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Lipopolysaccharide alters CEBPβ signaling and reduces estradiol production in bovine granulosa cells

Mackenzie J. Dickson[1], I. Martin Sheldon[2] and John J. Bromfield[1*]

Abstract

Background: Bacterial infection of the uterus in postpartum dairy cows limits ovarian follicle growth, reduces blood estradiol concentrations, and leads to accumulation of bacterial lipopolysaccharide (LPS) in ovarian follicular fluid. Although treating granulosa cells with LPS in vitro decreases the expression of the estradiol synthesis enzyme *CYP19A1* and reduces estradiol secretion, the molecular mechanisms are unclear. The transcription factor CCAAT enhancer binding protein beta (CEBPβ) not only facilitates the transcription of LPS regulated cytokines, but also binds to the promoter region of *CYP19A1* in humans, mice, and buffalo. We hypothesized that LPS alters CEBPβ signaling to reduce *CYP19A1* expression, resulting in decreased estradiol secretion.

Methods: Bovine granulosa cells were isolated from small/medium or large follicles and treated with LPS in the presence of FSH and androstenedione for up to 24 h.

Results: Treatment with LPS increased *CXCL8* and *IL6* gene expression and reduced estradiol secretion in granulosa cells from both small/medium and large follicles. However, LPS only reduced *CYP19A1* expression in granulosa cells from large follicles. Treatment with LPS increased *CEBPB* expression and reduced CEBPβ nuclear localization in granulosa cells from small/medium follicles, but not granulosa cells from large follicles.

Conclusions: Although LPS reduces estradiol synthesis in bovine granulosa cells, the effects of LPS on *CYP19A1* and CEBPβ are dependent on follicle size.

Keywords: Granulosa cell, Estradiol, Lipopolysaccharide, Inflammation

Background

Bacterial infections are ubiquitous in the postpartum uterus of the cow and uterine disease develops in up to 40% of cows within three weeks following calving (Sheldon et al. 2009). Disease caused by uterine infection is associated with subfertility and increases the likelihood of cows leaving the herd (Carvalho et al. 2019; LeBlanc et al. 2002). Interestingly, the ovary is rarely a site of bacterial infection, but uterine disease limits dominant follicle growth and reduces estradiol production (Sheldon et al. 2002). Even after the resolution of uterine disease, the granulosa cells that line ovarian follicles exhibit an altered transcriptome compared to healthy cows (Horlock et al. 2020; Piersanti et al. 2019). In parallel, oocytes collected from cows with uterine infection have a reduced capacity to develop to the morula stage after in vitro fertilization and embryo culture (Dickson et al. 2020). Although these observations suggest that uterine infection is associated with a perturbed intrafollicular environment and compromised fertility in cows, the molecular mechanisms are unclear.

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Bacterial components, including lipopolysaccharide (LPS) derived from Gram-negative bacteria cell wall, accumulate in the follicular fluid of cows diagnosed with uterine disease (Herath et al. 2007; Piersanti et al. 2019). Granulosa cells express Toll-like receptor 4 (TLR4), which is the receptor for LPS (Herath et al. 2007), and treating granulosa cells with LPS stimulates the synthesis of interleukin (IL)- 1β, IL-6, IL-8, and tumor necrosis factor alpha (TNFα) (Bromfield and Sheldon 2011; Herath et al. 2007; Price et al. 2013; Shimizu et al. 2012; Williams et al. 2008). Additionally, granulosa cells treated with LPS in vitro reduce estradiol secretion (Herath et al. 2007; Price et al. 2013; Williams et al. 2008), which is likely due to the concurrent reduction of aromatase (*CYP19A1*) expression in granulosa cells of small follicles (< 5 mm), medium follicles (4–8 mm) and dominant follicles (> 8 mm) (Herath et al. 2007; Li et al. 2017; Onnureddy et al. 2015; Price et al. 2013; Shimizu et al. 2012; Yenuganti et al. 2017). However, the molecular mechanisms by which LPS treatment decreases *CYP19A1* expression and subsequently reduces estradiol secretion in cattle remain elusive.

Estradiol production is a coordinated process involving both theca and granulosa cells (Fortune 1986). Steroidogenic acute regulatory protein (STAR) transports cholesterol into the inner mitochondrial matrix of granulosa and theca cells which is then converted to progesterone (Miller 2007); however, only theca cells can convert progesterone to androstenedione which is then utilized by granulosa cells to convert androstenedione to testosterone via 17-beta-hydroxysteroid dehydrogenase (HSD17B1). Granulosa cells then convert testosterone to estradiol via aromatase (CYP19A1) (Yoshimoto and Guengerich 2014). Previous research testing the effects of LPS on steroidogenic capacity of theca cells are inconclusive, as work has focused on the ability of granulosa cells to convert androstenedione to estradiol (Herath et al. 2007; Magata et al. 2014; Shimizu et al. 2016).

The transcription factor, CCAAT enhancer binding protein beta (CEBPβ), is modulated by LPS and also promotes leukocyte transcription of LPS-induced cytokines like IL-6 and TNFα (Greenwel et al. 2000; Stein and Yang 1995). In addition, CEBPβ has been shown to bind a consensus sequence in the *CYP19A1* promoter of buffalo granulosa cells (Yenuganti et al. 2017) and human endometriosis stromal cells (Yang et al. 2002). Interestingly, *Cebpb* knockout results in the upregulation of *Cyp19a1* expression in the mouse ovary (Sterneck et al. 1997). However, there is no consensus about whether CEBPβ activity increases or decreases *CYP19A1* expression. It is unknown if LPS influences the action of CEBPβ or if CEBPβ can modulate *CYP19A1* expression and estradiol secretion in bovine granulosa cells.

Here, we aimed to determine the mechanism by which LPS treatment downregulates granulosa cell *CYP19A1* expression and results in reduced estradiol production in bovine granulosa cells. We hypothesized that LPS alters CEBPβ signaling to reduce *CYP19A1* expression, resulting in decreased estradiol secretion. To test this hypothesis, we employed in vitro culture of bovine granulosa cells from small/medium (2–8 mm) and large (> 8 mm) follicles to determine the role of CEBPβ in LPS-mediated changes to *CYP19A1* expression and estradiol secretion.

Methods

General procedures for granulosa cell isolation, culture and challenge with LPS are derived from previous reports (Bromfield and Sheldon 2011; Price et al. 2013; Horlock et al. 2022; Horlock et al. 2021). Bovine ovaries from cattle of undetermined breeds were obtained as part of the commercial operation of a local abattoir (Florida Beef, Inc., Zolfo Springs, FL) and transported to the laboratory for use within 6 h of collection. Ovaries were transported at 22°C in 0.9% saline containing 1% penicillin/streptomycin (Thermo Fisher Scientific; Walton, MA). At the laboratory, ovaries were washed three times in warm (38.5°C) saline containing 1% penicillin/streptomycin. The use of abattoir tissues for experimentation does not require animal ethics approval from the local University of Florida Institutional Animal Care and Use Committee (IACUC).

Granulosa cell culture

Between 10 and 15 ovaries from 5 to 10 cows were processed together to provide cells for each biological replicate. Cells from small/medium diameter (2–8 mm) follicles were collected by slicing the surface of ovaries with a scalpel blade and vigorously rinsing the ovary in collection medium (Minitube; Verona, WI). Resultant collection medium was then filtered using a sterile 100 µm cell strainer (Corning Inc; Corning, NY) to remove cumulus oocyte complexes and tissue debris. The remaining filtrate was then passed through a sterile 40 µm filter (Thermo Fisher Scientific) to collect granulosa cells. Granulosa cells were retained in the filter and rinsed using complete cell culture medium (Medium 199 (Gibco; Thermo Fisher Scientific), with a final concentration of 10% fetal calf serum (FCS; Corning Inc), 1% insulin–transferrin–sodium selenite (ITS; 10 mg/L human recombinant insulin, 5.5 mg/L human recombinant transferrin, 6.7 µg/L selenious acid; Corning Inc), 1% penicillin/streptomycin (50 IU/mL penicillin and 50 µg/mL streptomycin; Thermo Fisher Scientific), and 1% L-glutamine (2 mM L-alanyl-L-glutamine dipeptide in 0.85% NaCl; GlutaMAX; Thermo Fisher Scientific) (Bromfield and Sheldon 2011). The resultant cell suspension

was centrifuged at 500×*g* for 10 min. A red blood cell lysis was performed on the cell pellet by the addition 900 µL of cell culture grade H_2O (Hyclone; Chicago, IL), immediately followed by the addition of 100 µL of sterile 10× phosphate buffered saline (PBS). Cells were washed with Dulbecco's PBS (DPBS) without calcium or magnesium (Hyclone) by centrifugation at 500×*g* for 10 min. The resultant cell pellet was resuspended in 1 mL of complete culture medium containing hyaluronidase (100 U/mL; Millipore Sigma; Burlington, MA) and vortexed for 10 s every 3 min for 10 min. Cells were again washed by centrifugation at 500×*g* for 10 min in complete cell culture medium. Cell concentration was adjusted to 1.5×10^6 cells/mL and plated (TPP, Trasadingen, Switzerland) in 500 µL (24-well plates for RNA isolation and supernatant) or 2 mL (6-well plates for protein isolation) of complete cell culture medium and cultured at 38.5°C with 5% CO_2 in humidified air.

Cells from large diameter (> 8 mm) follicles were aspirated using a sterile needle and syringe into granulosa cell collection medium (Medium 199, 0.5% BSA (Thermo Fisher Scientific), 20 mM HEPES (Hyclone), 2 mM sodium pyruvate (Gibco), 50 µg/mL heparin (Thermo Fisher Scientific), and 1% penicillin/streptomycin (Bromfield and Sheldon 2011). Following initial aspiration, granulosa cells were treated in the same manner as granulosa cells isolated from small/medium diameter follicles, with the exception of the treatment with hyaluronidase.

For cells from small/medium follicles, non-adherent cells were aspirated from the culture medium after 12–14 h of culture, and adherent granulosa cells were washed in warm DPBS and cultured for a further 24 h in complete culture medium. For cells from large follicles, granulosa cells were cultured undisturbed for 48 h to allow for cell adherence. Immediately prior to the application of treatment, cells were washed in warm DPBS, and all treatments were applied using complete medium (phenol red-free Medium 199 (Gibco) with a final concentration of 10% charcoal-stripped FCS (Corning), 1% ITS, 1% penicillin/streptomycin (50 IU/mL penicillin and 50 µg/mL streptomycin), 1% L-glutamine (GlutaMAX), 1 ng/mL porcine follicle stimulating hormone (Folltropin-V; Vetoquinol, Lavaltrie, Canada), and 1 µM androstenedione (Thermo Fisher Scientific) (Bromfield and Sheldon 2011).

Testing various concentrations of LPS on granulosa cell responses

To determine the impact of LPS concentration on estradiol production, granulosa cells from small/medium follicles were treated with control medium or with medium containing ultrapure LPS (*E. coli* 0111-B4; tlrl-3pelps, Invivogen, San Diego, CA) in sequential ten-fold increasing concentrations, from 1 to 10^4 ng/mL for 24 h (Bromfield and Sheldon 2011). Granulosa cells from large follicles were treated with control medium or with medium containing LPS at concentrations of 10^3 or 10^4 ng/mL for 24 h (Bromfield and Sheldon 2011). The experiment was repeated using 10 independent biological replicates of granulosa cells from small/medium follicles and 9–14 independent biological replicates of granulosa cells from large follicles. Each biological replicate consisted of 10–15 ovaries. Following treatment, supernatants were collected and stored at -20°C, and cells were stored at -80°C in RLT lysis buffer (Qiagen, Hilden, Germany) for RNA isolation or PhosphoSafe extraction buffer (Millipore Sigma) with protease inhibitor (Halt Protease Inhibitor Cocktail; Thermo Fisher Scientific) for protein isolation.

Assessing the temporal effects of LPS on granulosa cell responses

To assess estradiol production over time, granulosa cells from small/medium follicles were treated with either medium alone or medium containing 10^4 ng/mL LPS for 0.5, 2, 4, 8, or 12 h (Bromfield and Sheldon 2011). This experiment was repeated using six independent biological replicates. Following treatment, supernatants were collected and stored at -20°C, and cells were stored in RLT lysis buffer or PhosphoSafe extraction buffer with protease inhibitor at -80°C.

Testing the capacity of excess androstenedione to ameliorate LPS mediated effects on granulosa cells

To determine the impact of androstenedione availability on granulosa cell secretion of estradiol, granulosa cells from small/medium follicles were treated with medium containing a final concentration of 0, 1 or 100 µM androstenedione in the presence or absence of 10^3 ng/mL LPS for 24 h (Gutiérrez et al. 1997). This experiment was performed in six independent biological replicates. Following treatment, supernatants were collected and stored at -20 °C, and cells were stored at -80°C in RLT lysis buffer.

RNA isolation and real time RT-PCR

Total RNA was isolated from granulosa cells using the RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. Quality and quantity of RNA was assessed by an ultraviolet–visible spectrophotometer, Nanodrop2000 (Thermo Fisher Scientific). For verification of cell culture purity, total RNA was isolated from cells at the time of cell isolation prior to plating, at the time of initial treatment (36 or 48 h after initial plating), and 24 h after treatment (60 or 72 h after initial plating). Reverse transcription was performed on 1 µg of RNA using the Verso cDNA synthesis kit (Thermo

Fisher Scientific). Primers for PCR were designed using the NCBI database and are detailed in Table 1. All primers were validated to ensure they met the MIQE guidelines of $r^2 > 0.98$ and efficiency of 90–110% (Bustin et al. 2009), and product size was verified by agarose gel electrophoresis and melt curve analysis. Each PCR reaction consisted of 20 µL containing cDNA, iTaq Universal SYBR green master mix (Bio-Rad, Hercules, CA) and 500 nM of each sequence specific primer with exception of *CYP17A1* which included 300 nM of each primer. A Bio-Rad CFX Connect light cycler was employed with an activation step at 95°C for 30 s followed by 40 cycles consisting of denaturation at 95°C for 5 s followed by annealing and extension at 60°C for 30 s for the two-step protocol. Three genes (*AMH*, *CYP17A1*, *STAR*) required a three-step protocol with an activation step at 95°C for 30 s followed by 40 cycles consisting of denaturation at 95°C for 5 s, primer specific annealing for 5 s (*AMH*, 58°C; *CYP17A1*, 57°C; *STAR*, 54°C) and extension at 60°C for 30 s. A no template negative control, replacing cDNA with water was included for each primer set. Relative mRNA expression for each gene of interest was calculated using the $2^{-\Delta Ct}$ method relative to the geometric mean of the reference genes (*ACTB* and *GAPDH*) after verification of stable expression (Khan et al. 2016).

Standard RT-PCR using Dream Taq Hot Start green PCR master mix (Thermo Fisher Scientific) was performed to evaluate the presence of hematopoietic immune cells (*PTPRC*) and theca cells (*CYP17A1*) in cell preparations. A thermocycler (MultiGene OptimMax Thermal Cycler, Labnet International; Edison, NJ) was employed to perform RT-PCR using an activation step of 95°C for 2 min, and 30 cycles consisting of 95°C for 30 s, specific annealing temperature for 30 s (*PTPRC*, 60°C; *CYP17A1*, 57°C), and extension at 72°C for 1 min, followed by a final extension step of 72°C for 10 min. Amplification products were visualized after agarose gel electrophoresis using Diamond Nucleic Acid Dye (Promega, Madison, WI) and a Gel Doc EZ Gel Documentation System (Bio-Rad).

Table 1 Primer sequences used for real time RT-PCR

Gene	Primer sequence	Accession number
AMH	5′-GTGGTGCTGCTGCTAAAGATG 3′-TCGGACAGGCTGATGAGGAG	NM_173890.1
ACTB	5′-CAGAAGCACTCGTACGTGGG 3′-TTGGCCTTAGGGTTCAGGG	NM_173979.3
CEBPB	5′-ACAGCGACGAGTACAAGATCC 3′-GACAGTTGCTCCACCTTCTTCT	NM_176788.1
CXCL8	5′-GCAGGTATTTGTGAAGAGAGCTG 3′-CACAGAACATGAGGCACTGAA	NM_173925.2
CYP17A1	5′-CTCCAGCATTGGCGACCTTA 3′-GAAGCGCTCGGGCATGAA	XM_024985958.1
CYP19A1	5′-CGCAAAGCCTTAGAGGATGA 3′-ACCATGGCGATGTACTTTCC	NM_174305.1
FSHR	5′-GCAGTCGAACTGAGGTTTGTT 3′-TTGGAGAACACGTTTGCCTCT	NM_174061.1
GAPDH	5′-AGGTCGGAGTGAACGGATTC 3′-ATGGCGACGATGTCCACTTT	NM_001034034.2
HDAC1	5′-TTACGACGGGGATGTTGGAA 3′-GGCTTTGTGAGGGCGATAGA	NM_001075460.1
HDAC10	5′-CTCGGCTTCACTGTCAACCT 3′-TCAGGGTCGAACTCAAAGGC	NM_001037444.2
HSD17B1	5′-CGTGAGGGATGCAGATTCCA 3′-GTTACACACCAGCACGTCCA	NM_001102365.1
IL6	5′-ATGACTTCTGCTTTCCCTACCC 3′-GCTGCTTTCACACTCATCATTC	NM_173923.2
LHCGR	5′-TGCCTTTGACAACCTCCTCAAT 3′-GATGCTTAGGTATTTTAACCGAGG	NM_174381.1
PTPRC	5′-CTCGATGTTAAGCGAGAGGAAT 3′-TCTTCATCTTCCACGCAGTCTA	NM_001206523.1
STAR	5′-AGAAGGGTGTCATCAGAGCG 3′-TGGTCCTTGAGGGACTTCCA	NM_174189.3

Protein extraction and immunoblotting

Samples were isolated in PhosphoSafe extraction buffer with protease inhibitor before quantifying protein concentration using a bicinchoninic protein assay (Thermo Fisher Scientific). Equal concentrations of protein (10 µg) were loaded into 10% precast polyacrylamide gels (Mini-Protean TGX; Bio-Rad) and subjected to electrophoresis at 100 V for 1 h. Separated proteins were then transferred to nitrocellulose membranes using wet transfer for 4 h at 45 V at 4°C. The Revert 700 total protein stain (Li-cor, Lincoln, NE) was employed to verify equal protein loading using a Li-Cor Odyssey CLx infrared imager (Li-cor). For immunoblotting, membranes were blocked overnight in 5% BSA in tris-buffered saline (TBS) with 0.1% Tween (TBS/T, pH 7.6) or 3% milk in TBS/T. Primary antibodies were diluted in 5% BSA in TBS/T (mouse anti-human aromatase, 1:250; MCA2077S; Bio-Rad) or TBST alone (rabbit anti-human CEBPβ, 1:1000; NBP1-46,179 Novus Biologicals, Littleton, CO) and incubated for 6 h at 4°C with agitation. Membranes were washed three times with agitation for 5 min in TBS/T and incubated with an appropriate secondary antibody (1:5000, Li-COR) conjugated with infrared dye 680RD or 800CW in blocking solution for 1 h at room temperature (20–22°C) with agitation. Membranes were washed three times for 5 min in TBS/T and visualized on a Li-COR Odyssey CLx infrared imager. Target protein was normalized to total protein stain (Additional file 1: Fig. S1) from the same blot using the western blot function on the Li-COR Odyssey CLx infrared imager.

Immunocytochemistry for nuclear localization of CEBPβ

Chamber slides (Thermo Fisher Scientific) were used to culture granulosa cells for immunocytochemistry. Slides were preincubated with 100 µL of FCS for 30 min at room temperature (20–22°C) prior to aspiration and subsequent addition of granulosa cells. Granulosa cells were plated directly onto slides at a concentration of 2×10^5 cells/mL in complete culture medium. Before application of treatments, granulosa cells isolated from small/medium follicles were cultured for 24 h and granulosa cells isolated from large follicles were cultured for 48 h to allow cells to adhere to the serum coated glass slide. After pre-incubation in complete culture medium, granulosa cells were washed in DPBS and medium was replaced with serum-free medium overnight prior to application of treatment to ensure a baseline quiescent localization of CEBPβ. Immediately prior to treatment, cells were washed in DPBS and treated with either complete medium alone (containing 10% FCS) or complete medium containing 10^4 ng/mL LPS for 6 h or 24 h. Immediately following the treatment period, cells were fixed in 2% paraformaldehyde for 15 min at room temperature (20–22°C). Cells were washed three times in DPBS and stored at 4°C. Slides were washed in PBS with 0.1% Tween (PBS/T) twice for 5 min each, and permeabilized in PBS containing 0.1% Triton-X for 10 min. Cells were washed three times in PBS/T and incubated in blocking solution containing 1% normal goat serum and 1% BSA in PBS for 1 h at room temperature (20–22°C). Cells were then incubated with rabbit anti-human CEBPβ antibody (1:500, Novus Biologicals) overnight with agitation at 4°C before washing three times in PBS/T and incubation with anti-rabbit Alexa Fluor 488 secondary antibody (1:800, Thermo Fisher Scientific) for 1 h at room temperature. Cells were washed and mounted using 50% glycerol in PBS containing 1.5 µg/mL Hoechst 33342 (Thermo Fisher Scientific). Slides were imaged using a Zeiss Axio Observer 7 (Zeiss, Jena, Germany) fitted with an Andor DSD2 Confocal Unit and Zyla Plus 4.2-megapixel camera using a Plan-Apochromat 40× objective lens. A minimum of seven independent fields of view were quantified in granulosa cells of five replicates from small/medium follicles and seven replicates from large follicles. A no primary control was included to assess background staining. Nuclear localization of CEBPβ was quantified using ImageJ by splitting the image into individual color channels, converting to a binary image and overlaying nuclear Hoechst location with CEBPβ labeling and calculating the mean fluorescence intensity of CEBPβ for each nucleus (Schneider et al. 2012).

Quantification of estradiol production

Estradiol accumulation in supernatants was measured by enzyme immunoassay (Estradiol sensitive ELISA, DRG International, Springfield, NJ) according to the manufacturer's instructions, and previously validated for cell culture supernatants (Bromfield et al. 2013). All samples were run in duplicate, and the limit of detection was 10.6 pg/mL. Samples were diluted if needed in standard zero buffer. The intra-assay coefficient of variation ranged from 1.49 to 5.6% depending on the experiment. The ELISA is reported to have 0% cross reactivity to androstenedione, corticosterone, progesterone or testosterone.

Statistical analysis

All statistical analyses were performed using SPSS v26 (IBM Corporation, Armonk, NY). A general linear model was used to analyze estradiol, gene expression, protein abundance, and fluorescent intensity data. Gene expression data were log transformed for normality following a Shapiro–Wilk test for normality. Fixed effects depended on experiment, but replicate was always considered a random effect. For dose-dependent experiments, dose was used as the fixed effect and the least significant difference function was used for pairwise comparisons between doses. For time-course experiments and immunocytochemistry, time, LPS and the interaction between time and LPS were used as fixed effects and the least significant difference function was used for pairwise comparisons within a single timepoint. For the androstenedione experiment, androstenedione supplementation, LPS, and the interaction between androstenedione and LPS were used as fixed effects and the least significant difference function was used for pairwise comparisons within a single androstenedione dose. Statistical significance was set at $P \leq 0.05$. Graphs were made using GraphPad Prism v9 (San Diego, CA) and depict estimated marginal means ± standard error of the mean, unless otherwise stated.

Results

Lipopolysaccharide increases mRNA expression of inflammatory mediators and decreases estradiol secretion in granulosa cells from small/medium follicles

The purity of granulosa cells at the time of isolation from small/medium follicles was assessed to determine the presence of contaminating theca (*CYP17A1*) or $CD45^+$ hematopoietic immune cells (*PTPRC*) (Herath et al. 2007; Richards et al. 2018). Expression of *PTPRC* (Additional file 1: Fig. S3A) or *CYP17A1* (Additional file 1: Fig. S3B) was not detected in granulosa cells at the time of isolation from small/medium follicles, suggesting granulosa cell isolation yielded pure cultures. However, moderate

CYP17A1 expression was observed in granulosa cells isolated from large follicle after a total of 72 h of culture, suggesting that a small number of contaminating theca cells have proliferated during the culture of granulosa cells from large follicles (Additional file 1: Fig. S3B).

Granulosa cells from small/medium follicles were treated with increasing concentrations LPS for 24 h in the presence of 1 ng/mL FSH and 1 μM androstenedione (Fig. 1; $n=10$). Exposure of granulosa cells to 10^4 ng/mL LPS increased mRNA expression of both *CXCL8* and *IL6* compared to controls (Fig. 1A, B; $P \leq 0.05$). Granulosa cell mRNA expression of *CYP19A1* or *HSD17B1* was not affected by treatment with LPS for 24 h (Fig. 1C, D). In the absence of LPS, granulosa cells accumulated 11.3 ± 2.5 ng/mL of estradiol in culture medium by 24 h. Interestingly, granulosa cells treated with either 10^3 or 10^4 ng/mL of LPS decreased estradiol accumulation by 29.8% and 47.6%, respectively compared to controls (Fig. 1E; $P \leq 0.05$). Treatment of granulosa cells with 10^4 ng/mL LPS for 24 h did not affect aromatase abundance measured by western blot (Fig. 1F; $n=4$). As granulosa cells from small/medium follicles accumulated less estradiol in response to LPS in the absence of altered *CYP19A1* or *HSD17B1*, the mRNA expression of factors known to contribute to estradiol synthesis were evaluated (Fig. 2; $n=10$). Expression of *AMH*, *ESR1*, *FSHR*, *LHGHR*, *STAR*, *HDAC1*, or *HDAC10* mRNA were all detected in granulosa cells from small/medium follicles but were not significantly affected by treatment with LPS for 24 h (Fig. 2A–G).

Because estradiol accumulation decreased after exposure of granulosa cells to 10^3 ng/mL of LPS for 24 h with no change to the expression of *CYP19A1, HSD17B1* mRNA or aromatase abundance, we evaluated gene expression in granulosa cells from small/medium follicles treated with 10^4 ng/mL of LPS for 0.5, 2, 4, 8 and 12 h anticipating temporal changes to gene expression

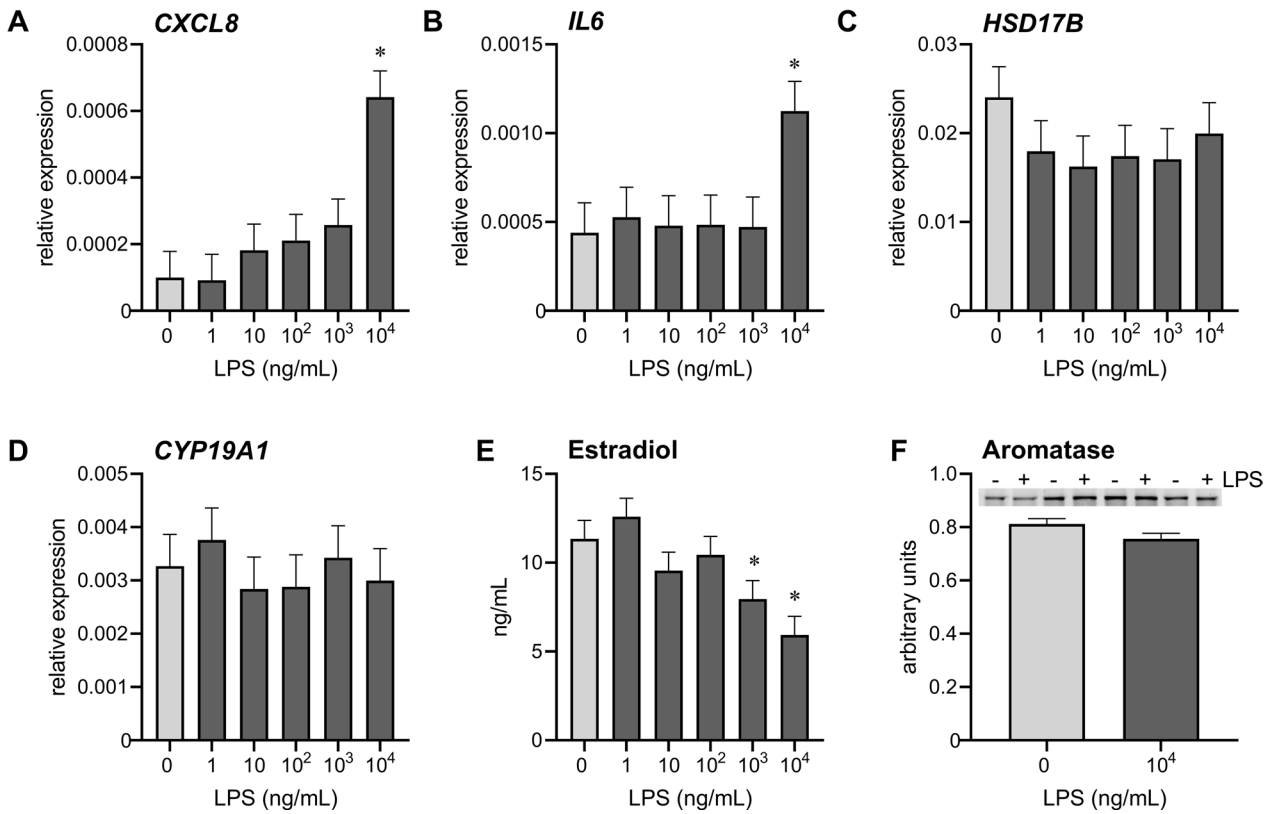

Fig. 1 Effect of LPS on mRNA expression of inflammatory mediators and estradiol synthesis in granulosa cells from small/medium follicles. Bovine granulosa cells from small/medium diameter (2–8 mm) follicles were exposed to control medium (light bars) or LPS (1–10^4 ng/mL; dark bars) for 24 h. Gene expression of *CXCL8* (**A**), *IL6* (**B**), *HSD17B* (**C**) and *CYP19A1* (**D**) were quantified by qPCR ($n=10$), accumulation of 17β-estradiol (**E**) was quantified using ELISA ($n=10$), and abundance of aromatase (**F**) was evaluated by western blot ($n=4$). Gene expression data were log transformed for normality and a general linear model with fixed effect of LPS dose and random effect of replicate followed by least significant difference pairwise comparisons were used to analyze each dose compared to the medium alone control. Data are presented as the estimated marginal means ± S. E. M. * indicates $P \leq 0.05$ compared to medium alone control. Western blot of total protein is presented in Additional file 1: Fig. S1A and uncropped blots are presented in Additional file 1: Fig. S2A

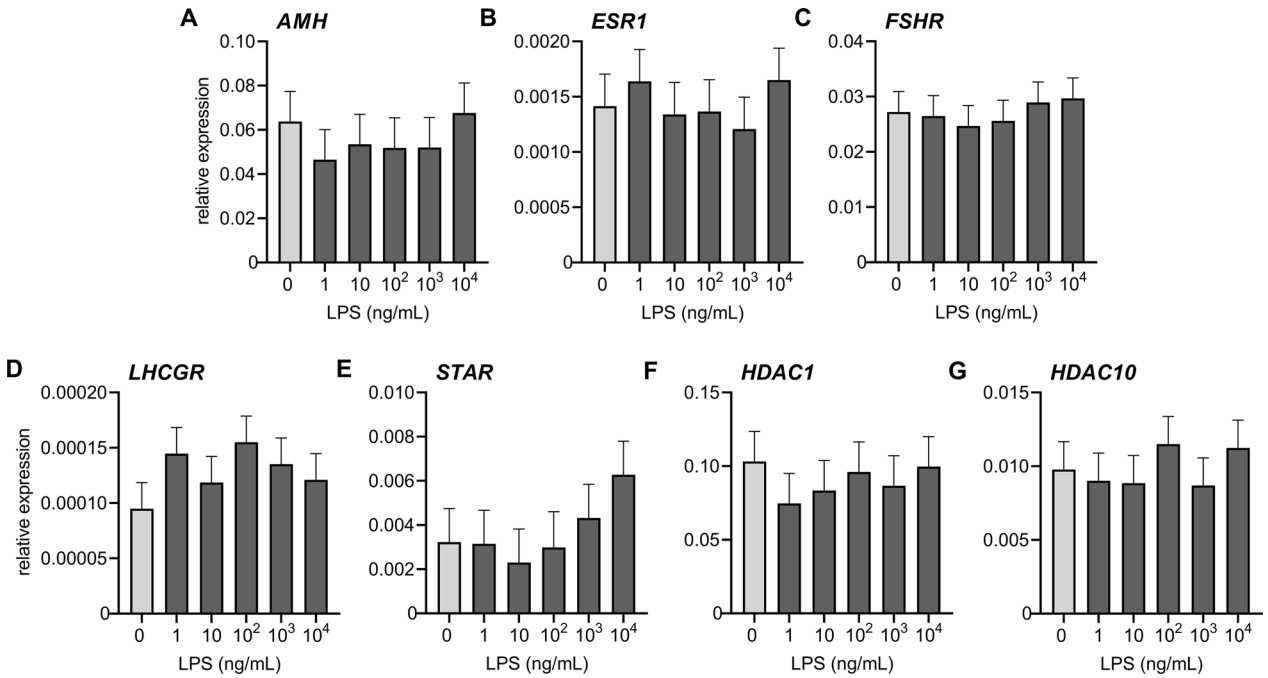

Fig. 2 Effect of LPS on mRNA expression of factors that mediate estradiol synthesis in granulosa cells from small/medium follicles. Bovine granulosa cells from small/medium diameter (2–8 mm) follicles were exposed to control medium (light bars) or LPS (1–10^4 ng/mL; dark bars) for 24 h. Gene expression of *AMH* (**A**), *ESR1* (**B**), *FSHR* (**C**), *LHCGR* (**D**), *STAR* (**E**), *HDAC1* (**F**), and *HDAC10* (**G**) were quantified by PCR ($n=10$). Data were log transformed for normality and a general linear model with fixed effect of LPS dose and random effect of replicate followed by least significant difference pairwise comparisons were used to analyze each dose compared to the medium alone control. Data are presented as the estimated marginal means ± S. E. M. There was no statistical differences detected for any gene

that were not observed after 24 h (Fig. 3; $n=6$). Treatment of granulosa cells from small/medium follicles with LPS increased mRNA expression of *CXCL8* by 0.5 h compared to controls and remained elevated by 12 h (Fig. 3A; $P \leq 0.05$). Treatment with LPS increased mRNA expression of *IL6* after 4 h compared to controls that remained elevated by 12 h (Fig. 3B; $P \leq 0.05$). Expression of *CYP19A1*, *HSD17B1*, and *STAR* (Fig. 3C–E) mRNA was not significantly affected by LPS treatment compared to controls; however, the mRNA expression of *CYP19A1* did increase over time ($P \leq 0.05$) independent of LPS treatment (Fig. 3D). Interestingly, LPS did not affect estradiol accumulation after 12 h compared to controls (Fig. 3F).

Collectively, granulosa cells of small/medium follicles responded to LPS by increasing mRNA expression of inflammatory mediators, and reduced estradiol secretion in the absence of altered *CYP19A1* expression or aromatase abundance.

Excess androstenedione does not ameliorate LPS mediated reduction to estradiol accumulation in granulosa cells from small/medium follicles

Androstenedione supplementation is required for cultured granulosa cells to synthesize estradiol. As we observed decreased estradiol secretion after treatment with LPS for 24 h with no significant change in *CYP19A1* or *HSD17B1* mRNA expression or aromatase abundance, we aimed to determine if androstenedione availability contributed to LPS-mediated estradiol decreases in the presence of FSH (Fig. 4; $n=6$). In the absence of androstenedione, estradiol accumulation of granulosa cells was 0.04 ± 0.03 ng/mL after 24 h and was similar (0.08 ± 0.04 ng/mL) following treatment with 10^3 ng/mL of LPS. Overall, androstenedione increased 24 h estradiol accumulation independent of LPS (Fig. 4A; $P \leq 0.05$). Interestingly, 100 μM androstenedione decreased 24 h estradiol accumulation compared to 1 μM androstenedione, regardless of treatment with LPS (Fig. 4A; $P \leq 0.05$). Supplementation with androstenedione (1 or 100 μM) decreased expression of *STAR* mRNA compared to cells with no androstenedione (Fig. 4D; $P \leq 0.05$), while supplementation with 1 μM androstenedione, but not 100 μM androstenedione, increased the mRNA expression of *CYP19A1* compared to cells cultured in the absence of androstenedione (Fig. 4C; $P \leq 0.05$). Androstenedione supplementation did not affect *HSD17B1* mRNA expression (Fig. 4B). Overall, treatment with LPS had no effect on mRNA expression of *HSD17B*, *CYP19A1*

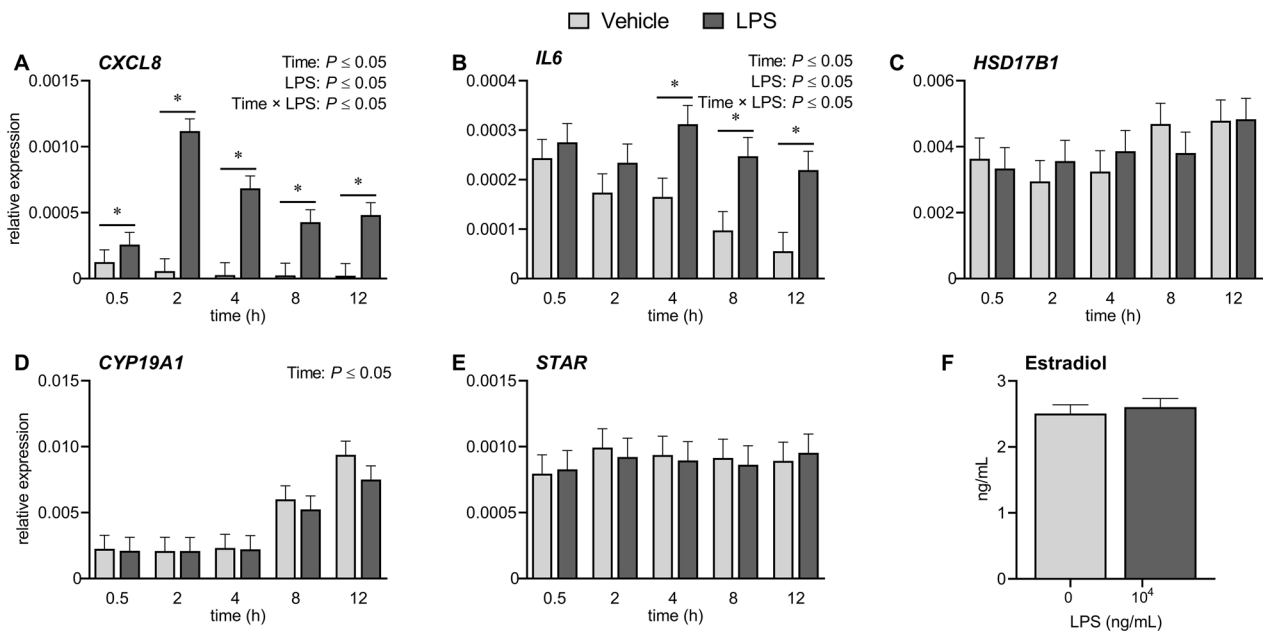

Fig. 3 Acute effects LPS on mRNA expression of inflammatory mediators and estradiol synthesis in granulosa cells from small/medium follicles. Bovine granulosa cells from small/medium diameter (2–8 mm) follicles were exposed to control medium (light bars) or 10^4 ng/mL LPS (dark bars) for 0.5, 2, 4, 8, or 12 h. Gene expression of *CXCL8* (**A**), *IL6* (**B**), *HSD17B1* (**C**), *CYP19A1* (**D**) and *STAR* (**E**) were quantified by qPCR ($n=6$). Accumulation of 17β-estradiol (**F**) was quantified at 12 h using ELISA ($n=6$). Gene expression data were log transformed and data were analyzed using a general linear model with replicate as a random effect and the fixed effects of LPS, time, and the interaction of LPS and time followed by least significant difference pairwise comparisons. Data are presented as the estimated marginal means ± S. E. M. Statistical significance for each test is presented when significant. * indicates $P \leq 0.05$ compared to medium alone at a specific timepoint

or *STAR* (Fig. 4B-D). These data suggest that androstenedione supplementation is required by granulosa cells from small/medium follicles for estradiol accumulation, excess androstenedione impairs estradiol accumulation, and LPS mediated reductions in estradiol accumulation is independent of androstenedione availability.

Lipopolysaccharide increases mRNA expression of inflammatory mediators and decreases estradiol secretion in granulosa cells from large follicles

To assess the impact of follicle size on the capacity of granulosa cells to respond to LPS, granulosa cells were isolated from large diameter (<8 mm) follicles and treated with 10^3 or 10^4 ng/mL LPS or control medium for 24 h in the presence of 1 ng/mL FSH and 1 μM androstenedione (Fig. 5; $n=9$–14). After initial isolation from large follicles, cells displayed moderate *PTPRC* mRNA expression which was negligible 48 h after initial isolation and prior to the application of treatment and was absent 72 h after initial isolation (Additional file 1: Fig. S3C). The mRNA expression of *CYP17A1* was absent at the of initial isolation, but steadily increased up to 72 h after the end of the treatment period, suggesting a very small number of contaminating theca cells that proliferated during culture (Additional file 1: Fig. S3D).

Treatment with LPS for 24 h increased mRNA expression of *CXCL8* and *IL6* in granulosa cells from large follicles compared to controls (Fig. 5A, B; $P \leq 0.05$). Expression of *HSD17B1* mRNA was decreased after LPS treatment compared to controls (Fig. 5C; $P \leq 0.05$), while treatment with 10^4 ng/mL LPS reduced *CYP19A1* mRNA expression by 44.2% compared controls (Fig. 5D; $P > 0.05$). In the absence of LPS, granulosa cells from large follicles accumulated 9.5 ± 4.7 ng/mL of estradiol in 24 h which was reduced by 27.7% in cells treated with 10^4 ng/mL of LPS (Fig. 5E; $P \leq 0.05$). Despite LPS reducing estradiol accumulation and *CYP19A1* gene expression, LPS had no effect on aromatase abundance measured by western blot (Fig. 5F; $n=4$).

Lipopolysaccharide alters the expression of CEBPB mRNA and intracellular localization of CEBPβ in granulosa cells

Expression of *CEBPB* mRNA was increased in granulosa cells of small/medium follicles treated with 10^4 ng/mL of LPS in the presence of 1 ng/mL FSH and 1 μM androstenedione for 24 h compared to controls (Fig. 6A; $P \leq 0.05$), but not in granulosa cells from large follicles (Fig. 6C; $n=6$–9). While CEBPβ protein was detected in granulosa cells of small/medium and large

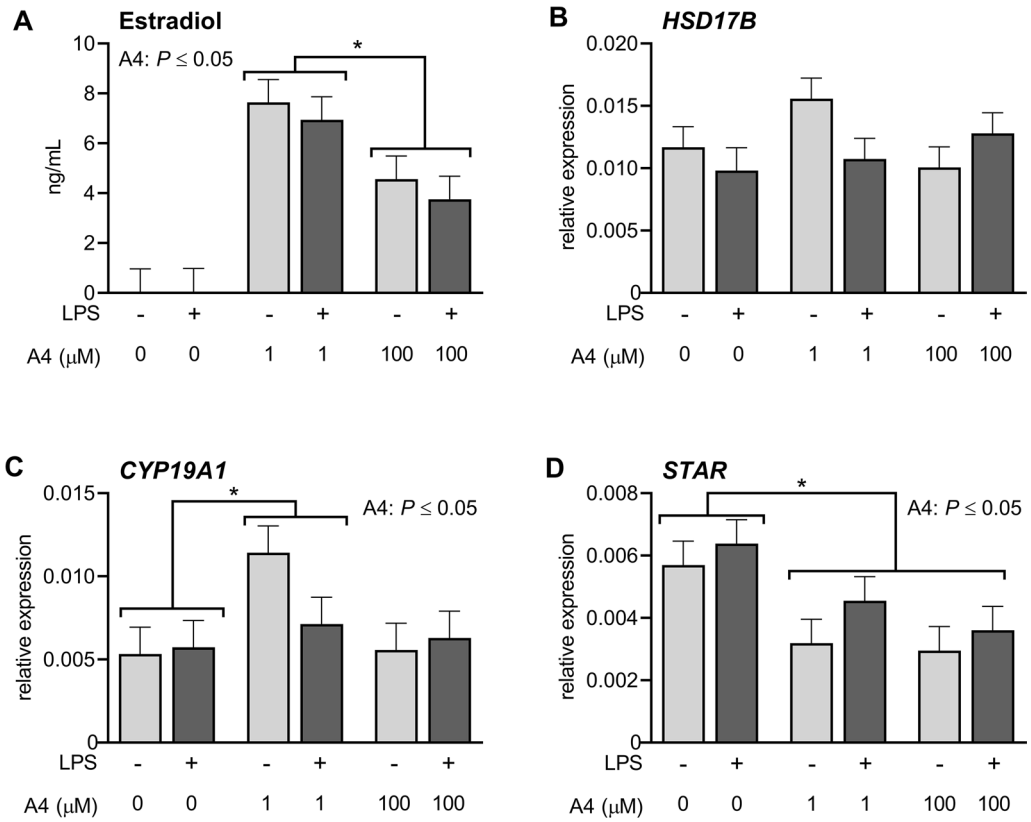

Fig. 4 Excess androstenedione does not prevent LPS-mediated estradiol accumulation of granulosa cells from small/medium follicles. Bovine granulosa cells from small/medium diameter (2–8 mm) follicles were exposed to control medium (light bars) or 10^3 ng/mL LPS (dark bars) for 24 h in medium supplemented with 0, 1 or 100 μM androstenedione (A4). Accumulation of 17β-estradiol (**A**) was quantified using ELISA and gene expression of *HSD17B* (**B**), *CYP19A1* (**C**) and *STAR* (**D**) were quantified by qPCR ($n=6$). Data were analyzed using a general linear model with replicate as a random effect and the main effects of LPS, androstenedione, and the interaction of LPS and androstenedione followed by least significant difference pairwise comparisons. Data are presented as the estimated marginal means ± S. E. M. Statistical significance for each test is presented when significant and * indicates $P \leq 0.05$

follicles by western blot, treatment with LPS did not alter total CEBPβ abundance (Fig. 6B and D; $n=4$).

The effect of LPS on CEBPβ nuclear localization was evaluated in granulosa cells treated with 10^4 ng/mL of LPS or medium alone for 6 or 24 h in the presence of 1 ng/mL FSH and 1 μM androstenedione. Nuclear localization of CEBPβ was evaluated in 163.8 ± 24.8 granulosa cells from small/medium follicle per replicate (Fig. 7A; $n=5$). Treatment of granulosa cells from small/medium follicles with LPS decreased nuclear abundance of CEBPβ by 27.3% compared to controls, regardless of the treatment time (Fig. 7B; $P \leq 0.05$). Nuclear localization of CEBPβ was evaluated in 104.5 ± 77.3 granulosa cells from large follicles per replicate (Fig. 8A; $n=7$). Treatment of granulosa cells of large follicles with LPS tended to decrease nuclear abundance of CEBPβ by 37.9% compared to controls, regardless of the treatment time (Fig. 8B; $P=0.09$). Collectively, these data suggest that LPS treatment reduces CEBPβ nuclear localization without altering total CEBPβ abundance.

Discussion

The mechanistic link between LPS treatment and reduced *CYP19A1* gene expression and estradiol synthesis in bovine granulosa cells is unknown. Here, bovine granulosa cells were isolated from small/medium follicles or large follicles and treated with LPS in vitro. As expected LPS increased gene expression of the pro-inflammatory mediators, *IL6* and *CXCL8*, and decreased estradiol accumulation after 24 h in both granulosa cell populations. Interestingly, LPS only reduced *CYP19A1* gene expression in granulosa cells from large follicles after 24 h, but not in granulosa cells from small/medium follicles, while aromatase protein abundance was not affected by LPS in either granulosa cell population. In parallel, gene expression of *CEBPB* was increased after LPS treatment in granulosa cells from small/medium follicles but remained

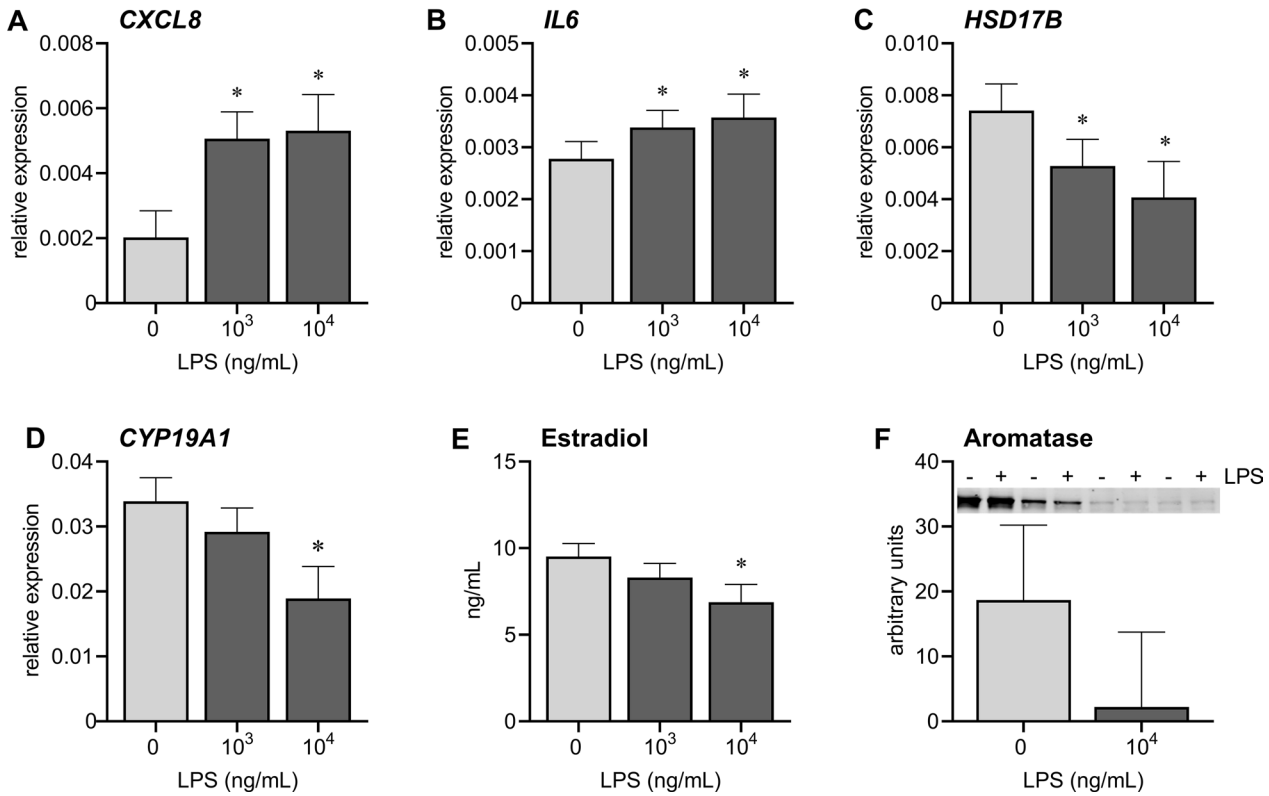

Fig. 5 Effect of LPS on mRNA expression of inflammatory mediators and estradiol synthesis in granulosa cells from large follicles. Bovine granulosa cells from large diameter (> 8 mm) follicles were exposed to control medium (light bars) or LPS (10^3 or 10^4 ng/mL; dark bars) for 24 h. Gene expression of *CXCL8* (**A**), *IL6* (**B**), *HSD17B* (**C**) and *CYP19A1* (**D**) were quantified by qPCR ($n = 9$–14), accumulation of 17β-estradiol (**E**) was quantified using ELISA ($n = 9$ or 12), and abundance of aromatase (**F**) was evaluated by western blot ($n = 4$). Gene expression data were log transformed for normality and a general linear model with fixed effect of LPS dose and random effect of replicate followed by least significant difference pairwise comparisons were used to analyze each dose compared to medium alone control. Data are presented as the estimated marginal means ± S. E. M. Statistical significance is depicted by * and indicates $P \leq 0.05$ compared to medium alone control. Western blot of total protein is presented in Additional file 1: Fig. S1B and uncropped blots are presented in Additional file 1: Fig. S2B

unchanged in granulosa cells from large follicles, while total protein abundance of CEBPβ was not altered by LPS in either granulosa cell population. Most interesting, LPS reduced nuclear translocation of CEBPβ in granulosa cells from small/medium follicles within 6 h which was still evident after 24 h.

While the cause of uterine infection associated subfertility is unknown, it likely involves organs other than the uterus, including the ovary. Despite the rarity of ovarian infection, uterine diseases are associated with reduced dominant follicle growth and estradiol production (Sheldon et al. 2002). Cows with a uterine infection accumulate LPS in dominant follicles, with LPS concentrations positively correlated with the severity of disease (Herath et al. 2007; Piersanti et al. 2019). Under culture conditions, purified granulosa cells mount a response to LPS via TLR4 to increased synthesis of inflammatory mediators such as IL-1β, IL-6, and IL-8 (Bromfield and Sheldon 2011; Price et al. 2013), and reduce estradiol secretion and *CYP19A1* gene expression (Herath et al. 2007; Price et al. 2013; Shimizu et al. 2012). Furthermore, the transcriptome of granulosa cells from cows with prior uterine disease is altered months after the resolution of uterine disease, suggesting long-term effects of infection on the follicular environment (Horlock et al. 2020; Piersanti et al. 2019). In the buffalo, histone deacetylases and CEBPβ regulate *CYP19A1* expression (Mehta et al. 2015; Yenuganti et al. 2017), while in mice, *Cebpb* knock out renders females infertile because granulosa cells cannot luteinize and corpus luteum function fails (Sterneck et al. 1997). Therefore, we hypothesized that LPS alters CEBPβ signaling to reduce *CYP19A1* expression and decrease estradiol secretion in bovine granulosa cells.

Granulosa cells begin expressing *CYP19A1* mRNA after follicle recruitment (4 mm) and continue to increase expression and estradiol synthesis until follicle are large

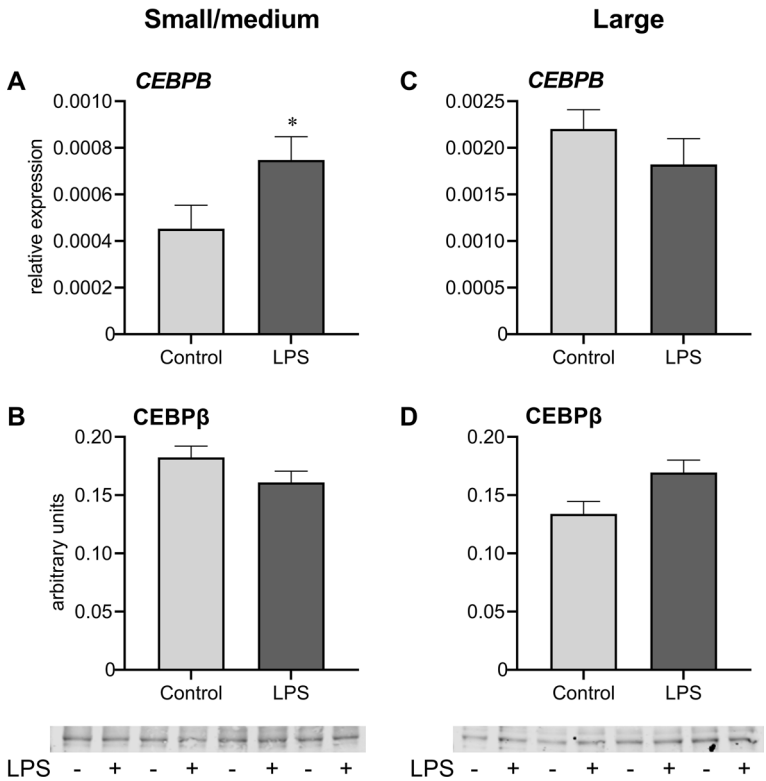

Fig. 6 Effect of LPS on granulosa cell *CEBPB* mRNA expression and CEBPβ abundance. Bovine granulosa cells from small/medium (**A**, **B**) follicles or large (C-D) follicles were exposed to control medium (light bars) or 10^4 ng/mL LPS (dark bars) for 24 h. Gene expression of *CEBPB* (**A**, **C**) was quantified by qPCR ($n = 6$–9), and protein abundance of CEBPβ (**B**–**D**) was evaluated by western blot ($n = 4$). Gene expression data were log transformed for normality and a general linear model with fixed effect of LPS and random effect of replicate. Data are presented as the estimated marginal means ± S. E. M. * indicates $P \leq 0.05$ compared to medium alone control. Western blot of total protein is presented in Additional file 1: Fig. S1C, D and uncropped blots are presented in Additional file 1: Fig. S2C, D

and preovulatory (Xu et al. 1995). In response to the preovulatory surge of luteinizing hormone, granulosa cells rapidly decrease *CYP19A1* mRNA expression (Komar et al. 2001; Richards 1994). Previous studies demonstrate that granulosa cells from small (< 5 mm), medium (4–8 mm) or large (> 8 mm) follicles reduce estradiol synthesis in response to LPS (Herath et al. 2007; Price et al. 2013; Shimizu et al. 2012). In parallel, granulosa cells from large follicles but not small or medium follicles decrease *CYP19A1* mRNA expression in response to LPS (Herath et al. 2007). As such, developmental stage of follicles predicates the response to LPS regarding estradiol synthesis.

The transcription factor CEBPβ modulates cellular processes involved in inflammation and fertility (Poli 1998; Sterneck et al. 1997). Synthesis of CEBPβ is induced by cytokines such as IL-6 or TNFα (Akira et al. 1990; Greenwel et al. 2000), while inflammatory mediators, including *IL6*, *CXCL8*, and *TNF* have consensus sequences for CEBPβ in their promoter regions (Matsusaka et al. 1993; Stein and Yang 1995; Wedel et al. 1996), suggesting regulation by feedback loops. Indeed, while CEBPβ has the capacity to regulate cytokine expression and possibly aromatase expression, the proinflammatory cytokines IL-6 or TNFα that are increased after LPS exposure can themselves also regulate estradiol synthesis (Taylor and Terranova 1996; Basini et al. 2002).

The role for CEBPβ in granulosa cell function varies between species. In mice and rats, human chorionic gonadotropin (hCG) induces *Cebpb* expression in granulosa cells from antral follicles (Sirois and Richards 1993), and aromatase expression remains elevated in *Cebpb* knockout mice (Sterneck et al. 1997), suggesting a regulatory role for CEBPβ on aromatase in the mouse. Conversely, in bovine granulosa cells from large follicles (8–12 mm) CEBPβ abundance decreases following treatment with hCG (Liu et al. 1999) when estradiol synthesis and *CYP19A1* mRNA expression decrease.

Expression of *CYP19A1* mRNA is regulated by multiple promoters depending on the specific tissue type, with the proximal promoter II acting as the primary regulator of *CYP19A1* mRNA expression in granulosa cells

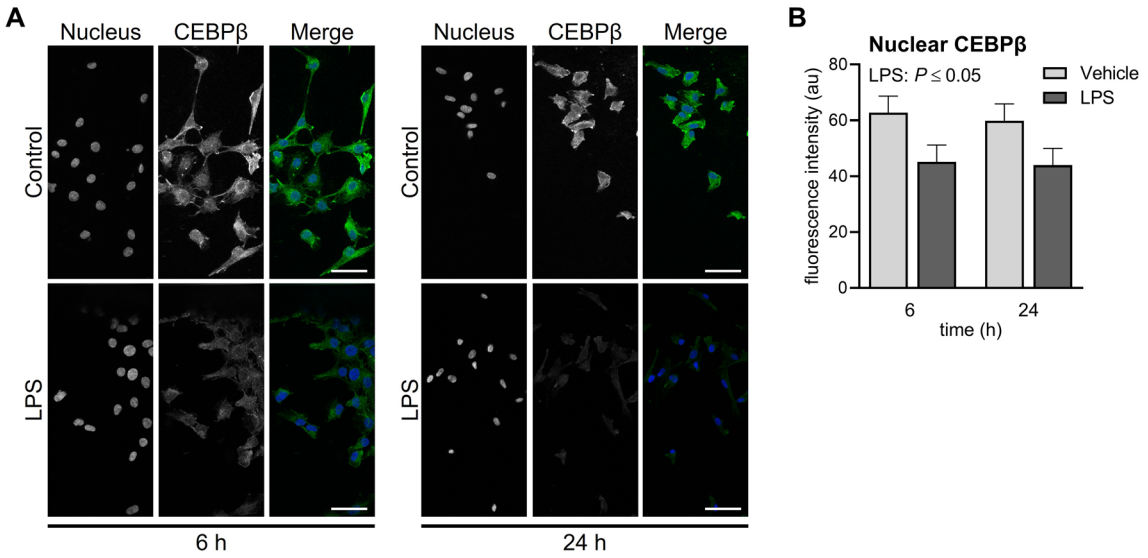

Fig. 7 Effect of LPS on CEBPβ nuclear translocation in granulosa cells from small/medium follicles. Bovine granulosa cells from small/medium diameter (2–8 mm) follicles were exposed to control medium (light bars) or 10^4 ng/mL LPS (dark bars) for 6 or 24 h. Nuclear localization of CEBPβ was assessed using immunocytochemistry immediately after the treatment period ($n = 5$ for each time point). (**A**) Representative images show individual nuclear and CEBPβ staining and a merge image showing nuclear (blue) and CEBPβ staining (green). Each scale bar represents 50 µm. (**B**) Nuclear translocation of CEBPβ was quantified using ImageJ. Data were analyzed using a general linear model with replicate as a random effect and the main effects of LPS and time, and presented as the estimated marginal means ± S. E. M

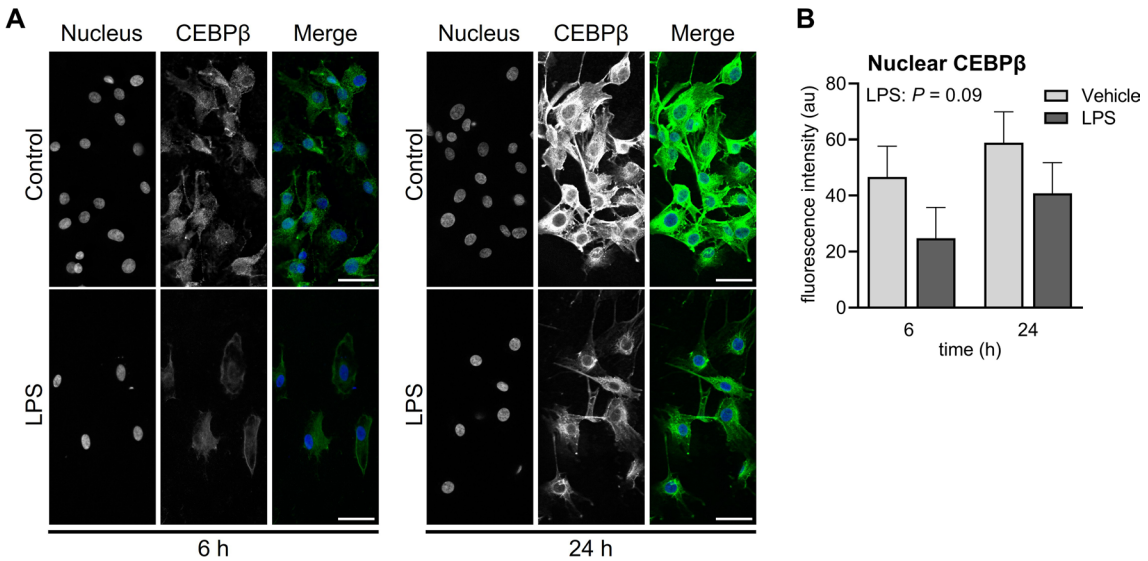

Fig. 8 Effect of LPS on CEBPβ nuclear translocation in granulosa cells from large follicles. Bovine granulosa cells from large diameter (> 8 mm) follicles were exposed to control medium (grey bars) or 10^4 ng/mL ultrapure LPS (black bars) for 6 h or 24 h. Nuclear localization of CEBPβ was assessed using immunocytochemistry immediately after the treatment period ($n = 7$ foreach time point). (**A**) Representative images show individual nuclear and CEBPβ staining and a merge image showing nuclear (blue) and CEBPβ staining (green). Each scale bar represents 50 µm. (**B**) Nuclear translocation of CEBPβ was quantified using ImageJ. Data were analyzed using a general linear model with replicate as a random effect and the main effects of LPS and time, and presented as the estimated marginal means ± S. E. M

of mice, humans, and cows (Fürbass et al. 1997; Golovine et al. 2003; Means et al. 1991), however, promoter I.1 also contributes to *CYP19A1* mRNA expression in bovine granulosa cells (Fürbass et al. 1997; Lenz et al. 2004). Interestingly, there is strong sequence homology (80%) between humans and cows for promoter II but

not for promoter I.1 (< 50%). The bovine *CYP19A1* promoter I.1 contains two consensus sequences for CEBPβ and four CAAT transcription elements which are predicted to act as binding sites for CEBPβ, while promoter II contains three CAAT elements (Fürbass et al. 1997). The regulatory role of CEBPβ on promoter II of *CYP19A1* is conflicting. Binding of CEBPβ to promoter II in human adipose fibroblasts (Zhou et al. 2001), or promoter I.3/II sites in uterine leiomyomas cells stimulates *CYP19A1* gene expression (Ishikawa et al. 2008), while *CEBPB* knockdown decreases *CYP19A1* mRNA expression (Ishikawa et al. 2008), suggesting CEBPβ is a positive regulator of *CYP19A1* gene expression. Conversely, overexpression of *CEBPB* reduces *CYP19A1* promoter II activity in endometrial stromal cells (Yang et al. 2002). As the role for CEBPβ in the regulation of *CYP19A1* is not consistent amongst cell types or species, the relationship between a CEBPβ and *CYP19A1* expression in bovine granulosa cells cannot be inferred.

There is a binding site for CEBPβ in proximal promoter II of the *CYP19A1* gene in buffalo granulosa cells, and LPS increases CEBPβ binding to promoter II, increases total abundance and nuclear localization of CEBPβ, reduces *CYP19A1* mRNA expression and reduces estradiol synthesis in granulosa cells from small/medium buffalo follicles (Yenuganti et al. 2017). In contrast, our data show that LPS increased *CEBPB* gene expression, reduced CEBPβ nuclear localization, decreased estradiol accumulation, but did not affect *CYP19A1* mRNA expression in bovine granulosa cells from small/medium follicles; however, in granulosa cells from large bovine follicles LPS reduced nuclear localization of CEBPβ and estradiol accumulation while *CYP19A1* mRNA expression was again not affected. Although both cows and buffalo are part of the Bovidae family, there may be differences in CEBPβ activity in response to LPS and subsequent control of estradiol synthesis. Biological variation between experimental replicates may also play a role in the interpretation of data presented here, as such increasing the number of biological replicates may produce different statistical findings while not necessarily addressing the biological mechanisms of LPS affected estradiol synthesis.

To demonstrate a mechanistic link between CEBPβ and estradiol synthesis, a knockdown of *CEBPB* mRNA in bovine granulosa cells is required. We have been successful in knocking down targets in bovine granulosa cells previously using siRNA (Bromfield and Sheldon 2011), and while we could knockdown *CEBPB* mRNA in bovine endometrial cells (data not shown) we failed in our attempts to knockdown *CEBPB* in bovine granulosa cells here using siRNA, GapmeR or shRNA technologies. The future use of chromatin immunoprecipitation (ChIP) sequencing would allow us to identify the genes CEBPβ is regulating in response to LPS and to determine if CEBPβ is directly involved in *CYP19A1* regulation in bovine granulosa cells. The role for other LPS mediated factors in estradiol regulation also needs to be considered. For example, ERK1/2 signaling is a primary driver of LPS/TLR4 responses, and mice lacking *Erk1* and *Erk2* in granulosa cells have erroneous *Cyp19a1* downregulation following the preovulatory LH surge (Fan et al. 2011, 2009). Perhaps, there are intermediary molecules, such as ERK1/2, that contribute to *CYP19A1* regulation. In the absence of a specific *CEBPB* knock down in bovine granulosa cells, caution must be taken to interpret associations described here in place of mechanistic causation.

Conclusions

In conclusion, LPS increased inflammatory responses, and decreased estradiol accumulation of bovine granulosa cells from small/medium and large follicles. However, only granulosa cells of large follicles decreased expression of *CYP19A1* mRNA after treatment with LPS. Interestingly, LPS decreased CEBPβ nuclear translocation in granulosa cells of small/medium follicles. These data suggest that while LPS reduces estradiol synthesis in bovine granulosa cells, there are likely follicle stage specific mechanisms of estradiol regulation at play that may include CEBPβ.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s43170-022-00133-3.

Additional file 1: Figure S1. Total protein stains from western blots. Each protein sample (10 µg) was loaded into a 10% gel and run for 1 h at 100 V. Proteins were transferred to nitrocellulose and stained for total protein. Membranes were imaged and quantified on the Li-Cor Odyessy CLx. Membranes used for western blot of granulosa cells from small/medium follicles (A, C) or large follicles (B, D) to detect aromatase (A-B) or CEBPβ (C-D) are shown and correspond to blots presented in the results section. **Figure S2.** Uncropped western blots. Each protein sample (10 µg) was loaded into a 10% gel and run for 1 h at 100 V. Proteins of granulosa cells from small/medium follicles (A, C) or large follicles (B, D) were transferred to nitrocellulose and blotted for aromatase (A-B) or CEBPβ (C-D). Membranes were imaged and quantified on the Li-Cor Odyessy CLx. Membranes shown below correspond those shown as cropped images in the results section (Fig. 1F, 5F, 6B and 6D). **Figure S3.** Assessment of granulosa cell culture purity. Bovine granulosa cells (GC) from small/medium (A-B) follicles or large (C-D) follicles were tested for the expression of the immune cell marker *PTPRC* (A, C) or the luteal cell marker *CYP17A1* (B, D). Expression was evaluated after initial isolation (A) or following a period of culture, as indicated. Whole blood, testis, liver, corpus luteum (CL), peripheral blood mononuclear cells (PBMC) and water (H_2O) were used as controls.

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Author contributions
MJD, IMS, JJB conceived the experiments and wrote and proofread the manuscript, MJD conducted the experiments. All authors read and approved the final manuscript.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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