Effects of LPA2R, LPA3R, or EP4R agonists on luteal or endometrial function *in vivo* or *in vitro* and sirtuin or EP1R, EP2R, EP3R or EP4R agonists on endometrial secretion of PGE and PGF_{2α} *in vitro*.

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1.0 Abstract

In previous work, an EP2 prostanoid receptor (EP2R) agonist *in vivo* increased mRNA expression of luteal LH receptors (LHR), unoccupied and occupied luteal LHR, and circulating progesterone, while an EP3R or FPR agonist decreased mRNA expression of luteal LHR, unoccupied and occupied luteal LHR, and circulating progesterone. An EP4R and lysophosphatidic acid (LPA) LPA2R and LPA3R agonists were reported to inhibit luteal function and sirtuins have been proposed to increase prostaglandin synthesis. The objectives were to determine whether an EP4R, LPA2R, or LPA3R agonist affect ovine luteal function *in vivo* or *in vitro*. In addition, whether sirtuin (SIRT)-1, 2, or 3; LPA2R or LPA3R; or EP1R, EP2R, EP3R, or EP4R agonists affect caruncular endometrial PGF_{2α} or PGE (PGE1+PGE2) secretion *in vitro*. Day-10 nonpregnant ewes received a single injection of Vehicle (N=5); an LPA2R (N=5); LPA3R (N=6); or EP4R (N=5) agonist given into the interstitial tissue of the ovarian vascular pedicle adjacent to the luteal-containing ovary to determine effects on circulating progesterone, mRNA expression of luteal LHR, and luteal unoccupied and occupied LHR. In addition, agonists for LPA2R, LPA3R, EP1R, EP2R, EP3R, or EP4R or SIRT-1, SIRT-2, or SIRT-3 activators were incubated with caruncular endometrial slices *in vitro* to

determine their effect on caruncular endometrial $\text{PGF}_{2\alpha}$, or PGE secretion. LPA2R, LPA3R, or an EP4R agonist *in vivo* did not affect ($P \geq 0.05$) luteal weight, circulating progesterone, or occupied luteal LHR. However, an LPA2R or EP4R agonist, but not LPA3R agonist, *in vivo* increased ($P \leq 0.05$) mRNA expression of luteal *LHR*. An LPA2R, LPA3R, or EP4R agonist increased ($P \leq 0.05$) luteal unoccupied LHR, but not occupied LHR. An LPA2R, LPA3R, or an EP4R agonist did not affect ($P \geq 0.05$) luteal progesterone secretion *in vitro*. An LPA2R or LPA3R agonist did not affect ($P \geq 0.05$) luteal $\text{PGF}_{2\alpha}$, or PGE secretion *in vitro*. However, an EP4R agonist tended to decrease ($P < 0.066$) luteal $\text{PGF}_{2\alpha}$ secretion and increased ($P \leq 0.05$) luteal PGE secretion *in vitro*. EP1R, EP2R, EP3R, or an EP4R agonist did not affect ($P \geq 0.05$) caruncular endometrial $\text{PGF}_{2\alpha}$ secretion *in vitro*. However, EP1R, EP3R, or an EP4R agonist increased caruncular endometrial PGE secretion *in vitro*, while two different EP2R agonists did not affect ($P \geq 0.05$) caruncular endometrial PGE secretion. A SIRT-1 activator, but not SIRT-2 or SIRT-3 activators, increased ($P \leq 0.05$) caruncular endometrial PGE secretion, while sirtuin 1, 2, or 3 activators did not affect ($P \geq 0.05$) caruncular endometrial $\text{PGF}_{2\alpha}$ secretion.

In conclusion, receptors for EP4, LPA2, and LPA3 do not appear to be involved in luteolysis, but EP4R and LPA2R might participate in preventing luteolysis by maintaining luteal mRNA expression for *LHR* and preventing loss of unoccupied luteal LHR. In addition, SIRT-1, EP1R, EP3R, and EP4R might be involved in regulating caruncular endometrial PGE secretion, but not $\text{PGF}_{2\alpha}$ secretion.

Key words: LPA2; LPA3; EP1, 2, 3, 4; sirtuin; corpus luteum, endometrium

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2.0 Introduction

2.1 Prostaglandins E-luteolysis and pregnancy

The corpus luteum is the source of progesterone during the estrous cycle (reviewed in [1, 2]). Loss of progesterone secretion at the end of the cycle is initiated via uterine $\text{PGF}_{2\alpha}$ secretion (reviewed in [1, 2]). In the ewe, $\text{PGF}_{2\alpha}$ in the endometrium, uterine or ovarian venous blood, or luteal tissue, binding of $\text{PGF}_{2\alpha}$ to luteal membranes, or transport of $\text{PGF}_{2\alpha}$ from the uterine vein to the ovarian artery adjacent to the luteal-containing ovary are not decreased to explain prevention of luteolysis during early pregnancy (reviewed in [1, 2]). Uterine $\text{PGF}_{2\alpha}$ secretion is not decreased until after Day-50 of pregnancy in ewes (reviewed in [1]). The embryo-caruncular endometrial interaction provides resistance to $\text{PGF}_{2\alpha}$ -induced luteolysis during early pregnancy (reviewed in [2]). Resistance to $\text{PGF}_{2\alpha}$ is due at least in part to increases in caruncular endometrial PGE secretion and PGE in uterine venous blood during early pregnancy to prevent luteolysis (reviewed in [1, 2]). PGE_1 or PGE_2 infused chronically into the ovine uterine lumen adjacent to the luteal-containing ovary prevented a spontaneous [3-5] or a premature luteolysis induced by estradiol- 17β given at midcycle [6, 7], or an intrauterine device [8, 9], progesterone [10], or oxytocin [11] given early in the estrous cycle, or $\text{PGF}_{2\alpha}$ given at midcycle [12, 13]. Chronic infusion of PGE_1 or PGE_2 into the uterine lumen opposite the luteal-containing ovary did not prevent spontaneous luteolysis [3, 4]. Acute treatment with PGE_1 into the interstitial tissue of the ovarian vascular pedicle of the ovine luteal-containing ovary increased circulating progesterone for a longer duration than treatment with PGE_2 [12, 13]. In cows, a single intramuscular

137 treatment with PGE₁ at midcycle increased circulating progesterone for the 72 h
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139 sampling period [14]. Thus, PGE₁ and PGE₂ have been reported to be major
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141 direct signals from the uterine horn containing an embryo to the adjacent luteal-
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143 containing ovary to prevent luteolysis during early pregnancy in the ewe (reviewed
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145 in [1, 2]).

146 Both PGE₁ and PGE₂ are vasodilators [15], increase luteal progesterone secretion
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148 *in vitro* in cows [16] and ewes [17], and *in vivo* in ewes [12, 13] and cows [14], while
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150 PGF_{2α} is a vasoconstrictor [15]. The common PGE₁ or PGE₂ antiluteolytic
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152 mechanism in the cow [18] or ewe [5] is preventing loss of luteal mRNA expression
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154 for *LHR* and loss of luteal unoccupied and occupied LHR. In addition, PGE₁, but
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156 not PGE₂, increased luteal mRNA expression for *LHR* [5]. Maintenance of luteal
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158 progesterone secretion is paramount to prevent luteolysis. Luteolysis in both small
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160 luteal cells (SLC) or large luteal cells (LLC) is via an increase in cellular calcium
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162 uptake [19]. Progesterone inhibited PGF_{2α} calcium uptake by LLC and oxytocin
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164 stimulated calcium uptake by SLC *in vitro* [20].

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166 There is one FP and four EP prostanoid receptor subtypes (EP1, EP2, EP3, and
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168 EP4). EP2R and EP4R agonists increase cAMP [21], the second messenger for LH-
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170 stimulated small luteal cell progesterone secretion [19]. An EP2R agonist given into
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172 the interstitial tissue of the ovarian vascular pedicle adjacent to the luteal-
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174 containing ovary increased mRNA expression of luteal *LHR* and occupied and
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176 unoccupied LHR, while an EP3R agonist or PGF_{2α} decreased circulating
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178 progesterone, luteal mRNA for LH receptors, and luteal unoccupied and occupied
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180 LHR [22]. An EP1R agonist had no effect on luteal function *in vivo* in ewes [22]. In
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cows, intraluteal silastic implants inserted on Day-13 postestrus containing PGE₁ or PGE₂ prevented loss of circulating progesterone, luteal mRNA expression for *LHR* and luteal unoccupied and occupied LHR on Day-19 postestrus, [23]. However, PGE₁ or PGE₂ upregulated luteal mRNA expression of *FPR*, but luteal mRNA expression for *EP2R* and *EP4R* in these same cows was downregulated by PGE₁ or PGE₂ [24]. A single intramuscular injection of a PGF_{2α} analogue (500 µg Cloprostenol; Estrumate, Intervet, Germany) at midcycle in cows decreased circulating progesterone, but increased mRNA expression of luteal EP4 and EP4R at 48 h, which was interpreted that EP4 receptors are involved in luteolysis (B. Berisha and D. Schams, unpublished data).

2.2 Lysophospholipids

LPA is derived from arachidonic acid in phospholipids as are PGF_{2α} and PGE [25]. Four LPA receptors LPA1R, LPAR2, LPA3R, and LPA4R have been identified [26]. LPA increased cyclooxygenase-2 (COX-2 mRNA) and increased prostaglandin secretion in several ovarian cancer cell lines [27]. In addition, primary human bronchial epithelial cells cultured with LPA increased COX-2 protein expression and PGE₂ secretion [28, 29]. Moreover, LPA inhibited LH-induced progesterone secretion by midcycle bovine luteal cells *in vitro* [30] via inhibition of cAMP production [31, 32].

2.3 Sirtuins

Sirtuins (silent information regulators) are histone deacetylase proteins that reduce apoptosis, transcription, and energy metabolism, and are proposed to alter prostaglandin synthesis [33]. Seven sirtuins (SIRT-1-7) are expressed in human

cells and are classified according to their location [33]. SIRT-1, SIRT-6, and SIRT-7 are in the nucleus, SIRT-3, SIRT-4, and SIRT-5 are in the mitochondria, and SIRT-2 is found primarily in the cytoplasm [34-37]. Sirtuins are especially interesting, since these three sites are locations of enzymes regulating progesterone production from cholesterol [34]. SIRT-1, 2 and 3 receptor signaling has been implicated in regulation of timing of implantation and prostaglandin secretion [38]. SIRT-1 has been proposed to prevent apoptosis [33]. The tumor suppressor p53 activates the transcriptional program that promotes apoptosis and has several acetylation sites. Deacetylation of p53 leads to its degradation; therefore, it seems that SIRT-1 acts as an antiapoptotic factor by reducing the amount of p53 in the cell [34]. SIRT-1 deacetylates RelA/p65, the primary form of NF- κ Beta, inhibiting its transactivation and sensitizing cells to TNF- α -induced apoptosis [33-37]. SIRT-1 has been proposed to play a role in development of the embryo to blastocyst formation [38] and in pregnancy and parturition via PGE₂ and PGF_{2 α} [39]. In addition, SIRT-1 may play a role in male fertility through the hypothalamic-hypophyseal axis [40].

2.4 Objectives

Therefore the objective of these experiments was to determine whether treatment with LPA2R, LPA3R, or EP4R agonists *in vivo* alter circulating progesterone, luteal weight, luteal mRNA expression for *LHR*, or luteal unoccupied and occupied LHR in nonpregnant ewes. The second objective of these experiments was to determine whether LPA or EP receptor agonists, or sirtuin activators alter PGE, or PGF_{2 α} secretion by caruncular endometrial tissue of nonpregnant ewes *in vitro*.

3.0 Materials and methods

3.1. Animals and surgery

The experimental protocol was approved by the University of Hawaii Institutional Animal Care and Use Committee guidelines. Mature Merino crossbred ewes were checked twice daily (0800 and 1700 h) for estrus (Day-0) with brisket-painted vasectomized rams. Only ewes with two consecutive interestrous intervals of 16-17 days were used. Ewes were fed alfalfa cubes and given water *ad libitum* except for 12 h prior to surgery. Ewes received one mL (0.54 mg) atropine sulfate (Pheonix Pharmaceuticals, Inc., St. Joseph, MO) intramuscularly, as a preanesthetic 30 minutes prior to induction of anesthesia. A polyvinyl catheter (Cole-Palmer, Chicago, IL) was inserted into the jugular vein and secured with 000 silk sutures. Ewes were laparotomized under aseptic conditions via a midventral incision under pentobarbital anesthesia (AnthonyProducts Co., Arcadia, CA).

3.2. Experiment 1

Day-10 ewes received 500 µg of treatment in one mL PBS given into the interstitial tissue of the ovarian vascular pedicle adjacent to the luteal-containing ovary. Treatments were: 1. Vehicle (N=5); 2. Dodecylphosphate (DP) (BML-LP-105- Enzo Life Sciences International, Inc., Plymouth Meeting, PA) an LPA2 receptor agonist and an LPA3 receptor antagonist (N=5); 3. (2*S*) [(hydroxymercaptoposphoinyl)oxy] -2-methoxy-propyl ester, 9 Zoctadecenoic acid, triethyl ammonium salt (OMPT; Cayman Chemical Inc., Ann Arbor, MI) an LPA3 receptor agonist (N=6); or 4. 2-(3-hydroxyoctyl)-5-oxo-1-pyrrolidineheptanoic acid, (CAY 10580; Cayman Chemical Inc., Ann Arbor, MI), an EP4R agonist (N=5). Jugular venous blood was collected hourly from 0 to 6 h and every six h from 6 to 48

h for analysis for progesterone by RIA [41]. Corpora lutea were collected at 48 h, bisected into halves, weighed, and frozen in liquid nitrogen until analysis for mRNA for LHR and unoccupied and occupied LHR.

3.3. Real-time PCR luteal mRNA expression for *LHR* analysis

Procedures for ovine luteal mRNA expression for LHR were validated and published previously by our laboratories [5, 42-44]. Individual RNA samples were DNase digested and cleaned by incubation with RNase-Free DNase kit (Qiagen, Valencia, CA, USA) in RNeasy spin columns (RNeasy Midi Kit, Qiagen, Valencia, CA, USA) according to manufacturer's instructions. Concentrations of RNA were determined by spectrophotometry (Nano Drop ND-100, Technologies, Inc., Wilmington, DE, USA) and integrity of RNA was verified by denaturing gel electrophoresis in 1% agarose and 37% formaldehyde in the presence of ethidium bromide. Samples were normalized to a total RNA concentration of 22 ng/ μ L. Total RNA (300 ng) was reverse transcribed into cDNA using the qScript cDNA Synthesis Kit (cat # 95047-500; Quanta Biosciences, Inc., Gaithersburg, MD, USA) following the manufacturer's instructions containing 5 μ L master Mix ($2^{\times}$), 0.5 M forward and reverse (1 μ L) specific primers, and 4 μ L of the RT product. As a negative control for reverse transcription some samples did not include RNA or reverse transcriptase whereas RNase–DNase-free water replaced cDNA for the real-time step. Primers were designed in the NCBI Primer BLAST software to span intron–exon boundaries and to obtain amplicons with a similar size between the gene of interest (GOI) and the reference gene (RG). Accession numbers for oLHr and ovine ribosomal protein L19 (bRP) were K4JY44 and H2E6Z6, respectively. For

oLHR : forward and reverse primers were
TCTGGCACACACCTTCTAC AND GGTCATCTTCTCACGGTTGG and
for bRP CACAAGCTGAAGGCAGACAA and CGGGCTTCCTTGGTCTTAGG.
PCR products for oLHR were cloned into A topo VECTOR (# k4595-40, TOPO TA
CLONING KIT, Invitrogen, Carlsbad, CA, USA) according to the manufacturers
instructions to generate calibration curves with a linear dynamic range from 10 to
 10^{10} copies per μL of standard. PCR products and plasmid containing amplicons
were sequenced at Protonomics and Metabolomics Facility, Colorado State
University (<http://macromolecular.colostate.edu/dnasequencing.html>).
Amplification efficiencies of target and reference genes were estimated from serial
dilution of at least three samples in triplicate. Sample cDNAs were assayed in
duplicate and standards were assayed in triplicate. Specificity of the primers were
further corroborated by evaluation of melting curves. Evidence of nonregulation of
RG by the treatments tested here has not been documented. As a precautionary
step, RNA samples from sheep were normalized initially to total RNA. Therefore,
two sets of qPCR plates were assayed under two conditions (i. e. normalization to
total RNA and normalization first to total RNA and then to cDNA). The influence of
qPCR method (calibration curves or absolute quantification vs relative
quantification) on the final outcome was evaluated.

3.4. Analysis of luteal unoccupied and occupied LHR

Quantification of unbound LH receptors was based upon binding of ^{125}I -hCG
to luteal membrane fractions as adapted from Hichens et al. [45], Grinwich et al.
[46], and published previously by our laboratory [12, 13]. Unoccupied luteal LHR

were quantified from 50 mg of the luteal membrane preparation from one half of the corpus luteum incubated with 20,000 CPM of 125 I-hCG. LH bound to luteal membranes was prepared from 10 mg tissue from each corpus luteum as reported previously by our laboratory [12, 13]. Endogenously bound LH was eluted from a membrane preparation of each corpus luteum by mild heat treatment (60° C, 10 min). Eluted LH was quantified in the supernatant by RIA as reported previously by our laboratory [12, 13].

3.5 *In vitro* luteal and caruncular endometrium experiment

Ewes were laparotomized under aseptic conditions via a midventral incision under pentobarbital sodium anesthesia on Day-12 postestrus. Corpora lutea and endometrial caruncles from the anterior third of the uterine horn adjacent to the luteal-containing ovary were collected. Luteal and caruncular endometrial slices were weighed, diced, and incubated in 3mL of medium 199 (Invitrogen; Carlsbad, CA) *in vitro* in a shaking water bath at 39.5° C under 95% air and 5% CO₂ at pH 7.2 in 5 mL of M-199 containing 25 mM HEPES buffer and Earle's salts (Life Technologies, Rochester, NY), 0.1 % BSA (Fraction V; Sigma Chemical Co., St. Louis, MO), 25-hydroxycholesterol (20 µg/mL; Sigma Chemical Co., St. Louis, MO), 100 IU penicillin (Sigma Chemical Co., St. Louis, MO) and 0.2 mg/mL streptomycin sulfate (Sigma Chemical Co., St. Louis, MO) for one h in the absence of treatments and for 4 and 8 h with treatments. Media were collected after the 4 h incubation, replaced with fresh media containing treatments, and incubated for another 4 h, after which media were collected again, for a total incubation time of 8 h. Treatments were randomized within each ewe. Tissue media collected at 4 and 8 h

were stored at -20° C until analysis for progesterone (41), PGE [47], and PGF_{2α} [47] by RIA. Treatments (100 ng/mL; N=7/treatment group) for Day-12 luteal slices *in vitro* were: 1. Vehicle; DP (LPA2 agonist); 3. OMPT (LPA3 agonist); or EP4 receptor agonists. Treatments were 100 ng/mL unless otherwise noted; N=7/treatment group).

3.6. Treatments for EP prostanoid receptor agonists for endometrial caruncles

Treatments (100 ng/mL) in 3 mL medium 199 (N=7/treatment group) were: 1. Vehicle; 2. 9-oxo-11a, 15S-dihydroxy-17 phenyl-18, 19, 20-trinor-prosta-5Z, 13E-dien-1-oic acid (17PT-PGE₂; Cayman Chemical Inc., Ann Arbor, MI)-EP1R agonist -synthetic analog of PGE₂; 3. 9-oxo-11a, 15S, 19R-trihydroxy-prosta-5Z, 13E-dien-1-oic acid (19(R) OH PGE₂; Cayman Chemical Inc., Ann Arbor, MI)-selective EP2R agonist; 4. 9-oxo-11a, 16S-dihydroxy-17-cyclobutyl-prosta-13E-en-1-oic acid; (±)-15-deoxy-16S-hydroxy-17-cyclobutyl-PGE₁ (Butaprost; Cayman Chemical Inc., Ann Arbor, MI)-selective EP2R agonist; 5. N-(methylsulfonyl)-9-oxo-11a,15R-dihydroxy-16-phenoxy-17, 18, 19, 20-tetranor-prosta-5Z, 13E-dien-1-amide (Sulprostone; Cayman Chemical Inc., Ann Arbor, MI)-selective EP3R agonist and metabolism-resistant synthetic analog of PGE₂; 6. 2-(3-hydroxyoctyl)-5-oxo-1-pyrrolidineheptanoic acid (CAY10580; Cayman Chemical Inc., Ann Arbor, MI) selective EP4R agonist; and 7. N-[[4'[[3-Butyl-1, 5-dihydro-5-oxo-1-[2-(trifluoromethyl)phenyl]-4H-1, 2, 4-triazol-4-yl]methyl][1, 1'biphenyl]-2-yl]sulfonyl]3-methyl-2-thiophenecarboxamide (L-161982; Cayman Chemical Inc., Ann Arbor, MI)-potent and selective EP4R antagonist. .

3.7. Sirtuin caruncular endometrial treatments *in vitro*

Treatments in 3 mL medium 199 were: 1.human recombinant SIRT-1; 2 U/mL (CAYMAN #10011190); Cayman Chemical Inc., Ann Arbor, MI)—nuclear sirtuin, regulates p53 activity, inhibits apoptosis via NAD-dependent deacetylase-1 [18]; 2. human recombinant SIRT-2; 2 IU/mL (CAYMAN #10011191; Cayman Chemical Inc., Ann Arbor, MI)—cytoplasmic protein with histone NAD-dependent deacetylase-2 activity, functions as part of a mitotic checkpoint to prevent chromosomal instability; and 3.human recombinant SIRT-3; 2 IU/mL (CAYMAN #10011194; Cayman Chemical Inc., Ann Arbor, MI)—mitochondrial protein, inhibits NAD-dependent deacetylase-3, induced by cold exposure, modulates adaptive thermogenesis in brown adipose tissue.

3.8. Radioimmunoassays

Progesterone was analyzed by RIA validated previously [41]. PGE (equal crossreactivity with PGE₁ or PGE₂) and PGF_{2α} were analyzed by RIA validated previously [47]. The interassay and intrassay coefficients of variation for progesterone in jugular venous plasma were 4.9 and 4.2%, respectively. The interassay and intrassay coefficients of variation for progesterone assays performed on luteal tissue culture media *in vitro* were 2.6 and 2.6%, respectively. The interassay and intrassay coefficients of variation for PGE assays performed on luteal culture media *in vitro* were 5.5 and 1.1%, respectively. The interassay and intrassay coefficients of variation for PGF_{2α} assays performed on luteal culture media *in vitro* were 2.8 and 2.1%, respectively. The interassay and intrassay coefficients of variation for PGE (PGE₁+PGE₂) in caruncular culture media *in vitro* were 4.4 and 2.2%, respectively. The interassay and intrassay coefficients of

variation for $\text{PGF}_{2\alpha}$ in caruncular cultured media *in vitro* were 2.7 and 1.2%, respectively.

3.9. Statistical analyses

All data analyses were carried out using Statistical Analysis Software (SAS) version 9.1 unless otherwise noted [48]. All data were examined for homogeneity of variance using Bartlett's Box F test. When a data set was not homogenous, data were transformed before statistical analysis. Data for luteal weights from nonpregnant ewes *in vivo* in Experiment 1 were analyzed by a completely randomized design for a one way ANOVA. Jugular profiles of progesterone from nonpregnant ewes *in vivo* in Experiment 1 were analyzed using a general linear mixed effect model [48] for ANOVA for repeated measures using a split-plot design. Treatments served as the main plot and ewe within treatment served as the error term for the detection of treatment effects. Time and treatment x time interaction were included in the subplot. Data for progesterone secretion were transformed ($\log[X*10]$) prior to analysis to meet the assumption of homogeneity of variance. Relative gene expression for mRNA was analyzed by randomization test using the Relative Expression Software Tool 2008 (REST 2008) (<http://www.genequantification.de/pfaffl-nar-2001.pdf> [43, 49, 50]. This software normalizes the gene of interest by the reference gene and includes an efficiency (E) correction using the following mathematical model: $\text{Ratio} = (\text{E}_{\text{target}})^{\Delta\text{CP}} \text{target} (\text{control-sample}) / (\text{E}_{\text{reference}})^{\Delta\text{CP}}$. For absolute quantification, copy number was calculated as described by others. Copy number of calibration curves was Log_{10} transformed, plotted against their Cp values and subjected to linear regression

analysis using Prism 5 (GraphPad Prism 5 software, Inc.). Copy number of samples was estimated from interpolation of Cp values in the slope, then powered, adjusted to ng of total RNA, and subjected to Mixed Procedures of SAS (SAS, 99 1987).

Data for unoccupied and occupied receptors for LH in ovine luteal tissue collected during *in vivo* Experiment 1 were analyzed by a completely randomized design for a one way ANOVA. Data for unoccupied and occupied LHR were transformed using Log₁₀ and square root transformation, respectively. Treatment differences were tested using Duncan's multiple range test. Data for progesterone, PGE, and PGF_{2α} secretion by luteal tissue *in vitro* were analyzed using a 2x4 factorial completely randomized design for ANOVA. When significant effects of treatment or treatment x time interaction were detected, Tukey's Honest Significant Difference test was used to make individual treatment or treatment x time comparisons. Data for PGF_{2α} secretion by luteal tissue *in vitro* were transformed (log[X*1000]) prior to analysis to meet the assumption of homogeneity of variance. Data for progesterone and PGE secretion by luteal tissue *in vitro* met the assumption of homogeneity of variance. When data from the 4 and 8 h did not differ, data were pooled and reanalyzed by a one way ANOVA.

4.0. Results

Weights of corpora lutea 48 h after treatment *in vivo* with Vehicle, DP (LPA2R), OMPT (LPA3R), or EP4R agonists did not differ ($P \geq 0.05$; data not shown). Profiles of circulating progesterone of ewes treated *in vivo* with Vehicle, DP (LPA2R), OMPT (LPA3R), or EP4R agonists did not differ ($P \geq 0.05$) throughout the 48 h sampling period and there was no treatment x time interaction (Figure 1). There

was no effect ($P \geq 0.05$) of treatment with DP (LPA2R), OMPT (LPA3R), or EP4R agonists on Day-12 luteal progesterone secretion *in vitro* (data not shown).

Treatment with DP, the LPA2R agonist ($P \leq 0.01$), or the EP4R agonist CAY 10580 ($P \leq 0.05$) *in vivo* upregulated the relative Vehicle:mRNA luteal expression ratio for *LHR* at 48 h. Treatment with OMPT, the LPA3R agonist, had no effect ($P \geq 0.05$) on the relative Vehicle:mRNA expression ratio of luteal *LHR* (Figure 2).

Occupied LHR on luteal membranes of ewes 48 h after treatment with Vehicle, DP (LPA2R), OMPT (LPA3R), and EP4R agonists *in vivo* were similar ($P \geq 0.05$; Figure 3). However, unoccupied LHR on luteal membranes 48 h after treatment with DP (LPA2R), OMPT (LPA3R), and EP4R (CAY 10580) agonists *in vivo* was greater ($P \leq 0.05$) when compared to Vehicle-treated ewes (Figure 3). Luteal unoccupied LHR of DP (LPA2R) agonist-treated ewes at 48 h was greater ($P \leq 0.05$) than any other treatment group (Figure 3).

There was no treatment x time interaction ($P \geq 0.05$) on Day-12 ovine luteal $\text{PGF}_{2\alpha}$ secretion *in vitro* by ewes treated with DP (LPA2R), OMPT (LPA3R), or EP4R (CAY10580) agonists (Figure 4). Therefore, data for 4 and 8 h were pooled and treatment with the EP4R agonist (CAY 10580) tended ($P = 0.066$) to decrease luteal $\text{PGF}_{2\alpha}$ secretion *in vitro* (Figure 4). There was no treatment x time interaction ($P \geq 0.05$) on Day-12 luteal PGE secretion and data for DP (LPA2R), OMPT (LPA3R), or EP4R (CAY 10580) agonist for 4 and 8 h *in vitro* were pooled (Figure 5). Treatment with the EP4R (CAY 10580) agonist increased ($P \leq 0.05$) luteal PGE secretion seven fold by Day-12 luteal tissue *in vitro* while the LPA2R or LPA3R agonist had no effect on luteal PGE secretion (Figure 5).

No LPA receptor agonist tested affected ($P=0.81$) Day-12 caruncular endometrial secretion of $\text{PGF}_{2\alpha}$ or PGE ($P=0.73$) *in vitro* (data not shown). No EP prostanoid receptor subtype agonist tested affected ($P=0.79$) caruncular endometrial $\text{PGF}_{2\alpha}$ secretion *in vitro* (data not shown). There was no treatment x time interaction by PGE receptor subtype agonist on secretion of PGE by Day-12 caruncular endometrial tissue. Thus, data for caruncular endometrial PGE secretion for 4 and 8 h were pooled (Figure 6). 17PT-PGE₂, an EP1R agonist; sulprostone, an EP3R agonist; and CAY 10580 an EP4R agonist increased ($P\leq 0.0001$) ovine caruncular endometrial PGE secretion *in vitro*. The two EP2R receptor agonists, 19-R-OH-PGE₂ and Butaprost, or L-161982 a selective EP4R antagonist did not affect ($P\geq 0.05$) ovine caruncular endometrial PGE secretion *in vitro* ($P\geq 0.05$; Figure 6).

There was no effect ($P\geq 0.05$) of treatment or a treatment x time interaction for SIRT-1, SIRT-2, or SIRT-3 activators on Day-12 ovine caruncular endometrial $\text{PGF}_{2\alpha}$ secretion *in vitro* (data not shown). However, there was a significant SIRT-1 treatment effect ($P\leq 0.0001$) and a treatment x time interaction ($P\leq 0.01$) at 8 h on ovine caruncular endometrial PGE secretion *in vitro* (Figure 7). SIRT-2 or SIRT-3 activators did not affect ($P\geq 0.05$) ovine caruncular endometrial PGE secretion at 4 or 8 h (Figure 7)..

5.0. Discussion

5.1. EP4R agonist

EP4R agonists *in vivo* were not luteolytic, since the EP4R agonist did not decrease luteal weight, circulating progesterone, luteal mRNA expression of *LHR*, or luteal unoccupied or occupied LHR, which are decreased during spontaneous

luteolysis in ewes [5]. Further evidence that the EP4R agonist was not luteolytic is the failure to decrease luteal progesterone secretion *in vitro*. These data are in contrast to data in the cow where Estrumate, a PGF_{2α} analogue, given at midcycle increased luteal mRNA for *EP4R* and *EP4R*, which provided evidence that EP4R may be involved in luteolysis (B. Berisha and D. Schams-personal communication). Instead, the upregulation of luteal mRNA expression for *EP2R* and *EP4R* during a PGF_{2α}-induced premature luteolysis may be interpreted that luteal tissue was attempting to resist luteolysis by increasing blood flow to deliver LH to luteal tissue [51]. Blood flow to the luteal-containing ovary decreases during luteolysis in part by PGF_{2α} being a vasoconstrictor [51], while PGE₁ and PGE₂ are vasodilators [15, 52]. Jugular venous progesterone in cows declines to less than 1 ng/mL within 4 h after lutectomy at midcycle, which was similar to an exogenous dose of PGF_{2α}-induced luteolysis given at midcycle [53]. Thus, a single injection PGF_{2α}-induced luteolysis is a pharmacological effect, since circulating progesterone during natural luteolysis decreases slowly over 5 to 6 days to less than 1 ng/ml [54]. Bovine luteal tissue at midcycle or pregnancy secretes much PGE and PGF_{2α} *in vitro* at a 1.1:1 ratio [55, 56], while PGE and PGF_{2α} secretion *in vitro* by midcycle luteal tissue of ewes is barely detectable [17, 57]. In cow luteal tissue undergoing natural luteolysis, the luteal PGE:PGF_{2α} ratio changes to 0.2:1.4 [Weems, unpublished data].

Synchronization of estrus in cows altered the response to agonists by bovine luteal or endometrial tissue *in vitro* [58, 59]. Further evidence that the EP4R agonist was not luteolytic is the increase in luteal mRNA expression of luteal *LHR* and the increase in luteal unoccupied LHR without increasing luteal occupied LHR. Instead,

EP4R may be antiluteolytic, because the EP4R agonist *in vitro* tended to decrease luteal PGF_{2α} secretion, but increased luteal PGE secretion six fold *in vitro*.

5.2. LPA2R and LPA3R agonists

Treatment with DP (LPA2R) or OMPT (LPA3R) agonists *in vivo* did not decrease ovine luteal weight, circulating progesterone, luteal mRNA expression of *LHR*, or luteal unoccupied or occupied luteal LHR, each of which occurs during luteolysis. In addition, DP (LPA2R) or OMPT (LPA3R) agonists did not inhibit progesterone secretion by ovine luteal tissue *in vitro* reported herein indicating that LPA2R or LPA3R agonists are not involved in luteolysis. This is in contrast to reports that luteal mRNA expression of *LPA2R* increased transiently in bovine and that LPA inhibited LH-stimulated progesterone secretion by these cells *in vitro* via inhibition of cAMP production [29-31]. Instead, DP (LPA2R) may enhance luteal function, since the LPA2R agonist *in vivo* increased luteal mRNA expression of *LHR*, and OMPT the LPA3R agonist did not alter luteal mRNA expression of *LHR*. Moreover, both LPA2R and LPA3R agonists increased luteal unoccupied LHR, but had no effect on occupied LHR. These data do not support involvement of LPA2R or LPA3R involvement in luteolysis. A hallmark of luteolysis in the ewe [5] or cow [18] is decreases in circulating progesterone, luteal mRNA expression of *LHR*, and luteal unoccupied and occupied LHR.

The LPA2R or LPA3R agonists did not affect luteal or caruncular endometrial PGF_{2α} or PGE secretion *in vitro*. In contrast, LPA increased PGF_{2α} and PGE₂ secretion by ovine trophectoderm cells in a dose dependent manner [60] and could play a role in preventing luteolysis during maternal recognition of pregnancy.

However, LPA did not affect PLA2 and COX-2 gene expression in the ovine trophoctoderm [60]. That study found that LPA1R and LPA3R expression in the ovine conceptus peaked on Day-17 of pregnancy, two days after COX-2 peak expression, leading researchers to conclude that LPA signaling does not regulate prostaglandin synthesis by the ovine conceptus [60]. During early pregnancy in the cow, a positive correlation was reported between PGE₂ synthase expression and mRNA expression of LPA1R and a negative correlation between PGF_{2α} synthase expression and mRNA expression of LPA1R [61]. Additionally, cows receiving a single infusion of LPA into the abdominal aorta on Day-16 of the estrous cycle increased concentrations of PGE₂ in peripheral blood over the 24 h posttreatment period when compared to vehicle [61]. Treatment with Ki16425 (LPA1R and LPA3R antagonist) decreased peripheral concentrations of PGE₂ compared to Saline in cows [61]. LPA infusion also increased PGFM concentrations, the catabolite of PGF_{2α}, in peripheral blood on Day-16 in cows, but to a lesser extent compared to PGE₂ [61]. Thus, there are insufficient data to indicate LPA is involved in luteolysis. However, LPA3 may play a role in early development of the peri-implantation porcine conceptus [74].

5.3. EP receptor agonists on caruncular endometrium PGE and PGF_{2α} secretion

Receptors for PGE₂ were reported to be present in the epithelium of the ovine uterus and embryo using immunohistochemical analysis and thus may play roles in implantation and elongation of ovine embryos [62]. None of the prostanoid receptor agonists for EP1, EP2, EP3, or EP4 receptors affected ovine endometrial PGF_{2α} secretion *in vitro*. However, an EP1R (17 PT-PGE₂), EP3R (Sulprostone), and

an EP4R (CAY 10580) agonist increased ovine caruncular endometrial PGE
 secretion *in vitro*, while two EP2R agonists (Butaprost or 19-OH-PGE2) did
 not increase caruncular endometrial PGE secretion. These same two EP2R agonists
 given via the same route increased ovine circulating progesterone *in vivo*, while
 Sulprostone an EP3R agonist decreased circulating progesterone [22]. Thus,
 the endometrial EP4R agonist (CAY10580) may be more important than caruncular
 endometrial EP2R to regulate caruncular endometrial PGE secretion in the ewe.
 However, luteal EP2R may be more important than EP4R prostanoid receptors to
 mediate the antiluteolytic effects reported for PGE₁ and PGE₂ [5]. PGE₂ has been
 reported to be essential for decidualization of the uterus and pregnancy in rodents,
 because inhibitors of prostaglandin synthesis block uterine decidualization, which
 can be overcome with PGE₂ [62]. Interferon τ (INF τ) may regulate regulate
 endometrial PGE₂ secretion during early pregnancy in the ewe, but it may not be
 driven solely by INF τ [63]. INF α is not the only stimulator of ovine uterine PGE
 secretion and uterine INF τ was not detected after Day-25 of pregnancy in the ewe
 [64]. Chronic intrauterine infusion of a nitric oxide donor during the estrous cycle of
 the ewe prevented luteolysis *in vivo* via stimulating uterine PGE secretion [65, 66].
 Ovine uterine PGE secretion by the uterine/placenta complex increased by Day-12
 postbreeding and continues to increase to Day-90 of pregnancy *in vitro* or *in*
vivo [67, 68], while PGF_{2 α} secretion does not decrease until after Day-50 of
 pregnancy [67, 68].

5.4 Sirtuins 1, 2, and 3 caruncular endometrium

SIRT-1, SIRT-2, or SIRT-3 activators did not affect caruncular secretion of

PGF_{2α} at any time, or PGE at 4 h; however, SIRT-1, increased PGE at 8 h, which
 was 4 fold greater than Vehicle controls. Location of SIRT-1 within the nucleus is
 interesting, because cyclooxygenase-2 (COX-2), the inducible form of COX is
 located adjacent to the nuclear membrane [69]. SIRT-1 did not increase PGF_{2α}
 secretion, but increased caruncular endometrial PGE secretion. Thus, SIRT-1 is
 probably activating PGE synthase. SIRT-1 activated cyclooxygenase (COX-2), but
 not COX-1 in kidney cells. Moreover, SIRT-1 increased EP1R, EP3R, and EP4R,
 but not EP2R in renal medulla cells [70]. In ovine caruncular endometrial tissue
 reported herein, EP1R (17-PT-PGE₂), EP3R (Sulprostone), and EP4R (CAY10580)
 agonists also increased PGE secretion *in vitro*, but was not increased by EP2R
 agonists (Butaprost, 19-R-OH-PGE₂). SIRT-1 activators inhibited development of
 experimental endometriosis *in vivo* and reduced endometrial stromal cell
 invasiveness *in vitro* [71]. In addition to activating COX-2, SIRT-1 and activators of
 SIRT-1 are of particular interest in prostaglandin synthesis by reproductive tissue
 [38, 39] because they increase fatty acid release [32-37]. SIRT-1 binds to and
 represses genes controlled by PPAR-γ, including several genes regulating fat
 mobilization, resulting in mobilization of fatty acids in adipocytes[32-37]. SIRT-1
 inhibited apoptosis and reduced NF Kappa β (NFκβ), transforming growth factor β
 (TGF β), and peroxisome proliferator activated receptors (PPARγ) action on
 lipolysis [34]. The tumor suppressor p53 activates the transcriptional program that
 promotes apoptosis and has several acetylation sites in its C-terminus that are
 excellent substrates for SIRT-1 [36]. Deacetylation of p53 is thought to lead
 to its degradation; therefore, it seems that SIRT-1 might act as an anti-apoptotic

factor by reducing the amount of p53 in the cell [36]. SIRT-1 decreased lipopolysaccharide, TNF α , interleukin 6 and 8, mRNA expression of prostaglandin synthase, PGE₂ and PGF_{2a} release by human placental tissue [38], and SIRT-6 is decreased during preterm labor [72]. These data indicate that SIRT-1 may protect tissues from apoptosis because NF κ B, TGF β , and PPAR γ are involved in luteal regression in which apoptosis is the final stage [73]. Roles for SIRT-1 on inhibition of NF κ B, TGF β , and PPAR γ are needed in luteal tissue.

5.5 Summary

In summary, EP4R and LPA2R or LPA3R do not appear to be involved in luteal regression, since circulating progesterone, luteal mRNA expression of *LHR*, and luteal unoccupied and occupied LHR were not decreased. In addition, luteal mRNA expression for LHR was increased by EP4R and LPA2R agonists. EP1R, EP3R, and EP4R might play a role in regulating endometrial caruncular PGE secretion, while EP2 agonists did not affect caruncular endometrial PGE secretion. Moreover, data presented herein might indicate that SIRT-1 induced increases in PGE might reduce apoptosis in reproductive tissue.

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8. List of figures

Figure 1. Profile of circulating progesterone of ewes treated *in vivo* with Vehicle, DP (LPA2R), OMPT (LPA3R), or CAY10580 (EP4R) receptor agonist.

Figure 2. Luteal tissue:mRNA:Vehicle expression ratio of luteal *LHR* of ewes treated *in vivo* with Vehicle, DP (LPA2R agonist), OMPT (LPA3R agonist), or CAY10580 (EP4R agonist).

Figure 3. Ovine luteal unoccupied and occupied receptors for LH of ewes treated *in vivo* with Vehicle, DP (LPA2R), OMPT (LPA3R), or CAY10580 (EP4R) receptor agonist.

Figure 4. Ovine luteal tissue secretion of PGF_{2α} treated *in vitro* with Vehicle, DP (LPA2R), OMPT (LPA3R), or CAY10580 (EP4R) receptor agonist.

Figure 5. Ovine luteal tissue secretion of PGE *in vitro* treated with Vehicle, DP (LPA2R), OMPT (LPA3R), or CAY10580 (EP4R) receptor agonist.

Figure 6. Ovine caruncular endometrial tissue secretion of PGE treated *in vitro* with the prostanoid EP (1-4) receptor agonists: Vehicle, 17PT PGE₂ (EP1R agonist); 19-R-OH-PGE₂ (EP2R agonist); butaprost (EP2R agonist; sulprostone (EP3R agonist) or CAY10580 (EP4 R agonis) receptor agonists; or L-61982 (EP4R) receptor antagonist.

Figure 7. Ovine endometrial tissue secretion of PGE treated *in vitro* with Vehicle, SIRT-1, .SIRT-2, or SIRT-3 activators.