

Ovarian follicular cells have innate immune capabilities that modulate their endocrine function

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Abstract

Oestrogens are pivotal in ovarian follicular growth, development and function, with fundamental roles in steroidogenesis, nurturing the oocyte and ovulation. Infections with bacteria such as *Escherichia coli* cause infertility in mammals at least in part by perturbing ovarian follicle function, characterised by suppression of oestradiol production. Ovarian follicle granulosa cells produce oestradiol by aromatisation of androstenedione from the theca cells, under the regulation of gonadotrophins such as FSH. Many of the effects of *E. coli* are mediated by its surface molecule lipopolysaccharide (LPS) binding to the Toll-like receptor-4 (TLR4), CD14, MD-2 receptor complex on immune cells, but immune cells are not present inside ovarian follicles. The present study tested the hypothesis that granulosa cells express the TLR4 complex and LPS directly perturbs their secretion of oestradiol. Granulosa cells from recruited or dominant follicles are exposed to LPS *in vivo* and when they were cultured in the absence of immune cell contamination *in vitro* they produced less oestradiol when challenged with LPS, although theca cell androstenedione production was unchanged. The suppression of oestradiol production by LPS was associated with down-regulation of transcripts for aromatase in granulosa cells, and did not affect cell survival. Furthermore, these cells expressed TLR4, CD14 and MD-2 transcripts throughout the key stages of follicle growth and development. It appears that granulosa cells have an immune capability to detect bacterial infection, which perturbs follicle steroidogenesis, and this is a likely mechanism by which ovarian follicle growth and function is perturbed during bacterial infection.

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Introduction

Oestrogens are intimately involved in the growth and development of ovarian follicles from the early antral stage to ovulation, with fundamental roles in steroidogenesis, nurturing the oocyte, ovulation and the subsequent formation of the corpus luteum (CL; Matzuk *et al.* 2002, Schams & Berisha 2002). Oestradiol is produced by granulosa cell aromatisation of androgens secreted by the theca cells (Fortune 1994). Oestradiol synergises with the gonadotrophins to regulate the expression of follicle-stimulating hormone (FSH) and luteinising hormone (LH) receptors on granulosa cells, which are important developmental checkpoints in the lifespan of the follicle at recruitment and selection respectively (Dierich *et al.* 1998, Ma *et al.* 2004). Towards the end of the ovarian cycle, secretion of oestradiol by the dominant follicle stimulates the

pituitary LH surge, which induces ovulation (Moenter *et al.* 1990). However, cattle that ovulate a smaller follicle with lower oestradiol concentrations form a smaller CL with lower peripheral plasma progesterone concentrations (Perry *et al.* 2005, Robinson *et al.* 2005). Progesterone is critical for implantation, and the recognition and maintenance of pregnancy (Spencer *et al.* 2004). Hence, perturbation of follicle growth and oestradiol production has important consequences for ovulation, conception and pregnancy.

Uterine bacterial infections are commonly acquired by humans and animals during coitus and after parturition. Each year 350 million new, mainly bacterial, sexually transmitted infections occur in adults of reproductive age (http://www.who.int/topics/sexually_transmitted_infections), the majority of which are initially asymptomatic, but the consequences range from subfertility to severe pelvic inflammatory disease

(PID; Quayle 2002, Butler 2004). Mammalian fertility is also compromised by PID associated with *post partum* bacterial infections. *Bos taurus* is an economically important species, where considerable infertility is caused by bacterial contamination of the uterus, which is ubiquitous after parturition (Dohmen *et al.* 2000, Sheldon *et al.* 2002), with 40% of animals developing PID and infection persisting >3 weeks in 15% of cows as endometritis (Sheldon & Dobson 2004). The gram-negative *Escherichia coli* is the first and the most common pathogenic bacteria isolated from the uterus (Dohmen *et al.* 2000, Sheldon *et al.* 2002); and lipopolysaccharide (LPS), the main pathogenic moiety of *E. coli*, is detectable in the plasma of cows with uterine infection (Mateus *et al.* 2003). Infertility is not only associated with uterine damage but also with perturbation of the ovarian cycle. Disease associated with *E. coli* or infusion of LPS suppresses oestradiol production and follicular growth in a range of mammals (Xiao *et al.* 1998, Battaglia *et al.* 2000, Sheldon *et al.* 2002). However, the availability of suitable tissue for research is a major constraint to advancing knowledge of the effects of bacterial infection on ovarian function in humans. Monovulatory domestic ruminants such as *B. taurus* represent a physiologically relevant model to study these basic mechanisms (Campbell *et al.* 2003). Furthermore, organisms infecting the uterus, such as *Escherichia* and *Tritrichomonas* species, are similar in humans and cattle (Sheldon *et al.* 2002, Kamiyama *et al.* 2004, Singh *et al.* 2004, Herath *et al.* 2006a), making the cow a good model for studying uterine disease and immunity.

The host response to infection depends on innate immunity in which intrinsic mechanisms are responsible for recognising and responding to pathogen challenge (Janeway *et al.* 2001). Toll-like receptors (TLRs), which are usually associated with professional immune cells, recognise pathogen-associated molecules (Beutler 2004, Akira *et al.* 2006) and engagement with TLRs initiates a signalling cascade that stimulates the production of immune mediators, which orchestrate the immune response (Akira & Takeda 2004, Beutler 2004). However, the presence of physical barriers and the limitation of antigen at immune privileged sites raise the question whether cells in isolated areas express TLRs. Granulosa cells reside within the basement membrane of the ovarian follicle, separating them from the local vasculature and preventing immune cell transmigration (Petrovska *et al.* 1996). However, the basement membrane of the ovarian follicle is highly porous to molecules like LPS and the molecular mass cut-off is calculated to be 100–850 kDa (Rodgers *et al.* 1999). Since *E. coli* infection or LPS perturbs ovarian follicle development and suppresses oestradiol production *in vivo*, the present study tested the hypothesis that granulosa cells express the TLR4 complex and LPS directly perturbs their secretion of oestradiol.

Results

Presence of LPS in ovarian follicular fluid

The concentrations of LPS in follicular fluid collected from normal postpartum animals were below the limits of detection in 16 of 19 animals (mean 0.06 ± 0.04 ng/ml; range 0–0.8 ng/ml). However, concentrations of LPS were higher in seven cows with clinical endometritis (mean 176.1 ± 112 ng/ml, $P < 0.05$; range 4.3–875.2 ng/ml). The follicular fluid concentrations in animals with subclinical endometritis were intermediate between the normal and clinical cases, with 4 of 8 cows below the limit of detection for mild cases (mean 0.7 ± 0.3 ng/ml; range 0–0.8 ng/ml) and 8 of 24 cows below the limit of detection for moderate cases (mean 4.8 ± 1.8 ng/ml; range 0–40.0 ng/ml). For follicles soaked in LPS *in vitro*, the concentrations of LPS in follicular fluid were higher than control follicular fluid for medium-sized follicles (0.5 ± 0.7 ng/ml versus 0; $P < 0.05$) or large follicles (74.2 ± 31.1 ng/ml versus 0; $P < 0.01$).

LPS does not affect androstenedione production by theca cells

Production of oestradiol by granulosa cells is dependent on the aromatisation of theca-derived androstenedione and the stage of follicle growth or development (Fortune 1994). Hence, cultured theca cells were challenged with LPS to determine whether *E. coli* affects androstenedione production. However, LPS treatment for 48 h did not affect the production of androstenedione, regardless of the follicle size from which the cells were isolated (Fig. 1A–C). In addition, there was no effect of LPS after 96 h treatment (data not shown) and the LPS challenge did not affect cell numbers ($P > 0.05$; Fig. 1D–F).

Oestradiol production is directly inhibited following LPS treatment of granulosa cells

Since peripheral plasma oestradiol concentrations are lower following uterine infection *in vivo* (Sheldon *et al.* 2002), and LPS does not modulate the production of androstenedione by theca cells *in vitro*, we investigated whether LPS had a direct effect on oestradiol production by granulosa cells. In the presence of optimum FSH and androstenedione concentrations as determined previously (Gutierrez *et al.* 1997), LPS inhibited oestradiol production by bovine granulosa cells, with the greatest impact on cells from dominant (> 8 mm diameter) and recruited (4–8 mm) follicles (Fig. 2A–C). In addition, LPS also inhibited progesterone production by bovine granulosa cells from dominant and recruited follicles (Fig. 2D–F). The inhibition of oestradiol (Fig. 3A–C), but not progesterone (Fig. 3D–F), was still observed at 96 h after LPS treatment. However, LPS treatment was not associated with significant changes in cell numbers (Fig. 3G–I). To determine if granulosa cells were sensitive to lower concentrations of LPS associated

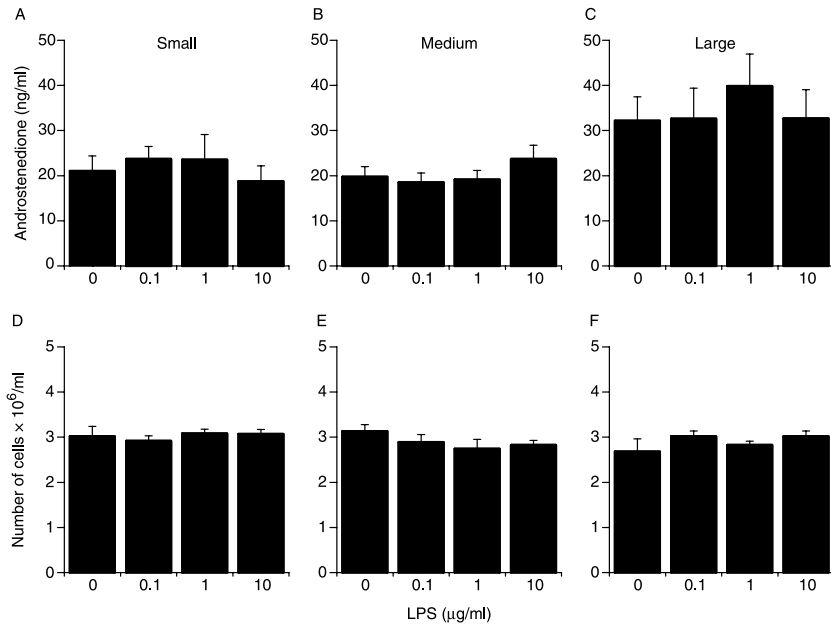


Figure 1 Androstenedione production of theca cells isolated from (A) small (<4 mm diameter), (B) medium (4–8 mm diameter) or (C) large (>8 mm diameter) bovine follicles. Theca cells were treated with LPS at the concentrations indicated. After 48 h treatment, supernatants were harvested and androstenedione production was measured by RIA. The number of theca cells ($\times 10^6$ /ml) after 96 h culture are indicated (D to F). Numerical values are presented as the mean \pm S.E.M. of three experiments.

with subclinical uterine disease, cells were treated with 0.1 ng/ml LPS, which reduced oestradiol production by granulosa cells from dominant follicles (2.6 ± 0.8 vs 4.1 ± 0.8 ng/ml, $P < 0.05$) and tended to reduce oestradiol production by cells from recruited follicles (1.9 ± 0.5 vs 3.4 ± 0.8 ng/ml, $P = 0.09$) compared with control. Treatment of granulosa cells with 0.1, 1 and 10 μ g/ml of a detoxified form of LPS had no effect on oestradiol secretion by cells from dominant follicles (1.4 ± 0.3 , 1.4 ± 0.2 and 1.5 ± 0.2 ng/ml) compared with control cells (1.5 ± 0.2 ng/ml).

To determine whether the decrease in oestradiol production in granulosa cells from medium and large

follicles was due to changes in responsiveness to FSH and/or the down-regulation of the enzyme required for oestradiol synthesis, expression of mRNA for the FSH receptor (FSHR) and aromatase were analysed. Following LPS challenge of granulosa cells isolated from dominant (>8 mm) follicles, only aromatase mRNA levels were down-regulated (Fig. 4A and C; FSHR, $P = 0.11$). Analysis of oestradiol receptor α (ER α), ER β and LH receptor (LHR) mRNA expression was determined to explore the effects of LPS on granulosa cell function. Granulosa cells isolated from recruited and dominant follicles expressed ER α mRNA, while only granulosa cells isolated from dominant follicles

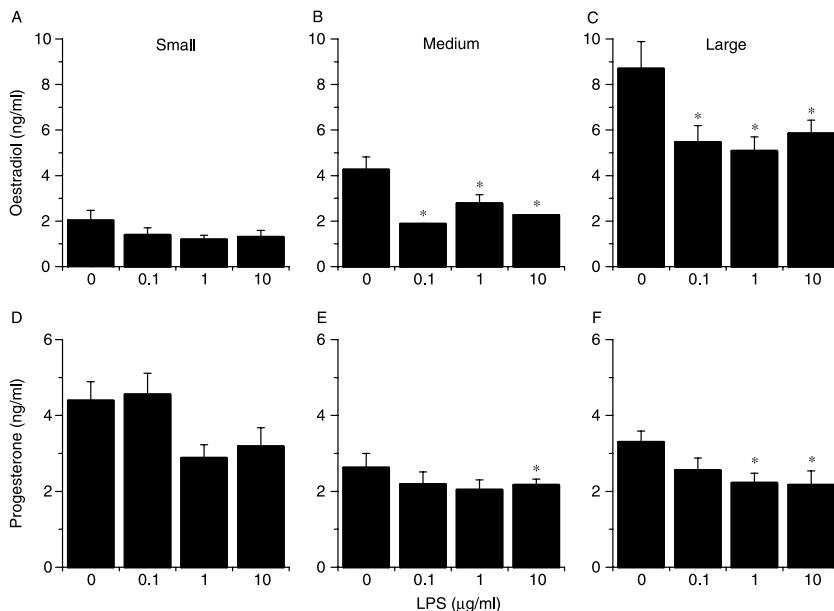


Figure 2 Oestradiol and progesterone production of granulosa cells isolated from (A and D) small (<4 mm diameter), (B and E) medium (4–8 mm diameter) or (C and F) large (>8 mm diameter) bovine follicles. Granulosa cells were treated with LPS at the concentrations indicated. After 48 h treatment, supernatants were harvested and steroid production was measured by RIA. * $P < 0.05$ compared with control, within follicle size. Numerical values are presented as the mean \pm S.E.M. of three experiments.

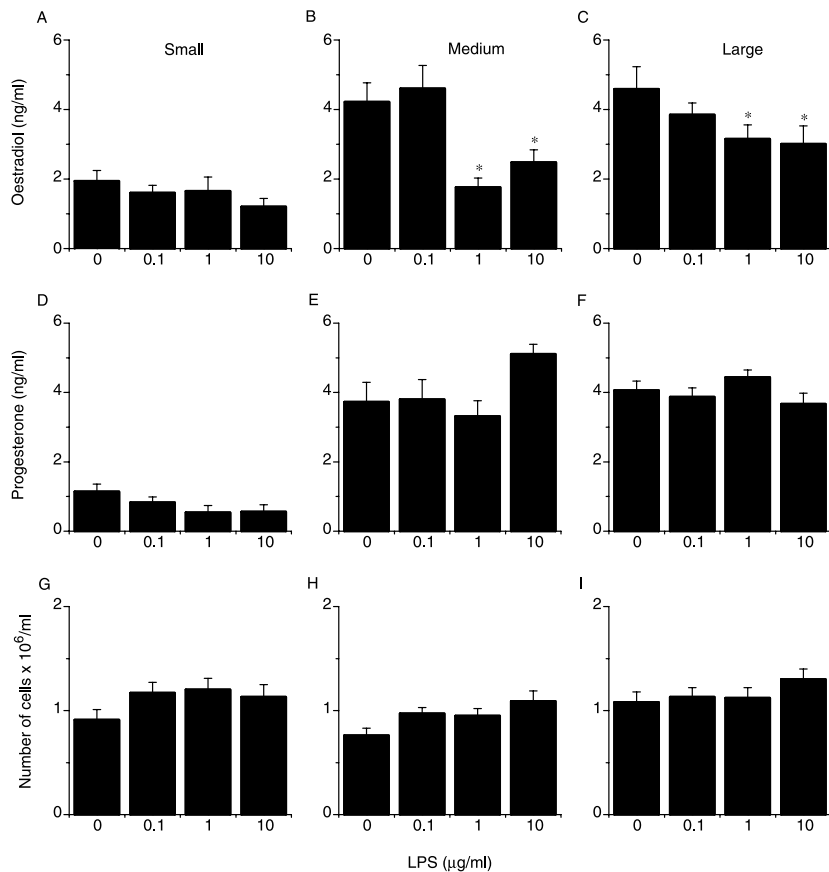


Figure 3 Oestradiol and progesterone production of granulosa cells isolated from (A and D) small (<4 mm diameter), (B and E) medium (4–8 mm diameter) or (C and F) large (>8 mm diameter) bovine follicles. Granulosa cells were treated with LPS at the concentrations indicated. After 96 h treatment, supernatants were harvested and steroid production was measured by RIA. The number of granulosa cells ($\times 10^6/\text{ml}$) after 96 h culture are indicated (G to I). * $P < 0.05$ compared with control, within follicle size. Numerical values are presented as the mean \pm s.e.m. of three experiments.

expressed LHR mRNA and expression was not affected by LPS treatment (Fig. 4A). Transcripts for ER β mRNA were not detectable in control or LPS-treated granulosa cells at 48 h, but were expressed at 0 h by freshly isolated granulosa cells (data not shown).

Granulosa cells express immune mediators

Interleukin-1 (IL-1) plays an important role in immunity as well as in ovarian cell function (Spicer & Alpizar 1994, Janeway *et al.* 2001, Gerard *et al.* 2004). Consequently, the expression of IL-1 α and IL-1 β mRNA by granulosa cells was determined following LPS challenge. Granulosa cells isolated from medium and large follicles expressed transcripts for IL-1 α (Fig. 4B) but not IL-1 β mRNA (data not shown). There was no significant difference in the IL-1 α mRNA expression following LPS treatment of cells isolated from the large follicle ($P = 0.12$, Fig. 4C).

In response to LPS treatment, immune cells produce TNF α and NO (Janeway *et al.* 2001, Akira & Takeda 2004, Beutler 2004). Since granulosa cells responded to LPS, TNF α and NO were measured in the supernatants of cell cultures treated with LPS, but concentrations were below the limits of detection. Consequently, granulosa cells were analysed for the expression of TNF α and NO

synthase (NOS2, formerly iNOS, inducible NOS) mRNA. Granulosa cells expressed TNF α but not NOS2 mRNA (Fig. 4B). As observed with IL-1 α , TNF α mRNA was detected in granulosa cells isolated from medium and large follicles, but there was no significant regulation of TNF α mRNA following LPS treatment ($P = 0.14$, Fig. 4C).

Granulosa cells express TLR4, MD-2 and CD14 mRNA

To ensure that the response to LPS was not mediated via contaminating immune cells, the expression of the pan-leukocyte marker, CD45, was determined by PCR. Granulosa cell cultures were free of contaminating immune cells (Fig. 5A). As granulosa cells responded to LPS challenge directly, we analysed the mRNA expression of the LPS receptor complex: TLR4, CD14 and MD-2. Granulosa cells isolated from medium and large follicles expressed TLR4, CD14 and MD-2 mRNA (Fig. 5B).

Discussion

Uterine infection with *E. coli* or infusion of LPS perturbs ovarian antral follicle growth and function (Xiao *et al.* 1998, Battaglia *et al.* 2000, Sheldon *et al.* 2002). In the

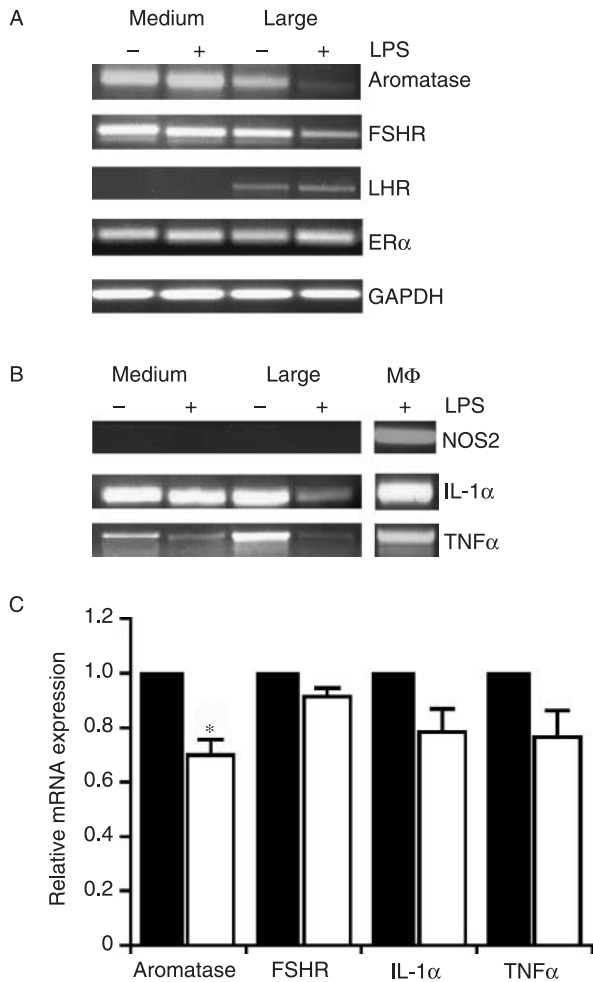


Figure 4 Analysis of gene expression by granulosa cells associated with reproductive function. Granulosa cells isolated from medium (4–8 mm diameter) or large (>8 mm diameter) follicles were stimulated with 10 μ g/ml LPS for 48 h and harvested, RNA was isolated and reverse transcribed as described in Materials and Methods. cDNA was analysed for the presence of (A) aromatase, FSHR, LHR, ER α , and (B) NOS2, IL-1 α and TNF α transcripts using the indicated primer pairs (Table 1). A representative result is shown ($n=3$). (C) PCR bands were analysed and are represented as LPS treatment (open bar) relative to nil control (closed bar) for granulosa cells isolated from large (>8 mm diameter) follicles. Results are presented as the mean \pm s.e.m. of three experiments. * $P<0.05$ compared with control.

present study, we found LPS in follicular fluid from animals with uterine disease and treatment of granulosa cells with LPS *in vitro* suppressed oestradiol production in cells from recruited and dominant follicles. Furthermore, the pure populations of granulosa cells from the different stages of follicle growth expressed TLR4, CD14 and MD-2 mRNA transcripts, which constitute the specific receptor complex for LPS. The observations that theca cell androstenedione production was unaffected by LPS treatment but that granulosa cell aromatase transcripts were regulated provides an insight into a mechanism by which LPS may mediate its effect on

follicle growth, development and function in the mammalian ovary.

In the whole animal, the effects of bacteria or LPS are most evident in large or pre-ovulatory antral follicles of several species (Xiao *et al.* 1998, Battaglia *et al.* 2000, Sheldon *et al.* 2002). In cattle with uterine infection, ovarian follicle growth is suppressed from 8 mm diameter, around the time of dominant follicle selection (Sheldon *et al.* 2002, Campbell *et al.* 2003). The lower peripheral plasma oestradiol concentrations are evident in the days before ovulation, but as oestradiol concentrations are in the pg/ml range, it would be difficult to detect subtle differences in the whole animal before this (Sheldon *et al.* 2002). However, infusion of LPS suppresses plasma oestradiol concentrations within 24 h, delaying the LH surge and ovulation (Suzuki *et al.* 2001).

To substantiate the concept that LPS is present in the follicular fluid of animals with uterine disease, ovarian follicular fluid was aspirated *in vivo* from animals in which the severity of uterine disease had been characterised (Moussavi *et al.* 2007). Animals with clinical disease had concentrations of LPS that ranged up to 0.8 μ g/ml and normal animals did not have measurable concentrations of LPS in their ovarian follicular fluid, while animals with subclinical disease had intermediate concentrations. The high concentrations of LPS in ovarian follicular fluid in disease animals is lower than LPS concentrations reported in the uterus but higher than those reported in peripheral plasma (Dohmen *et al.* 2000, Mateus *et al.* 2003, Williams *et al.* 2007). This suggests that there may be some localised transfer of LPS by the intimate vasculature that links the uterus and ovary, as is the case for prostaglandin F $_{2\alpha}$ (Ginther 1974, Ford *et al.* 1979). To support further the concept that LPS can cross the basement membrane of the ovarian follicle, dissected follicles were maintained in culture media containing 10 μ g/ml LPS for 18 h *in vitro*. Despite the lack of active vasculature, LPS was found in the follicular fluid of medium and particularly large follicles, probably reflecting the surface area available for diffusion of LPS. Taken together these data support the *in vivo* observations that LPS has a localised effect in the ovarian follicle of cattle and sheep (Battaglia *et al.* 2000, Sheldon *et al.* 2002).

Oestradiol is produced by granulosa cell aromatisation of theca-derived androgens under the stimulation of gonadotrophins (Fortune 1994). In the present study, treatment of theca cells with LPS did not affect androstenedione production or cell survival, whereas oestradiol secretion was suppressed by LPS treatment of granulosa cells, and the effect was most marked in cells from medium and large follicles, concurring with the whole animal observations. Androstenedione and oestradiol secretion from the theca and granulosa cells respectively were maintained for 96 h under serum-free

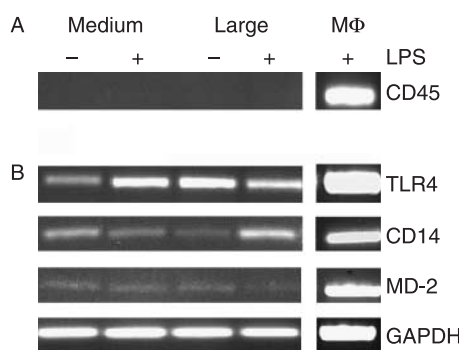


Figure 5 Analysis of LPS receptor complex and immune mediator gene expression by granulosa cells. Granulosa cells isolated from medium (4–8 mm diameter) or large (>8 mm diameter) follicles were stimulated with 10 µg/ml LPS for 48 h and harvested, RNA was isolated and reverse transcribed as described in Materials and Methods. cDNA was analysed for the presence of (A) CD45, (B) TLR4, CD14, MD-2, GAPDH, using the indicated primer pairs (Table 1). A representative result is shown ($n=3$).

culture conditions, as previously described (Gutierrez *et al.* 1997, Glistler *et al.* 2005); and the LPS suppression of granulosa cell oestradiol secretion was also maintained in the present study. This would appear to be a direct effect of LPS on granulosa cells as they were cultured in the presence of previously determined optimal concentrations of androstenedione and FSH and no further addition of androstenedione or FSH was made to the treatment cultures (Gutierrez *et al.* 1997); furthermore, cell survival was unaffected even after 96 h LPS treatment. The sensitivity of granulosa cells was confirmed further by the reduced oestradiol secretion when cells were treated with 0.1 ng/ml LPS and the specificity of the LPS response confirmed by treatment with detoxified LPS, which did not affect oestradiol secretion. The present bovine data contrast somewhat with the rat where LPS suppressed theca cell androstenedione production, although LPS also perturbed LH-stimulated oestradiol production from rat granulosa cells without affecting cell viability, similar to the present study (Taylor & Terranova 1995, 1996). However, there are likely to be differences between species and different stages of follicular development. The strength of the present study is that granulosa cells were derived from follicles reflecting the physiological stages of development in a biologically relevant species where disease causes infertility.

Oestradiol secretion by granulosa cells is dependent on gonadotrophins binding to G-protein-coupled receptors driving aromatisation of androstenedione (Dierich *et al.* 1998, Ma *et al.* 2004). In the whole animal, LPS can perturb ovarian follicle function by disruption of pituitary LH secretion directly or by suppressing peripheral plasma oestradiol concentrations (Battaglia *et al.* 2000, Suzuki *et al.* 2001, Karsch *et al.* 2002). However, effects on the pituitary are only part of the explanation as peripheral plasma oestradiol

concentrations are lower in the face of normal LH concentrations (Battaglia *et al.* 2000); and plasma FSH concentrations are unaffected by uterine infection (Sheldon *et al.* 2002). In the present study, FSHR mRNA was expressed in granulosa cells from medium and large follicles but LHR was only present in cells from large follicles, in agreement with previous observations (Bao *et al.* 1997). However, the expression of FSHR and LHR was unaffected by LPS in granulosa cells. On the other hand, aromatase transcript expression in granulosa cells was down-regulated in the cells from the dominant follicles following incubation with LPS, which may affect steroidogenesis (Fortune 1994, Richards 1994). In the rat, LPS similarly inhibits gonadotrophin-induced aromatisation of androgens (Taylor & Terranova 1996). However, it is not clear why the expression of aromatase was not affected in cells from the recruited follicles. Freshly isolated granulosa cells expressed ER α and ER β , in agreement with previous observations (Berisha *et al.* 2002). Transcripts for ER α were unchanged by LPS treatment and ER β was not detected in control or treated granulosa cells, appearing to be down-regulated by culture. Thus, the effect of LPS on oestradiol biosynthesis was not due to the inability of the cells to respond to oestradiol.

The present study used CD45 expression to demonstrate the absence of contaminating leukocytes in the granulosa cell cultures, suggesting a direct response of granulosa cells to LPS. This is important because the granulosa cell compartment within the basement membrane of the ovarian follicle is devoid of immune cells *in vivo* (Petrovska *et al.* 1996). The TLR4, CD14 and MD-2 complex is required for binding LPS to leukocytes, leading to signal transduction and activation of the innate immune response (Akira & Takeda 2004, Beutler 2004, Akira *et al.* 2006). However, TLR4 has been identified on cells other than leukocytes, including uterine epithelial and stromal cells (Hirata *et al.* 2005, Herath *et al.* 2006b). Indeed, analysis of mRNA transcripts in the present study indicated that granulosa cells from medium and large ovarian follicles expressed mRNA for TLR4 and the accessory molecules, CD14 and MD-2. Regulation of these genes in granulosa cells following LPS stimulation requires further validation and was beyond the scope of the present study. Interestingly, a recent study showed that murine granulosa cells around the time of ovulation increased the expression of TLR4 transcripts in response to LPS challenge (Shimada *et al.* 2006). Thus, it appears that granulosa cells have immune capabilities.

Recognition of LPS by immune cells results in the production of pro-inflammatory molecules such as IL-1 β , TNF α and NO (Janeway *et al.* 2001, Akira & Takeda 2004, Beutler 2004). Although these pro-inflammatory molecules play an important role in ovulation in many species (Machelon & Emilie 1997, Bornstein *et al.* 2004, Gerard *et al.* 2004), they also

suppress granulosa cell aromatase expression and oestrogen biosynthesis (Adashi *et al.* 1989, Spicer & Alpizar 1994, Ghersevich *et al.* 2001). Since granulosa cell oestradiol production was impaired following LPS challenge in the present study, we investigated whether LPS enhanced the production of inflammatory mediators that could compromise steroidogenesis. However, LPS did not increase IL-1 α or TNF transcripts in granulosa cells isolated from dominant follicles. Thus, the perturbation of granulosa cell steroidogenesis appears to be a direct effect of LPS.

In conclusion, bovine granulosa cells express the LPS innate immune receptor gene complex, comprising TLR4, MD-2 and CD14, throughout follicle recruitment and dominance. The treatment of granulosa cells with LPS *in vitro* suppressed the secretion of oestradiol but did not affect the secretion of androstenedione from theca cells or the survival of either granulosa or theca cells. This impairment of granulosa cell function may in part be mediated via the down-regulation of aromatase gene expression. As granulosa cell oestradiol production is pivotal during follicle development and these cells nurture the oocyte until ovulation, the immune capability of these granulosa cells and their response to patho-physiological concentrations of LPS *in vivo* is likely to be an important mechanism underlying the infertility associated with bacterial infection.

Materials and Methods

LPS measurement in follicular fluid

Follicular fluid was aspirated using a sterile needle guided by transrectal ultrasonography, from postpartum (40–60 days) dairy cows ($n=58$) in which the uterine disease had been evaluated as part of an independent study (Moussavi *et al.* 2007). Briefly, the uterine disease cytology evaluates the level of inflammation from 0 (normal; no inflammation), 1 (subclinical; mild inflammation), 2 (subclinical; moderate inflammation) to 3 (clinical endometritis). Samples were stored in endotoxin-free glass or polystyrene tubes (Lonza, Basel, Switzerland) at -20°C until analysed. Concentrations of bacterial LPS were measured in samples using the QCL-1000 Chromogenic Limulus Amebocyte Lysate (LAL) Endpoint Assay Kit (Lonza) following the manufacturer's guidelines. Samples were thawed, diluted in endotoxin-free 0.05 M Tris and tested for non-specific LAL inhibition by comparing samples spiked with a known concentration of LPS with unspiked samples. Samples with evidence of LAL inhibition were heated in a water bath at 75°C for 30 min using temperatures and times validated in our laboratory to remove non-specific inhibitors of the LAL reaction (Williams *et al.* 2007). Samples were then mixed with the LAL substrate reagent and assayed in duplicate in 96-well endotoxin-free microplates (Corning, Lowell, MA, USA) alongside standard curve LPS concentrations of 0.01, 0.25, 0.5, 1.0 and 5 endotoxin units/ml (10 eu=1 ng LPS) in serum. Serial dilutions were made in 50 mM Tris until concentrations

were measurable in the linear part of the standard curve. Internal recovery as determined using positively spiked serum samples was $>80\%$ and the intra- and inter-assay coefficients of variation were 4.0 and 7.2% respectively and the limit of detection was 0.01 ng/ml. To establish further that LPS crosses the ovarian follicle basement membrane, bovine ovaries were obtained from a slaughterhouse and eight medium (4–8 mm diameter) and large follicles (>8 mm diameter) dissected and maintained in 6 ml Dulbecco's modified Eagle's medium (DMEM)/F12 (Sigma) containing 10 $\mu\text{g/ml}$ LPS (Sigma: *E. coli* serotype 055:B5) for 18 h at 37°C . Ovaries were washed six times in water and endotoxin-free Tris, and follicular fluid aspirated using a sterile needle (25 G) and syringe. Concentrations of LPS were measured as previously described.

Cell cultures

Granulosa and theca cells were obtained and cultured separately in serum-free media as previously described (Gutierrez *et al.* 1997, Glistler *et al.* 2005). Briefly, bovine ovaries were collected at a local abattoir immediately after slaughter and returned to the laboratory within 1 h. Follicles were isolated manually by dissection and selected for isolation of cells if they had a translucent appearance, a well-vascularised theca and clear follicular fluid with no visible debris or blood. Follicles were measured using a grid or callipers and classed by external diameter as small (<4 mm diameter), medium (4–8 mm diameter) or large (>8 mm diameter), reflecting their gonadotrophin dependence and changes in the expression of steroidogenic enzymes and LH receptors (Fortune 1994, Campbell *et al.* 2003). At 4 mm diameter, follicles are recruited into follicle waves in cattle and become responsive to FSH, with increased expression of aromatase (Xu *et al.* 1995). From 8 mm diameter, granulosa cells express LH receptors and these selected dominant follicles require pulsatile LH stimulation to continue growing (Xu *et al.* 1995). Follicles were hemisected and granulosa cells obtained by flushing the hemisected shells and collecting the cell-rich supernatant (Gutierrez *et al.* 1997). Theca cells were then obtained by manually peeling the basal lamina from the hemisected follicular shells and digesting for 45 min at 37°C in digestion medium containing 1 mg/ml collagenase (Sigma) and 3 mg/ml trypsin inhibitor (Sigma) in a moving water bath, and then collecting the cell-rich supernatant as previously described (Glistler *et al.* 2005). Cells were $>80\%$ viable as determined by Trypan blue exclusion. Both granulosa and theca cells were plated at a density of 1.5×10^6 cells/ml in 96-well plates (Nunc, Lutterworth, UK) using serum-free media, with 10^{-7} M androstenedione for granulosa cells, and maintained at 37°C , 5% CO_2 in air, in a humidified incubator (Gutierrez *et al.* 1997, Glistler *et al.* 2005). Oestradiol and androstenedione production by granulosa and theca cells respectively were induced and maintained in response to physiological concentrations of FSH (1 ng/ml) or LH (160 pg/ml) respectively as previously described (Gutierrez *et al.* 1997, Glistler *et al.* 2005).

Macrophages were isolated from peripheral blood mononuclear cells (PBMC) using blood collected from the local abattoir. PBMCs were separated by density gradient centrifugation on 1.083 g/ml Histopaque (Sigma) and cell viability

was >95%, as determined by Trypan blue exclusion. Cells were plated in six-well plates at a density of 5×10^6 cells/ml in DMEM (low glucose; Sigma) supplemented with 10% FBS (PAA Laboratories GmbH, Pasching, Austria), 50 IU/ml penicillin, 50 µg/ml streptomycin, 2.5 µg/ml amphotericin B and 240 U/ml Nystatin (Sigma). After 2 h, non-adherent cells were removed and adherent cells (monocytes) were differentiated in culture for a further 3 days to yield macrophages. Macrophages, which were used as positive controls for PCR, were cultured in the presence or absence of 1 µg/ml LPS (Sigma: *E. coli* serotype O55:B5) for 24 h, at which time cells were collected for RNA extraction.

Cell culture challenge

After an initial 48-h establishment period, the ovarian cell culture media were removed and replaced with fresh media containing 0, 0.1, 1 or 10 µg/ml LPS (Sigma: *E. coli* serotype O55:B5). These concentrations are similar to those in follicular fluid of animals with clinical disease and LPS concentrations used for immune cell work (Poltorak *et al.* 1998, Shell *et al.* 2005, Tsatsanis *et al.* 2006), and preliminary studies with bovine cells covering the range from 1 ng/ml to 10 µg/ml where clear effects were seen with concentrations >0.1 µg/ml. Indeed, in the original descriptions of the role of TLR4, maximal responses were obtained at 10 µg/ml *E. coli* O55:B5 LPS (Hoshino *et al.* 1999). After 48 h treatment, media were carefully removed and stored at -20°C until assayed and fresh media containing 0, 0.1, 1 or 10 µg/ml LPS (Sigma: *E. coli* serotype O55:B5) were added for a further 48-h treatment

period (termed 96 h) to confirm that the granulosa cells maintained physiological function under serum-free conditions and determine if any effects of LPS on granulosa cells also persisted. At 96 h, media were removed and stored at -20°C until assayed, the number of viable cells determined by neutral red dye uptake as previously described (Campbell *et al.* 1996), and where the maximum responses were observed (10 µg/ml LPS), cells were collected for RNA isolation. To explore if granulosa cells responded to concentrations of LPS in follicular fluid of animals with subclinical uterine disease *in vivo*, the experiments were repeated using 0.1 ng/ml LPS (Sigma: *E. coli* serotype O55:B5) for a 48-h treatment period. To confirm further that the effects of treatments were associated with LPS, granulosa cells from dominant follicles were treated with 0.1, 1 and 10 µg/ml of a detoxified form of LPS (Sigma: detoxified from *E. coli* serotype O55:B5), media alone as a negative control, and 0.1 µg/ml LPS (Sigma: *E. coli* serotype O55:B5) as a positive control.

Hormone assays

Culture supernatants were analysed by RIA as previously described (Abayasekara *et al.* 1993), adapted for androstenedione, oestradiol or progesterone. Samples were diluted in 0.05 M Tris buffer containing 0.1% gelatin and 0.01% sodium azide. Standards, antiserum and tritiated tracer were purchased from Sigma, Biogenesis (Biogenesis, Kidlington, UK) and Amersham International PLC (Amersham) respectively. The limit of detection for oestradiol, progesterone and

Table 1 Primer sequences for Toll-like receptor-4 (TLR4), CD14, MD-2, CD45, oestradiol receptor α (ER α), ER β , aromatase, follicle-stimulating hormone receptor (FSHR), luteinising hormone receptor (LHR), interleukin-1 α (IL-1 α), IL-1 β , nitric oxide synthase (NOS2, formerly inducible NOS), tumour necrosis factor α (TNF α) and GAPDH.

Gene	Primer	Sequence (5'-3')	Size	T _m (°C)	Accession no.
TLR4	Sense	CTT GCG TAC AGG TTG TTC CTA A	153	56	NM174198
	Antisense	CTG GGA AGC TGG AGA AGT TAT G			
CD14	Sense	GGG TAC TCT CTG CTC AAG GAA C	199	56	NM174008
	Antisense	CTT GGG CAA TGT TCA GCA C			
MD-2	Sense	GGG AAG CCG TGG AAT ACT CTA T	204	54	DQ319076
	Antisense	CCC CTG AAG GAG AAT TGT ATT G			
CD45	Sense	CTC GAT GTT AAG CGA GAG GAA T	185	56	AJ400864
	Antisense	TCT TCA TCT TCC ACG CAG TCT A			
ER α	Sense	TCA GGC TAC CAT TAC GGA GTT T	169	60	AY538775
	Antisense	CCA CTT CAT AGC ACT TGC GTA G			
ER β	Sense	CTT CGT GGA GCT CAG CCT GT	262	60	NM174051
	Antisense	GAG ATA TTC TTT GTG TTG GAG TTT			
Aromatase	Sense	CGC AAA GCC TTA GAG GAT GA	211	53	U18447
	Antisense	ACC ATG GCG ATG TAC TTT CC			
FSHR	Sense	GCC AAG TCA ACT TAC CGC TT	193	56	NM174061
	Antisense	TGA CCC CTA GCC TGA GTC AT			
LHR	Sense	TGT TCT CCT GAC CAG TCG TTA CAA	743	56	BTU20504
	Antisense	AAT GCC TTT GTG AAA ATT GCG TAC			
IL-1 α	Sense	AGA GGA TTC TCA GCT TCC TGT G	224	54	NM174092
	Antisense	ATT TTT CTT GCT TTG TGG CAA T			
IL-1 β	Sense	GAG GAG CAT CCT TTC ATT CAT C	229	56	X54796
	Antisense	TTC CTC TCC TTG TAC GAA GCT C			
NOS2	Sense	GGA CAG TAA AGA CGT CTC CAG AA	197	54	AF340236
	Antisense	TAT GGT CAA ACT TTT GGG GTT C			
TNF α	Sense	ACT CAG GTC CTC TTC TCA AGC C	774	56	BTTNFG
	Antisense	ATG ATC CCA AAG TAG ACC TGC C			
GAPDH	Sense	GGG GTG AAC CAC GAG AAG TAT AA	120	56	BC102589
	Antisense	CCC TCC ACG ATG CCA AAG T			

androstenedione was 80 pg/ml. The respective intra- and inter-assay coefficients of variation were 8.8 and 9.9% for oestradiol, 3.1 and 12.6% for progesterone, and 3.6 and 16.5% for androstenedione.

Determination of immune mediators

Concentrations of bioactive tumour necrosis factor α (TNF α) were measured as previously described (Herath *et al.* 2006b). Briefly, rat fibroblast L929 cells were cultured in DMEM supplemented with 12.5% FBS, 50 IU/ml penicillin and 50 μ g/ml streptomycin. Cells were plated at a density of 2.5×10^4 cells per 100 μ l medium in 96-well plates (Nunc). Cytotoxicity was determined by the colorimetric MTT assay involving the addition of 0.1 μ g/ml MTT dye (Sigma–Aldrich) to each well and incubating for 2–4 h at 37 °C in a 5% CO₂ atmosphere. The cells were lysed using 100 μ l DMSO (Sigma–Aldrich) per well and colour development read at 560 nm on a Spectra Max 250 (Molecular Devices, Wokingham, UK). The limit of detection was 10 pg/ml; standards were made using recombinant human TNF α (Sigma) and cross-reactivity was confirmed using recombinant bovine TNF α (kindly provided by Prof. C Howard, Institute for Animal Health, Compton, UK).

Concentrations of nitric oxide (NO) were measured using the Greiss Reagent System (Promega) according to the manufacturer's instructions. The limit of detection was 2.5 μ M.

PCR

Total RNA was isolated from cell cultures using the RNeasy Mini Kit (Qiagen) and quantitated using a NanoDrop spectrophotometer (ND-1000 Spectrophotometer, NanoDrop Technologies Inc., Wilmington, DE, USA). Following DNase treatment (Promega), RNA was reverse transcribed into first-strand cDNA using SuperScript II RNase H⁻ Reverse Transcriptase (Invitrogen, Life Technologies) according to the manufacturer's protocols. Amplification of 50 ng cDNA used the following conditions, denaturation for 5 min at 94 °C, followed by 94 °C for 30 s, 54–56 °C (Table 1) for 30 s and 72 °C for 30 s, followed by a final extension of 5 min at 72 °C. Each primer was optimised for T_m and cycle number to ensure that semi-quantitative differences in expression could be detected. A control reaction, omitting cDNA template, was performed to confirm the absence of contamination and macrophage cDNA was used as an external control to ensure optimal PCR conditions. Primer combinations were designed using the Primer 3 software package (<http://frodo.wi.mit.edu/>), and were chosen on the criteria that the amplified product would traverse an exon/intron boundary and that the product was short enough to ensure optimum amplification. Primers were purchased from MWG (<https://ecom.mwgdna.com>) and were analysed for primer set-specific gene amplification using the nucleotide BLAST database (<http://www.ncbi.nlm.nih.gov/blast>). Primer sequences are presented in Table 1 and housekeeping gene primer conditions were optimised as previously described (Fenwick *et al.* 2006). PCR bands were analysed by densitometry using Quantity One 1-D Analysis Software version 4.6.2 (Bio-Rad). Products were sequenced using an ABI 3100 genetic analyzer and BigDye Terminator 3.1

from ABI (Foster City, CA, USA) and were verified using the BLAST database.

Statistical analysis

Results are reported as the arithmetic mean \pm S.E.M., and significance ascribed when $P < 0.05$. Hormone data were log transformed to yield variance homogeneity and the effects of treatments were explored using General Linear Model Univariate analysis in SPSS version 14.0 (SPSS Inc., Chicago, IL, USA), where treatment was the fixed variable and animal was fitted as a random variable. *Post hoc* comparisons between treatment and control were made using the Dunnett's *t*-test. Densitometry and LPS values were compared using independent *t*-test.

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