmed by the absence of any increase in plasma PGFM levels after the intramammary LPS challenge in the present study. Consistently, endotoxin caused elevated systemic PGFM concentrations when administered intravenously (Gilbert et al., 1990; Jackson et al., 1990; Herzog et al., 2012), but not when administered into the mammary gland (Jackson et al., 1990). In contrast, cows with natural cases of clinical mastitis (mainly due to gram-negative pathogens) were twice as likely to have altered interestrus intervals (Moore et al., 1991) and had a significantly higher rate of premature luteolysis (Huszenicza et al., 2005) compared with healthy cows. We assume that the prolonged LPS release from gram-negative bacteria in natural cases of mastitis stimulates

a sufficient $PGF_{2\alpha}$ output of the endometrium to induce luteal regression. In the present study, no difference in plasma PGE_2 concentrations after intramammary LPS challenge was noted compared with control cows. As PGE_2 is the most potent luteotropic and antiluteolytic factor in the bovine CL (Rekawiecki et al., 2005; Skarzynski et al., 2007), this result is consistent with the fact that no difference was noticed between the 2 groups in luteal morphology and function.

Luteal *TNFA* mRNA was significantly more abundant following intramammary LPS challenge compared with the control group. The increased concentrations of circulating TNF- α during mastitis may have originated from the mammary gland or could have been produced in response to signals from the mammary gland (Hansen et al., 2004). Production of TNF- α by the bovine CL is of particular interest because TNF- α can exert a direct cytotoxic effect on luteal cells (Petroff et al., 2001).

Intramammary application of LPS induced a tremendous increase in luteal mRNA expression of *TLR2* but had no effect on *TLR4* mRNA. At first sight this result seems contradictory because LPS of *E. coli* and other gram-negative bacteria is recognized by *TLR4* (Poltorak et al., 1998), whereas *TLR2* binds peptidoglycan and lipoteichoic acid from gram-positive bacteria (Takeda and Akira, 2005). However, previous studies (Hirvonen et al., 1999; Hoebe et al., 2000) provide evidence that LPS is not directly released from the mammary gland into the general circulation. Still, it is the proinflammatory cytokines such as TNF- α that enter the circulation and cause systemic effects. Studies in mice (Matsumura et al., 2000) and humans (Davanian et al., 2012) have clearly demonstrated that TNF- α increase *TLR2* mRNA without altering mRNA expression of *TLR4*. Therefore, we suggest that LPS-induced TNF- α contributes to the observed upregulation of luteal *TLR2* mRNA.

In the present study, luteal mRNA expressions of steroidogenic and prostaglandin-related factors were not affected by intramammary application of LPS. This was consistent with the fact that no differences in luteal P_4 production and plasma PGFM concentrations were noticed. In contrast, cows with increased PGFM and reduced P_4 levels due to intravenous LPS challenge had decreased mRNA expressions of *STAR* (Herzog et al., 2012) and *HSD3B* (J. Lüttgenau, unpublished data). Likewise, an increased luteal expression of prostaglandin synthases and a reduction in *PTGFR* mRNA and *STAR* and *HSD3B* activities were associated with an exposure to increased amounts of $PGF_{2\alpha}$ (Shirasuna et al., 2010; Atli et al., 2012).

Luteal mRNA expression of *CASP3* was increased after treatment in both LPS and control groups. As

CASP3 is an important mediator of apoptosis, which is activated by $\text{PGF}_{2\alpha}$ during luteal regression (Stocco et al., 2007), it can be suggested that the physical insult of the CL due to the biopsy can induce luteal $\text{PGF}_{2\alpha}$ release and apoptosis. However, it was clearly demonstrated that a sequential collection of luteal tissue for biopsy alone does not induce luteal regression (Tsai et al., 2001; Atli et al., 2012). Furthermore, the remaining results from mRNA expression analysis in the present study do not support the assumption of apoptosis due to luteal regression.

Apart from the local inflammation of the mammary gland, intramammary LPS treatment induced severe systemic symptoms, including pyrexia, tachycardia, and tachypnea. Likewise, an acute phase response, characterized by clinical disturbances such as pyrexia and tachycardia, was induced by IMI with *E. coli* or treatment with LPS (Bannerman et al., 2004; Hertl et al., 2010; Lavon et al., 2011c). In the present study, peak of body temperature was observed 6 h after LPS challenge. The same elevation of rectal temperature was noticed after IMI with *E. coli* and was coincident with the maximum concentrations of proinflammatory cytokine $\text{TNF-}\alpha$ (Ma et al., 2011). Following intravenous LPS treatment, 3 out of 7 cows had increased body temperature, but all cows showed tachycardia and tachypnea (Herzog et al., 2012). The higher percentage of cows with pyrexia (100 vs. 43%) and the higher peak temperatures (41.1 to 42.3 vs. 39.5 to 40.4°C) after the intramammary compared with the intravenous application of LPS was unexpected. However, the infections of the mammary gland have been linked particularly with an abundant release of inflammatory mediators and the stimulation of pyrexia (Hertl et al., 2010).

Furthermore, cortisol concentrations were increased after LPS challenge, consistent with previous reports on cows with clinical mastitis (Hockett et al., 2000) and on cows with intramammary (Vernay et al., 2012) and intrauterine (Peter et al., 1989) infusion of *E. coli* LPS. It is noteworthy that increased cortisol levels in these studies were associated with anovulation. In the present study, 3 out of the 8 cows with intramammary application of LPS during diestrus did not ovulate during the subsequent estrus. Likewise, ovulation was delayed 4 to 14 d in cows with intravenous LPS treatment during proestrus (Suzuki et al., 2001) and diestrus (Herzog et al., 2012), respectively. In cows with clinical (Barker et al., 1998) and subclinical mastitis (Schrick et al., 2001), anovulation and delayed ovulation were explained by increased cortisol concentrations and suppression of the LH surge. Lavon et al. (2010, 2011b,c) suggested that *E. coli* LPS in the mammary gland activates the adrenal system that leads to suppression of pulsatile LH secretion and decreased estradiol concentrations,

and subsequently to delayed LH surge and ovulation. However, in the present study, cortisol was transiently elevated after LPS challenge on d 9 (i.e., when the potential preovulatory follicle was still small, <5 mm). Therefore, a direct effect of cortisol on follicular capacity to ovulate is questionable. It seems more likely that LPS or LPS-induced secretion of inflammatory mediators impair the development of the preovulatory follicle, as even small follicles are susceptible to endotoxins or cytokines. Previous studies have shown that LPS disturbed the estradiol production of immature granulosa cells isolated from small follicles (Shimizu et al., 2012). Therefore, the LPS-induced damage of small follicle function during folliculogenesis was carried over to the preovulatory follicles (Furman et al., 2014).

In contrast to the short-term increase in cortisol concentrations that peaked 4 h after LPS treatment, haptoglobin level increased steadily, reaching its peak 3 d after LPS challenge. Elevated haptoglobin concentrations have been shown to indicate an acute infectious process in dairy cows, and the long-lasting increase was consistent with previous observations in cows with metritis (Huzzey et al., 2009). Therefore, the acute phase response was not only characterized by pyrexia and increased production of inflammatory cytokines, but also by elevated levels of acute phase proteins such as haptoglobin (Uthaisangsook et al., 2002).

In conclusion, LPS-induced mastitis triggers a severe systemic inflammatory response and alters the mRNA abundance of *TLR2* and *TNFA* in the bovine CL. Although intramammary (in contrast to intravenous) LPS challenge was not associated with increased PGFM and did not induce luteolysis, the observed alterations in luteal gene expression may contribute to the reduced fertility in cows with mastitis.

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