RESEARCH ARTICLE

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Evidence for a Novel, Local Acute-Phase Response in the Bovine Oviduct: Progesterone and Lipopolysaccharide Up-regulate Alpha 1-Acid-Glycoprotein Expression in Epithelial Cells In Vitro

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SUMMARY

Little is known about the local production and function of alpha 1-acid glycoprotein (AGP), an acute-phase protein, in the female reproductive tract. This study aimed to investigate the regulation and immune function of AGP in cultured bovine oviduct epithelial cells. Analysis by Western blotting and immunohistochemistry revealed that bovine oviduct tissue expresses AGP protein in epithelial cells and the smooth muscle layer. Stimulation of bovine oviduct epithelial cells in culture with either progesterone (1 ng/ml) or lipopolysaccharide (LPS, 10 ng/ml) induced both mRNA expression and secretion of AGP. Estradiol (1 ng/ml), progesterone (1 ng/ml), and luteinizing hormone (10 ng/ml), which are observed during the peri-ovulatory period in oviduct tissues (steroids) or in circulation (luteinizing hormone), suppressed LPS-induced expression and secretion of AGP, which in turn induced the expression of Toll-like receptor-4 (*TLR-4*) and interleukin-1β (*IL-1B*), but suppressed *TLR-2* and tumor necrosis factor-α (*TNFA*) expression. AGP also inhibited LPS-induced *TLR-2* and *TNFA* expression, but had no effect on LPS-induced *TLR-4* and *IL-1B* expression. These findings suggest that oviductal epithelial cells can participate in antimicrobial processes through the secretion of AGP, which is partly regulated by ovarian steroids. Moreover, oviductal AGP may regulate the response of epithelial cells, thereby reducing the expression of the acute pro-inflammatory cytokine *TNFA*, which could contribute to the local homeostasis during the acute response to endotoxin release in the oviduct's anti-infection process.

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INTRODUCTION

Epithelial cells are among the first cells exposed to pathogens, and their mucosal surfaces function not only as a physical barrier but also as a regulator for both innate and adaptive immunity (Bulek et al., 2010). In the female

Abbreviations: AGP[R], alpha1-acid glycoprotein [receptor]; E2, estradiol; IL, interleukin; LH, luteinizing hormone; LPS, lipopolysaccharide; OEC, oviduct epithelial cell; P4, progesterone; TLR, Toll-like receptor; TNFA, tumor necrosis factor-α.

reproductive tract, oviductal epithelial cells (OECs) are critical for the establishment of a successful pregnancy (Wira et al., 2005a). OECs are exposed to pathogens and endotoxins from the uterus and peritoneal cavity, resulting in oviduct infection (Rizos et al., 2010). Thus, the mucosal OECs sense and respond to potential pathogens while providing an optimal microenvironment for fertilization and fetal survival. An in vitro model of cultured uterine or oviductal epithelial cells may, at least in part, help us to gain basic information regarding the role of epithelial cells in the uterus or oviduct as these isolated cell populations have been shown to develop into polarized monolayers with distinct apical (luminal) and basolateral surfaces (Fahey et al., 1999). Of course, the contribution of other cells, such as stromal cells and immune cells, and their interaction with epithelial cells must also be considered when interpreting anti-pathogen responses.

Antimicrobial peptides, such as β-defensins, secretory leukocyte protease inhibitor (SLPI), and acute-phase proteins, help resolve local inflammation, repair injured tissue, re-establish homeostasis, and provide non-specific protection against pathogens (Hochepied et al., 2000; Veas, 2011; Wira et al., 2005b). The serum concentration of acute-phase proteins dramatically increases in response to systemic inflammation or infection. Acute-phase proteins such as alpha 1-acid glycoprotein (AGP, or orosomucoid) and haptoglobin are primarily synthesized by the liver, but can also be produced in extra-hepatic organs (Lavery et al., 2003; Lecchi et al., 2009). Thus, an acute-phase response may occur in extra-hepatic cell types.

AGP is one of the main acute-phase proteins in humans, rats, mice, and other species (Hochepied et al., 2002; Petersen et al., 2004), whose expression can be regulated by sex steroids (Hochepied et al., 2000). The AGP system is present in the bovine ovary and uterus, and in rat and human alveolar epithelial cells (Crestani et al., 1998; Lecchi et al., 2009). The exact biological and local function of AGP remains largely unknown, but several lines of evidence indicate a modulatory action on the immune response as it has been shown to possess pro- and anti-inflammatory functions (Pukhal'skii et al., 2001; Hochepied et al., 2003). For example, AGP enhances the release of pro-inflammatory cytokines by mononuclear leukocytes stimulated with lipopolysaccharide (LPS) (Boutten et al., 1992). On the other hand, AGP reduces lymphocyte proliferation and IL-2 secretion, thus acting as an anti-inflammatory molecule (Elg et al., 1997). AGP can increase secretion of the interleukin-1 (IL-1) receptor antagonist, inhibit chemotaxis and activation of neutrophils, and inhibit the pro-angiogenic effect of tumor necrosis factor-α (TNFA) (Bories et al., 1987; Lain et al., 1990; Ligresti et al., 2012).

The present study tested the hypothesis that cells of the bovine oviduct can express and secrete the non-specific effector molecule AGP. The objectives were first to investigate the local protein expression and secretion of AGP in bovine OEC cultures challenged by LPS and the ovarian steroids, progesterone (P4), estradiol (E2), as well as luteinizing hormone (LH). Second, we examined the effect of AGP alone, or in combination with LPS, on the expression of inflammatory-response genes (*TNFA*, *IL-1B*, and Toll-like receptors 2 and 4 [*TLR-2* and *TLR-4*]) in bovine OEC cultures. These data indicate that AGP expression in bovine oviductal tissue is induced by LPS and P4, and that AGP elicits both pro- and anti-inflammatory effects in bovine OEC culture.

RESULTS

Expression of AGP Protein in the Bovine Oviduct

To analyze if the bovine oviduct expresses AGP protein, immunohistochemistry and Western blotting were carried out on oviduct tissue from pre-ovulatory, post-ovulatory, and mid-luteal samples. No stage-dependent differences were observed, so representative results from the pre-ovulatory phase are displayed (Fig. 1). Immunohistochemistry revealed the expression of AGP by endothelial cells, smooth muscle cells, and epithelial cells of the oviduct, with stronger expression of AGP in the epithelium of the ampulla (Fig. 1C-D) compared to the isthmus (Fig. 1A-B). Western blotting (Fig. 1E) confirmed the specificity of the antibody used (PAA816Bo01), as characteristic bands at 40–45 kDa (multiple bands at 40–45 kDa, possibly due to the state of N-glycosylation) were detected in bovine oviduct and liver (positive control), whereas the fibroblast sample (negative control) has no reactive AGP.

Up-regulation of the AGP System by P4 in Bovine OEC Cultures

To assess the effect of ovarian steroids, E2 and P4, as well as LH on the AGP system, confluent bovine OEC cultures were stimulated after two passages (Fig. 2) with ovarian steroid hormones detected in oviduct tissue (E2, 1 ng/ml; P4, 1 ng/ml) or with circulating levels of LH (10 ng/ml) that were previously observed during the pre-ovulatory phase (Wijayagunawardane et al., 1998; Wijayagunawardane et al., 2005). P4, but not E2, induced expression of the AGP receptor (*AGPR)* as well as the mRNA expression and secretion of *AGP* (Fig. 3). LH, on the other hand, did not affect transcription of *AGP* or *AGPR*, but tended to induce AGP secretion by bovine OEC cultures. Media of control, unstimulated cultures contained approximately 400 ng/ml AGP.

Stimulation of the AGP System by LPS in Bovine OEC Cultures

LPS challenge at 10 or 100 ng/ml revealed no significant differences in cell viability compared to control in the tetrazolium reduction assay. LPS at 1000 ng/ml did, however, significantly reduce cell viability to 74% of control (data not shown). We previously reported that bovine OECs differentially respond to doses of LPS: Low concentrations (10 ng/ml) induced a pro-inflammatory response, such as up-regulation of *TLR-4*, *IL-1B*, and *TNFA*, but higher levels of LPS (100 ng/ml) stimulated an anti-inflammatory response, such as up-regulation of *IL-10* and *IL-4* (Kowsar

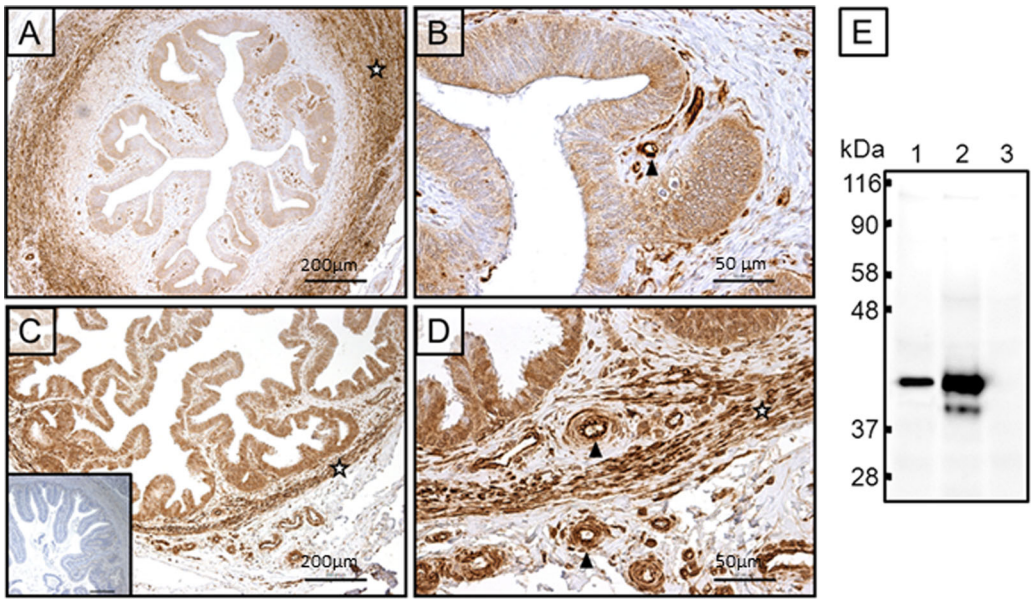

Figure 1. Localization of AGP in bovine oviductal tissue during the preovulatory period. AGP is localized in endothelial cells and in the surrounding vascular smooth muscle cells (black arrowheads) as well as in the smooth muscle cells of the tunica muscularis (stars) in both the isthmus **A,B**: and ampulla **C, D**. Staining is also observed in the oviductal epithelium, with a stronger AGP signal in the epithelium of the ampulla. Insert in C shows a control section after processing without the primary antibody. Scale bars, 200 μm **A, C**; 50 μm **B, D**. **E**: Specificity of AGP antibody was tested on extracts of bovine liver (positive control, (1), bovine oviduct (2), and bovine fibroblasts (negative control, (3) by Western blotting. Characteristic bands at 40–45 kDa (different N-glycosylations) were detected in bovine oviduct and liver extracts.

et al., 2013). To determine the effect of LPS on the AGP system, bovine OEC cultures were stimulated with 10 ng/ml or 100 ng/ml LPS for 24 hr. From this, 10 ng/ml of LPS stimulated the expression of *AGPR* and transcription and secretion of *AGP*, whereas 100 ng/ml LPS did not induce the expression of genes involved in the AGP system (Fig. 4).

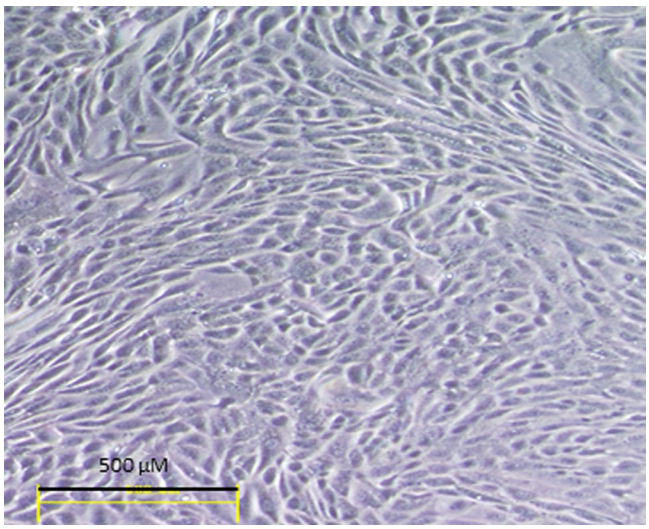

Figure 2. Confluent layer of the cultured bovine OECs after the second passage. Magnification, ×100; scale bar, 500 μm.

Ovarian Steroids and LH Reverse the Stimulatory Effect of LPS on the AGP System in Bovine OEC Cultures

We recently showed that the ovarian steroids and LH completely inhibit the LPS-induced immune response (Kowsar et al., 2013), whereas P4 induces the AGP system (as shown above). Therefore, to understand the effect of E2, P4, and LH on LPS-induced AGP upregulation in bovine OECs, the cultures were stimulated with LPS (10 ng/ml) together with ovarian steroid hormones (1 ng/ml E2; 1 ng/ml P4) or with LH (10 ng/ml). In all cases, simultaneous exposure to ovarian steroids or LH completely suppressed LPS-induced mRNA expression and secretion of AGP (Fig. 5). Moreover, E2 and LH reversed the stimulatory effect of LPS on *AGPR* mRNA expression.

Immunological Response of Bovine OECs to AGP in Culture

To explore the possible immune response of cultured bovine OECs to AGP alone, a dose-response study was performed in which epithelial cells were cultured with increasing levels of AGP (0, 1, 10, 100, and 1000 ng/ml) for 24 hr. Control cultures contained approximately 400 ng/ml AGP after 24 hr, while P4 enhances AGP concentration to 700 ng/ml (Fig. 3). Lower concentrations of AGP (1, 10, or 100 ng/ml) stimulated *TLR-4* and *IL-1B* expression, whereas AGP (1–1000 ng/ml) completely suppressed the expression of *TLR-2* and *TNFA* (Fig. 6).

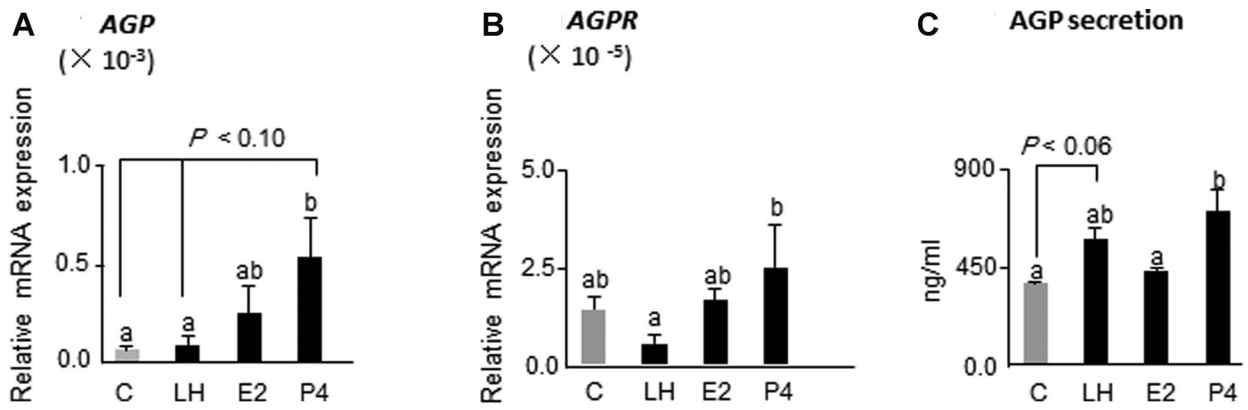

Figure 3. **A-B**: Relative mRNA expression of *AGP* and *AGPR* in, and **C**: AGP secretion from bovine OEC cultures stimulated for 24 hr with ovarian steroids or LH. C, control without any stimulant; LH (10 ng/ml); P4 (1 ng/ml); E2 (1 ng/ml); and EPLH (E2 + P4 + LH) at the same concentrations. Numerical values are presented as the mean ± standard error of the mean from 5 experiments. Different letters indicate significant differences between treatments at $P < 0.05$, as determined by ANOVA followed by Bonferroni's multiple-comparison test.

Interaction Between AGP and LPS in the Bovine OEC Response

To determine if AGP can also modulate the LPS-mediated immune response, bovine OEC cultures were stimulated with 10 or 100 ng/ml AGP together with either 10 or 100 ng/ml LPS for 24 hr. Neither concentration of AGP altered the stimulatory effect of 10 ng/ml LPS on *TLR-4* and *IL-1B* expression, whereas both AGP concentrations completely suppressed LPS-induced *TNFA* and *TLR-2* expression (Fig. 7).

DISCUSSION

The results of this study provide the first evidence that the bovine oviduct expresses the *AGP* gene and secretes the AGP protein. Such acute-phase proteins have been shown to be involved in regulating and amplifying the immune response and restoring homeostasis (Veas, 2011). The present data suggest that extra-hepatic cell types and tissue might contribute to local changes in acute-phase protein abundance, especially during the initial phases of an inflammatory response.

We recently reported that ovarian steroids and LH suppressed the stimulatory effect of LPS on the expression of pro-inflammatory cytokines (Kowsar et al., 2013). In the present study, however, similar concentrations of P4 or LH increased the secretion of AGP. Moreover, AGP secretion by bovine OECs occurred without any stimulant (to a concentration of approximately 400 ng/ml). Therefore, constitutive secretion of AGP by bovine OECs may help to suppress future infections, which is consistent with its function in preventing Gram-negative infections (Hochepied et al., 2000) by binding *Eschericia coli* LPS

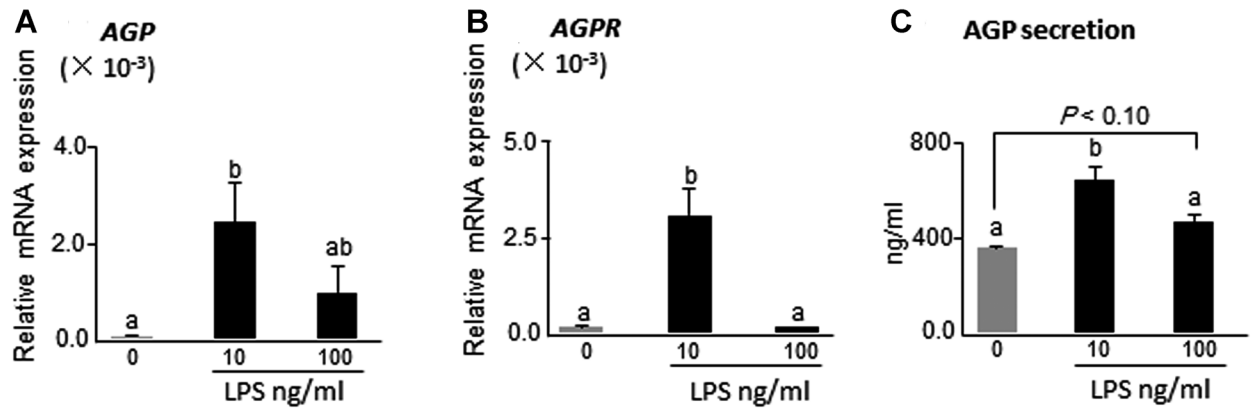

Figure 4. **A-B**: Relative mRNA expression of *AGP* and *AGPR* in, and **C**: AGP secretion from bovine OEC culture stimulated for 24 hr with different levels of O55:B5 *E. coli* LPS (0, 10, or 100 ng/ml). Numerical values are presented as the mean ± standard error of the mean from 5 experiments. Different letters indicate significant differences between treatments at $P < 0.05$, as determined by ANOVA followed by Fisher's multiple-comparison test.

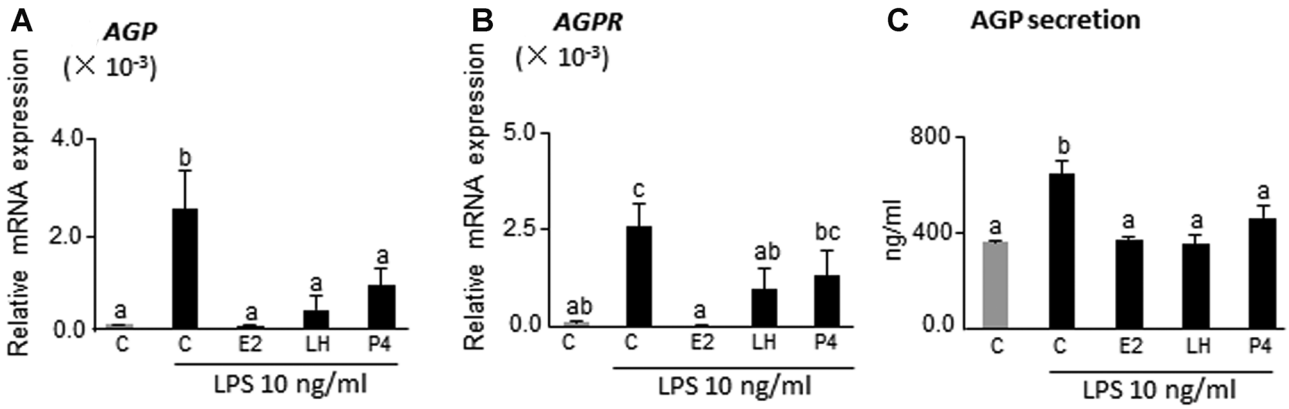

Figure 5. **A-B**: Relative mRNA expression of *AGP* and *AGPR* in and **C**: AGP secretion from bovine OEC cultures stimulated for 24 hr with O55:B5 *E. coli* LPS (10 ng/ml) together with LH (10 ng/ml), P4 (1 ng/ml), or E2 (1 ng/ml). C (gray bar), control without any stimulant. Numerical values are presented as the mean ± standard error of the mean from 5 experiments. Different letters indicate significant differences between treatments at $P < 0.05$, as determined by ANOVA followed by Bonferroni's multiple-comparison test.

(Moore et al., 1997). Within the bovine OEC culture system, P4 and LH may act to suppress LPS-induced pro-inflammatory cytokines (Kowsar et al., 2013) that are otherwise deleterious to the epithelial cells, allogeneic sperm, and the semi-allogeneic embryo. As these hormones also induce AGP secretion, an alternative role could be to establish non-specific resistance to a Gram-negative infection (Moore et al., 1997).

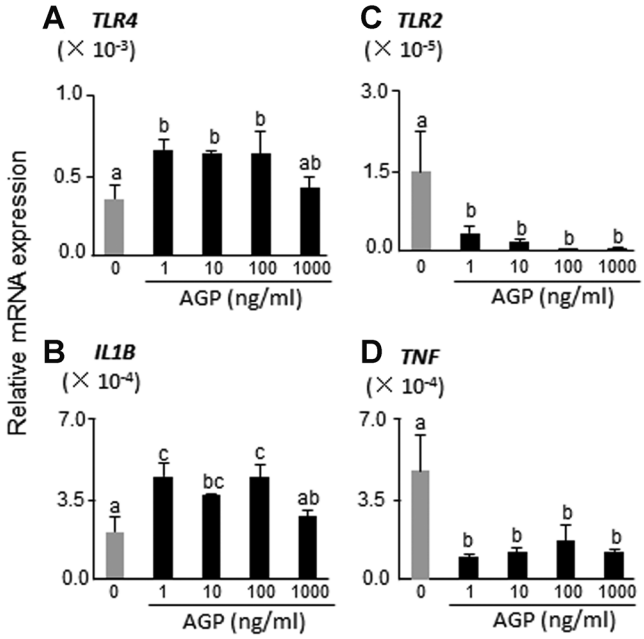

Figure 6. Relative mRNA expression of *TLR−4*, *IL−1B, TNFA*, and *TLR-2* in bovine OEC cultures stimulated for 24 hr with different levels of AGP (0, 10, 100, or 1000 ng/ml). Numerical values are presented as the mean ± standard error of the mean from 5 experiments. Different letters indicate significant differences between the treatments at $P < 0.05$, as determined by ANOVA followed by Bonferroni's multiple-comparison test.

The present data showed that 10 ng/ml, but not 100 ng/ml, of LPS induced the AGP system. As LPS can increase acute-phase protein production via TLR-4 in the liver after injury (Cho et al., 2004), these results suggest that the early induction of AGP by LPS could contribute to mucosal defense in the oviduct epithelial cells, and may initiate an auto-regulatory feedback loop that limits hyperactive LPS-induced pro-inflammatory responses. Moreover, high levels of LPS (100 ng/ml) are able to overcome this immune response in bovine OEC cultures.

Whereas P4 or LPS individually induced *AGP*, a combination of P4 and LPS inhibited LPS-induced *AGP* expression. The bovine oviduct and bovine OECs have been shown to express receptors for E2 and P4 (Ulbrich et al., 2003), thus these findings collectively suggest that ovarian steroid hormones may exert their effects in the oviduct microenvironment via their specific receptors; however, a suppressed immune response may lead to susceptibility of the host to infection (Kaushic et al., 2003).

P4 can inhibit the NF-κB pathway, which plays a crucial role in regulating inflammatory and innate immune responses (Hardy et al., 2006; Giannoni et al., 2011). P4 can rapidly induce mRNA and protein levels of inhibitor of κBa, a protein that blocks NF-κB translocation (Hardy et al., 2006; Giannoni et al., 2011). Thus, it is likely that during an infection or under LPS stimulation, P4 may inhibit LPS-induced *AGP* expression by inducing an inhibitor of κBa.

Of note, a very low level of AGP (1 ng/ml) affected bovine OEC gene expression. We do not have a clear explanation for why only 1 ng/ml AGP changed mRNA abundance in bovine OEC cultures that accumulate 400 ng/ml AGP after 24 hr. One clue, however, may be related to the observation that the exact biological function of AGP depends on its carbohydrate composition, and different forms of AGP can

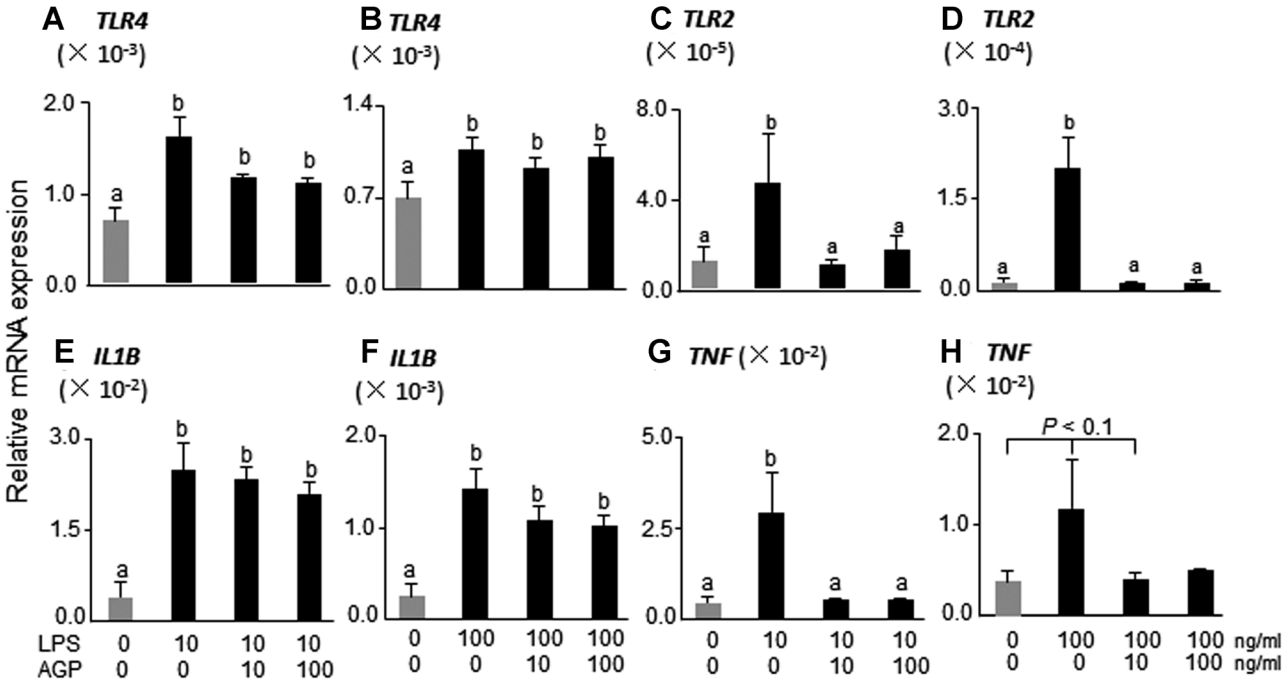

Figure 7. Relative mRNA expression of *TLR-4*, *IL−1B*, *TNFA*, and *TLR-2* in bovine OEC cultures stimulated for 24 hr with different levels of O55:B5 *E. coli* LPS (10 or 100 ng/ml) together with different levels of AGP (10 or 100 ng/ml). Numerical values are presented as the mean ± standard error of the mean from 5 experiments. Different letters indicate significant differences between treatments at $P < 0.05$, as determined by ANOVA followed by Bonferroni's multiple-comparison test.

be distinguished in serum and liver, depending on the type of glycosylation and multiple amino acid substitutions (Schmid et al., 1977; Yoshima et al., 1981). Thus, the AGP purified from bovine serum that we used for in vitro stimulation of might contain forms of AGP that differ from those secreted endogenously by bovine OECs.

Interestingly, AGP (1−100 ng/ml) up-regulated both *TLR-4* and *IL-1B* expression, while down-regulating *TLR-2*, which could be a strong mediator of anti-inflammatory effects (Netea et al., 2004). Moreover, the administration of 10 or 100 ng/ml AGP did not affect LPS-induced *TLR-4* and *IL-1B* expression, whereas it suppressed the LPS-induced *TLR-2* expression. To our knowledge, there is no information on the effect of AGP on *TLR-4* and *TLR-2* expression. Yet AGP can potentiate LPS-induced IL-1B production by human monocytes and macrophages (Boutten et al., 1992). This could result in a positive-feedback loop, as local concentrations of AGP can induce the expression of *TLR-4* and the pro-inflammatory cytokine *IL-1B*. The present data imply a protective and pro-inflammatory role for AGP that occurs by up-regulating *TLR-4* and *IL-1B* in the bovine oviduct, and suggest that the secretion of AGP in vitro at a low dose of LPS might activate a pro-inflammatory response.

All tested concentrations of AGP suppressed the expression of *TNFA* in bovine OEC cultures, whereas administration of moderate doses of AGP (10 or 100 ng/ml) also inhibited the stimulatory effect of LPS on *TNFA* expression. TNFA is considered to be the major mediator of endotoxin activity (Vassalli, 1992). AGP has been shown to protect animals from the lethal shock induced by TNFA or LPS by reducing TNFA production by stimulated mononuclear leukocytes and thus suppressing the inflammatory response (Libert et al., 1994; Moore et al., 2000). Our results suggest that a similar repressive function may be occurring in the oviduct, namely that AGP could also play an anti-inflammatory role by suppressing *TNFA* expression in bovine OECs to prevent tissue damage.

In conclusion, this study identifies the expression of a novel, acute-phase protein, AGP, in the bovine oviduct that is partly regulated by ovarian steroids and LH in cultured OECs. The results suggest that the immune responsiveness of epithelial cells to LPS is affected by AGP, which may be involved in maintaining local homeostasis in the acute response to endotoxin in the oviduct.

MATERIALS AND METHODS

Immunohistochemistry

In the local slaughterhouse, the female reproductive tracts were opened and macroscopically examined for health (free of inflammation, pus, and abnormal color). Healthy oviducts were transported to the laboratory immersed in a 0.9% saline solution in ice. In the laboratory, the ovaries were also examined to be free of any cystic follicles, and without inflammation, swelling, abscesses, or pus. The

oviducts were used from the pre-ovulatory, post-ovulatory, and mid-luteal phases, which were identified as previously reported (Miyamoto and Schams, 1991), based on the appearance of the corpus luteum, weight, color of the corpus luteum, and follicle diameter.

Paraffin-embedded tissue sections (4 μm thick) of bovine ampulla or isthmus were mounted on silane-treated glass slides (Histobond Superior; Paul Marienfeld Laboratory Glassware, Laud-Königshofen, Germany), and dried at 37°C for 24 hr. Afterwards, they were deparaffinized in xylene and rehydrated in a series of graded alcohol concentrations. Sections were incubated for 30 min in 80% alcohol solution containing 2% hydrogen peroxide to block endogenous peroxidase activity. After rinsing the sections three times for 5 min in phosphate buffered saline (PBS, pH 7.2), antigen retrieval was performed by boiling the sections in 10 mM citric acid (pH 6) for 15 min. They were subsequently incubated for 20 min in 20% normal goat serum (in PBS) at room temperature to saturate any sites for non-specific binding of proteins. The primary antibody used for immunohistochemistry was rabbit anti-bovine AGP (1:200) (PAA816Bo0; Uscn Life Science Inc.; China). The antibody was diluted in PBS containing 1% bovine serum albumin, and applied to sections that were incubated in a humidified chamber overnight at 4°C. The EnVision™ anti-rabbit immunoglobulin conjugated to peroxidase-labeled dextran polymer system (DAKO, Glostrup, Denmark) was used to detect the primary antibody, in accordance with the manufacturer's protocol. Finally, sections were washed with PBS, and peroxidase activity was detected with 3, 3′-diaminobenzidine (DAB) substrate (Sigma, Steinheim Germany) for 5 min at room temperature. Sections were counterstained with hemalum, dehydrated, and mounted with DPX (Fluka, Buchs, Switzerland). To analyze non-specific binding, primary antibody was replaced by non-immune rabbit IgG (Sigma) at the same concentration of the primary antibody.

Western Blotting

Tissue samples from bovine oviduct and liver were homogenized in protein extraction buffer (50 mM Tris HCl, 150 mM NaCl, 40 mM NaF, 5 mM EDTA, 5 mM EGTA, 1 mM Na_3VO_4, 1% (v/v) NP-40, 1% (w/v) sodium desoxycholate, and 1 mM PMSF). The total amount of extracted protein was quantified by the DC Protein Assay Kit (BioRad, Munich, Germany). Twenty micrograms of total protein were electrophoretically separated on a 12% SDS-polyacrylamide gel under reducing conditions in a BioRad electrophoresis unit. Separated proteins were subsequently transferred onto a nitrocellulose membrane (0.2 mm; Roth, Karlsruhe, Germany) using a tank-blotting device (BioRad). After blocking non-specific binding sites with 5% non-fat dry milk in Tris-buffered saline (TBS)/Tween-20 for 60 min at room temperature, the membrane was incubated with a solution containing rabbit anti-bovine AGP (1:1000) (PAA816Bo0; Uscn Life Science Inc.; China) overnight at 4°C. Afterwards, the membrane was rinsed 3 times for 10 min in TBS/Tween-20 at room temperature, and then exposed to a solution with a horseradish peroxidase-conjugated secondary antibody for 60 min at room temperature. Visualization was achieved with SuperSignal West Pico (Pierce, Rockford, IL, USA) according to the manufacturer's protocol. Negative-control bovine fibroblasts (formed by spontaneous immortalization) were isolated from caruncles and, therefore, were of bovine maternal placental origin; they were isolated at passage number 25.

Bovine OEC Isolation and Culture

Oviducts from 15 cows were transported on ice from the local slaughterhouse to the laboratory, with the oviducts immersed in $PBS^{-/-}$ (Sigma, St. Louis, MO. USA) with 0.3% gentamicin (Sigma) and amphotericin B (Illkirch, Graffenstaden, France). They were cut and separated from the connective tissue, then washed twice with PBS. Epithelial cells were isolated and cultured as previously described (Wijayagunawardane et al., 2005; Way, 2006; Kowsar et al., 2013). In brief, the oviductal lumen was flushed with 15 ml PBS, and the epithelial cells were mechanically dislodged while being flushed with the same volume of PBS. The isolated epithelial cells from 3 animals were pooled and cultured together in D-MEM/F12 medium containing 10% fetal calf serum (FCS) (Bio Whittaker, Walkersville, MD), 0.1% gentamicin, 1% amphotericin, 2.2% $NaHCO_3$ using 6-well culture dishes (Nalge Nunc International, DK-4000 Roskilde, Denmark) at 38°C in 5% CO_2 and 95% air. The following day, the bovine OEC culture was washed twice with PBS, and incubated with the supplemented DMEM/F12 medium containing 5% FCS. After monolayer formation, cells were trypsinized (0.05% trypsin EDTA; Amresco, Solon, OH) until single cells appeared, and these cells were plated in 12-well culture dishes at a density of 1.5×10^4/ml. Cells were cultured at 38°C in 5% CO_2 and 95% air in supplemented DMEM/F12 with 5% FCS until the bovine OEC monolayer reached 70%−80% confluency (Fig. 2).

LPS, AGP, and Hormone Treatments

After the second passage, but before the bovine OECs reached 70%−80% confluency, the monolayer was washed twice with PBS and cultured using a medium supplemented with 0.1% FCS and incubated for 24 hr with one of the following: (i) AGP (1, 10, 100, or 1000 ng/ml,); (ii) LPS, serotype *E. coli* 055:B5 (10 or 100 ng/ml) (Sigma); (iii) LPS (10 or 100 ng/ml) + AGP (10 or 100 ng/ml); (iv) LH (10 ng/ml) (USDA-bLH-B-6, USDA Animal Hormone Program, Bethesda, MD, USA); (v) E2 (1 ng/ml) (Sigma); (vi) P4 (1 ng/ml) (Sigma); (vii) LPS (10 or 100 ng/ml) + LH; (viii) LPS (10 or 100 ng/ml) + P4; or (ix) LPS (10 or 100 ng/ml) + E2. Hormone concentrations were the same for all experiments. As a control, culture medium without any stimulant was added. The concentrations of ovarian steroid hormones used in these bovine OEC cultures were based on their endogenous levels in the bovine oviduct tissue during the pre-ovulatory period or the circulating pre-ovulatory level of LH, although the exact levels of these hormones in oviduct

fluid are unclear (Wijayagunawardane et al., 1998; Wijayagunawardane et al., 2005). This was done to mimic the local hormonal conditions around the time of ovulation in the oviduct.

In a preliminary study, the bovine OEC monolayer was stimulated with 4 doses (1, 10, 100, and 1000 ng/ml) of LPS (serotype *E. coli* 055:B5; Sigma) for 24 hr. In this dose-response study, 1000 ng/ml of LPS had lethal effects on cell viability, so we stimulated the bovine OEC cultures with 10 or 100 ng/ml of LPS (Kowsar et al., 2013). The purity of epithelial cell preparations was evaluated by reacting the cultured cells with monoclonal antibodies to cytokeratin (anti-cytokeratin-CK1) and immunostaining. The cells in culture medium showed characteristic epithelial morphology. Approximately 98% of the cells were positive for cytokeratin (CK1).

Cell Viability Assay

Cell viability was estimated using Trypan-blue staining, and was confirmed to be more than 90% at each time of plating as well as at the end of the culture experiment. The cell counting kit-8 assay (Dojindo Laboratories, Kumamoto, Japan) was also used to quantify the toxicity of LPS based on the reduction of the tetrazolium salt to a water-soluble formazan product by the cellular dehydrogenase, as previously described (Tominaga et al., 1999). Basically, 100 μl of growing bovine OECs suspension (5000 cells/well) was dispensed in a 96-well plate and incubated for 24 hr in at 38°C and 5% CO_2. Then, 10 μl of LPS (0, 10, 100, or 1000 ng/ml) was added to the plate and incubated for 2 hr. Ten microliters of the cell counting kit-8 reagent solution was added to each well, and incubated for an additional 2 hr. Absorbance per well was measured at 450 nm using a microplate reader (Thermo, Labsystems Multiskan MS plate reader, Vantaa, Finland).

Extraction of RNA, Production of cDNA, and Real-Time Reverse-transcriptase Polymerase Chain Reaction

Total RNA was extracted from oviduct tissue and bovine OEC cultures using TRIzol reagent (Invitrogen, Carlsbad, USA), as described previously (Chomczynski and Sacchi, 1987; Kowsar et al., 2013). DNase treatment was carried out using an RQ1 RNase-Free DNase kit (Promega, Madison, WI, USA), as described previously (Kowsar et al., 2013). The synthesized cDNA was stored at −30°C. Quantifications of mRNA expression were performed using synthesized cDNA via real-time PCR with a LightCycler (Roche Diagnostics, Mannheim, Germany) using a QuantiTect™ SYBR Green PCR Master Mix (QIAGEN GmbH, Hilden, Germany).

We analyzed the following genes: *AGP*, *AGPR*, *TLR-4*, *TLR-2*, *TNFA*, *IL-1B*, and *β-actin*. The primers were designed using Primer 3 based on bovine sequences (Table 1). The amplification program consisted of 15 min activation at 95°C, followed by 40 cycles of 15 sec denaturation at 95°C, 30 sec annealing at 54−58°C, and 20 sec extension at 72°C). The values of mRNA expression were normalization to *β-actin* as an internal control. The expression of *β-actin* was stable in all experiments, and no significant difference was detected in the levels of *β-actin* expression between treatments.

Measurement of AGP in Medium From Cultured Bovine OECs

Conditioned media were collected from bovine OEC cultures after 24 hr, centrifuged at 1000 *g*, and the supernatants were stored at −30°C until analyzed using an ELISA kit from Uscn (Life Science Inc. WUHAN, China), according to the manufacturer's protocol. The amount of AGP was quantified based on a standard curve after optical density measurements at 450 nm on a microplate reader (Thermo, Labsystems Multiskan MS plate reader). Coefficients of variance for intra- and inter-assay were 10 and 12%, respectively. The ranges of the standard curve for these assays were 15.6−1000 ng/ml.

Statistical Analysis

Data are presented as the mean ± standard error of the mean of 5 experiments. Statistical analyses were performed with Stat View 5.0 (SAS Institute Inc.). One-way

TABLE 1. Bovine Primers Were Used in Real-Time Reverse-Transcriptase PCR

Gene		Sequence of nucleotide[a]	Tm (°C)	Accession No.	Product size (bp)
TLR-4	F	5′-CTTGCGTACAGGTTGTTCCTAA	56	NM_174198	153
	R	5′-CTGGGAAGCTGGAGAAGTTATG			
TLR-2	F	5′-GCTCCTGTGACTTCCTGTCC	54	NM_174197	501
	R	5′-CCGAAAGCACAAAGATGGTT			
AGP	F	5′-CCAACCTGATGACAGTGGC	58	NM_001040502	194
	R	5′-GCCGACTTATTGTACTCGGG			
AGPR	F	5′-ATGACAAAGGAGTATCAA	52	NM_001037590	108
	R	5′-AGCTTGGAGTTCTGGGAT			
IL-1B	F	5′-ATGAAGAGCTGCATCCAACA	56	NM_174093	196
	R	5′-ATGGAAGACATGTGCGTAGG			
TNFA	F	5′-TGACGGGCTTTACCTCATCT	56	NM_173966	221
	R	5′-TGATGGCAGACAGGATGTTG			
β-actin	F	5′-CCAAGGCCAACCGTGAGAAAAT	58	K00622	256
	R	5′-CCACATTCCGTGAGGATCTTCA			

[a]F, forward; R, reverse.

ANOVA followed by multiple comparison tests, Fisher (for 3 groups) or Bonferroni (for more than 3 groups), were performed, and all results were considered to be statistically significant at $P < 0.05$.

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