Expression and Function of Toll-like Receptor 4 in bovine endometrium[1]

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Abstract

The uterine endometrium, like other mucosal surfaces, is the first line of defence against invading pathogens and must play a stringent role in preventing the establishment of infection in the uterus. However, the bovine endometrium invariably succumbs to bacterial contamination following parturition, most commonly by *Escherichia coli*. The aim of the present study was to determine whether bovine endometrial cells responded specifically to LPS *in vitro* and whether they expressed the CD14 and TLR4 transcripts. In addition, since reproductive steroid hormones play an important role in the establishment of infection in the postpartum uterus, the role of hormones in the response to LPS was also investigated. Endometrial stromal and epithelial cells did express CD14 and TLR4 mRNA, and were able to respond to LPS by producing PGE_2 and $PGF_{2\alpha}$ respectively, in the absence of immune cells. Furthermore, this functional response was TLR4 mediated since neutralisation of LPS with polymyxin B abrogated the production of PGs. In addition, estradiol and progesterone inhibited the production of PGs by endometrial cells in response to LPS indicating a possible role for steroidal hormones in the response to LPS. Thus, bovine endometrial cells express members of the LPS-receptor complex, are capable of responding to bacterial products but this response is dependent on and influenced by the reproductive hormone milieu.

Keywords

Bacterial; inflammation; reproductive immunology; other animals

Introduction

The uterus is a unique immunological site as it must maintain immunity to constantly invading pathogens yet still remain tolerant to the foetus during pregnancy (1). Like other mucosal surfaces, the endometrium is the first barrier of defence against pathogens and must, therefore, distinguish non-self antigen (2). The ability of the endometrium to carry out this function depends on innate immune receptors recognising conserved sequences on pathogens, known as pathogen-associated molecular patterns. A key group of receptors are the toll-like receptors (TLRs) and nine TLRs are expressed at the mRNA level in the human endometrium with TLR 2, 4, 5 and 9 playing important roles in the recognition and clearance of bacterial infections (2-8).

Female fertility is dependent on the ability of the uterus to counter and eliminate microbial infections. Indeed, there are an estimated 350 million new cases of sexually transmitted diseases each year (www.who.int/topics/sexually_transmitted_infections) with 5% of US women of reproductive age infected and signs ranging from asymptomatic to pelvic inflammatory disease (PID) (9-11). Mammalian fertility is also compromised by PID associated with bacterial infections after parturition, and *Bos taurus* is a particularly extreme phenotype in which bacterial contamination of the uterus is ubiquitous after parturition (12-15). Bacterial infections in cattle often persist for more than 3 weeks after parturition, causing clinical disease ranging from PID to asymptomatic disease in 15% of animals, resulting in infertility (13). Due to its size and easy accessibility, the bovine uterus provides consequently an excellent model for studying the effects of uterine infection on fertility in mammals.

Amongst the wide range of bacteria that contaminate the bovine postpartum uterus, *Escherichia (E.) coli* are abundant and are associated with high concentrations of LPS in the uterine lumen and with clinical disease (12, 14, 15). Uterine infection provokes a strong immune response illustrated by the influx of neutrophils into the lumen and increased peripheral plasma concentrations of acute phase proteins (14-16). Indeed, endometrial epithelial cells exhibit immune function and are capable of modifying immune cell recruitment and function at the site of infection (10, 17).

The uterus is influenced by continually changing concentrations of the female sex hormones estradiol and progesterone, which have a profound effect on the establishment of infections (18, 19). For example, in humans, rodents and cattle progesterone suppresses uterine immune function by decreasing the proliferative capacity of lymphocytes, thereby increasing the susceptibility to bacterial infection (18, 19). Furthermore, increased production of progesterone results in decreased production of $PGF_{2\alpha}$, a pro-inflammatory molecule that enhances neutrophil chemotaxis (18, 20, 21). Conversely, estradiol plays a role in the recruitment of immune cells as studies in rodents have shown that macrophages ($M\Phi$[3]) are present in large numbers in the endometrium when estradiol levels are high. In addition, IFN-γ secretion is significantly higher in estradiol-supplemented cell cultures from mice. However, estradiol shows biphasic properties as studies in humans have shown that estradiol downregulates cytotoxic activity and suppresses IL-2 production by human peripheral blood lymphocytes suggesting that the effects of estradiol are dependent on estradiol levels (19). However, the effect of estradiol on the immune response to infection in the bovine uterus is less clearly defined and, therefore, our model provides a system to investigate cross-talk between immunity and reproduction.

The objective of the present study was to determine whether bovine endometrial cells respond to LPS and express the mRNA for molecules involved in LPS recognition and binding. Furthermore, since the endometrium is under the continual influence of steroidal

hormonal control, which play a role in the establishment of infection, the influence of estradiol and progesterone on the *in vitro* immune response of stromal and epithelial cells to LPS was investigated.

Materials and Methods

Tissue explants, cell isolation and culture

Bovine uteri were collected from post-pubertal non-pregnant animals with no evidence of genital disease at a local abattoir and kept on ice until further processing in the laboratory. The physiological stage of the reproductive cycle for each genital tract was determined by observation of the ovarian morphology (22). Genital tracts with an ovarian Stage I corpus luteum were used for endometrial culture and only the horn ipsilateral to the corpus luteum was used. Working under sterile conditions, the endometrium was cut into strips and placed into serum-free RPMI-1640 (Sigma) supplemented with 50 IU/ml of penicillin, 50 μg/ml of streptomycin and 2.5 μg/ml of Amphotericin B (Sigma). The strips were then chopped into 1 mm^3 pieces using a mechanical tissue chopper (McIlwain Laboratory Engineering, UK) and placed into HBSS (Sigma) (23). 50 mg of tissue were weighed in triplicate for each experimental group within the study and then transferred onto sterile tissue-lined metal grids in 6 well plates (Nunc) with 4.25 ml serum-free RPMI-1640 per well. Plates were incubated at 37°C, 5% CO_2 in air, in a humidified incubator (24). Following overnight incubation, supernatants were removed and replaced with fresh media. Endometrial tissue was used for cell isolation as previously described (25, 26) with the following modifications. Briefly, tissue was digested in 25 ml sterile filtered digestive solution, which was made by dissolving 50 mg trypsin III (Roche), 50 mg collagenase II (Sigma), 100 mg BSA (Sigma) and 10 mg DNase I (Sigma) in 100ml HBSS. Following a 1.5 h incubation in a shaking water bath at 37°C, the cell suspension was filtered through a 40 μm mesh (Fisher Scientific) to remove undigested material and the filtrate was resuspended in HBSS containing 10% FBS (Sigma) and 3 μg/ml trypsin inhibitor (Sigma)(Washing medium). The suspension was centrifuged at $100 \times g$ for 10 min and following two further washes in washing medium the cells were resuspended in RPMI-1640 containing 10% FBS, 50 IU/ml of penicillin, 50 μg/ml of streptomycin and 2.5 μg/ml of Amphotericin B. The cells were plated at a density of 1×10^5 cells per 2 ml per well in 24-well plates (Nunc). To obtain separate stromal and epithelial cell populations, the cell suspension was removed 18 h after plating, which allowed selective attachment of stromal cells (25). The removed cell suspension was then replated and incubated allowing epithelial cells to adhere (27). Stromal and epithelial cell populations were distinguished by cell morphology as previously described (25). The culture media was changed every 48 h until the cells reached confluence. All cultures were maintained at 37°C, 5% CO_2 in air, in a humidified incubator.

Tissue explant and cell culture challenge

After 24 h culture, tissue explants were challenged with different concentrations of oxytocin (OT[3], 3-300 nM, Bachem), LPS (0.03-1 μg/ml, Sigma, *E. coli* serotype 055.B5) and polymyxin B (2 μg/ml, Sigma) individually or in combination as indicated. Once confluence had been reached, stromal and epithelial cells were challenged with different concentrations of arachadonic acid (AA[3], 10-300 μM, Sigma), OT (100 nM), LPS (0.1 –3 μg/ml), heat-killed *E. coli* (10^2 –10^5 CFU/ml, isolated from a case of clinical bovine endometritis associated with pyrexia (14)), polymyxin B (2 μg/ml), 17-β estradiol (3 pg/ml, Sigma) or progesterone (5 ng/ml, Sigma) individually or in combination and for the period of time indicated. Culture supernatants were harvested and frozen prior to cytokine and PG determination.

Prostaglandin radioimmunoassays

Supernatants were analysed for PGE_2 and $PGF_{2\alpha}$ by RIA as previously described (28). Samples were diluted in 0.05 M Tris buffer containing 0.1% gelatin and 0.01% sodium azide. Standards and tritiated tracers for the PGs were purchased from Sigma and Amersham International PLC (Amersham, UK), respectively. The antisera were a generous gift from Professor N.L. Poyser (University of Edinburgh, UK) and their cross-reactivities have been reported previously (29). The limit of detection for PGE_2 and $PGF_{2\alpha}$ was 2 pg/tube and 1 pg/tube, respectively.

RT – PCR

Total RNA was isolated from cell cultures using the RNeasy Mini Kit (Qiagen, UK) and First Strand cDNA was prepared using SuperScript II RNase H$^-$ Reverse Transcriptase (Invitrogen, Life Technologies, UK) according to the manufacturers' protocols. Amplification of cDNA used the following conditions: denaturation for 5 min at 94°C, followed by 30 cycles of 94°C for 30 sec, 54-56°C (depending on primer T_m) for 30 sec and 72°C for 30 sec, followed by a final extension of 5 min at 72°C. Primer combinations were designed using the Mac Vector™ software package and were purchased from MWG (https://ecom.mwgdna.com). Primer sequences are presented in Table 1. All PCR products were sent for sequencing (MRC Geneservice, Camb.) and products showed >98% homology to published sequences on the NCBI database (http://www.ncbi.nlm.nih.gov/blast).

Monoclonal antibodies and flow cytometry

The sources of mouse mAb and isotypes, secondary reagents and methods for flow cytometry have been described in detail (30). The defined surface antigens assessed and mAb used to detect the molecules were: CD14 (CCG33; IgG_1) and CD45 (CC171; IgG_{2a}). Isotype matched controls were murine mAb against chicken surface proteins AV20 (Bu-1, IgG_1), and AV37 (chicken spleen cell subset, IgG_{2a}) (kindly provided by F. Davison, institute for Animal Health, Compton). Bound mAb were detected with FITC and PE-labelled mouse isotype-specific reagents (Southern Biotechnology Associates (SBA), Birmingham, AL, USA). 10,000 cells were analysed on a FACSAria and immunofluorescent staining was analysed using the FC Express® software (DeNovo Software).

Determination of TNF-alpha (TNFα) and NO

Levels of bioactive TNFα were determined using L929 cells as previously described (31), with the following modifications. L929 cells were cultured in DMEM (Sigma) supplemented with 12.5% FBS, 50 IU/ml penicillin, 50 µg/ml streptomycin and 20 mM HEPES buffer (Sigma). Cells were plated at a density of 2.5×10^4 cells/well in a 96 well plate (Nunc) in 100 µl medium. Cytotoxicity was determined by the colorimetric MTT assay involving the addition of 0.1 µg/ml MTT dye (Sigma) to each well. The cells were then lysed using 100 µl DMSO (Sigma) per well and colour development was read at 560 nm on a Spectra Max 250 (Molecular Devices). The limit of detection was 10 pg/ml and standards were made using recombinant human TNFα (Sigma) and cross-reactivity was determined using recombinant bovine TNFα (kindly provided by Prof C. Howard, Institute for Animal Health, Compton, UK).

NO was measured using the Greiss Reagent System (Promega) and was carried out according to the manufacturer's instructions. The limit of detection was 2.5 µM nitrite.

Statistical analysis

Data were analysed using Mixed Model ANOVA in SAS version 9.1. Results are quoted as mean + SEM, and significance attributed when P<0.05.

Results

Endometrial tissue responds to OT in vitro with the production of prostaglandins

During the normal ovarian cycle, OT release from the ovary or hypothalamus results in the production of PGs from AA by the endometrium (32, 33). To determine whether the endometrial tissues and cells were functional *in vitro*, the production of PGE_2 and $PGF_{2\alpha}$ in response to OT challenge, was measured. Bovine endometrial explants produced both PGE_2 and $PGF_{2\alpha}$ in response to OT stimulation (data not shown). Furthermore, stromal and epithelial cells isolated from these explants by tissue digestion released PGE_2 and $PGF_{2\alpha}$ respectively in response to OT challenge, though media required supplementation with AA (Fig 1). Interestingly, stromal cells produced little $PGF_{2\alpha}$ and epithelial cells produced no PGE_2 (stromal cells: 0.5ng/ml ± 0.1ng/ml $PGF_{2\alpha}$, and epithelial cells: PGE_2 levels below limits of detection). This polarised production of PGs by stromal and epithelial cells supports previous observations (21, 25).

Bacterial products drive PG response of endometrial explants

Following parturition, the bovine uterus becomes contaminated with micoorganisms, with *E. coli* being the most commonly isolated pathogen (14, 15). Since LPS is the main endotoxin of *E. coli* and a strong activator of the innate immune response, LPS was added to tissue explant cultures and the production of PGE_2 and $PGF_{2\alpha}$ was measured. Polymyxin B was used to neutralise LPS and to confirm that the response was LPS mediated (34). Endometrial explants produced increasing amounts of PGE_2 and $PGF_{2\alpha}$ in response to LPS in a dose dependent manner (Fig 2). Moreover, this response was dependent on LPS since addition of polymyxin B abrogated the LPS-induced PG production.

Endometrial cells produce PG in response to LPS independent of leukocytes, but fail to produce detectable amounts of other immune mediators

Prostaglandins can be produced by a variety of cells, including fibroblasts and MΦ in response to pathogenic stimulation (35). As, it cannot be excluded that other cell types, present in endometrial tissues explants, contributed to the production of PGs, endometrial stromal and epithelial cells cultures were established and analysed for the pan-leukocyte marker CD45 by PCR and flow cytometry. No levels of CD45 mRNA were detected in samples derived from endometrial cell cultures, and this result was confirmed by flow cytometric analysis showing the absence of $CD45^+$ cells (Fig 3). After verification of the absence of immune cells in the endometrial cell preparations, the capacity of these cells to produce PGs in response to pathogenic stimulation was investigated. Both stromal and epithelial cells challenged with heat-killed *E. coli* produced increased concentrations of PGE_2 and PGF_{2a} respectively in response to bacterial challenge (Fig 4) and this response was abrogated by the addition of polymyxin B to the cultures (Fig 4). As LPS was considered the active component the production of PG by stromal and epithelial cells was analysed after incubation of the cells with LPS alone. Both stromal and epithelial cells produced increasing amounts of PGE_2 and PGF_{2a} respectively, in a dose dependent manner (Fig 5) and this response was significantly abrogated by neutralising LPS with polymyxin B.

As LPS induces the production of other immune mediators, such as NO and TNFα, by MΦ (36), we consequently analysed levels of NO and TNFα produced by endometrial cell subsets in response to LPS to determine further immune function. However, no detectable levels of NO or TNFα were measured (data not shown). These results show that stromal and epithelial cells respond to pathogenic stimulation, but that this response may be limited or more important for directly stimulating neighbouring cells.

Bovine endometrial cells express members of the LPS-receptor complex

The ability of cells to respond to LPS is dependent on the expression on, and signalling through the receptor complex (5, 37-44). Since stromal and epithelial cells responded to LPS with an increase in PG production (Fig 5), we analysed the expression of two members of the LPS-receptor complex, TLR4 and CD14 by PCR. Using primers designed for the bovine TLR4 and CD14, mRNA transcripts for both were detected in samples derived from endometrial stromal and epithelial cells (Fig 6) indicating that these cells posses the required LPS-receptor complex to respond to LPS.

iNOS and COX-2 are induced in endometrial cells following LPS challenge

Stimulation of the LPS-receptor complex subsequently leads to activation of NFκB and the activation of genes whose products contribute to the inflammatory process, such as the inducible from of nitric oxide synthase (iNOS) leading to the production of NO, and the production of PGs by activating cyclooxygenase enzymes COX-1 and COX-2 (36, 37). However, while COX-1 is expressed constitutively to maintain homeostatic processes, COX-2 is inducible and is involved in the regulation of inflammation (35). Despite the fact that we were unable to detect measurable amounts of NO in stromal and epithelial cell cultures, LPS challenge up-regulate iNOS expression (Fig. 7). Furthermore, an LPS-dependent up-regulation of COX-2 mRNA expression was observed (Fig.7), suggesting that both iNOS and COX-2 are stimulated in stromal and epithelial cells via an LPS-receptor complex pathway involving NFκB.

Sex hormones abrogate LPS-induced PG production by endometrial cell subsets

Several publications indicate that changes in steroid hormone secretion during the estrous cycle change the response of endometrial cells to bacterial stimuli (18, 19). To characterise further the LPS response of stromal and epithelial cells, we investigated the effect of physiological concentrations of ovarian steroid hormones on the response of endometrial cells to LPS. In both cell types, pre-incubation of stromal and epithelial cells with either estradiol or progesterone for 48 hr resulted in a significantly reduced production of PGs in response to LPS challenge (Fig 8).

Discussion

The uterus represents an organ within the body, which is constantly exposed to commensals and pathogenic bacteria. Furthermore, uterine infections, either as sexually transmitted diseases or as asymptomatic infections such as PID are on the increase worldwide. However, research on the interactions of uterine cells with invading pathogens is often hampered by the lack of cell-accessibility or established and evaluated models. As the innate immune systems of humans and cattle seem to be closely related, and as uterine infections play an important role in the cattle industry, bovine uterine tissue explants were used as an *in vitro* model to study the innate immune response to pathogens.

In the present publication, we analysed the ability of bovine tissue explants and cell cultures to produce PGs, as markers of the innate response, in response to OT stimulation. Moreover, the results showed that although tissue explants produced both PGE_2 and $PGF_{2\alpha}$, individual endometrial cell phenotypes showed a more polarised PG production, with stromal cells producing predominantly PGE_2 and epithelial cells producing $PGF_{2\alpha}$. Tissue explants, stromal and epithelial cells produced PGs in response to *E. coli* and LPS challenge demonstrating that bovine endometrial tissues and cells have the ability to respond to microbial stimulation, indicating that cultures were functional *in vitro*. This production of PG was LPS mediated as neutralisation of LPS with polymyxin B resulted in the abrogation of PG production. The ability of bovine endometrial cells to respond to LPS suggested the

presence of members of the LPS receptor complex, which was confirmed by RT-PCR and our results describe for the first time the expression of CD14 and TLR4 mRNA by bovine endometrial stromal and epithelial cells. Unfortunately, limitations in the availability of bovine TLR4 and CD14 antibodies does not allow us to identify the protein expression of the LPS recognition complex. Interestingly, addition of estradiol and progesterone, hormones involved in the ovarian cycle, to the cultures influenced the production of PGs in response to LPS implicating a direct mechanism of cross-talk between the immune and reproductive system.

Prostaglandins are lipid molecules that are synthesised from AA by cyclooxygenase enzymes, which regulate numerous processes in reproduction as well as immune function and are produced by various cells (32). The role of $PGF_{2\alpha}$ in reproduction is to provide the luteolytic signal required for corpus luteum regression during the oestrus cycle and is produced by epithelial cells in response to oxytocin (21, 32). Conversely, PGE_2 is produced by stromal cells and is thought to be responsible for the maintenance of the corpus luteum during pregnancy as well as changes in collagen and vascular permeability (21, 32). The antagonistic functions of $PGF_{2\alpha}$ and PGE_2 are further demonstrated in their roles in immunity, where $PGF_{2\alpha}$ has been shown to be pro-inflammatory and PGE_2 to be anti-inflammatory (18, 35, 45). In the present study, we challenged bovine endometrial explants to a range of concentrations of LPS found in the plasma of cattle with uterine infections (12, 46). Surprisingly, tissue explant cultures produced both the pro-inflammatory $PGF_{2\alpha}$ and anti-inflammatory PGE_2 in response to LPS challenge, although there were higher concentrations of PGE_2. However, these explants consist of a variety of cells including resident MΦ, which produce large quantities of PGE_2 in response to LPS (35, 47). Therefore, it could not be excluded that the high levels of PGE_2 detected in endometrial explant cultures were produced by MΦ. For further investigations, individual endometrial cell subsets were purified and analysed for the absence of CD45^{+} cells.

The most commonly isolated pathogen from the bovine postpartum uterus is *E. coli* (12, 15). Using heat-killed *E. coli*, which we isolated from the uterus of a case of bovine metritis and, was therefore associated with clinical disease as determined by uterine pyrexia (14), we stimulated endometrial stromal and epithelial cells with a range of bacterial concentrations. Endometrial stromal and epithelial cells produced PGs in response to bacterial stimulation and interestingly, epithelial cells responded to bacterial challenge at much lower concentrations compared to stromal cells. This, in part, may be due to the position of stromal and epithelial cells in relation to each other in the endometrium. Since epithelial cells are in contact with the lumen, their location suggests that they may be more sensitive to lower levels of bacteria, ensuring a rapid immune response and clearance of the pathogen. Indeed, epithelial cells are a source of anti-bacterial activity following *E. coli* or *Staphylococcus aureus* challenge and therefore their direct contact with the uterine lumen plays an important role in immunity (48). The production of PGs in response to bacterial challenge was in part LPS mediated since addition of polymyxin B abrogated the production of PGs. However, it cannot be excluded that other bacterial products, such as lipoproteins and peptidoglycans, stimulated the production of PGs by endometrial cells via other TLRs. Thus, stromal and epithelial cells were stimulated with the main *E. coli* endotoxin, LPS.

Interestingly, stromal cell cultures challenged with LPS produced similar concentrations of PGE_2 as compared to that of tissue explant cultures. This suggested that bovine endometrial stromal cells were the source of PGE_2 in endometrial tissues, capable of producing high levels of PGE_2 in response to LPS stimulation. Although epithelial cell cultures produced $PGF_{2\alpha}$, they produced less $PGF_{2\alpha}$ in response to LPS challenge as compared to tissue explants, suggesting that production of optimal levels of $PGF_{2\alpha}$ was dependent on cross-talk between epithelial cells and other factors found in the bovine endometrium.

The role of $PGF_{2\alpha}$ in immunity is less clearly defined as compared to the role of PGE_2 (35, 47). PGE_2 results in the down-regulation of Th1 cytokines, such as TNFα, and NO production by MΦ and up-regulates the production of IL-10 (35, 47). The deactivation of MΦ and the down-regulation of pro-inflammatory mediators by PGE_2 plays an important role in reproduction as the latter is produced following conception, and therefore plays an important role in suppressing the immune response to the implanting conceptus. In addition, the down-regulation of TNFα and NO by PGE_2 may explain the absence of these immune mediators in tissue explant and stromal cell cultures. However, *iNOS* gene expression was up-regulated following LPS challenge so it cannot be excluded that stromal cells may release NO in concentrations below the detection limits to the assay applied, and the same phenomenon was observed for epithelial cell cultures. Our findings, and those of others (49-51), suggest that despite a clear up-regulation of iNOS mRNA, NO is released only in low concentrations, which may play an important role for the cellular microenvironment. Indeed, NO plays an important role in the production of PGs by bovine uterine stromal and epithelial cells (52, 53).

Stimulation of epithelial cells with LPS resulted in the up-regulation of TLR4, CD14 and COX-2, the enzyme required for the conversion of AA to PGs, which suggested the initiation of a pro-inflammatory response. Since LPS-stimulated stromal cells produced high levels of PGE_2, an anti-inflammatory molecule, down-regulation of TLR4 may prevent the cells from further recognising LPS and producing PGE_2, thereby preventing suppression of an immune response. These data suggest that a balance in the production of PGs following infection is required such that $PGF_{2\alpha}$, which encourages a pro-inflammatory response, is prevented from becoming overwhelming by the production of PGE_2. Indeed, the balance of $PGF_{2\alpha}$ and PGE_2 during infection may have implications on the length of the estrous cycle and is therefore an important factor in cross-talk between immunity and reproduction. Our results therefore showed that endometrial tissue explants, but more importantly pure stromal and epithelial cell cultures, were capable of producing PGs in response to heat-killed *E. coli* and LPS. Moreover, the response was LPS mediated since neutralisation of LPS with polymyxin B abrogated the production of PGs. Importantly, the balance of PG production following infection determines the length of the reproductive cycle and illustrates a mechanism of cross-talk between immunity and reproduction.

In addition to PGs, the female sex steroids, estradiol and progesterone, have also been shown to play a role in influencing the immune response (18, 19, 45, 48, 54-58). While progesterone suppresses uterine immune responses and can therefore result in the increased susceptibility of the uterus to infection (18, 45, 54, 55), the role of estradiol in infection is dependent on the species, tissue, and concentration of the sex hormone (19). As expected, incubation of stromal and epithelial cells with progesterone resulted in a decrease in the production of PGs following LPS challenge. In addition, incubation of stromal and epithelial cells with estradiol also resulted in a decrease in the production of PGs as previously described (59) although the mechanism of abrogated PG production is not, as yet, known. Others have demonstrated that estradiol inhibits antigen presentation by stromal cells (56). This suggests down-regulation of MHC class I and II, and subsequently estradiol may play a role in the down-regulation of other receptors such as TLRs, which may explain the decreased production of PGs following LPS stimulation. Currently, research in our group is investigating the role of differing concentrations of steroid sex hormones on the response of endometrial cells to LPS stimulation.

Taken together, our results show for the first time that bovine endometrial stromal and epithelial cells respond specifically to LPS stimulation and that this response is possibly based on a pathway involving CD14 and TLR4. Moreover, the response to LPS is dependent on the hormonal milieu, which has important implications for the establishment and

treatment of uterine infections. However, it must be noted that the only a single concentration of estradiol and progesterone was used in the present experiment, whereas different concentrations may determine the outcome of cell function (19, 58). Therefore, further work is required to assess the role of cycle-phase specific hormone concentrations on the LPS response of isolated uterine cells.

Acknowledgments

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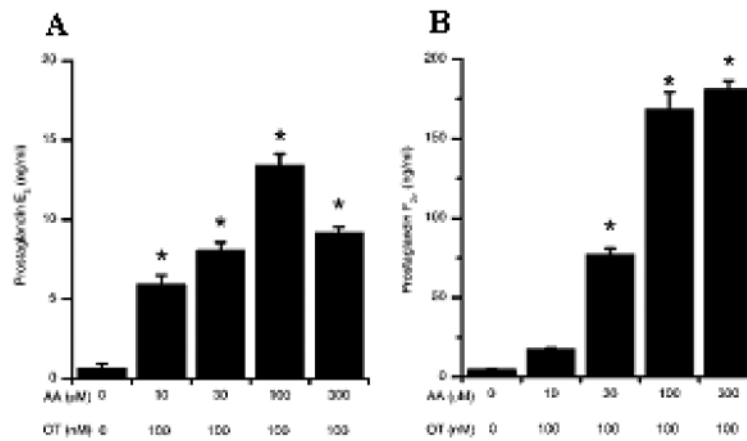
Figure 1.
Prostaglandin production by stromal and epithelial cells following OT and AA stimulation.
Stromal (A) and epithelial (B) cells were stimulated with OT (100 nM) and AA at the
concentrations indicated. After 24 h in culture, supernatants were harvested and
prostaglandin production was measured by RIA. *Differences were statistically different at
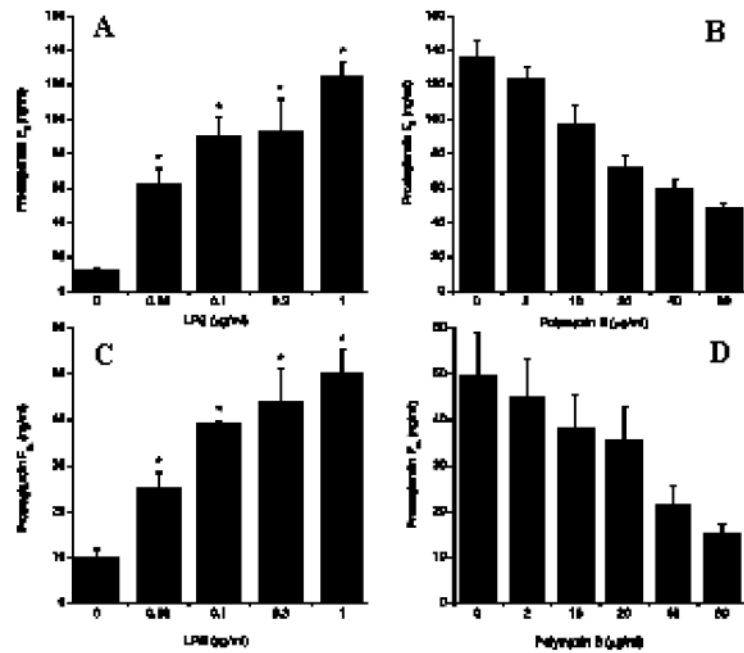P<0.05 as compared to control. Numerical values are presented as the mean + SEM of three
experiments.

Figure 2.
Prostaglandin F_{2a} and E_2 production by tissue explants following LPS and polymyxin B challenge. LPS (A and C) and Polymyxin B (B and D) were added at the indicated concentrations. After 24 hours in culture, supernatants were harvested and prostaglandin production was measured by RIA. * Differences were statistically different at $P<0.05$ as compared to control. Numerical values are presented as the mean + SEM of three experiments.
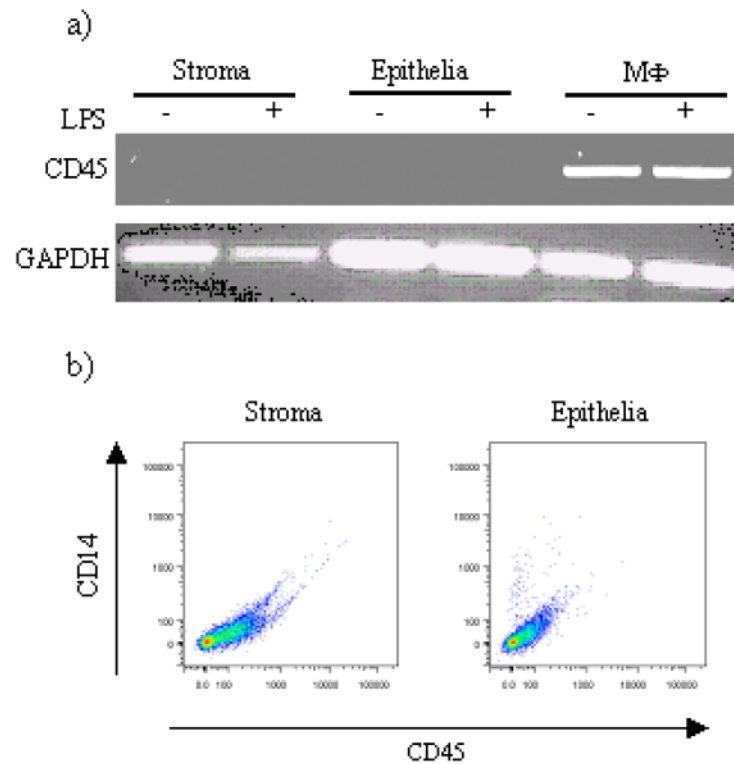
Figure 3.
Analysis of CD45 expression by stromal and epithelial cells. A) Cells were stimulated with
LPS for 24h, harvested and RNA was isolated as described and the resulting cDNA analysed
for the presence of CD45 transcripts using the indicated primer pairs. cDNA from bovine
MΦ were used for comparative reason. B) stromal and epithelial cells were stained with
antibodies to bovine CD45 and surface expression of these molecules analysed by flow
cytometry. A representative staining result is shown (n=3).
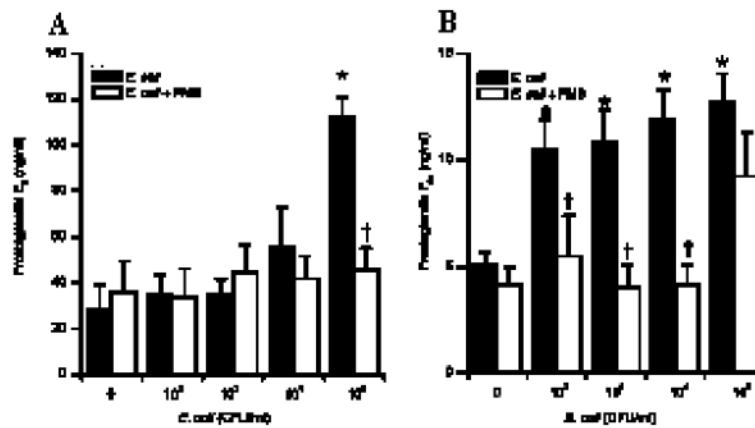
Figure 4.
Prostaglandin production by stromal and epithelial cells following *E. coli* challenge. Stromal (A) and epithelial (B) cells were stimulated with *E. coli* in the presence (open bar) or absence (closed bar) of polymyxin B (2µg/ml). After 24h in culture, supernatants were harvested and prostaglandin production was measured by RIA. * Differences were statistically different at P<0.05 as compared to control. † Differences were statistically different at P<0.05 as compared to corresponding LPS stimulation. Numerical values are presented as the mean + SEM of three experiments.
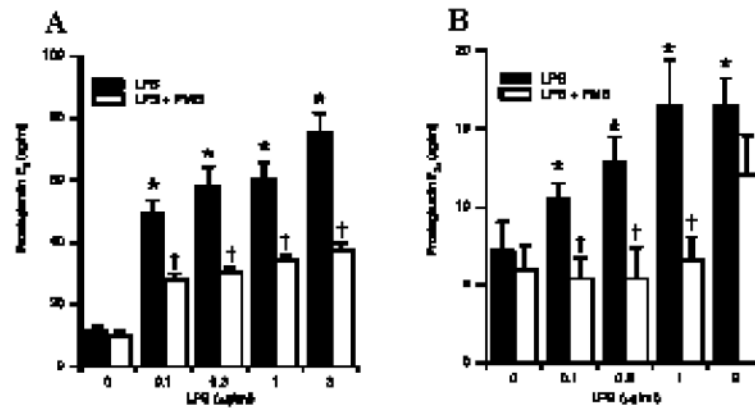
Figure 5.
Prostaglandin production by stromal and epithelial cells following LPS challenge. Stromal (A) and epithelial (B) cells were stimulated with LPS in the presence (open bar) or absence (closed bar) of polymyxin B (2μg/ml). After 24h in culture, supernatants were harvested and prostaglandin production was measured by RIA. * Differences were statistically different at P<0.05 as compared to control. † Differences were statistically different at P<0.05 as compared to corresponding LPS stimulation. Numerical values are presented as the mean + SEM of three experiments.
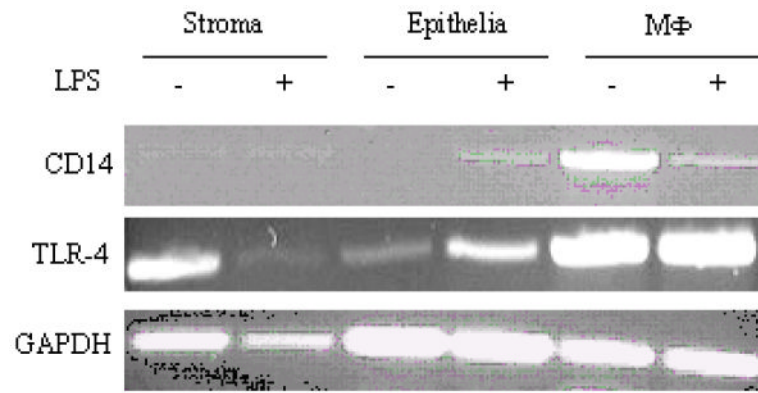
Figure 6.
Analysis of CD14 and TLR4 mRNA expression by stromal and epithelial cells. Cells were stimulated with LPS for 24h, harvested and RNA was isolated as described and the resulting cDNA analysed for the presence of CD14 and TLR4 transcripts using the indicated primer pairs. cDNA from bovine MΦ were used for comparative reason.
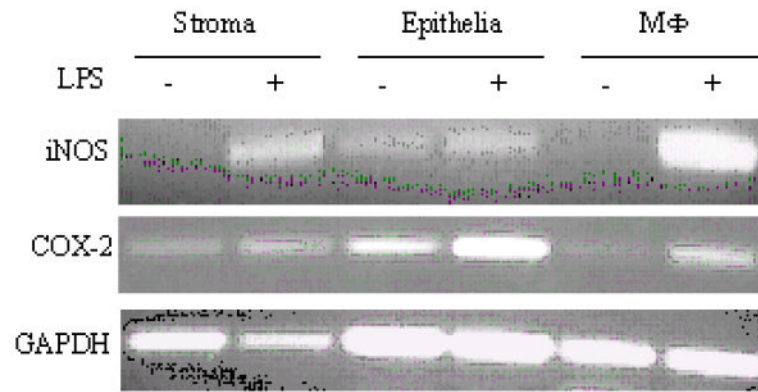
Figure 7.
Analysis of iNOS and COX-2 mRNA expression by stromal and epithelial cells. Cells were stimulated with LPS for 24h, harvested and RNA was isolated as described and the resulting cDNA analysed for the presence of iNOS and COX-2 transcripts using the indicated primer pairs. cDNA from bovine MΦ were used for comparative reason.
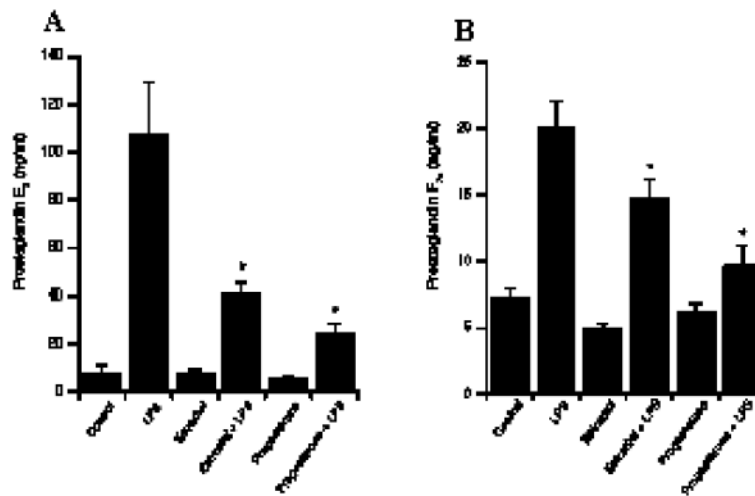
Figure 8.
Influence of sex hormones on LPS-stimulated PG production by endometrial cells.
Prostaglandin production by stromal (A) and epithelial (B) cells following LPS challenge (1
µg/ml) in the presence of estradiol (3 pg/ml) or progesterone (5 ng/ml). . After 24h in
culture, supernatants were harvested and prostaglandin production was measured by RIA.
, * Differences were statistically different at P<0.01 and P<0.001 respectively as
compared to LPS stimulation. Numerical values for stromal and epithelial cells are presented
as the mean + SEM of three and nine experiments respectively.

Table I

Primer sequences: CD45, CD14, TLR4, inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), and GAPDH

Gene	Primer	Sequence	bp
CD45	Sense	5′-TGC AAC CGC TCT CTC AAC CAT A-3′	845
	Anti-sense	5′-CTT GCT TGG CTT TGC TGG ATC T-3′	
CD14	Sense	5′-GAC GAC GAT TTC CGT TGT GT-3′	600
	Anti-sense	TGC GTA GCG CTA GAT ATT GGA-3′	
TLR4	Sense	5′-AAC CAC CTC TCC ACC TTG ATA CTG-3′	452
	Anti-sense	5′-CCA GCC AGA CCT TGA ATA CAG G-3′	
iNOS	Sense	5′-CGG CAT GTG AGG ATC AAA AAC TG-3′	270
	Anti-sense	5′-CTC CTT TGT TAC TGC TTC CAC CC-3′	
COX-2	Sense	5′-GGG TGT GAA AGG GAG GAA AGA G-3′	128
	Anti-sense	5′-TGA AGT GCT GGG CAA AGA ATG-3′	
GAPDH	Sense	5′-GGC GTG AAC CAC GAG AAG TAT AA-3′	120
	Anti-sense	5′-CCC TCC ACG ATG CCA AAG T-3′	