Effects of IL8 and immune cells on the regulation of luteal progesterone secretion

Heather Talbott1,2,*, Abigail Delaney2,*, Pan Zhang2, Yangsheng Yu3, Robert A Cushman4, Andrea S Cupp5, Xiaoying Hou2 and John S Davis6

1Department of Biochemistry and Molecular Biology, University of Nebraska Medical Center, Omaha, Nebraska 68198-5870, USA, 2Department of Obstetrics and Gynecology, Olson Center for Women’s Health, University of Nebraska Medical Center, Omaha, Nebraska 68198-3255, USA, 3Department of Pathology and Microbiology, University of Nebraska Medical Center, Omaha, Nebraska 68198-5900, USA, 4United States Department of Agriculture-U.S. Meat Animal Research Center, Clay Center, Nebraska 68933-0166, USA, 5Department of Animal Science, University of Nebraska-Lincoln, Lincoln, Nebraska 68583-0908, USA and 6VA Nebraska Western Iowa Health Care System and Olson Center for Women’s Health, Department of Obstetrics and Gynecology, University of Nebraska Medical Center, 983255 Nebraska Medical Center, Omaha, Nebraska 68198-3255, USA

Correspondence should be addressed to J S Davis; Email: jsdavis@unmc.edu

*(H Talbott and A Delaney are co-first authors)

Abstract

Recent studies have suggested that chemokines may mediate the luteolytic action of prostaglandin F2α (PGF). Our objective was to identify chemokines induced by PGF in vivo and to determine the effects of interleukin 8 (IL8) on specific luteal cell types in vitro. Mid-cycle cows were injected with saline or PGF, ovaries were removed after 0.5–4 h, and expression of chemokine was analyzed by qPCR. In vitro expression of IL8 was analyzed after PGF administration and with cell signaling inhibitors to determine the mechanism of PGF-induced chemokine expression. Purified neutrophils were analyzed for migration and activation in response to IL8 and PGF. Purified luteal cell types (steroidogenic, endothelial, and fibroblast cells) were used to identify which cells respond to chemokines. Neutrophils and peripheral blood mononuclear cells (PBMCs) were cocultured with steroidogenic cells to determine their effect on progesterone production. IL8, CXCL2, CCL2, and CCL8 transcripts were rapidly increased following PGF treatment in vivo. The stimulatory action of PGF on IL8 mRNA expression in vitro was prevented by inhibition of p38 and JNK signaling. IL8, but not PGF, TNF, or TGFβ1, stimulated neutrophil migration. IL8 had no apparent action in purified luteal steroidogenic, endothelial, or fibroblast cells, but stimulated ERK phosphorylation in neutrophils. In coculture experiments neither IL8 nor activated neutrophils altered basal or LH-stimulated luteal cell progesterone synthesis. In contrast, activated PBMCs inhibited LH-stimulated progesterone synthesis from cultured luteal cells. These data implicate a complex cascade of events during luteolysis, involving chemokine signaling, neutrophil recruitment, and immune cell action within the corpus luteum.


Introduction

The corpus luteum develops after ovulation and secretes progesterone, a steroid hormone essential for the establishment and maintenance of early pregnancy (Niswender et al. 2000, Stocco et al. 2007). In the absence of hormonal cues or pregnancy, the corpus luteum will regress in a process termed luteolysis. In many species, luteolysis is mediated by uterine and/or intraluteal release of prostaglandin F2α (PGF; Davis & Rueda 2002, Wiltbank & Ottobre 2003, Niswender et al. 2007, Bogan et al. 2008). PGF has been shown to act indirectly at the vascular level to cause disruption of luteal capillaries (Maroni & Davis 2011) and apoptosis of capillary endothelial cells (Henkes et al. 2008). PGF has also been implicated in the initiation of luteal cell apoptosis in vivo (Davis & Rueda 2002, Quirk et al. 2013); however, PGF alone cannot directly reduce the viability of luteal cells in vitro (Davis & Rueda 2002, Kawaguchi et al. 2013). Thus, other mechanisms must be activated for luteolysis to proceed through both the functional (loss of progesterone secretion) and structural (apoptosis and tissue remodeling) stages of regression.
Immune cells and their effector cytokines participate in various reproductive processes (Pate & Landis Keyes 2001, Skarzynski 2008, Shirasuna et al. 2012a, b), including ovulation (Vinatier et al. 1995, Ujioka et al. 1998) and endometrial function (Braudmeier et al. 2012, Care et al. 2013), as well as corpus luteum formation and regression (Erlebacher et al. 2004, Skarzynski 2008, Shirasuna et al. 2012a, b, c, Care et al. 2013). Interleukin 8 (IL8, also known as CXCL8) is a known chemotactic cytokine secreted by a variety of cells in response to inflammatory stimuli. IL8 secretion is implicated in the recruitment and activation of neutrophils (Mukaida 2000, 2003), including within the corpus luteum (Polec et al. 2009, Jiemtaweeboon et al. 2011, Shirasuna et al. 2012a). In rabbits, neutralization of IL8 suppresses neutrophil activation and ovulation (Ujioka et al. 1998). Recent studies have also indicated that neutrophils and IL8 are involved in establishment of the corpus luteum following ovulation. IL8 and neutrophils are known to promote angiogenesis (Heidemann et al. 2003, Li et al. 2003), findings that have been recently extended to the developing corpus luteum (Jiemtaweeboon et al. 2011, Nitta et al. 2011, Shirasuna et al. 2012b, c). IL8 is also capable of stimulating progesterone secretion by luteinizing granulosa (Shimizu et al. 2012) and theca cells (Shimizu et al. 2013).

Our objective was to identify chemokines induced by PGF in vivo and to determine the effect of IL8 on specific luteal cell types in vitro. We also employed cocultures to evaluate the effects of immune cells on luteal progesterone synthesis. The present study demonstrates that PGF stimulates the expression of IL8, CCL8, CCL2, and CXCL2. While IL8 was effective at recruitment of neutrophils, neither IL8 nor activated neutrophils reduced LH-stimulated luteal progesterone synthesis. In contrast, activated peripheral blood mononuclear cells (PBMCs) inhibited LH-stimulated progesterone by luteal cells in vitro. The activation of immune cells during luteolysis may be involved in the regression of the bovine corpus luteum.

Materials and methods

In vivo studies

All animal procedures were conducted under an IACUC-approved protocol and carried out at the University of Nebraska-Lincoln, Animal Sciences Department. Post-pubertal female calves of composite breeding age received an i.m. injection at mid-cycle (days 9–10) with saline (n = 3) or 25 mg of the PGF analog, Lutalyse (Pharmacia and Upjohn Company, New York, NY, USA) or 25 mg of the PGF analog, Lutalyse (Pharmacia and Upjohn Company, New York, NY, USA) (days 9–10) with saline (n = 3) or 25 mg of the PGF analog, Lutalyse (Pharmacia and Upjohn Company, New York, NY, USA). Ovariectomies were performed at 0.5, 1, 2, and 4 h after PGF treatment and RNA was isolated from the corpora lutea using the Absolutely mRNA Purification Kit (Agilent Technologies, Inc., Santa Clara, CA, USA). The tissue was obtained from cows during early pregnancy (fetal crown rump length < 10 cm) to ensure luteal function (Ireland et al. 1980). The luteal tissue was dissected from the ovary and dissociated with 103 IU/ml collagenase (Atlanta Biologicals, Norcross, GA, USA) as described previously (Chakravorty et al. 1993). Luteal cell viability was determined using trypan blue exclusion, and luteal cell preparations with more than 90% viability were used. Enriched bovine steroidogenic luteal cells (1 × 107 cells/cm2) were plated as described previously (Hou et al. 2010). The cells were incubated overnight in medium 199 (M199, Lonza, Basel, Switzerland) supplemented with 5% fetal bovine serum (FBS, Valley Biomedical, Winchester, VA, USA). The next day the medium was changed and the incubations were continued for 1 day in FBS-free media. On the day of the experiment, the medium was replaced with fresh FBS-free medium for 2–3 h to pre-equilibrate before administering the treatments described in the figure legends.

Table 1 Bovine primers for qPCR.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Primers for qPCR</th>
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<tbody>
<tr>
<td>ACTB</td>
<td>F: ACACCCGAAACCAGTTCCGCGAT &lt;br&gt; R: AAGACCGGCCCGCGGAGGACAT</td>
</tr>
<tr>
<td>IL8</td>
<td>F: TGTCAGACGTGCAATTCGTCGACAG &lt;br&gt; R: TGACCCACATTTTCTTCCGGGT</td>
</tr>
<tr>
<td>GAPDH</td>
<td>F: AAGTTGGAAGCGGAGTCAGT &lt;br&gt; R: GAGCTCGTGCTCCTGAGAAT</td>
</tr>
<tr>
<td>CCL2</td>
<td>F: TGGCTCCGTACCCAGCATGGAAT &lt;br&gt; R: GGGACACCGTGCTGCTGACGCGA</td>
</tr>
<tr>
<td>CCL8</td>
<td>F: TCTCAGGCTGGAAGCCGGCGCTC &lt;br&gt; R: ACTGAATCTGGCTGAGCGACG</td>
</tr>
<tr>
<td>CXCL2</td>
<td>F: GGGCCCGTGCTGCAAGCCACT &lt;br&gt; R: AGACTGCGCATGACTGCTGGTTTGGT</td>
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**Isolation of bovine endothelial and fibroblast cells**

Endothelial cells were isolated from bovine corpus luteum of early pregnancy and purified as described previously (Maroni & Davis 2011). The endothelial cells were positive for vascular endothelial cell cadherin (VE-cadherin) and negative for steroidogenic enzymes and prolyl 4-hydroxylase (antibodies are listed in Table 2). The cells were grown to ~80% confluence in DMEM (Corning CellGro, Corning, NY, USA) containing 10% FBS and 20 µg/ml endothelial cell growth supplement (ECGS, Millipore, Bedford, MA, USA). The medium was changed to serum-free DMEM containing 20 µg/ml ECGS 2 h before administring treatments described in the figure legends.

Fibroblasts were isolated from the bovine corpus luteum and characterized as described previously (Maroni & Davis 2012). The fibroblasts were positive for prolyl 4-hydroxylase and collagen 1 and negative for steroidogenic enzymes and VE-cadherin (antibodies listed in Table 2). Luteal fibroblasts were grown to ~80% confluence and changed to serum-free DMEM 2 h before treatment with IL8 as described in the figure legends.

**Isolation of bovine neutrophils and migration assays**

Potassium EDTA (Sigma–Aldrich)-anticoagulated bovine blood samples were collected from a local abattoir (XL Four Star Beef), centrifuged, and subjected to Percoll gradient (Sigma–Aldrich) separation to isolate neutrophils. The remaining erythrocytes were lysed by rapid treatment with dH2O and samples were collected from a local abattoir (XL Four Star Beef). Blood was then diluted 1:2 in cold Hank’s Balance Salt Solution (HBSS, Corning CellGro) with 2 mM EDTA (Sigma–Aldrich) and 5% FBS. Diluted blood was underlayed with an equal volume of Histopaque (specific gravity 1.083, Sigma–Aldrich) and centrifuged at 900 g for 30 min. PBMCs were collected from an interface between the plasma and Histopaque. The cells were washed in HBSS three times before use.

Enriched bovine steroidogenic luteal cells were plated (~1 x 10^5 cells/cm^2) in basal M199 medium containing 5% FBS in 48-well plates overnight as described earlier.

**Table 2** Antibodies used for cell signaling, western blots, and flow analysis.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Vendor</th>
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<tr>
<td>VE-cadherin</td>
<td>Pierce</td>
</tr>
<tr>
<td>STAR</td>
<td>Douglas Stocco, PhD</td>
</tr>
<tr>
<td>3B-HSD</td>
<td>Ian Mason, PhD</td>
</tr>
<tr>
<td>P450ccc</td>
<td>Millipore</td>
</tr>
<tr>
<td>Prolyl 4-hydroxylase</td>
<td>Acris</td>
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<td>Collagen 1</td>
<td>Rockland Immunochemicals</td>
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<tr>
<td>Phospho ERK1/2</td>
<td>Cell Signaling</td>
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<td>Phospho p38</td>
<td>Cell Signaling</td>
</tr>
<tr>
<td>Phospho JNK</td>
<td>Santa Cruz, TX</td>
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<td>Phospho AKT</td>
<td>Cell Signaling</td>
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<tr>
<td>Phospho P65-NeFkB</td>
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<tr>
<td>IxBz</td>
<td>Cell Signaling</td>
</tr>
<tr>
<td>B-actin</td>
<td>Sigma–Aldrich, St. Louis, MO</td>
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</table>

To determine the signaling pathways used by IL8 in bovine neutrophils, we treated neutrophils with IL8 or TNF (R&D Systems), a modulator of immune function and activator of multiple signaling pathways. Western blot analysis was carried out to examine the MAPK (ERK1/2, p38, and JNK), AKT, and NFkB signaling pathways using phosho-specific antibodies. A complete list of antibodies used is given in Table 2.

**Isolation of human neutrophils and degranulation assays**

Human neutrophils were isolated from peripheral blood of healthy donors by density gradient centrifugation under an approved IRB at the University of Nebraska Medical Center, using polymorphrep (Axis-Shield, Oslo, Norway) in accordance with manufacturer’s instruction. Purified neutrophils were resuspended in RPMI +5% FBS. The neutrophils (3 x 10^6 cells) were incubated with different concentrations of IL8 at 37 °C for 1 h. Neutrophil degranulation was examined by florescence-activated cell sorting for increased cell surface expression of granule molecules: carcinoembryonic antigen-related cell adhesion molecule 8, CEACAM8 (CD66b) and integrin alpha M, ITGAM (CD11b). The cells were stained with FITC-conjugated mouse anti-human CD66b antibody and allophycocyanin (APC)-conjugated mouse anti-human CD11b antibody on ice for 30 min. After rinsing, cells were fixed with PBS plus 2% formaldehyde. Flow cytometry analysis was done using a Becton Dickinson (Franklin Lakes, NJ, USA) FACS Caliber flow cytometer and was carried out at the UNMC Cell and Tissue Analysis Facility.

**Isolation of bovine peripheral blood mononuclear cells**

Acid citrate dextrose-anticoagulated blood samples from cows were collected from a local abattoir (XL Four Star Beef). Blood was then diluted 1:2 in cold Hank’s Balance Salt Solution (HBSS, Corning CellGro) with 2 mM EDTA (Sigma–Aldrich) and 5% FBS. Diluted blood was underlayered with an equal volume of Histopaque (specific gravity = 1.083, Sigma–Aldrich) and centrifuged at 900 g for 30 min. PBMCs were collected from an interface between the plasma and Histopaque. The cells were then washed in HBSS three times before use.

**Coculture experiments**

Enriched bovine steroidogenic luteal cells were plated (~1 x 10^5 cells/cm^2) in basal M199 medium containing 5% FBS in 48-well plates overnight as described earlier.

**Neutrophil–luteal cell coculture:**

Neutrophils were isolated on the same day the luteal cells were prepared. Purified neutrophils were then cultured in RPMI (10% FBS) with or without 30 ng/ml IL8 and 20 nM phorbol myristate acetate (PMA, EMD Millipore Calbiochem, Billerica, MA, USA) overnight. After 24 h, the medium was replaced onto the luteal cell cultures. Neutrophils (250 000 cells/ml) were then added to the
luteal cells to give a final ratio of M199:RPMI (1:1) with 10% FBS 2 h before adding control media or 10 ng/ml bLH (Tucker Endocrine Research Institute, Atlanta, GA, USA). Medium from each well was collected 6 h after LH or control treatments for progesterone analysis.

**PBMC–luteal cell coculture**

Twenty-four hours after plating the luteal cells, the medium was removed from the culture wells and replaced with fresh M199. Then an equal volume of newly isolated bovine PBMCs in RPMI (100 000 cells/ml) was added to the luteal cell culture. The cocultures were incubated for 24 h in M199 and RPMI (1:1 ratio) + 10% FBS, and with or without 10 μg/ml concanavalin A (Sigma), to activate the PBMCs. After 24 h of coculture, the medium was replaced with M199:RPMI + 10% FBS for 2 h to pre-equilibrate the cells before the addition of control media or 10 ng/ml LH. The medium was removed from each well after 6 h of control or LH treatment for progesterone analysis.

**Western blot analysis**

Cultures of neutrophils, steroidogenic cells, luteal endothelial cells, and luteal fibroblasts were harvested with ice-cold cell lysis buffer (20 mM Tris–HCl (pH = 7), 150 mM NaCl, 1 mM Na₂EDTA, 1 mM EGTA, 1% Triton X-100 and protease and phosphatase inhibitor cocktails (Sigma–Aldrich)). Protein concentration was determined and 40–60 μg protein was subjected to 10% SDS–PAGE. After transfer to polyvinylidene fluoride (PVDF) membranes, the membranes were probed with appropriate amounts of primary antibodies, and bound antibodies were detected with a HRP-conjugated secondary antibody and the Femto Western Blotting Detection Kit (GE Healthcare Amersham, Cleveland, OH, USA). Signals were visualized using a Digital Sciences Image Station 440 (Kodak).

**Progesterone analysis**

Conditioned media were collected for progesterone determination using the Coat-A-Count Progesterone RIA kit (Siemens, Deerfield, IL, USA) according to the manufacturer’s instructions and as reported previously (Hou et al. 2010).

**Statistical analysis**

All experiments were carried out at least two times using different cell preparations with qualitatively comparable results. The data are presented as representative experiments or as the mean ± S.E.M. of the averages from multiple experiments. The differences in means were analyzed by t-test or ANOVA followed by multiple range testing. \( P < 0.05 \) was considered statistically significant.

**Results**

**PGF stimulates chemokine gene expression in vivo**

Treatment with PGF in vivo resulted in a 4.3 ± 1.0-fold increase in IL8 mRNA within 30 min and a 8.9 ± 2.2-fold increase in IL8 mRNA within 1 h of administration (Fig. 1A). Treatment with PGF also increased mRNA expression of CCL8, CXCL2, and CCL2 after 1 h (fold increases of 2.5 ± 0.6, 2.9 ± 0.7, and 3.1 ± 0.6 respectively). After a brief lag, the expression of chemokine mRNA increased dramatically after 4 h of treatment with PGF. A 35 ± 4-fold increase in IL8 mRNA expression was observed in response to a 4 h treatment with PGF. At the 4 h, PGF also markedly stimulated significant \( P < 0.05 \) increases in the mRNA expression of CCL8, CCL2, and CXCL2 (29 ± 3.8-, 12 ± 1.5- and 6.4 ± 1-fold respectively).

*Fig. 1* Induction of chemokines following treatment with PGF in vivo and in vitro. (A) Midluteal phase cows were treated with saline or the PGF analog Lutalyse (25 mg) for up to 4 h. Ovaries were surgically removed and RNA was isolated from corpora lutea. Quantitative real-time PCR was carried out. Results are shown as mean ± S.E.M., \( n = 3 \). (B) To determine the cellular signaling pathway leading to the induction of IL8 mRNA, bovine steroidogenic luteal cells were pretreated for 60 min with vehicle, the ERK1/2 inhibitor U0126 (20 μM), the p38 MAPK inhibitor SB203580 (10 μM), or the JNK inhibitor SP600125 (20 μM). Luteal cells were then treated with control media (open bars) or PGF (100 nM, solid bars) for 60 min. Quantitative real-time PCR for IL8 mRNA was carried out. Results are shown as mean ± S.E.M., \( n = 3 \). *\( P < 0.05 \); **\( P < 0.01 \); NS, not significant.
**PGF stimulates IL8 expression in vitro**

Treatment of steroidogenic luteal cells with PGF for 1 h *in vitro* also increased IL8 mRNA expression (2.7 ± 0.3-fold increase, P < 0.05; Fig. 1B). Luteal steroidogenic cells were pretreated in the presence or absence of specific inhibitors of the MAPK signaling cascade to determine which intracellular signals contribute to the stimulatory effect of PGF on the induction of IL8 gene expression (Fig. 1B). Pretreatment with the ERK1/2 inhibitor U0126 (Enzo Life Sciences, Farmingdale, NY, USA) failed to prevent the stimulatory effect of PGF on IL8 mRNA (Fig. 1B). In contrast, inhibition of the stress-activated protein kinase p38 MAPK with SB2037580 resulted in a complete inhibition of the response to PGF. Treatment with the JNK inhibitor SP600125 also resulted in a significant inhibition (77%, P < 0.05) of the PGF-induced increase in IL8 mRNA.

**IL8 induces migration of bovine neutrophils**

To determine whether IL8 would affect the function of bovine neutrophils, we purified neutrophils from the blood collected at slaughter of cows. As shown in Fig. 2A, neutrophils stained with hematoxylin and eosin had distinct multi-lobular nuclei, a characteristic of neutrophils. A Boyden chamber assay (Fig. 2A) was used to determine whether IL8 or other factors produced during luteolysis could increase migration of bovine neutrophils. We observed that treatment for 18 h with 30 ng/ml IL8 caused a 20-fold (P < 0.05) increase in neutrophil migration. However, treatment of neutrophils with 100 nM PGF under identical conditions had no effect on neutrophil migration (Fig. 2B). Migration assays were also carried out with other chemokines that have been implicated in luteal regression, namely TNF (Henkes et al. 2008, Skarzynski et al. 2009) and TGFβ1 (Maroni & Davis 2011, 2012). In experiments evaluating neutrophil migration during a 3 h treatment period, we found that 30 ng/ml IL8, but not 100 nM PGF, 10 ng/ml TNF, or 1 ng/ml TGFβ1, was capable of stimulating the migration of neutrophils (Fig. 2C).

Activation of neutrophils results in the rapid cell surface expression of molecules that allows for endothelium attachment for extravasation. Treatment of human neutrophils with increasing concentrations of human IL8 (0–100 ng/ml) resulted in the rapid expression of the cell adhesion molecules: ITGAM and CEACAM8 as determined by flow cytometry (not shown).

**IL8 selectively stimulates signaling in bovine neutrophils**

Treatment with IL8 for 15 min stimulated an increase (fivefold, P < 0.05) in ERK1/2 phosphorylation (Fig. 3A). The response was transient and returned to control levels within 120 min following IL8 treatment (Fig. 3). IL8 did not stimulate either the p38 or the JNK MAPK signaling pathways (Fig. 3A). TNF was used as a positive control because it activates many signaling pathways in immune cells. In contrast to IL8, TNF provoked sustained ERK phosphorylation, as well as p38 and JNK
phosphorylation, in bovine neutrophils throughout the 120 min investigated (Fig. 3). IL8 exerted a slight, but consistent, increase in the phosphorylation of p65-NFκB and AKT, whereas TNF stimulated a robust increase in p65-NFκB and AKT phosphorylation in neutrophils. To determine whether IL8 could similarly stimulate other cells of the corpus luteum, we treated bovine luteal fibroblasts, endothelial cells, and steroidogenic cells with IL8 under various treatment times and concentrations. IL8 did not stimulate the phosphorylation of AKT, ERK, or NFκB in any of these luteal cell types. As a positive control, we observed that TNF stimulated MAPK and NFκB signaling in each cell type examined (data not shown).

In view of the very prominent effect of IL8 on ERK signaling in neutrophils, we tested whether the IL8-induced increase in ERK phosphorylation was associated with the effect of IL8 on neutrophil migration. Pretreatment with 5 μM of U0126, completely blocked the induction of ERK phosphorylation (Fig. 4A), but did not prevent the stimulatory effect of IL8 on bovine neutrophil migration (Fig. 4B).

**Effect of IL8 and immune cells on progesterone secretion**

Experiments were carried out to determine whether IL8 altered progesterone secretion. Pretreatment of steroidogenic luteal cells with increasing amounts of IL8 (0–30 ng/ml) did not alter basal or LH-simulated progesterone production in luteal cells (Fig. 5A). Next, we cocultured neutrophils with steroidogenic cells and evaluated the ability of LH to stimulate progesterone secretion. We observed that cocultures of steroidogenic cells and neutrophils had no effect on the ability of LH to increase progesterone (Fig. 5B). Furthermore, cocultures of steroidogenic cells and activated neutrophils had no effect on basal or LH-stimulated progesterone production.

Cocultures of steroidogenic cells and PBMCs had no effect on the ability of LH to secrete progesterone (Fig. 6). However, LH-stimulated progesterone production was completely abrogated (P<0.05) in cultures of activated PBMCs and steroidogenic luteal cells (Fig. 6).

**Discussion**

For over 30 years, the immune system has been postulated as essential for fertility (Espey 1980). The present...
study provides additional insights into the expression and function of chemokines during luteal regression. We observed that induction of luteal regression in cows with a bolus of PGF in vivo resulted in a rapid increase in the expression of IL8, CCL8, CCL2, and CXCL2. Our findings confirm the recent findings reported by Shirasuna et al. (2012a) that PGF treatment of dairy cattle increased luteal IL8 mRNA by approximately fourfold within 30 min. In that study, the fold increase in IL8 mRNA remained constant over a 12-h period of treatment with PGF. In this study, using beef cattle we observed more robust increases in luteal IL8 mRNA expression: ninefold increases within 1 h and 35-fold increases after 4 h of PGF treatment. At present, it is not clear whether the differences in the magnitude of the responses are due to differences in the cattle breeds or other factors, because there are reported differences in the responses of beef and dairy cattle to synchronization protocols using PGF (Lucy et al. 2001). Based on the pronounced increase in IL8 expression, it was selected for further analysis. We found that IL8 acted directly on neutrophils but had little effect on other cell types in the mid-cycle corpus luteum. Furthermore, cocultures of luteal cells with activated neutrophils did not alter LH-stimulated progesterone synthesis, whereas cocultures with activated PBMCs suppressed LH-stimulated progesterone synthesis.

Activation of the PGF receptor rapidly induces calcium mobilization and activation of PKC (Davis et al. 1987). These initial signaling events lead to the activation of ERK1/2 (Chen et al. 1998, Arvisais et al. 2010), p38, and JNK (Yadav et al. 2002, Yadav & Medhamurthy 2006, Mao et al. 2013) in vivo and in vitro, with subsequent activation of multiple transcription factors. The MAPK signaling family induces early-response genes such as FOS and JUN (Chen et al. 2001), NR4A1 (Stocco et al. 2007, Atli et al. 2012), EGR1 (Hou et al. 2008, Atli et al. 2012), and ATF3 (Mondal et al. 2011, Mao et al. 2013) in the corpus luteum. To determine which intracellular signals contribute to the stimulatory effect of PGF on IL8 gene expression, luteal cells were treated with specific inhibitors of ERK1/2, p38, and JNK. We observed that the ERK1/2 inhibitor U0126 had no effect on IL8 mRNA expression in response to PGF, while the p38 MAPK inhibitor had a significant effect. The JNK inhibitor did not significantly affect IL8 expression.

Figure 5 IL8 and neutrophils do not inhibit luteal progesterone production. (A) Steroidogenic luteal cells were pretreated with increasing amounts of IL8 (0–30 ng/ml) for 30 min and then treated without (Control) or with LH (10 ng/ml) for 4 h. Progesterone in the media was measured by RIA. Results are shown as mean±s.e.m., n=4. (B) Steroidogenic luteal cells were cocultured with bovine neutrophils or activated bovine neutrophils as described in the Materials and methods. Cells were then treated without (Control) or with LH (10 ng/ml) for 4 h. Progesterone in the media was measured by RIA. Results are shown as mean±s.e.m., n=3. *P<0.05 vs Control; NS, not significant.

Figure 6 Cocultures of luteal cells with activated peripheral blood mononuclear cells (PBMCs) inhibit luteal progesterone production. Steroidogenic luteal cells were cocultured with bovine PBMCs or activated PBMCs as described in the Materials and methods. Cells were then treated without (Control) or with LH (10 ng/ml) for 4 h. Progesterone in the media was measured by RIA. Results are shown as mean±s.e.m., n=4. *P<0.05; NS, not significant.
inhibitor SB2037580 and the JNK inhibitor SP600125 significantly inhibited the PGF-mediated upregulation of IL8 mRNA. The results indicate that the stress-activated MAPKs, p38 and JNK, play an important and perhaps overlapping role in the induction of IL8 mRNA in response to PGF.

Chemokines such as IL8 are responsible for the recruitment of immune cells to chemokine-producing tissues. Our findings demonstrate that IL8 is chemotactic for bovine neutrophils, in agreement with previous literature (Mukaida 2000, 2003, Jientaweeboon et al. 2011, Shirasuna et al. 2012a). IL8 stimulated a sixfold increase in neutrophil migration within 3 h and after 24 h and IL8 treatment increased neutrophil migration nearly 20-fold. In contrast, treatment with PGF had no effect on neutrophil migration at either time point. These findings are consistent with the studies reported previously (Liptak et al. 2005, Shirasuna et al. 2012a), showing that immune cells are unresponsive to PGF because they do not express the PGF receptor. In this study, we also report that TNF and TGFβ1, two cytokines induced rapidly in the bovine corpus luteum in response to PGF and implicated in events associated with luteal regression (Henkes et al. 2008, Hou et al. 2008, Mondal et al. 2011, Maroni & Davis 2012), did not increase the migration of bovine neutrophils in the Boyden chamber assay. Our observations support the recent reports (Sales et al. 2009, Mondal et al. 2011, Atlì et al. 2012, Shirasuna et al. 2012a), showing that PGF induces IL8 mRNA and that the expression of IL8 is associated with the appearance of neutrophils in the bovine corpus luteum (Jientaweeboon et al. 2011, Shirasuna et al. 2012a,b). In addition, our studies indicate that IL8 stimulates the degranulation of human neutrophils which may be important for their migration from the blood into the corpus luteum. These findings correspond with the studies reported by Shirasuna et al. (2012a) demonstrating the rapid appearance of P-selectin on endothelial cells following treatment with PGF, which allows for adhesion of neutrophils to endothelial cells. Other chemokines (CCL8, CCL2, and CXCL2) are induced concomitantly with IL8, therefore it will be important to evaluate the contributions of each individual chemokine to the recruitment of specific immune cells into the regressing corpus luteum. Future experiments should also address how combinations of these chemokines signal the recruitment and activation of immune cells within the corpus luteum (Gouwy et al. 2008).

Treatment of neutrophils with IL8 stimulated a robust increase in ERK phosphorylation, a slight increase in AKT and NFκB phosphorylation, and had no effect on p38 and JNK signaling. In contrast, TNF activated all of these pathways simultaneously in neutrophils. As ERK signaling was the most prominent pathway activated following IL8 treatment of bovine neutrophils, we determined whether neutrophil migration could be blocked by treatment with the MEK1/2 inhibitor U0126. Interestingly, we found that inhibition of ERK signaling with U0126 had no inhibitory effect on IL8-stimulated neutrophil migration. These results suggest that another signaling pathway is responsible for IL8-stimulated chemotaxis, probably the PI3K or RAC signaling pathway (Neptune et al. 1999, Futosi et al. 2013). Further studies are required to determine the contributions of other signaling pathways to neutrophil activation and migration.

IL8 has been shown to induce diverse cellular responses in cells other than neutrophils (Mukaida 2003). Recent studies have suggested that IL8 may contribute to angiogenesis in the newly forming corpus luteum (Jientaweeboon et al. 2011) and progesterone secretion by granulosa (Shimizu et al. 2012) and theca (Shimizu et al. 2013) cells. Treatment with various IL8 concentrations and treatment times revealed no changes in cell signaling in steroidogenic cells, endothelial cells, or fibroblasts isolated from the bovine corpus luteum. However, IL8 stimulated a robust increase in ERK phosphorylation in neutrophils. Furthermore, IL8 did not affect basal or LH-stimulated progesterone secretion from cultured luteal cells. In contrast to the findings reported by Shimizu et al. (2012) using cells from the ovarian follicle, we found no evidence suggesting that IL8 acted directly on bovine luteal cells that are involved in luteal regression (e.g., endothelial cells, fibroblasts, and steroidogenic cells). Based on these findings, it appears that IL8 exerts specific effects on ovarian cell types depending on their stage of differentiation. Given that the corpus luteum is highly differentiated and undergoes regression in response to PGF, the lack of a stimulatory effect of IL8 on angiogenesis and steroidogenesis may be expected because the vasculature and steroid secretion are disrupted during regression (Davis & Rueda 2002, Niswender 2002, Stocco et al. 2007, Maroni & Davis 2012). It is possible that during luteal regression IL8-activated neutrophils contribute to phagocytosis during structural regression of the corpus luteum.

The increase observed in multiple chemokines suggests that immune cells other than neutrophils could be recruited into the corpus luteum following administration of PGF. In fact, studies from multiple laboratories have demonstrated an increase in neutrophils, T cells, or macrophages during the regression of the corpus luteum in rodents (Kuranaga et al. 1999), rabbits (Krusche et al. 2002), ruminants (Murdoch 1987, Penny et al. 1999), primates (Braundmeier et al. 2012), and women (Wang et al. 1992, Best et al. 1996, Gaytan et al. 1998, Suzuki et al. 1998). A previous report indicated that coculture of rat neutrophils with luteal cells resulted in a decrease in progesterone secretion, presumably as a result of oxidative stress (Behrman et al. 2001). However, under our experimental conditions cocultures of bovine neutrophils and steroidogenic luteal cells did not alter basal or LH-stimulated progesterone synthesis. Treatment of neutrophils with IL8 and PMA, alone or in combination, to activate
neutrophils was not sufficient to reduce progesterone secretion under coculture conditions. In addition to neutrophils, monocytes are immune effector cells that are also equipped with chemokine receptors and adhesion receptors that mediate migration from blood to tissues (Murray & Wynn 2011). As we observed that PGF rapidly induced the expression of other chemokines (CCL8, CCL2, and CXCL2), which could recruit other types of immune cells, we established a coculture system with PBMCs and luteal cells. Although unactivated PBMCs did not reduce progesterone secretion, we observed that activated PBMCs effectively reduced LH-driven progesterone secretion. These observations support our earlier findings (Liptak et al. 2005) that activated immune cells may contribute a factor (or factors) that impair steroidogenesis in response to LH. It is known that activated monocytes produce inflammatory cytokines, nitric oxide, and reactive oxygen species (Murray & Wynn 2011, Zhou et al. 2014) all of which may contribute individually or in combination to the inhibition of progesterone synthesis (Al-Gubory et al. 2012, Quirk et al. 2013, Skarzynski et al. 2013). In the in vivo setting, activated monocytes may also secrete matrix metalloproteinases that contribute to the degradation of the extracellular matrix (Murray & Wynn 2011), which could facilitate the recruitment of additional inflammatory cells to the regressing corpus luteum.

A complex interaction of endocrine and immune cells appears to be required to mediate the structural and functional regression of the bovine corpus luteum. As chemokines act synergistically to activate their target cells (Gouwy et al. 2008), additional studies are needed to examine the actions of chemokines as a complex cocktail rather than in isolation, as demonstrated in the present study. The current findings complement a recent review (Walasimbi & Pate 2013) that postulates that immune cells in the developing and functional corpus luteum play a supportive role, but once corpus luteum regression is triggered, the immune cells promote apoptosis, debris clearance, and tissue remodeling. Understanding these endocrine and immune events is important for increasing our ability to control reproductive function to facilitate full-term pregnancies in both humans and livestock.

Declarations of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This project was supported by Agriculture and Food Research Initiative Competitive Grant no. 2011-67015-20076 from the USDA National Institute of Food and Agriculture (J S Davis); the Department of Veterans Affairs (J S Davis), the National Institutes of Health 1 P01 AG029531; the Olson Center for Women's Health (A Delaney and J S Davis), and an Exceptional Incoming Graduate Student Award (H Talbott).

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Received 23 November 2013
First decision 4 February 2014
Revised manuscript received 14 March 2014
Accepted 31 March 2014