**Prostaglandin synthesis by the porcine corpus luteum; effect of Tumor Necrosis
Factor- α**

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Running or short title: Prostaglandins in the pig CL.

ABSTRACT

The porcine corpus luteum (CL) displays delayed sensitivity to PGF-2 α (Luteolytic sensitivity, LS) until days 12-13 of cycle. The control of LS is unknown, but it is temporally associated with macrophage (which secrete TNF- α) infiltration into the CL. Other studies showed that TNF- α induces LS in vitro and that Prostaglandins (PGs) may be involved in this mechanism. In Experiment 1, PGF-2 α and PGE secretion by luteal cells was measured on days 4-14 of the estrous cycle, and the expression of PTGFS/AKR1B1 and PTGES/mPGES-1, by Western blot, before (day 7), versus after (day 13), the onset of LS. Results showed that the PGF-2 α :PGE ratio increased significantly ($P<0.05$) from day 4 to 13-14, and PTGFS/AKR1B1 and PTGES/mPGES-1 were significantly increased ($P<0.05$) on day 13 (vs. day 7). In Experiment 2, luteal cells (LC) were collected from porcine CL at EARLY (~ days 4-6) or MID (~ days 7-12) stages of the estrous cycle and cultured with 0, 0.1, 1 or 10 ng/mL TNF- α . Results showed that TNF- α significantly increased ($P<0.05$) mRNA expression of COX-2 and mPGES1, but not AKR1B1. TNF- α had no significant effects on AKR1B1 or mPGES protein abundance. TNF- α significantly increased ($P<0.05$) PGE-2, but had no effect on PGF2 α , secretion, or on the PGF2 α :PGE2 ratio. In conclusion, while TNF- α increased COX2 and mPGES1 mRNA, and PGE-2 secretion in vitro, it did not increase the PGF2 α :PGE2 ratio. Studies are currently directed toward exploring other pathways (e.g. FP receptor signaling) by which TNF- α induces LS in the porcine CL.

Keywords: Pig, corpus luteum, prostaglandins, TNF- α , macrophages, luteolytic sensitivity

1. Introduction

It has been recognized for 40 yr or more that the porcine corpus luteum (CL) is “refractory” or “insensitive” to the luteolytic actions of PGF-2 α for the first 12-13 d of the estrous cycle [1]. However, we have demonstrated that multiple injections of PGF-2 α agonists induced premature luteolysis and reduced estrous cycle length by ~7 d [1, 2], indicating that the premature acquisition of “luteolytic sensitivity” to PGF-2 α (LS) could be induced by this approach. Since PGF-2 α treatment of cycling female pigs was shown to increase of macrophage infiltration into the CL [3], one possible explanation for these findings is that macrophages play a role in the acquisition of LS. Macrophages infiltrate the porcine CL throughout the estrous cycle [4] in confirmation of similar observations in other species [5-12]. Specifically in the pig, intra-luteal macrophage numbers increased ~4-fold between days 4-6 and days 7-12 of the estrous cycle [4], which coincides temporally with the development of LS during the normal estrous cycle [13]. Since porcine CL macrophages produce TNF- α [4] we hypothesized that macrophage-derived TNF- α may play a role in the premature acquisition of LS by PGF-2 α , described above [1,2], and provided some support for this hypothesis by showing that TNF- α “sensitized” porcine luteal cells to PGF-2 α in culture [13]. It is known that TNF- α increases luteal PGF-2 α synthesis by bovine luteal cells [14], and preliminary data (not shown) using inhibitors of PG synthesis suggested that PGs may be involved in the TNF- α induced “sensitization” pathway in porcine luteal cells. It has been shown in several species, that during the early luteal phase, luteal prostaglandin (PG) secretion favors the luteotropic PGs, e.g. PGE-2 (or PGI₂), but in the mid-late luteal phase, the synthesis of the luteolytic PG, PGF-2 α increases, resulting in an increased intra-luteal PGF-2 α :PGE ratio [15-19]. The intra-luteal PGF-2 α :PGE-2 ratio is thought to play an

important role in determining the luteolytic response of the CL to PGF-2 α [15-20]. Thus we hypothesized that TNF- α acts to induce LS by increasing PGF-2 α vs. PGE secretion by, and thus the PGF-2 α :PGE ratio in, the porcine CL.

Prostaglandins are derived from arachidonic acid (AA), which is released from cellular phospholipids via phospholipase A2 [21]. AA is then converted to PGH₂, the common precursor of the PGs, via cyclooxygenase (COX, PGG/H synthase or PTGS2). COX-1 is the constitutive form of this enzyme, whereas COX-2 is the inducible form and subject to control by hormones, cytokines (including TNF- α) and growth factors [22]. The final PG product of this pathway depends on the activities of the terminal *synthases* (*S*) (i.e. PTGES -> PGE-2; PTGFS -> PGF-2 α ; PTGDS -> PGD-2) [22]. A novel PTGFS sub-type of the aldose-keto-reductase (AKR) family, AKR1B1, was identified in CL of ruminants and localized to large luteal cells [15,18,23]. Although AKR1B1 has not previously been reported in the porcine CL, it does contain a different PTGFS (AKR1C3), the levels of which increased from days 2-4 to days 14-15 of the cycle [17]. The porcine CL also expresses PTGES (microsomal = mPGES1), the levels of which also increased from early to late cycle [17]. However, the PGF-2 α :PGE-2 (CL content of PGs) ratio ($\equiv$ functional activity of these enzymes), appeared to increase on days 9-15 versus days 2-8, coincident with the acquisition of LS [17].

Thus the objectives of this study were to: 1) examine the abundance (protein levels) of mPGES (PTGES1) and AKR1B1 (PTGFS) in, and the secretion of PGE and PGF-2 α by, CL or luteal cells collected during the normal estrous cycle (Experiment 1), and 2) to examine the effects of TNF- α on the expression (mRNA) and abundance (protein) of mPGES-1 and AKR1B1 in, and the secretion of PGE-2 and PGF-2 α by, luteal cells in culture (Experiment 2).

2. Materials and methods

2.1 Experiment 1

2.1.1 Animals and CL collection

Regularly cycling gilts (*Sus scrofa*, White Landrace x Blackline hybrid) were obtained from the herd of the NCSU Swine Educational Unit. Animals were checked daily for standing estrus with a mature boar. The first day of estrus was designated as day 0. Animals were subjected to surgical ovariectomy on days 4, 7-8, 10, 13-14 (n = 5-8 per day; PG secretion by luteal cells in vitro), or on days 7 and 13 (n = 4 and 6, per day respectively; PG synthetic enzyme protein levels in CL by Western blotting), as described previously [24-26]. Before surgery, animals were anesthetized with i.v. injection of ketamine (2.2 mg/kg; Webster Veterinary Supply, Sterling, MA) and xylazine (0.4 mg/kg; Webster), and a surgical plane of anesthesia was subsequently maintained with halothane (Webster) and an oxygen/nitrous oxide mixture. Ovaries were collected via mid-ventral laparotomy, and CL were dissected from ovarian tissue. Corpora lutea were either subjected to dissociation and culture (in vitro studies), or snap frozen in liquid nitrogen and then stored at -80C until further use (Western blotting). All animal use protocols were approved by the NCSU Institutional Animal Care and Use Committee (IACUC).

2.1.2 Protein extraction and analysis by Western blot – whole CL

CL protein extraction and Western blotting were performed using similar protocols to those previously reported [24-26]. In brief, total proteins were extracted from CL by homogenization in a “lysis buffer” (10mM Tris-HCl, 100 mM NaCl, 1mM EGTA, 1mM EDTA) containing 1% Triton-X, 0.5% NP-40, 1% P8340 protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) and 1% Halt Phosphatase inhibitor (Thermo-Fisher, Waltham, MA), on ice,

107 followed by sonication for 10-15 sec. Samples were then centrifuged at 14,000g to remove
108 cellular debris, the supernatants collected and frozen at -20C prior to Western blot
109 analysis. Protein concentrations were determined using the Micro-BCA™ protein assay
110 (Pierce, Rockford, IL) according to manufacturer's instructions. Between 15-40 µg of CL
111 protein lysates were boiled (95C for 5 mins) in Laemmli buffer and loaded on to 4-12% Bis-
112 Tris gels (Invitrogen, Carlsbad, CA) and subjected to electrophoresis at 180v for 50 min.
113 Following electrophoresis, proteins were transferred to PVDF membrane (Pall Company,
114 Pensacola, FL) using a Turbo-Blot trans-blot system (Bio-Rad, Hercules, CA). Blots were
115 subsequently blocked in 5% (w/v) milk dissolved in tris buffered saline (TBS)-Tween and
116 then incubated with primary antibody, both steps for 1h at room temperature with rocking.
117 Primary antibodies used were PGFS-AKR1B1 (1:2000; rabbit polyclonal, from Dr. Michel
118 Fortier, Laval University, Quebec, or rabbit polyclonal primary, Cat. No. ab71405, Abcam
119 Cambridge, MA), mPGES-1 (1:250; rabbit polyclonal primary, Cat. No. 160140I, Cayman
120 Chemical, Ann Arbor, MI) and β-actin (1:2000; mouse monoclonal primary, Cat. No. A2228,
121 Sigma-Aldrich). Membranes were washed in TBS-Tween, followed by incubation in
122 secondary antibody (1:1000-1:2000; goat anti-rabbit, Cat. No. sc-2004 or goat anti-mouse,
123 Cat. No. sc-2005, Santa Cruz Biotechnology, Santa Cruz, CA) for 1-2 h at room temperature,
124 and washed again with TBS-Tween. Chemiluminescent reagent (SuperSignal™ West Pico,
125 Thermo-Fisher) was added for 5 min, protein bands were visualized using a Bio-Rad,
126 Chemi-Doc (Bio-Rad) gel-imaging system, and bands were quantified with Image-Lab
127 software (Bio-Rad). The intensity (determined by densitometry) of chemiluminescent
128 specific protein bands were normalized to that obtained with the β-actin antibody and the
129 data expressed as a ratio [24-26].

2.1.3 CL dissociation and luteal cell culture

CL were dissected from the ovaries, minced (on ice) and subjected to dissociation with 0.1% collagenase, 0.1% hyaluronidase, 0.06% DNase in Calcium and Magnesium-free Hanks Balanced Salt Solution (HBSS; plus 10 mM HEPES, 0.35 g/L NaHCO₃, 50 µg/mL gentamicin, 50 IU penicillin and 50 µg/mL streptomycin) with 0.1% BSA at 37C, as described previously [27,28]. Luteal cells were washed in HBSS and incubated in 1 mL of Medium 199 (plus 10 mM HEPES, 0.35 g/L NaHCO₃, 50 µg/mL gentamicin, 50 IU penicillin and 50 µg/mL streptomycin and 0.1% BSA) at a concentration of 1 x 10⁶ luteal cells (based on Large Luteal Cell, LLC, count) in 13 x 100 mm glass test tubes, in shaking water bath (37C) with gassing hood under 95% oxygen: 5% carbon dioxide, for 6 h, in triplicate, per CL prep. After incubation, tubes were centrifuged, media collected and frozen at -20C prior to prostaglandin analysis by Radioimmunoassay (RIA).

2.1.4 Prostaglandin Radiomunoassays

All procedures were performed on ice except where noted. In brief, 100 µL of sample/standard, 100 µL antibody and 100 µL of [³H]-labeled tracer (~12,000 cpm per tube), diluted in assay buffer (0.01M Tris, pH 7.4), were incubated for 18-24h at 4C. A Norit A activated charcoal (2.5 mg/mL in 0.01M Tris, pH 7.4) suspension was added, and following mixing, separation of bound from unbound PG was achieved by centrifugation at 2500 rpm for 15 mins. Radioactivity was quantified by scintillation counting after addition of 12 mL of Ecolume (ICN, Costa Mesa, CA). Concentrations of PG in each sample was calculated from a standard line composed of the following concentrations of PG (1000, 333, 222, 111, 37.5 and 125 pg/tube; in triplicate)

PGE: The PGE-2 antibody used for these assays was obtained from ICN ImmunoBiologicals

(Lisle, IL), and displayed to following cross reactivities: PGE-2 100%, PGE-1 240%, PGA-1 10%, PGA-2 8%, PGF-1 α 3.2%, PGF-2 α 4.2%. *Based on this information, this assay measures both PGE-1 and PGE-2, and so the data obtained are described as "PGE"*. The antiserum was used at 1:15,000, and 50% binding was observed at 125-193 pg/tube. For this assay, samples were diluted 2-5 fold in assay buffer (0.01M Tris, pH 7.4). [3 H]-PGE-2 (145-170 Ci/mmol; New England Nuclear, Boston, MA) was used a tracer at ~12,000 cpm/tube. The lower limit of detection of this assay was 7 pg/tube. Intra-assay coefficient of variation (CV) for high (750 pg/tube), medium (250 pg/tube) or low (80 pg/tube) quality control standards were 8%, 8% and 9% respectively. Inter-assay CVs were, 22.1%, 7.9% and 29%.

PGF-2 α : The PGF-2 α antibody was generously provided by Dr. John Chenault of the UpJohn Co. (Kalamazoo, MI) and displayed the following cross reactivities: PGF-2 α free acid 100%, PGF-1 α 120%, 11 β -PGF-2 α 4.3%, Thromboxane B2 5.4%, 6-keto PGF-1 α 6.1%, and PGE-2 0.27%. The antiserum was used at 1:9,000 and 50% binding was observed at 91.6-113 pg/tube. For this assay, samples were diluted 2-3 fold in assay buffer (0.01M Tris, pH 7.4). [3 H]-PGF-2 α (195 Ci/mmol; New England Nuclear) was used as a tracer at ~ 12,000 cpm/tube. The lower limit of detection of this assay was 11.5 pg/tube. Intra-assay coefficient of variation (CV) for high (500 pg/tube), medium (125 pg/tube) or low (50 pg/tube) quality control standards were 13%, 10% and 15% respectively. Inter-assay CVs were, 16.7%, 5.9% and 15.1%.

2.2 Experiment 2

2.2.1 Luteal cell culture

Ovaries were collected from pigs at a local slaughterhouse (City Packing/Neese's Sausage Co., Burlington, NC) and assigned as EARLY (~days 4-6) or MID (~days 7-12) stages of the

estrous cycle based on overall gross morphology [4, 29]. Corpora lutea (CL) were dissociated [24-26] with 0.05-0.1% collagenase, 0.1% hyaluronidase, 0.06% DNase in medium (M) 199 containing 10 mM HEPES, 0.35 g/L NaHCO₃, penicillin (100 IU/mL), streptomycin (100 µg/mL), and gentamicin (50 µg/mL), at 37°C. Luteal cells (LC) were isolated by centrifugation at 800 x g for 5 mins, and washed with M199 to produce a mixed LC preparation [24-26]. LC were subjected to red blood cell lysis using a hypotonic buffer (10 mM KHC0₃, 150 mM NH₄CL, 0.1 mM EDTA, pH 8.0) prior to cell counting. Luteal cells (LC; 5 or 0.25 x 10⁵ LC per well, based on large luteal cell count, in 6 or 96-well plates respectively; 6 well plate cultures were used for mRNA and protein analysis, while 96 well cultures were used for examination of PG secretion) were plated on collagen type I coated culture plates (BD Biocoat, Bedford, MA) in M199 containing 5% fetal bovine serum (FBS), ITS (insulin 5 µg/mL, transferrin 5 µg/mL, selenium 5 ng/mL; Corning, Bedford, MA), penicillin (100 IU/mL), streptomycin (100 µg/mL), and gentamicin (50 µg/mL). After 24-48h to allow attachment of luteal cells, media were removed and replaced with fresh M199 (plating) with TNF-α (recombinant porcine TNF-α, R & D Systems, Minneapolis, MN: 0, 0.1, 1 or 10 ng/mL; n = 3-6 wells per dose; day 0 of treatment). On day 2 of culture (48 h after TNF-α treatment) media were collected and stored at -20°C for PGF-2α and PGE-2 assay EIA (Cayman). Luteal cells were also collected for mRNA, protein, or cell counting (see below). In preliminary studies (data not shown) TNF-α did not significantly affect cell viability as determined on day 2 of culture.

2.2.2 mRNA extraction and analysis by RT-PCR – luteal cells

Following media collection on day 2, cells were removed (by scraping) for mRNA extraction with 0.5 mL RNA extraction buffer and mRNA was isolated using the RNeasy

Micro kits (Qiagen, Valencia, CA, USA). After quantifying RNA levels by Nanodrop, RNA samples were DNase treated (DNA-free™, RNase-free DNase, Ambion, Austin, TX) to remove genomic DNA contamination. cDNAs were generated using the iScript advanced cDNA Synthesis Kit (Bio-Rad) and subjected to Q-PCR using the Sso Advanced Universal SYBER® Green Supermix (Bio-Rad) using the Roche LightCycler 480 system (Roche Diagnostics Corp. Indianapolis, IN). Porcine specific primers for each gene (Sigma-Genosys, Woodlands, TX, USA) were designed from the porcine sequences listed on Genbank, NCBI and validated (Table 1; COX-2/PTGS-2 [30]; PGFS/AKR1B1 [31]; m-PGES-1 [17]). Q-PCR was performed in a reaction volume of 20 µL in the 96-well format using 10 ng cDNA per reaction. Reaction conditions were as follows: pre-incubation (95°C for 2 min), amplification (45 cycles of 95°C for 5 sec, 57°C for 15 sec, 72°C for 30 sec), melt-curve (1 cycle; 95°C for 5 sec, 65°C for 1 min, 97°C continuous), and cooling (40°C for 30 sec). The steady state mRNA levels of each gene were quantified by comparison with GAPDH and expressed relative to 0 ng/mL TNF-α (Control) using the $\Delta\Delta CT$ method (Livak and Schmittgen, 2001; [32]).

2.2.3 Protein extraction and analysis by Western blot – luteal cells

In this Experiment, on day 2 of culture, following media removal, luteal cells were scraped from culture wells in ice-cold lysis buffer (see Experiment 1), homogenized to obtain a total cellular protein preparation (including membrane), and frozen at -70 until analysis. Protein extraction, electrophoresis and Western blotting of proteins for PG synthetic enzymes in cultured luteal cells was carried out as described above for whole CLs (Experiment 1) and as previously described [24-26].

2.2.4 DNA assay – cell number

Cell number in the 96-well cultures for PG secretion studies was estimated using the DNA assay procedure described previously [33]. In brief, after removal of spent culture medium, culture plates were frozen at -20 prior to analysis. For DNA assay, plates were thawed, distilled water added and the plates refrozen at -80C overnight. Culture plates were then thawed at room temperature, and 100 μ L from each well used for DNA assay using Hoechst 33258. Calf-thymus DNA standards (20-5000 ng/mL) were used to allow quantification of samples. Detection of fluorescence was carried out using excitation at 350 nm and emission at 460 nm. Conversion of DNA concentrations to cell number was performed using an estimate of 7 pg DNA per cell.

2.2.5 Prostaglandin EIA

Culture media samples were diluted 5-20-fold in assay buffer (supplied with EIA kits) and analyzed for PGE-2 and PGF-2 α concentrations using Enzyme Immunoassays from Cayman, following the manufacturers instructions. PG concentrations were corrected for cell number (see above) and expressed as pg/cell. These data were then expressed as fold-change compared to 0 ng/mL TNF- α controls. Data are also presented as PGF-2 α :PGE-2 ratio relative to TNF- α dose.

2.3 Statistical analysis

Protein abundance expressed as a ratio to that of β -actin, and prostaglandin secretion expressed as a PGF-2 α :PGE ratio, collected from Experiment 1 were compared for changes relative to stage of the estrous cycle by unpaired t-test (Protein, day 7 vs day 13) or by ANOVA (PGF-2 α :PGE ratio; all stages compared with each other) using GraphPad Prism 6 for Mac (GraphPad, La Jolla, CA). In Experiment 2, mRNA steady state expression

244 (gene/GAPDH) data, protein abundance (protein/ β -actin), PGF-2 α , PGE-2 and the PGF-
245 2 α :PGE-2 ratio were expressed relative to 0 ng/mL TNF controls, and presented as a ratio
246 (fold change vs. controls). All data were then subjected to analysis by ANOVA (all
247 treatments compared with each other) using GraphPad Prism 6 for Mac (GraphPad, La
248 Jolla, CA).

3. Results

3.1 Experiment 1

3.1.1 PGF-2 α :PGE ratio during the estrous cycle (Fig. 1)

The ratio of PGF-2 α :PGE displayed significant estrous cycle-related changes during the estrous cycle, increasing from day 4 through days 13-14, to reach a maximum PGF-2 α :PGE ratio of 3.2 ± 1.0 (mean $\pm$ SEM) on days 13-14 ($P < 0.05$ vs. day 4) of the cycle.

3.1.2 PG enzyme (AKR1B1 and mPGES-1) protein abundance during the estrous cycle (Fig. 2)

As shown in Figure 2, both PTGFS/AKR1B1 and PTGES-1/mPGES-1 protein abundance were significantly ($P < 0.05$) increased (AKR1B1, 2.5 fold; mPGES-1, 3.7 fold) on day 13 compared with day 7 of the estrous cycle.

3.2 Experiment 2

3.2.1 Effects of TNF- α on PG synthetic enzyme mRNA and proteins in culture (Figs. 3 and 4)

Early stage CL cultures (Fig. 3)

PTGFS/AKR1B1: TNF- α had no significant effects on mRNA expression (Fig. 3A). Protein levels appeared to increase with TNF- α dose, although this trend was not statistically significant (Fig. 3B).

COX-2/PTGS-2: TNF- α significantly ($P < 0.05$) increased mRNA expression ($\sim$ 4-fold vs. control) at the highest dose (10 ng/mL; Fig. 3C).

mPGES-1/PTGES-1: TNF- α significantly ($P < 0.05$) increased mRNA expression ($\sim$ 7-fold vs. control) at the highest dose (Fig. 3E), but while protein levels (Fig. 3F) appeared to increase numerically, no significant trends were observed.

Mid stage CL cultures (Fig. 4)

PTGFS/AKR1B1: TNF- α had no significant effects on mRNA expression (Fig. 4A) or protein abundance (Fig. 4B), although they both appeared to increase with TNF- α dose (1 and 10 ng/mL, mRNA; 10 ng/mL, Protein).

COX-2/PTGS-2: TNF- α significantly ($P<0.05$) increased mRNA expression (~2-fold vs. control) at the highest dose, 10 ng/mL (Fig. 4C).

mPGES-1/PTGES-1: TNF- α significantly ($P<0.05$) increased mRNA expression (~ 3.5-fold vs. control) at the highest dose (Fig. 4E), but protein abundance (Fig. 4F) displayed no significant trends.

3.2.2 Effects of TNF- α on Prostaglandin secretion by luteal cells in culture (Fig. 5)

In this Experiment, media from early stage luteal cells cultured with TNF- α were collected on day 2 of culture, assayed for PGE-2 and PGF-2 α concentrations by EIA, the values corrected for cell number (via DNA assay) and the data expressed as PGF-2 α or PGE-2 fold change vs. control, or PGF-2 α :PGE-2 ratio, relative to TNF- α dose. TNF- α had no significant effects on PGF-2 α (Fig. 5A) secretion at any dose, but did significantly ($P<0.05$) increase PGE-2 secretion (Fig. 5B) at the highest dose (10 ng/mL). When examined as a PGF-2 α :PGE-2 ratio, there was a numerical trend showing a decreased ratio with increasing TNF- α dose, but this trend was not significant.

4. Discussion

In first part of this study (Experiment 1) we set out to examine relative secretion of PGF-2 α compared to that of PGE (i.e. PGF-2 α : PGE ratio) by dissociated porcine luteal cells, and the abundance (protein) of PG synthetic enzymes in the porcine CL, at different stages of the

normal estrous cycle. These data were to serve as a physiological “base-line” to test our *hypothesis* (in Experiment 2) that TNF- α will induce similar changes in PG synthetic enzymes in, and PGF-2 α compared to that of PGE-2 secretion by, porcine luteal cells in culture, to that seen in the CL collected after (days 13-14), compared with before (days 4-10), the acquisition of luteolytic sensitivity (LS) during the estrous cycle (from Experiment 1). Overall these Experiments were designed to address our *overarching hypothesis* that infiltrating luteal macrophages and their secretion of TNF- α , play an important role in inducing the changes in intra-luteal PG synthesis/secretion seen during the normal estrous cycle, which is a key component of the pathway leading to the induction luteolytic sensitivity (LS) in the pig.

In Experiment 1, the data collected on PG secretion by luteal cells obtained at different stages of the estrous cycle showed stage-of-cycle dependent increases in the PGF-2 α :PGE ratio, with a significant increase observed days 13-14 (vs. day 4; Fig. 1), which is coincident with the acquisition of LS (day 13 and beyond). This pattern of luteal PG secretion was similar to that observed by Waclawik and colleagues, who showed that the PGF-2 α :PGE-2 (based on CL *content* of PGs) ratio also appeared to increase on days 9-15 versus days 2-8 of the estrous cycle in the pig [17]. In our studies of PG synthetic enzymes in the CL, we observed significant increases in PTGFS/AKR1B1 and PTGES-1/mPGES-1 protein levels in CL collected *after* the onset of LS (day 13) compared with those collected *before* the onset of LS (day 7) during the estrous cycle (Fig. 2). Our data confirm the findings of Waclawik and colleagues who also observed significantly elevated abundance of PTGES-1/mPGES-1 and PTGFS/AKR1C3 protein in porcine CL collected on days 14-15 (vs. days 2-4 or days 2-8, respectively), of the estrous cycle [17]. These data also suggest that

316 more than one PTGFS enzyme may be involved in increasing luteal PGF-2 α
317 synthesis/secretion in CL after acquisition of LS in the pig. The data on PG
318 secretion/content described above are *one* indicator of the functional activity of the PG
319 synthetic enzymes in the CL, and suggest that in spite of a significant elevation in both
320 PTGES-1/mPGES-1 and PTGFS/AKR1B1 (and PTGFS/AKR1C3 – [17]) protein levels in CL
321 which display LS, PTGFS activity appears to predominate over PTGES within the CL,
322 resulting in an increased PGF-2 α :PGE-2 ratio at this time. Although not measured in our
323 studies, it is possible that the enzyme Prostaglandin 9-ketoreductase/carbonyl reductase
324 (CBR1), which is capable of inter-converting PGE-2 and PGF-2 α , may also have a role to
325 play in determining the end product of CL PG secretion. In their studies Wacławik and
326 colleagues [17] found that neither CBR1 mRNA nor protein expression levels in the CL
327 changed during the estrous cycle, but since the functional activity of this enzyme is likely to
328 be determined by the relative oxidative or reductive conditions within the cells, a change in
329 ratio of PGF-2 α :PGE-2 secretion could occur, even in the absence of a change in enzyme
330 expression. Another enzyme that might influence the PGF-2 α :PGE-2 ratio is 15-
331 hydroxyprostaglandin dehydrogenase (HPGD) which metabolizes and thus inactivates both
332 PGF-2 α and PGE-2. This enzyme is present within the CL [18, 34, 35] and appears to be
333 most active (enzyme activity) in developing CL of the early cycle, compared with those
334 collected at the mid-late cycle, and thus has been linked to the mechanism underlying
335 luteolytic sensitivity in the ewe [34]. The fact that HPGD levels increase again in pregnant
336 animals, was taken to suggest that this enzyme may also play a role in de-sensitizing the CL
337 to PGF-2 α in pregnancy [34]. Although we did not examine this enzyme, it is possible that it
338 may also contribute to the increased PGF-2 α :PGE-2 ratio on days 13-14, in spite of elevated

mPGES and AKR1B1 seen in our studies in the pig at this time. The intra-luteal PGF-2 α :PGE-2 ratio is believed to play an important role in determining the luteolytic response of the CL to PGF-2 α [15-19], and its impact on luteal function has been demonstrated in vivo in the pig [20]. Thus the elevated PGF-2 α :PGE-2 ratio in CL towards the end of the estrous cycle, may contribute to an enhanced luteolytic response, and this may in part result from an activation of the “auto-amplification” mechanism within the CL by which initial PGF-2 α pulses are amplified in luteal cells by increased intra-luteal PGF-2 α synthesis [36]. The “auto-amplification mechanism” was originally described in the pig over 30 years ago [36], but the importance of this pathway in luteolysis is now widely accepted in several species [15,16,37,38]. While this mechanism may not be necessary for luteolysis in response to large exogenous PGF-2 α doses [39], it is believed to be critical [37, 38] to magnify locally, the responses to small physiological pulses of PGF-2 α secreted by the uterus during natural luteolysis [40], and thus plays an important role in the acquisition of LS during the normal estrous cycle.

In the second part of this study (Experiment 2), we set out to test the hypothesis that TNF- α plays a critical role in the acquisition of LS by promoting the development of PG synthetic enzymes leading to increased PGF-2 α :PGE-2 secretion ratio. In this Experiment, luteal cells were isolated from EARLY (~days 4-6) and MID (~days 7-12) stages of the estrous cycle and cultured for 2 d with increasing doses of TNF- α . Luteal cells showed significantly increased steady state mRNA levels for COX-2/PTGS-2 and mPGES-1/PGES-1 in a dose-dependent manner, in response to TNF- α in both EARLY and MID stage luteal cell cultures. AKR1B1/PGFS mRNA expression showed a numerical increase in MID (but not EARLY) stage cultures, but this increase was not significant. At the protein level,

AKR1B1/PGFS and mPGES-1/PGES-1 showed numerical increases in response to TNF- α doses, in EARLY and MID stage cultures, but these changes were not significant. There is little known about the direct effects of TNF- α on AKR1B1/PGFS in any tissue, but there are reports that LPS (which is presumed to act via TNF- α) increased AKR1B1 in macrophages [41]. As discussed above AKR1B1 has been identified and quantified in ovine [18] and bovine [15] CL, but to our knowledge no studies have been carried out to examine the effects of TNF- α on this enzyme in the CL. In studies on PG secretion and its regulation in the porcine uterus (luminal epithelial cells, LEC), it was found that TNF- α stimulated mPGES-1 mRNA and protein expression, but failed to have any impact on PTGFS (AKR1C3; mRNA or protein) [42]. However, in studies by the same group [43] it was reported that porcine LEC responded to TNF- α with the preferential secretion of PGF-2 α vs. PGE-2, in spite of elevated mPGES-1 and unchanging levels of PTGFS just described [42]. In our data on the patterns of PG secretion by porcine luteal cells in response to TNF- α , PGF-2 α secretion was unchanged while PGE-2 secretion was significantly elevated at the highest dose (10 ng/mL; Fig. 5). When these data were expressed as the PGF-2 α :PGE-2 secretion ratio, no significant differences were observed with increasing TNF- α dose, but there was a numerical trend in the decreasing direction. These findings do not appear to correlate with the mPGES-1 or AKR1B1 protein expression data, which while not significantly increased, did show numerical increases in response to TNF- α . Thus, in confirmation with the data described above for the porcine LEC, it would seem that PG enzyme expression at mRNA or protein in porcine luteal cells treated with TNF- α in culture, do not correlate with functional measures of enzyme activity, namely PG secretion patterns. As discussed above for Experiment 1, other enzymes such as CBR1 and HPGD likely play critical roles in

determining the final outcome of PG secretion. In preliminary studies, we observed no significant change in CBR1 mRNA expression, but we did not examine HPGD in our studies. These enzymes need to be examined in greater detail (e.g. measurements of enzyme activity) to allow us to understand more clearly the complex nature of PG secretion and its control by TNF- α in the porcine CL.

In conclusion, the observations of PG secretion by porcine luteal cells in response to TNF- α in vitro, do not support our hypothesis that TNF- α increases the PGF:PGE ratio, and suggest that TNF- α may not be the critical macrophage-derived factor responsible for inducing the increase PGF-2 α :PGE-2 ratio seen during the normal estrous cycle. Since porcine luteal macrophages appear to express multiple cytokines including IL-1 β , IL-6, IFN- γ , TGF- β and IL-10 (Chang, Frandsen and Gadsby, unpublished observations), it is possible that other cytokines acting alone or in the presence of TNF- α may be involved in inducing the increased intra-luteal PGF-2 α :PGE-2 ratio in CL displaying LS, and/or other critical pathways leading to the acquisition of LS. However, from other preliminary studies (Chang, Frandsen and Gadsby, unpublished observations) TNF- α was shown to increase the abundance of the PGF-2 α (FP) receptor protein in cultured porcine luteal cells, suggesting that this may be one mechanism by which TNF- α induces LS in vitro [13].

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Table 1. Primer sequences used for Q-PCR^a

Gene	Accession Number	Product Size	Primer F=Forward R= Reverse	Species
<i>AKR1B1</i>	NM001001539.2	166	F: AAGGAGCACAGTTCCAAGCAGTCA R: CCCGAAGAGCACTACCTGTAGATT	pig
<i>COX2/PTGS2</i>	AY028583.1	168	F: AGGCTGATACTGATAGGAGAAACG R: ATGCCAGTGGTAGAGTGTGTTAAA	pig
<i>mPGES-1</i>	AY857634	219	F: ACGCTGCTGGTCATCAAGA R: CCCAGGAAGGAGTAGACGAG	pig
	AF017079		F: TCAAGAAGGTGGTGAAGCAG	
<i>GAPDH</i>		214	R: TTGACGAAGTGGTCGTTGAG	Pig

^a All primers are listed 5'-3'

Figure legends

Fig. 1: Prostaglandin secretion by porcine luteal cells in culture, throughout the estrous cycle. Luteal cells (10^5 cells per tube) cultured at 37C for 6 h in 95% Oxygen: 5% carbon dioxide. Media were collected and assayed for “total” PGE (1 and 2; see Materials and Methods for explanation) and PGF-2 α concentrations by radioimmunoassay. The data are presented as the PGF-2 α :PGE ratio at each stage of the estrous cycle. N = the number of animals per stage of the estrous cycle. Different superscripts (a,b) represent significant differences ($P < 0.05$; ANOVA, Tukeys).

Fig. 2: Protein abundance of PG synthetic enzymes in CL collected at day 7 (CL lacking luteolytic sensitivity; no LS) and 13 (CL showing luteolytic sensitivity; + LS) of the estrous cycle. Proteins extracted from CL were subjected to Western blotting with antibodies against PTGFS/AKR1B1 (**A**) and PTGES-1/mPGES-1 (**B**). Data are presented as protein abundance relative to β -actin. N = number of animals per stage. *Represent significant differences between day 7 and day 13 ($P < 0.05$; T-test).

Fig. 3: mRNA expression (steady state concentrations; Q-PCR) and protein abundance (Western blot) of PG synthetic enzymes in luteal cells in response to TNF- α in culture (D.2). Luteal cells were obtained from EARLY (~ days 4-6 of cycle) cycle stage CL. (**A**) AKR1B1 mRNA, (**B**) AKR1B1 protein, (**C**) COX-2 mRNA, (**E**) mPGES mRNA and (**F**) mPGES protein. Data are presented as mRNA or protein abundance relative to 0 ng/mL TNF- α control (Fold change vs. control). N = number of cultures. *Represent significant differences relative to 0 ng/mL TNF- α control ($P < 0.05$; ANOVA, Tukeys). (**D**) Western blot composite, showing protein bands in representative blots for AKR1B1 (36 kD), mPGES (16 kD) and β -actin (42 kD), for each TNF- α treatment (0, 1 or 10 ng/mL).

Fig. 4: mRNA expression (steady state concentrations; Q-PCR) and protein abundance (Western blot) of PG synthetic enzymes in luteal cells in response to TNF- α in culture (day 2). Luteal cells were obtained from MID (~ days 7-12 of cycle) cycle stage CL. (A) AKR1B1 mRNA, (B) AKR1B1 protein, (C) COX-2 mRNA, (E) mPGES mRNA and (F) mPGES protein. Data are presented as mRNA or protein abundance relative to 0 ng/mL TNF- α (Fold change vs. control). N = number of cultures. *Represent significant differences relative to 0 ng/mL TNF- α control (P<0.05; ANOVA, Tukeys). (D) Western blot composite, showing protein bands in representative blots for AKR1B1 (36 kD), mPGES (16 kD) and β -actin (42 kD), for each TNF- α treatment (0, 1 or 10 ng/mL).

Fig. 5: Prostaglandin secretion by porcine luteal cells in culture, from EARLY (~days 4-6 of cycle) stage of the estrous cycle. After plating, luteal cells were cultured for 2 d (= day 2) with 0, 0.1, 1 or 10 ng/mL TNF- α . Media collected on day 2 and assayed for PGF-2 α or PGE-2 by EIA. PGF-2 α (A) or PGE-2 (B) concentrations were corrected for cell number (via DNA conc.) and expressed relative to 0 ng/mL TNF- α (Fold change vs. control). In panel (C), data were expressed as the PGF-2 α :PGE2 ratio for each TNF- α dose. N = the number of cultures. *Represent significant differences from 0 ng/mL TNF- α control (P<0.05; ANOVA, Tukeys).

FIGURES

Fig. 1: PG secretion in vitro – CL collected during estrous cycle.

Fig. 2: AKR1B1 (A) and mPGES (B) protein in CLs collected during the estrous cycle

A

B

Fig. 3: mRNA and protein for AKR1B1, COX-2 and mPGES – Early cultures

A

B

C

D

E

F

Fig. 4: mRNA and protein for AKR1B1, COX-2 and mPGES – Mid cultures

Fig. 5: PGF-2 α and PGE-2 secretion in response to TNF- α in vitro (Early)

A

B

C