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The effect of LPS on LH release and gene expression of *LH-β*, *GnRH-R* and *TLR4* in the anterior pituitary of follicular phase ewes – an *in vitro* study

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ABSTRACT. The study was designed to determine the *in vitro* effect of bacterial endotoxin – lipopolysaccharide (LPS) – on luteinizing hormone (LH) secretion from anterior pituitary (AP) explants collected from saline-treated 'healthy' and LPS-treated ewes in the follicular phase. In the AP explants, the expression of *LH-β*, gonadotropin-releasing hormone receptor (*GnRH-R*) and Toll-like receptor 4 (*TLR4)* genes was also assayed. It was found that explants incubated alone with LPS, as well as together with LPS-binding protein (LBP), abolished the stimulatory effect of GnRH on LH release. LPS added without LBP did not suppress *LH-β* gene expression in 'healthy' APs, however. Moreover, LBP intensified the inhibitory effect of LPS on gene expression in 'healthy' APs. These results show that LPS is a potent negative modulator of LH secretion and suggest that its direct action on the pituitary gland could be one of the mechanisms *via* which an immune/inflammatory challenge inhibits the reproductive process.

Introduction

Bacterial endotoxin, i.e. lipopolysaccharide (LPS), is a major component of Gram-negative bacterial cell walls. LPS is released from the surface of replicating and dying Gram-negative bacteria into the circulation (Rietschel et al., 1994). It is generally accepted that LPS from the outer membrane of this kind of bacteria is responsible for many of the clinical symptoms of sepsis by stimulating monocytes and macrophages to produce large amounts of pro-inflammatory mediators like tumor necrosis factor alpha (TNFα), interleukin (IL)-1β and IL-6 (Van Langevelde et. al., 1998). LPS induces this inflammatory effect acting via its corresponding receptor, Toll-like receptor 4 (TLR4) (Poltorak et al., 1998). LPS enters the bloodstream associated with a lipid-transfer protein known as lipopolysaccharide-binding protein (LBP), a necessary component of TLR4. This soluble, acute-phase protein binds to bacterial lipopolysaccharide to elicit immune responses by presenting LPS to TLR4 (Miyake, 2007; Leon et al., 2008). After activation by LPS, TLR4 can transduce its inflammatory signal through different signaling cascades leading to activation of either NF-κB or mitogen-activated protein kinases p38 and JNK pathways (Zhang and Ghosh, 2002; Leon et al., 2008) or inducing cell apoptosis (Leon et al., 2008).

The immune stress induced by endotoxin disturbs the homeostatic milieu and suppresses functions that are not essential to survival. One of the processes inhibited by immune/inflammatory challenge is reproduction. Our previous studies showed that peripheral administration of LPS decreased GnRH/LH release in

ewes (Herman et al., 2010; Herman and Tomaszewska-Zaremba, 2010). It is postulated that LPS affects reproductive function by acting indirectly at all levels of the hypothalamus-pituitary-gonadal (HPG) axis through such inflammatory mediators as pro-inflammatory cytokines (Rivest and Rivier, 1995; Yoo et al., 1997; Igaz et al., 2006), prostaglandins (PGs) (Breen et al., 2004), or *via* stress HPA axis components (Dobson et al., 2003; Maeda and Tsukamura, 2006). Previous studies have shown the presence of TLR4 in pituitary cells, however (Lohrer et al., 2000; Breuel et al., 2004). The existence of TLR4 on AP cells suggests that LPS could also directly affect its secretory activity and directly modulate reproduction at the pituitary level of the HPG axis.

The present study was designed to determine the effect of LPS on LH secretion from anterior pituitary explants collected from saline-treated ('healthy') and LPS-treated ewes in the follicular phase. The expression of LH-β, GnRH-R, and TLR4 genes was also assayed in the AP explants.

Material and methods

Animals

The studies were performed on adult, 3-year-old Blackhead ewes in the reproductive season (September–October). All animals were in good condition, their body condition score was estimated at 3 points (on a five-point scale). The animals were maintained indoors in individual pens and exposed to natural daylight. The ewes were well adapted to the experimental conditions; they always had visual contact with their neighbors, even during the experimental period, to prevent the stress of social isolation. The animals were fed a constant diet of commercial concentrates with hay and water available *ad libitum*.

All experimental procedures were conducted in accordance with the Polish Guide for the Care and Use of Animals (1997) and were approved by the Local Ethics Committee of the Warsaw University of Life Sciences.

Preparing animals for the experiment

The ewes were synchronized by the Chronogest® CR (Merck Animal Health, Boxmeer, the Netherlands) method using an intra-vaginal sponge impregnated with 20 mg of synthetic progesterone-like hormone. All ewes had Chronogest® CR sponges implanted for 14 days. After removing the sponges the animals received an intramuscular injection of 500 I.U. pregnant mare serum gonadotropin (PMSG) (Merck Animal Health, Boxmeer, the Netherlands). The experimental procedure was started 24 h after PMSG injection when all animals were in the follicular phase.

Experimental procedures

Inducing immune stress in the experimental animals. The animals (n = 12) were randomly assigned to two experimental groups: the NaCl-control group (n = 6) and the LPS-treated group (n = 6). In treated ewes, immune stress was induced by injection of an appropriate volume of LPS (400 ng · kg^{-1} body weight) from *E. coli* 055:B5 (Sigma-Aldrich, St Louis, MO, USA) dissolved in saline (0.9 % w/v NaCl) (Baxter, Deerfield, IL, USA) at a concentration of 10 mg · l^{-1} intravenously (i.v.) into the jugular vein. The maximum volume of LPS solution (10 · l^{-1}) administered to any animal was 2.5 ml. The control group, i.e. the 'healthy' ewes, received an equivalent volume of NaCl.

Incubation of the AP explants *in vitro*. The animals from both groups were slaughtered by decapitation 2 h after i.v. injection of LPS (n = 6) or saline (n = 6). The ovine brains were rapidly removed from the skulls and the anterior pituitary glands (AP) dissected. Each AP (n = 6) cut into four fragments and transfered to 24-well plates. The APs were then cut into four fragments, each of which was sectioned further and these sections were immediately transferred to 24-well plates (Becton Dickinson Labware, Franklin Lakes, NJ, USA). The *in vitro* incubation of the explants was performed in the 199 HEPES modification (Sigma-Aldrich, St. Louis, MO, USA) medium suitable for cell culture with penicillin-streptomycin at a dose of 10 ml · l^{-1} (Sigma-Aldrich, St. Louis, MO, USA) and carried out at 37°C with constant shaking. After collection, all tissues were pre-incubated for 1 h in 800 µl of 'pure' medium 199. During pre-incubation, the medium was replaced by fresh medium four times (every 15 min). The pre-incubation was performed to wash out blood and hormones from the pituitary fragments. Then, the explants collected from each 'healthy' ewe were divided into four experimental groups as follows: control ('native'): AP explants (n = 6) incubated in 600 µl of 'pure' medium 199; GnRH control: AP explants (n = 6) incubated in 600 µl of medium 199 with GnRH (100 pmol · ml^{-1}); GnRH + LPS: AP explants (n = 6) incubated in 600 ml of medium 199 with GnRH (100 pmol · ml^{-1}) and LPS (10 ng · ml^{-1}); GnRH + LPS + LBP: AP explants (n = 6) incubated in 600 µl of medium 199 with GnRH (100 pmol · ml^{-1}), LPS (10 ng · ml^{-1}), and LBP (120 ng · ml^{-1}). The explants collected from ewes in

immune stress were also divided into four experimental groups and treated analogously as described above. The *in vitro* experiment was carried out for 4 h. During 1 h of incubation all explants were treated with 600 µl of 'pure' medium 199. The medium was changed to fresh three times (every 20 min). After 1 h, all AP explants were incubated in the medium appropriate for each experimental group. Media were exchanged every 20 min and 600 µl samples were collected. After incubation was completed, all tissues were frozen in liquid nitrogen and stored at –80°C until assay.

Assays

Radioimmunoassay for LH. The concentration of LH in medium was assayed by the RIA double-antibody method using anti-ovine-LH and anti-rabbit-γ-globulin antisera and ovine standard (NIH-LH-SO18) as described by Stupnicki and Madej (1976). The sensitivity was 0.3 ng · ml^{-1}, intraassay and interassay coefficients of variation were 8.3% and 12.5%, respectively.

Relative gene expression assay. Total RNA from the AP tissues was isolated using NucleoSpin® RNA II Kits (MACHEREY-NAGEL Gmbh & Co; Düren, Germany) according to the manufacturer's protocol. The purity and concentration of isolated RNA were quantified spectrophotometrically by measuring optical density at 260 and 280 nm in a NanoDrop 1000 instrument (Thermo Fisher Scientific Inc., Waltham, USA). RNA integrity was verified by electrophoresis on 1% agarose gel stained with ethidium bromide. DyNAmo™ SYBR Green 2-Step qRT-PCR Kit (Finnzymes, Espoo, Finland) was used for cDNA synthesis, with 800 ng of total RNA as the starting material.

Real-time RT-PCR was carried out using SYBR® Green 2-Step qRT-PCR Kit (Finnzymes, Espoo, Finland) components and HPLC-grade oligonucleotide primers synthesized by Genomed (Poland). Specific primers for determining the expression of housekeeping genes and the genes of interest were designed using Primer 3 software. The sequences of the primers were as follows: *LH-β* primers: 5'-AGATGCTCCAGGGACTGCT-3' (forward) and 5'-TGCTTCATGCTGAGGCAGTA-3'(reverse) (Genebank accession no. X52488), generated product size, 184 bp; *GnRH-R* primers: 5'-TCTTTGCTGGACCACAGTTAT-3' (forward) and 5'-GGCAGCTGAAGGTGAAAAAG-3' (reverse) (Genebank accession no. NM-001009397), generated product size, 150 bp; *TLR4* primers: 5'-GGTTCCCAGAACTGCAAGTG-3' (forward) and 5'-GGATAGGGTTTCCCGTCAGT-3' (reverse) (Genebank accession no. AY957615), generated product size, 117 bp; β-actin (*ACTB*) primers: 5'-CTTCCTTCCTGGGCATGG-3' (forward) and 5'-GGGCAGTGATCTCTTTCTGC-3' (reverse) (Genebank accession no. U39357), generated product size, 168 bp; glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) primers: 5'-AGAAGGCTGGGGCTCACT-3' (forward) and 5'-GGCATTGCTGACAATCTTGA-3' (reverse) (Genebank accession no. NM-001034034), generated product size, 134 bp; cyclophilin C (*PPIC*) primers: 5'-ACGGCCAAGGTCTTCTTTG-3' (forward) and 5'-TATCCTTTCTCTCCCGTTGC-3' (reverse) (Genebank accession no. NM-001076910), generated product size, 131 bp. One tube contained: 10 µl PCR Master Mix (2x), 7 µl RNase-free water, 2 µl primers (1 µl each, the working concentration was 0.5 µM) and 1 µl cDNA template. The tubes were run on a Rotor-Gene 6000 (Qiagen, Duesseldorf, Germany). The following protocol was used: 95°C for 15 min to activate Hot Star DNA polymerase and PCR encompassing 30 cycles at 94°C for 5 sec for denaturation, 56°C for 20 sec for annealing, and 72°C for 15 sec for extension. After the cycles, final melting curve analysis under continuous fluorescence measurements was performed to confirm the specificity of the amplification.

Data analysis

LH concentrations. The results of LPS treatment on the concentrations of LH in all types of mediums were examined by two-way analysis of variance, ANOVA (STATISTICA; Stat-Soft, Inc. Tulsa, OK, USA). The least significant differences *post hoc* test was used for the comparison of LH concentrations between the 20-min periods of the *in vitro* experiment within and between the control and treated groups. The Mann-Whitney U-test was used to compare these values. All data are expressed as means ± SEM.

PCR data analysis. All data were analysed using Rotor Gene 6000 software 1.7. Relative gene expression was determined using the Relative Expression Software Tool 2008, first published by Pfaffl et al. (2002) and based on the PCR efficiency correction algorithm published by Pfaffl (2001). To compensate variation in cDNA concentrations and PCR efficiency between tubes, an endogenous control β-actin (*ACTB*) gene was included in each sample and used for normalization. Initially, three housekeeping genes: *GAPDH*, *β-actin*, and *PPIC* were tested. BestKeeper was used to determine the most stable housekeeping gene for normalizing the expression of the genes of interest. BestKeeper is based on pair-wise correlation analysis of all pairs

of candidate genes (Plaffl et al., 2004) and calculates variations of all reference genes (SD (± Ct)). *ACTB* was chosen as the best endogenous control gene. It had the lowest SD (± Ct) value and a good correlation coefficient with the remaining analysed housekeeping genes.

The results are presented as relative gene expression of the target gene vs housekeeping gene, relative expression value, and mean ± SEM. The significance of differences between the experimental groups was assessed by the Mann-Whitney U-test.

Results

The *in vitro* effect of LPS on luteinizing hormone (LH) release

In the AP explants collected from 'healthy' ewes, the GnRH treatment significantly ($p < 0.01$) stimulated LH release after 1 h of incubation. It is worth mentioning, however, that a statistically non-significant effect of GnRH on LH release was observed starting from 20 min. LH release from GnRH-treated explants remained elevated to the end of the experiment (Figure 1). In the AP explants collected from ewes in immune stress, GnRH treatment significantly ($p < 0.01$) stimulated LH release after 20 min of incubation. Similarly as in the 'healthy' group, LH release from GnRH-treated explants remained elevated to the end of the experiment (Figure 2). It was also found that LPS administered alone as well as together with LBP suppressed the stimulatory effect of GnRH treatment on LH release in both the 'healthy' and 'immune stress' groups (Figures 1 and 2).

Effect of LPS on *LH-β* gene expression in AP explants

In the AP explants collected from 'healthy' ewes, it was found that GnRH significantly ($P < 0.01$) stimulated *LH-β* gene expression in GnRH control and GnRH+LPS groups (mean exp. 1. 27 ± 0.03; 1.33 ± 0.09, respectively) compared with the 'native' control (mean exp. 1.00 ± 0.03). Concomitant LPS and LBP treatment inhibited the GnRH-induced elevation of *LH-β* gene expression. The relative *LH-β* mRNA level was significantly ($p < 0.05$) lower (mean exp. 1.03 ± 0.14) compared with the GnRH control and GnRH+LPS groups. In the AP explants collected from ewes in immune stress, GnRH significantly ($P < 0.01$) stimulated *LH-β* gene expression only in the GnRH control group (mean exp. 1.58 ± 0.24) compared with the 'native' control (mean exp. 1.00 ± 0.12). LPS alone

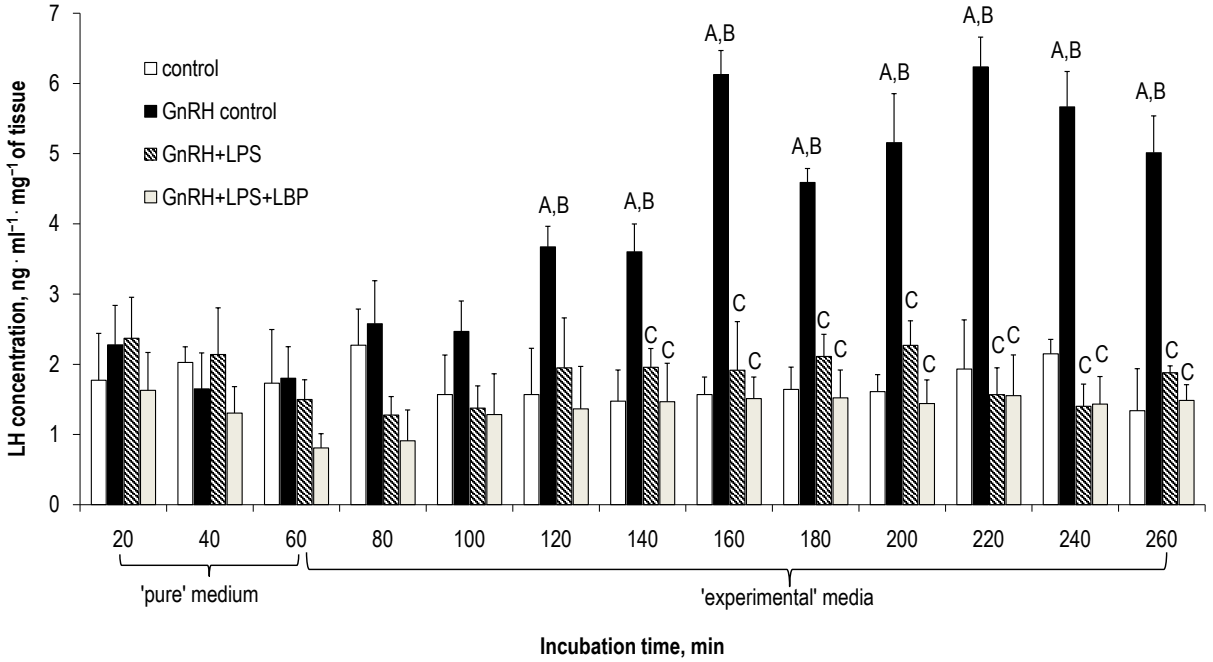

Figure 1. Distribution of mean concentrations of luteinizing hormone (LH) in four types of media (control medium 199 HEPES modification; GnRH control medium 199 HEPES modification with GnRH (100 pmol · ml^{-1}); GnRH+LPS medium 199 HEPES modification with GnRH (100 pmol · ml^{-1}) and LPS (10 ng · ml^{-1}); GnRH+LPS+LBP medium 199 HEPES modification with GnRH (100 pmol · ml^{-1}), LPS (10 ng · ml^{-1}) and LBP (120 ng · ml^{-1}), during the consecutive 20 min periods of the *in vitro* incubation of the AP explants collected from saline treated 'healthy' ewes. Each point represents mean ± SEM; A – $P < 0.01$ (letter indicates values that differ significantly from the basal level of LH release in GnRH group during incubation in 'pure' media according to Mann-Whitney U-test); B – $P < 0.01$ (letter indicates values that differ significantly from the control group according to the Mann Whitney U-test); C – $P < 0.01$ (letter indicates values that differ significantly from the GnRH control group according to the Mann Whitney U-test)

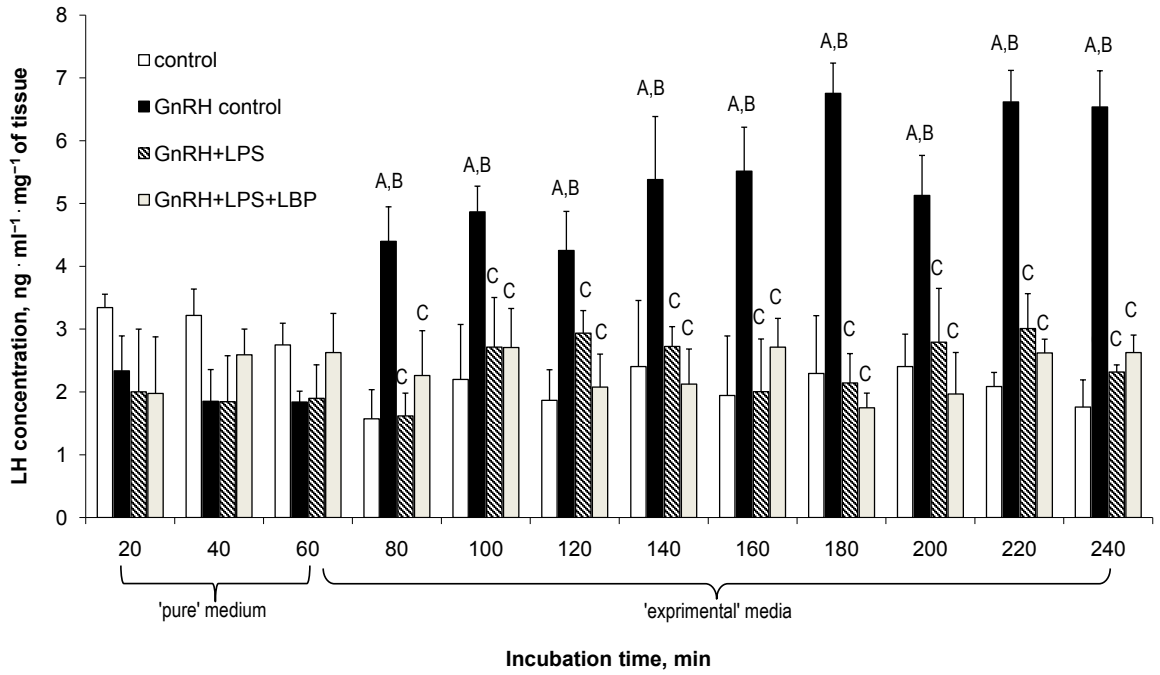

Figure 2. Distribution of mean concentrations of luteinizing hormone (LH) in four types of media (control medium, 199 HEPES Modification; GnRH control medium, 199 HEPES modification with GnRH (100 pmol · ml^{-1}); GnRH+LPS medium, 199 HEPES modification with GnRH (100 pmol · ml^{-1}) and LPS (10 ng · ml^{-1}); GnRH+LPS+LBP medium, 199 HEPES modification with GnRH (100 pmol · ml^{-1}), LPS (10 ng · ml^{-1}), and LBP [120 ng · ml^{-1}), during consecutive 20-min periods of *in vitro* incubation of AP explants collected from LPS-treated ewes. Each point represents mean ± SEM; A – $P < 0.01$ (letter indicates values that differ significantly from the basal level of LH release in the GnRH group during incubation in 'pure' media, according to the Mann-Whitney U test); B – $P < 0.01$ (letter indicates values that differ significantly from the control group according to the Mann-Whitney U-test); C – $P < 0.01$ (letter indicates values that differ significantly from the GnRH control group according to the Mann-Whitney U-test)

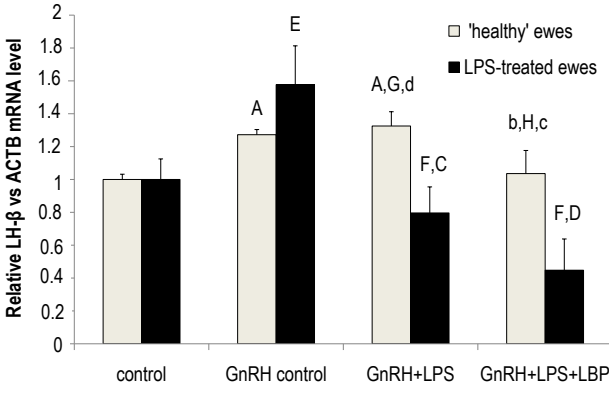

Figure 3. Relative luteinizing hormone subunit β (LH-β) mRNA level in ovine anterior pituitary explants collected from saline-treated ('healthy') and LPS-treated (400 ng · kg^{-1}) animals. The tissues were incubated in four types of media: control medium, 199 HEPES modification; GnRH control medium, 199 HEPES modification with GnRH [100 pmol · ml^{-1}); GnRH+LPS medium, 199 HEPES modification with GnRH (100 pmol · ml^{-1}) and LPS (10 ng · ml^{-1}); GnRH+LPS+LBP medium, 199 HEPES modification with GnRH (100 pmol · ml^{-1}), LPS (10 ng · ml^{-1}) and LBP (120 ng · ml^{-1}). Each bar represents mean ± SEM; Letters indicate values that differ significantly according to the Mann-Whitney U-test from the 'healthy control' (A – $P < 0.01$); 'healthy GnRH control' (b – $p < 0.05$); 'healthy GnRH+LPS' (C – $P < 0.01$; c – $p < 0.05$); 'healthy GnRH+LPS+LBP' (D – $P < 0.01$; d – $p < 0.05$); 'LPS-treated control' (E – $P < 0.01$); 'LPS-treated GnRH control' (F – $P < 0.01$); 'LPS-treated GnRH+LPS' (G – $P < 0.01$); 'LPS-treated GnRH+LPS+LBP' (H – $P < 0.01$), respectively

or together with LBP inhibited the GnRH-induced elevation of *LH-β* gene expression (mean exp. 0.8 ± 0.16; 0.45 ± 0.19, respectively), which was significanly ($P < 0.01$) lower than in the GnRH control (Figure 3).

Effect of LPS on *GnRH-R* gene expression in AP explants

In the AP explants collected from 'healthy' ewes, it was found that concomitant LPS and LBP treatment significantly ($p < 0.05$) decreased *GnRH-R* gene expression (mean exp. 0.7 ± 0.1) compared with other experimental groups (mean exp. 1 ± 0.17 control; 1.13 ± 0.23 GnRH control; 1.17 ± 0.24 GnRH+LPS). In the AP explants collected from ewes in immune stress, decreased *GnRH-R* gene expression was found in the GnRH+LPS group (mean exp. 0.79 ± 0.11; $p < 0.05$) compared with the GnRH control (mean exp. 1.19 ± 0.24; $p < 0.05$) and in the GnRH+LPS+LBP group (0.55 ± 0.07) compared with both 'native' and GnRH control groups (mean exp. 1.00 ± 0.19; 1.19 ± 0.24, respectively; $P < 0.01$). It is worth mentioning that *GnRH* gene expression in the group co-treated-with LPS and LBP was significantly ($P < 0.05$) lower than in the group treated with LPS alone (Figure 4).

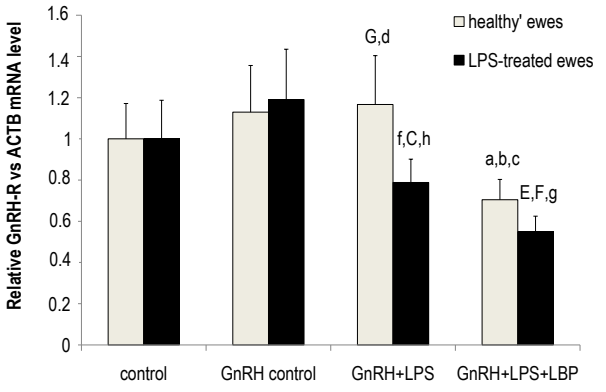

Figure 4. Relative gonadotropin-releasing hormone receptor (GnRH-R) mRNA level in the ovine anterior pituitary explants collected from saline-treated ('healthy') and LPS-treated (400 ng · kg^{-1}) animals. The tissues were incubated in four types of media: control – medium 199 HEPES modification; GnRH control – medium 199 HEPES modification with GnRH (100 pmol · ml^{-1}); GnRH+LPS – medium 199 HEPES modification with GnRH (100 pmol · ml^{-1}) and LPS (10 ng · ml^{-1}); GnRH+LPS+LBP – medium 199 HEPES modification with GnRH (100 pmol · ml^{-1}), LPS (10 ng · ml^{-1}) and LBP (120 ng · ml^{-1}). Each bar represents mean ± SEM; letters indicate values that differ significantly according to the Mann-Whitney U-test from 'healthy control' (a – $p < 0.05$); 'healthy GnRH control' (b – $p < 0.05$); 'healthy GnRH+LPS' (C – $P < 0.01$; c – $p < 0.05$); 'healthy GnRH+LPS+LBP' (d – $p < 0.05$); 'LPS-treated control' (E – $P < 0.01$); 'LPS-treated GnRH control' (F – $P <$ .01; f – $p < 0.05$); 'LPS-treated GnRH+LPS' (G – $P < 0.01$; g – $P < 0.05$); 'LPS-treated GnRH+LPS+LBP' (h – $P < 0.05$), respectively

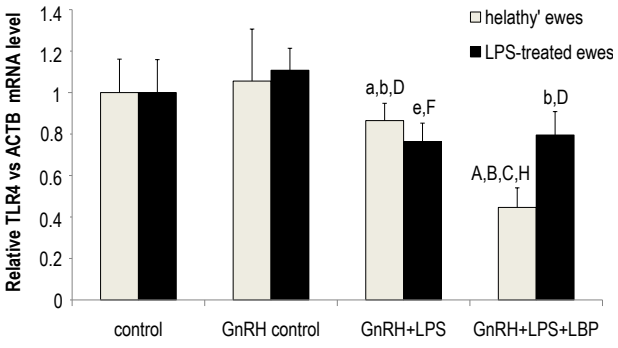

Figure 5. Relative Toll-like receptor 4 (TLR4) mRNA level in the ovine anterior pituitary explants collected from saline-treated ('healthy') and LPS-treated (400 ng · kg^{-1}) animals. The tissues were incubated in four types of media: control – medium 199 HEPES modification; GnRH control – medium 199 HEPES modification with GnRH (100 pmol – ml^{-1} GnRH+LPS – medium 199 HEPES modification with GnRH (100 pmol · ml^{-1}) and LPS (10 ng · ml^{-1}); GnRH+LPS+LBP – medium 199 HEPES modification with GnRH (100 pmol · ml^{-1}), LPS (10 ng · ml^{-1}) and LBP (120 ng · ml^{-1}). Each bar represents mean ± SEM; letters indicate values that differ significantly according to the Mann-Whitney U-test from 'healthy control' (A – $P < 0.01$; a – $p < 0.05$); 'healthy GnRH control' (B – $P < 0.01$; b – $p < 0.05$); 'healthy GnRH+LPS' (C – $P < 0.01$); 'healthy GnRH+LPS+LBP' (D – $P < 0.01$); 'LPS-treated control' (e – $p < 0.05$); 'LPS-treated GnRH control' (F –P < 0.01); 'LPS-treated GnRH+LPS+LBP' (H – $P < 0.01$), respectively

Effect of LPS on the *TLR4* gene expression in the AP explants

In the AP explants collected from 'healthy' ewes, it was found that *TLR4* gene expression was decreased in groups GnRH+LPS (mean exp. 0.81 ± 0.08; $p < 0.05$) and GnRH+LPS+LBP (0.45 ± 0.09; $P < 0.01$) compared with the 'native' control (mean exp. 1.00 ± 0.12) and the GnRH control (1.02 ± 0.23; $p < 0.05$, $P < 0.01$, respectively) groups. In the AP explants collected from ewes in immune stress, decreased *TLR4* gene expression was found in group GnRH+LPS (mean exp. 0.76 ± 0.09) compared with the 'native' (mean exp. 1.00 ± 0.16; $p < 0.05$) and GnRH control (mean exp. 1.11 ± 0.11; $P < 0.01$) groups. Decreased *TLR4* gene expression was also found in the GnRH+LPS+LBP group (mean exp. 0.8 ± 0.11; $p < 0.05$) compared with the GnRH control group (Figure 5).

Discussion

Our *in vitro* study showed that bacterial endotoxin administered either alone or together with LBP suppressed GnRH-induced LH release from AP explants collected from both 'healthy' and LPS-treated ewes. This is the first study described in the literature concerning the *in vitro* effect of LPS on LH release from the AP. *In vitro* studies performed on pituitary cells have shown, however, that LPS can directly affect growth hormone (GH) release by these cells. The stimulatory effect of LPS on GH synthesis and release has been described in pituitary cultures from sheep (Fry et al., 1998) and pigs (Mainardi et al., 2002). *In vitro* studies performed on rat AP cells showed that endotoxin stimulates GH release in a dose-dependent manner (Priego et al., 2003). The LPS-induced effect on GH was inversely proportional to the dose applied. The *in vivo* effect of LPS on reproductive function is well documented. Fergani et al. (2012) showed that peripheral endotoxin administration affects all three levels of the HPG axis, causing disorders in the GnRH/LH surge. Our previous studies demonstrated that i.v. injection of LPS affects LH release as well as *LH-β* gene expression in the AP of anoestrous ewes (Herman et al., 2010). Studies performed on ewes showed that LPS disturbs the preovulatory LH surge (Battaglia et al., 1999; Karsch and Battaglia, 2002). It was also found that endotoxin inhibits the plasma concentration of LH and the number of LH pulses without impact on pulse amplitude in castrated male sheep (Coleman et al., 1993; Daniel et al., 2003). On the other hand, studies performed on rats showed that lipopolysaccharide decreased the con-

centration of LH in intact males, affecting both LH pulse frequency and amplitude (Gow et al., 2001; Rivier, 2002). It is generally accepted that immune stress induced by LPS modulates LH secretion from the pituitary through indirect action *via* inflammatory mediators affecting GnRH secretion in the hypothalamus (Daniel et al., 2003; Watanobe and Hayakawa, 2003; Herman et al., 2012). Our previous studies showed that peripheral administration of LPS decreased *GnRH* gene expression in the hypothalamus (Herman and Tomaszewska-Zaremba, 2010). Other studies performed on ovariectomized ewes showed that endotoxin suppressed GnRH pulsatile release into hypophyseal portal blood (Battaglia et al., 1997). Furthermore, it was previously demonstrated in rats that LPS also suppressed the pulsatile LH secretion pattern (Refojo et al., 1998). This suggests that inhibition of reproduction processes during immune stress occurs in a large part at the hypothalamic level, however, it cannot be excluded that it also results from processes occurring directly at the pituitary level.

Our study also showed that LPS affects *LH-β* gene expression in the APs *in vitro*, although the effects observed in 'healthy' and LPS-treated animals differed. In 'healthy' APs, endotoxin affected *LH-β* gene expression only when administered together with LBP, whereas in APs from animals in immune stress, the pituitary response to LPS treatment was independent of LBP. Lowering of *LH-β* gene expression occurred in both LPS-treated groups. The ability of LBP to enhance the response to LPS has been demonstrated in both *in vivo* and *in vitro* studies (Knapp et al., 2006). LPS could, however, stimulate TLR4 alone, but the spontaneous diffusion of LPS monomers to the cellular-binding site is very slow and transfer by LBP enhances the immune response to LPS up to 1000-fold *in vitro* (Martin et al., 1992; Wurfel et al., 1997). It is worth mentioning that the biological role of LBP is closely dependent on its concentration. High LBP concentrations can inhibit LPS bioactivity *in vitro* and *in vivo* (Kitchens and Thompson, 2005). The differences in *LH-β* gene expression found in our study suggest that the APs collected from ewes during immune stress are more sensitive to LPS than those from 'healthy' animals. This could be due to increased *TLR4* expression in the AP in LPS-treated animals. It has been shown previously that LPS significantly stimulated *TLR4* gene expression in monocytes and polymorphonuclear leukocytes (Muzio et al., 2000) Although hepatocytes are the main source of LBP in the body, it is possible that activated AP cells also synthesized and released LBP into the culture media; this phenomenon may have enhanced the effect of LPS *in vitro*. Significant synthesis of LBP has previously been found in extrahepatic tissues (Wang et al., 1998; Dentener et al., 2000). Our results suggest that one of the mechanisms through which LPS treatment affects LH secretion from the AP is downregulating *GnRH-R* gene expression. The changes in the *GnRH-R* mRNA level were similar to those observed in *LH-β*. The level of gene expression does not, however, have to directly parallel the protein level, and it can be supposed that LPS treatment decreased the amount of GnRH-R resulting in the lower sensitivity to GnRH stimulation. It is known that the reduction of GnRH-R on pituitary cells can directly lead to reduced secretion of LH (Fox et al., 1987; Nett et al., 2002; Rispoli and Nett, 2005).

It was also found that LPS administered alone and together with LBP decreased *TLR4* gene expression in the AP. Downregulation of *TLR4* gene expression could be a mechanism contributing to the maintenance of homeostasis in AP cells during LPS treatment. A similar phenomenon was previously described in intestinal epithelial cells (Takahashi et al., 2009).

It is worth mentioning that the observed effects of LPS treatment could at least partially result from the action of pro-inflammatory cytokines on AP cells. It is known that the folliculostellate cells in the pituitary gland are the source as well as the target of pro-inflammatory cytokines, such as IL-1, IL-6 and TNF α (Bilezikjian et al., 2003; Meilleur et al., 2007). A previous report showed that LPS acting *via* the TLR4 receptor stimulated the folliculostellate cells to release proinflammatory cytokines such as IL-6 (Lohrer et al., 2000). These cytokines released from the folliculostellate cells could affect the paracrine secretion of LH from pituitary gonadotropes. It has recently been reported, however, that the pituitary can respond to an immune challenge within a few minutes, and the main pituitary cells that respond are the folliculostellate cells (Parnet et al., 2003). Although folliculostellate cells are non-hormone secreting cells, they do communicate directly with hormone-producing cells. They regulate pituitary function by intercellular communication and play a critical role in endocrine–immune regulation (Bilezikjian et al., 2003). Therefore, the decreased LH secretion from gonadotropes could be an effect of a signal transmitted *via* the folliculostellate cells activated directly by LPS and/or indirectly by the autocrine action of proinflammatory cytokines.

Conclusions

Summarizing, our studies showed that LPS is a potent negative modulator of LH secretion *in vitro*, affecting both its gene transcription and release. The reduced *GnRH-R* gene expression suggests that the decreased secretion of LH could result from lower sensitivity of AP cells to GnRH stimulation. Our results suggest that direct action of LPS on the pituitary gland could be one of the mechanisms *via* which an immune/inflammatory challenge inhibits the reproductive process. It could not be excluded, however, that the observed changes result, at least partially, from the autocrine action of proinflammatory cytokines synthesized and released by folliculostellate cells.

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