Lipopolysaccharide enhances apoptosis of corpus luteum in isolated perfused bovine ovaries in vitro

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Abstract

Lipopolysaccharide (LPS), the endotoxin of Gram-negative bacteria, has detrimental effects on the structure and function of bovine corpus luteum (CL) in vivo. The objective was to investigate whether these effects were mediated directly by LPS or via LPS-induced release of PGF2α. Bovine ovaries with a mid-cycle CL were collected immediately after slaughter and isolated perfused for 240 min. After 60 min of equilibration, LPS (0.5 μg/ml) was added to the medium of five ovaries, whereas an additional six ovaries were not treated with LPS (control). After 210 min of perfusion, all ovaries were treated with 500 iu of hCG. In the effluent perfusate, concentrations of progesterone (P4) and PGF2α were measured every 10 and 30 min, respectively. Punch biopsies of the CL were collected every 60 min and used for RT-qPCR to evaluate mRNA expression of receptors for LPS (TLR2, -4) and LH (LHCR); the cytokine TNFα; steroidogenic (STAR, HSD3B), angiogenic (VEGFA121, FGFR2) and vasoactive (EDN1) factors; and factors of prostaglandin synthesis (PGES, PGFS, PGFR) and apoptosis (CASP3, -8, -9). Treatment with LPS abolished the hCG-induced increase in P4 (P ≤ 0.05); however, there was a tendency (P = 0.10) for increased release of PGF2α at 70 min after LPS challenge. Furthermore, mRNA abundance of TLR2, TNFα, CASP3, CASP8, PGES, PGFS, and VEGFA121 increased (P ≤ 0.05) after LPS treatment, whereas all other factors remained unchanged (P > 0.05). In conclusion, reduced P4 responsiveness to hCG in LPS-treated ovaries in vitro was not due to reduced steroidogenesis, but was attributed to enhanced apoptosis. However, an impact of luteal PGF2α could not be excluded.

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Introduction

Fertility in dairy cows has decreased for more than a half century (Walsh et al. 2011) due, in part, to inflammatory diseases (Barker et al. 1998, LeBlanc et al. 2002). Cows with endometritis have prolonged calving-to-conception intervals (CCI) and are replaced sooner due to reproductive failure (LeBlanc et al. 2002). Furthermore, inflammation in non-genital tissues (e.g. mastitis) also prolongs CCI and increases the number of services per conception (Barker et al. 1998), attributed in part to activation of immune cells by bacterial cell wall components and production of cytokines (Hansen et al. 2004).

In previous studies (Suzuki et al. 2001, Herzog et al. 2012), lipopolysaccharide (LPS), the endotoxin from the outer membrane of Gram-negative bacteria, was used as a model to determine effects of inflammation on fertility in cows. Binding of LPS to toll-like receptor (TLR) 4 (Akira 2003) activates macrophages and triggers cytokine production. Furthermore, instillation of LPS in the mammary gland consistently increases systemic concentrations of cytokine tumor necrosis factor alpha (TNFα; Blum et al. 2000). In addition, exogenous LPS reduced fertility due to impairment of ovarian follicular development and ovulation (Suzuki et al. 2001, Lavon et al. 2008, Williams et al. 2008). The LH peak was significantly retarded or completely inhibited in cows given LPS during proestrus (Suzuki et al. 2001). Furthermore, LPS affected morphology and function of the bovine corpus luteum (CL). In that regard, intravenous LPS decreased blood progesterone (P4) concentrations (Giri et al. 1991, Herzog et al. 2012) and caused temporary reductions in both luteal size and blood flow (Herzog et al. 2012). Since PGF2α metabolism and cytokines during inflammation (Giri et al. 1991, Miyamoto et al. 2000, Skarzynski et al. 2000), it was speculated that enhanced uterine PGF2α induced premature luteolysis. It was noteworthy that TNFα consistently stimulated PGF2α production in endometrial cells during all stages of
the estrous cycle (Miyamoto et al. 2000). However, a more recent study (Mishra & Dhali 2007) suggested that LPS might impair the CL, independent of endometrium-derived PGF<sub>2α</sub>. In that study, which used a luteal monolayer culture, LPS induced oxidative stress and decreased cell viability, although an influence of endometrium-derived PGF<sub>2α</sub> was excluded.

In ruminants, the in vitro model of an isolated perfused ovary (IPO) was introduced several decades ago (Romanoff & Pincus 1962, Mills & Morrisette 1970, Stahler & Huch 1971, Sturm & Stahler 1971, Janson et al. 1978), mainly to investigate ovarian steroid biosynthesis and energy metabolism. For studying mechanisms of LPS action on the bovine CL, with special consideration of the impact of uterine-derived PGF<sub>2α</sub>, this model has important advantages. At first, compared to in vivo studies, the IPO model facilitates determining LPS effects on the CL, excluding any influence of PGF<sub>2α</sub> from extraluteal sources. Therefore, this model should be a good alternative to LPS, which has severe systemic effects on cows (Giri et al. 1990, Giri et al. 1991, Herzog et al. 2011). Secondly, compared to cell cultures, the IPO model enables interactions between various cell types (e.g. luteal, vascular, and stromal cells) of the bovine CL without changing their composition and arrangement, including an intact three-dimensional structure and intercellular communication (Brannstrom & Flaherty 1995). Furthermore, the structural integrity of the CL within the surrounding ovarian tissue was maintained in IPO (Stahler & Huch 1971) compared to tissue culture systems.

Using the IPO technique, determination of P<sub>4</sub> and PGF<sub>2α</sub> in the effluent perfusate enables direct assessment of release of these hormones from the CL. P<sub>4</sub> synthesis is the most important indicator of functional integrity of the CL (Rekawiecki et al. 2008). In that regard, increased P<sub>4</sub> synthesis following LH treatment is evidence of CL responsiveness (Skarzynski et al. 2008). Prostaglandin F<sub>2α</sub> is the most effective mediator of luteolysis in ruminants (McCracken et al. 1999). During physiological luteolysis in cattle, PGF<sub>2α</sub> is released from the endometrium as well as the CL (Shirasuna et al. 2004). Furthermore, gene expressions of steroidogenic, angiogenic, and vasoactive factors in luteal tissue provided relevant information regarding functionality of the bovine CL (Miyamoto et al. 2009, Shirasuna et al. 2010). The proinflammatory cytokine TNFA is present in bovine luteal cells, as well as in immune cells (mainly macrophages; Sakumoto et al. 2011), and is capable of reducing P<sub>4</sub> secretion, increasing PGF<sub>2α</sub> production, and inducing apoptosis in luteal cell cultures (Okuda & Sakumoto 2003, Skarzynski et al. 2005).

In this study, we hypothesized that LPS directly suppresses CL function via enhanced apoptosis. Thus, the isolated perfused ovary model was established, and the impact of LPS challenge using this in vitro system was evaluated in detail.

**Materials and methods**

**Ovaries**

Ovaries with mesovarium were harvested from the carcasses of clinically healthy cows (Bos taurus; including Holstein Friesian, Red Holstein, Swiss Fleckvieh and Brown Swiss) that were slaughtered at a commercial abattoir. Sixteen ovaries containing a CL with an estimated diameter of >20 mm (subsequently confirmed as a mid-cycle CL) and intact tunica albuginea as well as mesovarium with ovarian vessels were used.

**Preparation of ovaries**

Immediately after the ovary was recovered, the ramus uterinus and all branches of the ovarian artery with a similar diameter were ligated (Polysorb 0; Convidien, Dublin, Ireland). Since the ovarian artery splits into small vessels and forms a convolute that surrounds the ovarian vein before entering the ovary, catheterization was performed proximal to the location where the ramus uterinus branches from the ovarian artery (Fig. 1). The ovarian artery was bluntly dissected from the connective tissue, stretched, cut diagonally, and enlarged by catheterization with peripheral venous catheters (Terumo Surfloc; Terumo Europe, Leuven, Belgium) using increasing diameters (20 and 18 gauge) until a permanent 16-gauge venous catheter (VYGONüle T; VYGON, Écouen, France) could be installed. The catheter was fixed within the ovarian artery with two circular ligatures (Polysorb 0).

To avoid coagulation of blood within the vessels, ovaries were flushed with chilled (4°C), heparinized (Heparin Bichsel 5000 IE/ml; Bichsel AG, Interlaken, Switzerland; 150 iu heparin/ml medium) Tyrode’s solution, containing 136 mmol/l NaCl, 11.9 mmol/l NaHCO<sub>3</sub>, 5.5 mmol/l D(+)-glucose * H<sub>2</sub>O, 2 mmol/l KCl, 1.8 mmol/l CaCl<sub>2</sub> * 2H<sub>2</sub>O, 1.05 mmol/l MgCl<sub>2</sub> * 6H<sub>2</sub>O, 0.416 mmol/l NaH<sub>2</sub>PO<sub>4</sub>, and 6% (w/v) dextran 70 000 added to distilled water to a volume of 1 l. Flushing was

![Figure 1 Mesovarium stretched between the left ovary (A) and the left uterine horn (B) to display the ovarian artery (1) with its ramus uterinus (2) and ramus tubarius (3). Catheterization was performed at the marked position (→) proximally from the location where the ramus uterinus branched from the ovarian artery.](https://www.reproduction-online.org)
continued until the effluent perfusate was macroscopically clear and the color of the CL changed from red to yellow. Flushed ovaries were transported to the laboratory in ice-chilled heparinized Tyrode’s solution.

In the laboratory, ovaries with a vascular pedicle were weighed with a precision balance (Mettler PM 400; Mettler-Toledo, Greifensee, Switzerland), and average diameter of the CL was determined with ultrasonography (Aquila Esaote Pie Medical; Esaote Biomedica, Cologne, Germany). Only ovaries with a luteal diameter > 20 mm were used. Ovaries were fixed with two sutures (Polysorb 0) at their extremities tubaria and uterina on a bipod (Fig. 2). Gravity induced the margo liber of the ovary to point upwards and the margo mesovaricus to point downwards, with the free ends of the ovarian artery (catheterized) and vein (without catheter) positioned directly above a funnel.

**Isolated perfusion of ovaries**

Ovaries (fixed on the bipod) were put in an incubator for newborn humans (Atom Infant Incubator V-850; Atom Medical Corporation, Tokyo, Japan) to ensure perfusion under standard-ized microclimatic conditions (temperature, 37.5–38.5°C; relative humidity, 75–85%). A schematic illustration of the complete set-up is shown (Fig. 3).

As medium for the isolated perfusion of the ovary, a modified Tyrode’s solution was used (for detailed composition see Section ‘Preparation of ovaries’). Osmotically active dextran 70 000 was added to the original formula of Tyrode’s solution to reduce intercellular edema in the ovary, as suggested (Janson et al. 1978, Bjersing et al. 1981). Tyrode’s solution was oxygenated with carbogen (Oxycarbon medizinal; PanGas, Dagmersellen, Switzerland) using a hollow fiber type oxygenator (Membrana Oxyphan PP50/200; Membrana GmbH, Wuppertal, Germany). To ensure adequate oxygenation, pH and partial pressures of oxygen and carbon dioxide in the perfusion medium before and after its passage through the ovary were measured 10 min after the start of perfusion, and then every 30 min until the end of perfusion using a blood-gas analysis system (Rapidlap 248 TM, Siemens, Munich, Germany). According to the results of blood-gas analysis, the supply of carbogen and the flow rate were adjusted to sustain a physiologic pH (target value, 7.40). Temperature of the perfusion medium was controlled with a Liebig condenser (GB Kühler Liebig, NSK + H14/23 120 mm; UZH Glassblowing Factory, Zurich, Switzerland) connected to a tempered water circulation (Type SSB4; Grant Instruments, Cambridge, England). Directly before entering the ovarian artery, temperature of Tyrode’s solution was measured using a thermometer (WDT, Garbsen, Germany), every 5 min between 0 and 20 min after the start of perfusion, and then every 10 min to the end of perfusion. According to these measurements, temperature of the water circulation was adjusted to maintain the Tyrode’s solution between 37 and 38°C, as reported in similar studies (Koos et al. 1984, Holmes et al. 1985, Brannstrom & Flaherty 1995).

For perfusion, a volume- and pressure-controlled peristaltic pump (Storz Endomat n. Hamou; Karl Storz GmbH, Tuttingen, Germany) was used. Flow was manually determined with a chronograph and a volumetric flask after 15 and 40 min, and then every 30 min. Pressure was continuously controlled by means of an instrument for invasive blood pressure measurement (Cardiocap 5; Datex-Ohmeda GE Healthcare Systems, Little Chalfont, England), and pressure in the ovarian artery (before entering the ovary) was calculated concurrent with flow measurements. Pressure was adjusted to achieve a perfusion flow of ~2 ml/min per gram ovarian tissue, as proposed (Stahler & Huch 1971). To avoid artifacts caused by accumulation of metabolites or hormones, Tyrode’s solution was not recycled.

**Study design**

All ovaries were perfused for 240 min. During the first 60 min (equilibration), no agents were added. In eight ovaries, 0.5 μg/ml E. coli O55:B5 lipopolysaccharide (LPS O55:B5 lyophilized powder; Sigma-Aldrich, St Louis, MO, USA) was added to the medium for 180 min after equilibration, whereas the other eight ovaries were not treated with LPS throughout the entire experiment (control). For all ovaries, human chorionic gonadotropin (hCG, 500 iu; Chorulon 1500; MSD Animal Health GmbH, Luzern, Switzerland) was added to the perfusion medium 210 min after the start of perfusion.

**Glucose, lactate, lactate dehydrogenase, creatine kinase, P4, and PGF2α**

To ensure that the ovary remained in a physiological state, glucose and lactate concentrations and activities of lactate dehydrogenase (LDH) and creatine kinase (CK) were used as markers of hypoxia and cell death (Richter et al. 2000).

Before its passage through the ovary, influent perfusion medium was sampled at 0, 60, 120, and 180 min after the start of perfusion. In addition, effluent perfusion medium was sampled every 10 min throughout the entire perfusion period. At each time of sampling, three native aliquots and one aliquot for glucose analysis (containing sodium fluoride as a glycolysis

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Figure 2 Position of the ovary fixed at its extremities tubaria and uterina on a bipod with the vascular pedicle (including the ovarian artery and vein) hanging downwards. The yellow color of the corpus luteum confirmed adequate preparation after flushing with heparinized Tyrode’s solution.
inhibitor; Vacuette FX Sodium Fluoride/Potassium Oxalate; Greiner bio-one, Kremsmünster, Austria) were collected and stored at $-20^\circ$C ($P_4$) or $-80^\circ$C (glucose, lactate, LDH, CK, PGF$_{2\alpha}$), respectively.

Glucose concentrations were measured in all samples of influent perfusion medium. In the effluent perfusion medium, concentrations of glucose and lactate, as well as LDH and CK activities, were determined in samples collected after 10 min, and then every 30 min until the end of perfusion. For these measurements, the Cobas Mira Plus analyzer (Roche, Basel, Switzerland) with kits for glucose (Glucose RTU; Biomerieux, Lyon, France), lactate (Lactate PAP; Biomerieux), LDH (LDH IFCC; Axonlab, Baden, Switzerland), and CK (Enzyline CK NAC; Biomerieux) was used. Analyses of glucose, lactate, LDH, and CK had a range of standard concentrations of 0.18–22.2 mmol/l, 0.04–10 mmol/l, 5–1200 U/l, and 5–1000 U/l, respectively. Intra- and inter-assay coefficients of variation were $\leq 0.92$ and $\leq 2.55\%$ for glucose, $\leq 1.14$ and $\leq 3.20\%$ for lactate, $\leq 1.14$ and $\leq 1.41\%$ for LDH, and $\leq 2.40$ and $\leq 5.56\%$ for CK, respectively.

Concentrations of $P_4$ were measured in the effluent perfusate every 10 min throughout the duration of perfusion, using a radioimmunoassay (RIA kit IM1188; Beckman Coulter GmbH, Krefeld, Germany). The range of standard concentrations for this test was 0.05–50 ng/ml, intra- and inter-assay coefficients of variation were $\leq 6.5$ and $\leq 7.2\%$, respectively, and 50% of relative binding (ED50) occurred at 1.4 ng/ml.

Concentrations of PGF$_{2\alpha}$ were measured in effluent perfusate at 0, 10, 40, 70, 100, 130, 160, 190, 220, and 240 min of perfusion. A high-sensitivity PGF$_{2\alpha}$ ELISA kit (Enzo Life Sciences AG, Lausen, Switzerland) was used. For this test, the range of standard concentrations was 1.95–2000 pg/ml, intra- and inter-assay coefficients of variation were $\leq 7.2$ and $\leq 11.0\%$, respectively, and ED50 was 81 pg/ml.

**Corpus luteum biopsy and expression analysis**

A biopsy (~15x1 x 1 mm) was obtained from the maximum diameter (including cells from the periphery and the center) of the CL after 60, 120, 180, and 240 min of perfusion, using a semi-automatic, high-speed biopsy needle (TEMNO Evolution; Fa. Walter, Baruth/Mark, Germany) that was rendered free of RNase (RNase-ExitusPlus; AppliChem, Darmstadt, Germany). Tissue samples were immediately placed in a sterile DNase- and RNase-free cryo tube (Fa. Brand, Wertheim, Germany), frozen in liquid nitrogen (Dry Shipper Taylor-Wharton CX100; Jensons Scientific, Franklin, TN, USA), and stored at $-80^\circ$C until expression analysis was done. Immediately after the biopsy was collected, perforation points on the CL surface were closed with fibrin glue (Histoacryl; B. Braun Melsungen AG, Melsungen, Germany).

Luteal mRNA expression was determined for luteinizing hormone/choriogonadotropin receptor ($LHCGR$); steroidogenic acute regulatory protein (STAR); 3-beta-hydroxysteroid...
dehydrogenase (HSD3B); TLR2 and -4; caspase (CASP) 3, -8, and -9, prostaglandin E- (PGES/PTGES) and -F (PGFS/AKR1B1) synthases; prostaglandin F receptor (PTGFR/FPR); TNFA; vascular endothelial growth factor A isoform 121 (VEGFA121); fibroblast growth factor 2 (FGF2); and endothelin 1 (EDN1). Therefore, total RNA from luteal tissue samples was isolated and reverse-transcribed as described (Ulbrich et al. 2009) and luteal mRNA expression was determined in a two-step and reverse-transcribed as described (Ulbrich et al. 2009) and luteal mRNA expression was determined in a two-step

For included ovaries, the average interval between death of the cow and start of isolated perfusion was 79.1±4.2 min (range, 65–94 min), with no difference between ovaries of the control (77.5±4.6 min) and LPS groups (81.0±4.6 min). Furthermore, the ultrasonographically measured diameter of the CL was similar in control ovaries (27.8±1.4 mm) and ovaries treated with LPS (27.5±0.7 mm). Mean diameters and calculated cross-sectional areas of the CL ranged from 25.5 to 33.0 mm and 5.11 to 8.55 cm², respectively, with the exception of only one CL with a diameter and area of 22.5 mm and 3.98 cm², respectively (for the latter CL, P₄ concentration in a blood sample collected immediately before slaughter was 7.2 ng/ml). Based on cross-sectional areas (n=10) or plasma P₄ concentration (n=1), CL were designated as mid-cycle (Days 8–16; Day 1 = ovulation) according to Herzog et al. (2010).

During equilibration (0–60 min after start of perfusion), pressure in the ovarian artery decreased by similar values in the control (42.1±5.7 mmHg) and the LPS group (47.9±6.6 mmHg), with no changes in arterial pressure during the treatment period (60–240 min after start of perfusion). The mean temperature and flow of the perfusion medium for all ovaries was 37.9±0.1 °C (37.5–38.3 °C) and 37.7±1.4 ml/min (29.3–44.5 ml/min), respectively. During the treatment period, there were no differences between control and LPS groups for temperature (38.2±0.1 vs 38.2±0.1 °C) or flow (38.0±2.8 vs 36.5±1.3 ml/min) of the perfusion medium.

In five cows of each group, the vascular pedicle started to contract frequently after a similar duration of perfusion (11.0±1.0 and 12.0±2.6 min in the control and LPS groups, respectively). Total interval of frequent contractions did not differ between cows of the control (227.0±2.0 min) and the LPS group (228.0±3.6 min). During the last hour of perfusion, an intensification of contractions (not quantified) occurred in four ovaries of the LPS group, whereas contractions became less intense in the control group. The increase in weight of ovaries with vascular pedicle (due to edema in the mesovarium) did not differ between the control (30.3±7.3 g) and the LPS group (47.1±8.6 g).

Analysis of CK had a lower detection limit of 5 U/l. For measurements below this limit, 4 U/l was used as an arbitrary value to facilitate statistical analysis.

Distribution of the data was assessed visually for normality (PROC CHART) and by means of the Shapiro-Wilk-test (PROC UNIVARIATE). Because data were normally distributed, independent (between groups; PROC TTEST) and dependent (within groups; PROC UNIVARIATE) pairwise comparisons were done using a Student’s t-test, in due consideration of repeated measures. All statistical analyses were done with the Statistical Analysis SystemV9.1 (SAS Institute, Inc., Cary, NC, USA), and P≤0.05 was considered significant.

Results

Mean glucose consumption during the perfusion time did not differ between control ovaries (mean±s.e.m.; 0.2±0.3 mmol/l) and ovaries treated with LPS (0.2±0.02 mmol/l). On a per-time basis, glucose consumption was higher (P=0.03) in the LPS group compared to the
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.

<table>
<thead>
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<th>Gene</th>
<th>Gene symbol</th>
<th>Reference (acc. no.)</th>
<th>Forward primer (5′→3′)</th>
<th>Reverse primer (5′→3′)</th>
<th>PCR product (bp)</th>
<th>AT (°C)</th>
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</table>

*aDegenerate multispecies primer, R=A or G.*
control group at 180 min after the start of LPS challenge (Fig. 4A). Average lactate production during the perfusion period was not different between ovaries of the control (0.2 ± 0.04 mmol/l) and the LPS groups (0.2 ± 0.02 mmol/l), but on a per-time basis, lactate concentrations were increased 70 (P = 0.01) and 100 min (P = 0.03) after the start of treatment in ovaries of the LPS group compared to control ovaries (Fig. 4B). Mean enzyme activities of LDH and CK decreased (P < 0.05) during the equilibration time to nearly half of initial values, but thereafter remained relatively constant. For control and LPS ovaries, mean activities of LDH (12.3 ± 6.1 vs 7.3 ± 2.6 U/l, respectively) and CK (11.7 ± 2.3 vs 9.8 ± 1.1 U/l) were similar throughout the entire perfusion period. Furthermore, there was no difference in LDH and CK activities between these two groups on a per-time basis (Fig. 4C and D). In summary, parameters that characterized perfusion requirements (glucose consumption, lactate production, and activities of LDH and CK) were in a physiologic range at the start of the treatment period in both groups.

P₄ concentrations in the effluent perfusate remained constant during the first 150 min of LPS treatment (mean ± S.E.M.; 223.2 ± 41.3 ng/ml) and were similar to those of control ovaries (268.5 ± 35.0 ng/ml). However, after hCG challenge (150 min after the start of LPS treatment), P₄ concentrations increased in control, but not in LPS ovaries, and reached higher (P = 0.04) concentrations in control (402.4 ± 39.9 ng/ml) compared to LPS ovaries (241.9 ± 56.8 ng/ml) 30 min later (Fig. 5).

Mean concentrations of PGF₂α did not differ between the control (22.8 ± 6.9 pg/ml) and LPS groups (53.0 ± 18.5 pg/ml) throughout the entire treatment period. Furthermore, there was no significant difference in PGF₂α concentrations between ovaries treated with LPS and control ovaries on a per-time basis (Fig. 6) but PGF₂α concentrations tended (P = 0.10) to be higher in LPS compared to control ovaries at 70 min after LPS challenge. After hCG challenge, PGF₂α concentrations did not change within LPS and control ovaries.

Changes in mRNA expression of all investigated parameters are shown (Fig. 7). Luteal mRNA expression of steroidalogenic factors STAR and HSD3B did not differ between the control and the LPS group for any time of analysis (Fig. 7A). Furthermore, expression of LHCGR did not differ between groups at any time (Fig. 7A). However, mRNA expression of TNFA in the LPS group was higher at 60 (P = 0.0002) and 120 min (P = 0.004) after the start of treatment compared to the control group (Fig. 7B). Furthermore, luteal expression of TLR2 was higher (P = 0.03) 180 min after the start of treatment in the LPS compared to the control group, whereas expression of TLR4 did not differ between groups at any time (Fig. 7B).

Analysis of apoptotic factors revealed higher mRNA expression in the LPS group for CASP3 at 60 (P = 0.02), 120 (P < 0.0001) and 180 min after start of treatment (P < 0.0001; Fig. 7C). Higher expressions were also observed for CASP8 at 60 (P = 0.05) and 180 min (P = 0.01) after the start of LPS treatment compared to control ovaries, although CASP9 did not differ between groups at any time (Fig. 7C).

Whereas mRNA expressions of PGES and PGFS were higher in the LPS group at 120 (P = 0.008; only PGES) and 180 min (P = 0.03 and P = 0.05, respectively) compared to the control group, expression of PTGFR did not differ between groups during the treatment period (Fig. 7D). Luteal mRNA expression of angiogenic factors did not differ between groups at any time (Fig. 7E).
factor VEGF_{A2,1} was higher (P=0.05) at 60 min after the start of treatment in the LPS compared to the control group (Fig. 7E). In contrast, expressions of FGFR2 and EDN1 did not differ at any time between the control and the LPS group (Fig. 7E).

Discussion

In the present study, great efforts were made to maintain energy metabolism and viability of the ovaries similar to a physiological situation in vivo during the 3-h interval after equilibration. Therefore, the duration of ischemia was kept to a minimum, with an effort to maintain physiologic pressure in the ovarian artery, glucose consumption, lactate production, and LDH and CK activities to control vascular resistance (arterial pressure), oxygenation (glucose, lactate), and cytolytic tissue processes (LDH, CK), as suggested (Stahler & Huch 1971, Richter et al. 2000). Arterial perfusion pressure decreased markedly during equilibration but remained stable thereafter, indicating normalization of vascular resistance after re-perfusion. Furthermore, comparable metabolic activities in ovaries of the control and the LPS group were inferred, based on similar glucose consumption between groups. Lactate concentrations decreased directly after the start of perfusion in both groups. High lactate concentrations before perfusion were attributed to initial hypoxia (Ahren et al. 1972); therefore, re-perfusion with oxygenated medium decreased lactate production to physiologic values before the start of the treatment period. High concentrations of LDH and CK, as indicators of cell death during ischemia, decreased to a low and constant level within the equilibration period, consistent with a study on isolated perfused human uteri (Richter et al. 2000). In conclusion, there was good evidence that after equilibration, the general conditions were relatively physiologic, and therefore, results obtained during the treatment period were reliable.

Increased lactate concentrations 70 min after the start of LPS challenge and the higher values in the LPS compared to the control group indicated increased metabolic activity, attributed to LPS-induced activation of immune defense mechanisms. Higher glucose uptake in the LPS group at the end of the perfusion period, which also indicated increased metabolic activity, was probably due to intensified contractions of the vascular pedicle in the LPS group compared to the control group. Consistently, rhythmic contractions (2- to 3-min intervals) of the vascular pedicle and the hilus area of the ovary were clearly visible in isolated perfused sheep ovaries and the vascular pedicle was responsible for a non-negligible portion of glucose uptake (Janson et al. 1978).

Luteal mRNA expression of TNFA was significantly higher in ovaries treated with LPS than in control ovaries 60 and 120 min after the start of treatment. Consistently, LPS induces TNFA production and the activated cytokine cascade (involving TNFA) mediates the acute phase response to endotoxins (Kushibiki 2011). Functional TNF receptors were present in steroidogenic and endothelial cells of bovine CL (Okuda et al. 1999, Okuda & Sakamoto 2003). Therefore, the ability of TNF to modulate the lifespan of the bovine CL may largely depend on the direct action on CL cells (Skarzynski et al. 2007). In cultured steroidogenic luteal cells, TNFA inhibited gonadotropin-stimulated secretion (Benyo & Pate 1992), consistent with failure of LPS-treated ovaries to increase P4 concentrations after challenge with hCG in the present study. Furthermore, TNFA acting via TNF receptor-1 induced apoptotic death of steroidogenic and endothelial cells of the bovine CL through inactivation of the anti-apoptotic protein Bcl-2 and by stimulating expression and activity of CASP3 (Skarzynski et al. 2005). Consistently, CASP3 mRNA expression was increased 60, 120 and 180 min after the start of treatment in the LPS group compared to the control group. Furthermore, mRNA expression of CASP8 was increased 60 and 180 min after the start of LPS treatment, whereas CASP9 mRNA expression did not differ between LPS and control groups, suggesting the importance of the extrinsic (death receptor mediated) pathway of apoptosis.

P4 concentrations in the effluent perfusate did not significantly change during the first 150 min after treatment with LPS (similar values in the two groups). In a previous study on isolated perfused bovine ovaries (Sturm & Stahler 1971), P4 concentrations in the perfusate of untreated ovaries were 175–291 ng/ml, indicating adequate luteal release of P4 in the present study. Previously, measurements of P4 content in luteal tissue before and after in vitro perfusion indicated that changes in P4 concentrations of the effluent perfusate resulted from differences in P4 synthesis and release and FGF2 (pg/ml)

![Figure 6](image-url)
not from leakage of P₄ already present in the CL prior to perfusion (Dharmarajan et al. 1988). Furthermore, concentrations of P₄ in the perfusate corresponded well with those synthesized in the tissue of CL from isolated perfused ovaries (Brannstrom & Flaherty 1995), which were regarded as evidence of adequate
secretory capacity. However, our results were not consistent with an initial increase (within 30 min) and subsequent decline (until 9 h) of P₄ concentrations in an in vivo study involving cows given LPS by intravenous treatment (Herzog et al. 2012). Notwithstanding, our results supported the assertion that the initial increase in P₄ after treatment with LPS in vivo might be of adrenal origin due to activation of a neuroendocrine stress axis (Kujo et al. 1995, Herzog et al. 2012).

Since LH or hCG significantly increase P₄ production in bovine mid-cycle CL (Koos et al. 1984, Litch & Condon 1988), an hCG challenge was used in the present study to assess secretory capacity of the CL during isolated perfusion. It was noteworthy that P₄ increased significantly 30 min after application of hCG in the control, but not in the LPS group. In cattle, LH binds to its specific receptor LHCGR and ultimately increases synthesis of STAR and activity of cytochrome P450scc and HSD3B, followed by an increase in P₄ secretion (Rekawiecki et al. 2005). However, in control ovaries in the present study, mRNA expressions of LHCGR, STAR and HSD3B were not increased 30 min after hCG challenge, although P₄ concentrations were already higher at that time. Consistently, concentrations of mRNA for STAR, and HSD3B (measured at 3-h intervals) were not increased until 6 h after luteal cell stimulation with LH (Rekawiecki et al. 2005). Furthermore, LH responsiveness seemed to be more dependent on the extent of desensitization of luteal cells to LH (e.g., by extensive clustering and internalization of the receptor complex) than on the number of LH receptors, as during functional luteolysis, luteal cells apparently became desensitized to LH, despite maintenance of LHCGR (Amsterdam et al. 2002). However, we inferred that the absence of an increase in P₄ concentrations after LPS challenge was due to inhibited luteal release rather than synthesis of P₄. Release of P₄ from the ovine CL was regulated by calcium-dependent depolarization of the luteal cell membrane after stimulation with LH in vitro (Higuchi et al. 1976).

Moreover, luteal PGF₂α concentrations in LPS-treated ovaries did not increase significantly, although there was a trend toward significance. The release of PGF₂α from the uterus is essential to suppress P₄ concentrations during luteolysis at the end of the estrous cycle (McCracken et al. 1981). Whereas uterine PGF₂α has been clearly linked to luteolysis (Schams & Berisha 2004), it is a debatable point whether luteal PGF₂α had a luteolytic effect in the present study. However, changes in luteal mRNA expressions in ovaries of the LPS group do not resemble changes observed after exogenous PGF₂α treatment in cows, including decreased mRNA expressions of PTGFR, steroidogenic (STAR, HSD3B) and angiogenic factors (VEGF, FGf2), and increased vasoactive factor EDN1 mRNA expression (Shirasuna et al. 2010). In the present study, there were no significant differences in these parameters between control and LPS ovaries, except for a temporary increase in VEGFA121, indicating a non-specific response to LPS challenge. Therefore, we inferred that luteal PGF₂α was probably not primarily responsible for LPS-induced changes in the present study.

Tumor necrosis factor alpha is a potent stimulator of luteal synthesis of prostaglandins (including luteolytic PGF₂α and luteotrophic PGE₂), consistent with increased mRNA expressions of PGES at 120 and 180 min and PGFS at 180 min after the start of LPS treatment compared to control ovaries, similar to a previous report (Okuda et al. 1999). In bovine endometrial cells, PGE₂ and PGF₂α production were elevated after LPS challenge (Herath et al. 2006), in association with extended luteal phases and premature regression of the CL, respectively (Opsomer et al. 2000). The predominant effect (luteotropic or luteolytic) was dependent on PGE₂ to PGF₂α ratio (Herath et al. 2006) and luteal phase (Shirasuna et al. 2010), with PGE₂ primarily controlling the early and PGF₂α the mid- and late luteal phases, respectively. In the present study using mid-luteal CL, increased mRNA expression of PGFS in the LPS group compared to the control group might have contributed to lower P₄ concentrations after hCG challenge in LPS-treated ovaries. Bovine PGFS mRNA encodes for 20α-hydroxysteroid dehydrogenase (20α-HSD; Madore et al. 2003, Schuler et al. 2006) that is responsible for P₄ catabolism within the CL (Naidansuren et al. 2011), and therefore might decrease P₄ in the effluent perfusate. Furthermore, luteal cells that express 20α-HSD not only lose their capacity to secrete P₄, but also facilitate expression of the death receptor Fas on their surface (Stocco et al. 2007), thereby enhancing apoptosis.

The main receptor for recognition of LPS is TLR4 (Akira 2003), whereas TLR2 predominantly recognizes peptidoglycans and lipoteichoic acid (Takeuchi et al. 1999). Expression of both receptors was reported in bovine ovaries (Vahanan et al. 2008). Interestingly, mRNA expression of TLR4 did not differ significantly between control and LPS-treated ovaries in the present study, whereas expression of TLR2 was higher in the LPS than in the control group 180 min after the start of treatment. Binding of the respective pathogens to TLR2 and -4 initiated a signaling cascade that resulted in release of TNFα and other cytokines (Kannaki et al. 2011). Since TNFα increased TLR2 mRNA expression in various murine (Matsumura et al. 2000) and human (Davanian et al. 2012) tissues, an involvement of TNFα in higher mRNA abundance of TLR2 after LPS challenge may have occurred in the present study.

In conclusion, treatment with LPS inhibited hCG-induced P₄ secretion in the bovine CL. The reduced P₄ secretion seemed to be predominantly caused by an increase in LPS-induced apoptosis, but not by decreased steroidogenic factors. Regardless, an impact of luteal PGF₂α could not be excluded.
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
Benyo DF & Pate JL 1992 Tumor necrosis factor-α alters bovine luteal cell synthetic capacity and viability. Endocrinology 130 854–860. ( doi:10.1210/endo.130.2.1733731)
Giri SN, Stabenfeldt GH, Moebley TA, Graham TW, Bruss ML, BonDurant RH, Cullor JS & Osburn BI 1990 Effects of endotoxin infusion on circulating levels of eicosanoids, progesterone, cortisol, glucose and lactic acid, and abortion in pregnant cows. Veterinary Microbiology 21 211–231. ( doi:10.1016/0378-1135(90)90033-R)


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