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The effect of leptin on luteal angiogenic factors during the luteal phase of the estrous cycle in goats

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Abstract

Fibroblast growth factor 2 (FGF2), angiopoietin 1 (Ang1), and vascular endothelial growth factor (VEGF) are angiogenic factors implicated in the vascular development of the corpus luteum (CL). Each factor is regulated or influenced by leptin in non-ovarian tissues. Moreover, leptin and its receptor, ObRb, have been identified in luteal tissue throughout the luteal phase. Therefore, leptin is hypothesized to influence luteal vasculature through the regulation of FGF2, Ang1, and VEGF. Multiparous, cycling crossbred female goats (does) were allocated to early (n=12), mid (n=8), and late (n=11) stages of the luteal phase for CL collection. Luteal tissue was harvested and either snap frozen in liquid N₂, paraffin embedded, or cultured with leptin (0, 10⁻¹², 10⁻¹¹, 10⁻¹⁰, 10⁻⁹, 10⁻⁸ M). Tissue was analyzed for FGF2, Ang1, VEGF, ObRb, and leptin expression. Angiopoietin 1, FGF2, VEGF expression was higher (*P* 0.001) in the mid-luteal stage than the early stage. Expression decreased (*P* 0.001) during the late luteal stage with the exception of VEGF, which remained elevated. In contrast, leptin and ObRb were lowest (*P* 0.003) during the mid-luteal stage compared to the early and late stages. All factors were detected in and/or around vessels in early stage tissue compared to mid and late stages. Leptin stimulated (*P* 0.02) Ang1, FGF2, and VEGF

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Authors’ contributions JRW participated in study design, acquisition, analysis and interpretation of data, and in preparation of the manuscript. RAK, EAB, and CWO participated in acquisition and interpretation of the data. JME, DHK, and RLS participated in the analysis of data. As Principle Investigator, MRG participated in the intellectual and experimental design of the study, data acquisition, analysis and interpretation, as well as writing and revising the manuscript. All authors read and approved the final manuscript.

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expression only in early stage luteal cultures. Collectively, these data provide evidence that leptin may be involved in the luteal angiogenic process during the early stage of CL formation.

Keywords

leptin corpus luteum angiogenesis goats

1. Introduction

Abnormal vasculature of the CL is a defect that leads to abnormal development and decreased progesterone production (Glock and Brumsted, 1995; Hazzard and Stouffer, 2000). Identifying and understanding the underlying mechanisms of luteal angiogenesis may lead to a reduction in luteal-related infertility and alternate methodology for the regulation of reproductive cycles. In the ovary, angiogenesis is a cyclical, recurring process that takes place following ovulation. Upon ovulation, the basement membrane between the granulosa and thecal layers disassociates allowing the thecal capillaries to invade the avascular granulosa layer and antral space forming a nascent network of capillaries to nourish the developing CL. The luteal neo-vascularization process is attributed to the biological activity of FGF2, Ang1, and VEGF. Both FGF2 and VEGF promote capillary membrane destabilization, endothelial cell differentiation, proliferation, migration, and vascular tube formation in human, bovine, and ovine luteal tissue (Suzuki et al., 1998; Reynolds and Redmer, 1998). Maturation and stabilization of nascent vessels is then promoted by Ang1 through the recruitment of stromal support cells, including pericytes and smooth muscle cells (Shalaby et al., 1995). Interestingly, each of these angiogenic regulatory factors is influenced by the adipogenic hormone leptin (Aleffi et al., 2005, Cao et al., 2001).

Both leptin and its receptor (ObRb) have been identified in normal and polycystic ovaries of various species (Loffler et al., 2001; Ruiz-Cortez et al., 2000; Ryan et al., 2003; Munoz-Gutierrez et al., 2005), particularly in steroidogenically active follicles and luteal tissue. This would infer that leptin may be involved in steroidogenic processes (Kendall et al., 2004). However, in order to substantially modify steroid production cells must be incubated with leptin in the presence of a growth promotant (Spicer and Francisco 1997; Spicer and Francisco 1998; Zachow and Magoffin 1997; Karlsson et al., 1997; Brannian et al., 1999), which suggests that an ancillary role for leptin in ovarian tissue, such as angiogenesis, may exist. In support, ObRb has been identified in ovarian vascular endothelial cells in rodents (Ryan et al., 2003) and its expression in the porcine CL is highest during luteal development, a period of intense angiogenic activity (Ruiz-Cortez et al., 2000). Furthermore, leptin alone is capable of stimulating angiogenesis in the cornea of the eye (Sierra-Honigsmann et al., 1998) and upregulates the expression of angiogenic factors in non-ovarian tissue (Aleffi et al., 2005, Cao et al., 2001). Collectively, the evidence supports the supposition that leptin may be involved in ovarian angiogenesis, specifically vascularization of a developing CL. Therefore, it is hypothesized that leptin will influence the expression of angiogenic growth factors, FGF2, Ang1, and VEGF, in developing luteal tissue. The objectives were to: 1.) characterize FGF2, Ang1, VEGF, leptin, and ObRb expression at different stages of the luteal phase of the estrous cycle, and 2.) determine the effect of leptin

on the expression of FGF2, Ang1, and VEGF in dispersed luteal tissue during the early, mid, and late stages of the luteal phase. Understanding the mechanisms of the development of luteal tissue may lead to a future reduction in the occurrence of infertility associated with luteal defects.

2. Materials and methods

Institutional Animal Care and Use Committee at Texas A&M University-Kingsville (TAMUK) approved the animal care procedures used for this study.

2.1. Animals

Thirty-one mature, cycling crossbred (Boer × Spanish) female goats (does), *Capra aegagrus hircus*, averaging 11 months of age and weighing 38.2 ± 2.3 kg at date of tissue collection, from the Texas A&M University-Kingsville (TAMUK) farm were utilized. Animals were randomly allocated for CL tissue collection at either the early (n=12), mid (n=8), or late (n=11) stages of the luteal phase of the estrous cycle. Early, mid, and late stages of the luteal phase are defined by the day of the estrous cycle beginning with day of initial onset of estrus (D0). Therefore, 'early stage' CL were collected on D3 of the estrous cycle which is ~35-42 h post-ovulation, while 'mid stage' CL were collected on D10 of the estrous cycle, and 'late stage' CL were collected on D15 of the normally 21-day caprine estrous cycle. Tissue was collected to satisfy minimum requirements of 6 does/luteal stage, not exceeding 12 does/luteal stage, for tissue characterization analysis and cell cultures. The specifically defined luteal stages were based on classification by Arosh et al. (2002), which correlates well with the goat specie classification as reported by Camp et al. (1983). Briefly, early stage CL are smaller in diameter (< 5 mm), profuse with blood, red in color, are soft, and the bordering tissue is not well defined. The mid stage CL are larger in diameter (6-7 mm) than the early stage, have a more flesh-like pink color, are more compact in consistency, and are entirely covered with a distinct connective tissue layer. The late stage CL are a little larger in diameter (7-10 mm) than the mid stage, are less pink in color, and have a distinct connective tissue layer cover. All does were housed outdoors in covered facilities (9.5 × 15.5 meter pens) and were observed twice daily for classical behavioral estrus using an intact male. To ensure normal cycle length, tissue collection did not take place until does were well within the breeding season, i.e. cycling began in late August and tissue collection occurred between the months of November and February. At estrus, and daily thereafter, blood samples were collected, via jugular venipuncture, until day of CL tissue collection (D3, D10, or D15) for analysis of serum progesterone for confirmation of normal luteal function.

2.2. Corpus luteum tissue harvest

Does were fasted for 18-22 h and water was removed ~10 h prior to tissue collection. After the fasting period, does were transported to the surgical facility at the TAMUK farm where they were anesthetized using inhalation of isoflurane gas from a vaporizer set at 4%, mixed with an O₂ at a flow rate of 4 L/min. After initial induction, the vaporizer was set to 2.5%-3% with an O₂ flow rate of 2.8-3.2 L/min. A 3 to 4 inch vertical incision was made between the udder quarters to expose the reproductive tract. Ovaries were removed via blunt dissection and placed in ice-cold Hanks solution for transportation. Corpora lutea were

harvested, divided, and processed for paraffin embedding, RNA extraction, and/or cell culture.

2.3. Immunohistochemistry

Luteal tissue (n=6 animals/luteal stage) was placed in ice-cold (4°C) fixative solution (10% Formalin, ~4% formaldehyde; Sigma, St. Louis, MO) for 24 h at 4°C on a shaker and then rinsed twice in 1x phosphate buffered saline (PBS) for 1 h each. Tissue was then placed in a graded series of ethanol baths (40% (v/v), 60%, 80%, 95% (2x), and 100% (2x) ethanol) for 1 h each at 4°C and two separate 1 h rinses in xylene. Dehydrated luteal tissue was transferred to a paraffin (TissuePrep; Fisher Scientific, Pittsburgh, PA) bath for 2 h and then embedded in a paraffin mold. Tissue was sectioned (6 µm) and placed on glass slides (2 serial sections per slide). Tissue was deparaffinized and rehydrated in xylene (2x) for 5 min each and rehydrated through a graded series of ethanol baths (100% (2x), 95% (2x), and 70%) for 2 min each, rinsed in tap water for 5 min, and incubated in 3% (v/v) hydrogen peroxide for 10 min to block endogenous peroxidase activity. Following deparaffinization and rehydration, sections were boiled in 10 mM sodium citrate for antigen recovery, cooled to room temperature, and rinsed in buffer (1% (w/v) bovine serum albumin (BSA) in PBS) for 5 min. Slides were processed as per manufacturer's recommendations using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA). To block nonspecific binding, tissue was incubated for 30 min at room temperature with blocking solution [1% (w/v) BSA and 0.3% (v/v) Triton-X-100 in PBS], and normal goat or horse serum (1% v/v; Vector Laboratories). Following a rinse in buffer, slides were incubated in 3% BSA in PBS for blocking at 37°C for 1 h. Following another buffer rinse, slides were incubated with Ang1 (1:50; CA0636; Cell Applications, San Diego, CA), FGF2 (1:50; CA0259; Cell Applications), VEGF (1:50; SC-7269; Santa Cruz Biotechnology Inc., Santa Cruz, CA), leptin (1:500; PA1-052; Thermo Scientific, Waltham, MA), or ObRb (1:50; SC-8325; Santa Cruz) primary antibodies in buffer. For each slide incubated with primary polyclonal antibody, a consecutive slide was incubated in normal serum (1.5% v/v) as a control. Slides were rinsed and incubated with biotinylated secondary antibody (0.5% v/v; Vector Laboratories) for 30 min at room temperature. Following secondary antibody incubation, tissue sections were rinsed, incubated in an avidin biotinylated horseradish peroxidase complex (1% v/v) for 30 min at room temperature, rinsed, and incubated with peroxidase substrate solution (NovaRED Substrate Kit; Vector) for 10 min at room temperature. Each slide was rinsed, air dried, mounted in Permount medium and analyzed. To validate primary antibody binding anti-FGF2, anti-Ang1, anti-VEGF, and anti-leptin were pre-incubated with 1 µg of FGF2 (200-12; Shenandoah), Ang1 (100-46; Shenandoah Biotechnology, Inc., Warwick, PA), VEGF (10-663-45110; GenWay Biotech, San Diego, CA), leptin (CRL303B; Cell Sciences, Canton, MA), respectively, for 1 h and used during the primary antibody step. To verify anti-ObRb binding, sample slides were pre-incubated with leptin (Cell Sciences) for 1 h and rinsed then treated with antibody. Slides were observed at 20x for location of NovaRED staining, including small (5-20 µm) and large (>20 µm) luteal cells, and vessels (Kalender and Arikan, 2007).

2.4. Dispersed corpora lutea cell cultures

Luteal tissue (n=6 animals/luteal stage) was mechanically minced and enzymatically dissociated in digestion media (TypeIA collagenase, 1.27 mg/ml, 0.5% BSA in Dulbecco's Modified Eagle's Medium; DMEM) for 90 min at 37°C with gentle agitation in a shaking water bath. Cells were filtered through a metal mesh (80 µm) and centrifuged twice at 150 × g for 4 min and the enzymatic supernatant was removed. Cells were re-suspended and the numbers of viable cells were determined using the Trypan Blue Exclusion Test. Cell viability was greater than 85% for each luteal stage. The dispersed luteal cells were seeded in treated polystyrene 24-well cluster dishes (Sigma) at 3 × 10⁶ cells/well in attachment serum media [1 ml/well; DMEM supplemented with 10% (v/v) charcoal stripped fetal calf serum and 5,000 µg/mL penicillin/streptomycin] and incubated for 24 h in a humidified atmosphere of 5% CO₂ in air at 37°C to equilibrate cells to culture condition, allow aggregation, and promote adherence. Following the 24 h incubation, attachment serum media was aspirated and replaced with culture media (1 ml/well; DMEM, 0.1% (w/v) BSA, 2.0% (v/v) charcoal stripped fetal calf serum, 0.5 mM ascorbic acid, and 5,000 µg/mL penicillin/streptomycin) containing recombinant ovine leptin (0, 10⁻¹², 10⁻¹¹, 10⁻¹⁰, 10⁻⁹, 10⁻⁸ M; Cell Sciences) in triplicate (3 wells/dose/animal/luteal stage) for 24 h in a humidified atmosphere of 5% CO₂ in air at 37°C. Ovine leptin, which is >95% identical, at the amino acid level, to caprine leptin (Genebank access. #Q28603 and #CAJ38273), was substituted due to availability. After 24 h, the cell culture media was aspirated and stored at -80°C for measurement of secreted progesterone as a marker for effect of leptin in vitro. Denaturing solution [1 ml/well; 25 mM sodium citrate (pH 7.0), 0.5% (w/v) N-laurylsarcosine, 4 M guanidine thiocyanate with 0.7% (v/v) 2-mercaptoethanol] was added to cells, aspirated, pooled by dose, and stored at -80°C for total RNA extraction and mRNA analysis.

2.5. Gene expression

Total RNA was extracted from CL tissue (10-20 mg) (early stage, n=12; mid stage, n=8; late stage, n=11) using a phenol:chloroform based procedure, as previously reported (Garcia et al., 2002), and analyzed for FGF2, Ang1, VEGF, leptin, and ObRb (tissue only) mRNA using real-time polymerase chain reaction (PCR) with the DNA Engine Opticon II (Bio-Rad, Hercules, CA). All enzymes and PCR reagents were obtained from Promega (Madison, WI). Two µg of total RNA was treated for DNA contamination in a reaction mix containing moloney-murine leukemia virus reverse transcriptase (M-MLV RT), reaction buffer (50 mM Tris-HCL (pH 8.3), 75 mM KCL, 3 mM MgCl₂, and 10mM DTT), nucleotide mix (dATP, dCTP, dGTP, dTTP, 4 µM/µl), RNASIN (20-40 u/µl), RQ1 DNase (1 u/µl) and RNase free water in a total volume of 25 µl. Each reaction mix was incubated at 37°C for 30 min for DNase treatment, followed by an 85°C incubation for 10 min to terminate DNase activity. Following DNase treatment, Oligo dt15 (500 µg/ml) and M-MLV RT (200 u/µl) was added and incubated at 37°C for 1 h to RT mRNA into cDNA. Samples were then incubated at 85°C for 10 min to terminate the RT. Complementary DNA was amplified by real-time PCR using primers synthesized by Integrated DNA Technologies (Coraville, IA; Table 1) in a Takara SYBR® Green (Madison, WI) reaction mix. The VEGF primer pair was developed using the Bos taurus sequence for VEGFA mRNA (Genebank accession # NM_174216.1)

and does not differentiate among the 121, 165, and 189 isoforms reported in the bovine (Berisha et al., 2000), ovine (Redmer et al., 1996), rodent and human (Ferrara, 2004) CL. After a 10 min hot start, the PCR cycles were as follows: 94°C for 30 s, anneal temperature of 52°C for 30 s, 68°C or 72°C (relative to primer pair; Table 1) for 60 s for 36-44 cycles, ending with a 72°C 10 min extension. Cyclophilin mRNA from porcine liver was used to create a standard curve for relative quantification purposes, a scientifically accepted method for real time quantification (Bustin, 2000) that has been previously published by our laboratory (Matsumoto et al., 2006). To correct for procedural variability, L19 (housekeeping gene) mRNA was used to normalize targeted amplicons. Relative values of FGF2, Ang1, VEGF, leptin, and ObRb gene expression were quantitated from the relative standard curve. Values were then transformed to $\log_{10}$ and normalized with L19. Cultured luteal cell values are represented as a percentage of the control (0M). Agarose gel electrophoresis, containing ethidium bromide, was used to confirm the presence of a single band of the expected size of the PCR amplicon for each of the PCR primer pair reactions. The Ang1, VEGF, and ObRb PCR amplicons were electrophoresed on a 2% agarose gel stained with ethidium bromide, extracted, purified and cloned using the pGEM®-T Easy Vector Systems (Promega). Cloned products were sequenced using MCLab Sequencing (San Francisco, CA) to verify the amplicon sequence as the correct target of the primers.

2.6. Radioimmunoassay

Concentrations of progesterone in culture media and serum were determined by radioimmunoassay (RIA, Coat-A-Count®; Siemens Corp, New York, NY; Novak et al., 2003; Mao et al., 2001) to confirm cell viability during culture process, response to culture treatment (Fig. 4), and the presence of a functional CL at the time of tissue harvest. Progesterone concentrations were validated by the recovery of exogenous progesterone added in known amounts (1, 2.5, 5, 10, and 20 ng/mL) to pooled goat serum. Recovery of exogenous hormone was 100%. Inter- and intra-assay coefficients of variation for all assays were <10%. Serum concentrations of leptin were determined in a single RIA as described previously (Delavaud et al., 2000). Sensitivity of the assay was 0.1 ng/mL and intra-assay CV averaged 3.3%.

2.7. Statistical analyses

Messenger RNA was analyzed using the PROC MIXED procedure of SAS (2004). Source of variation was dose of leptin treatment. The ESTIMATE procedure of SAS was used to determine differences between treatment doses compared to control (0M) upon detection of model significance. Comparisons among sources of variation were conducted using the PDIFF option. Tissue mRNA values were analyzed using the PROC MIXED procedure of SAS with luteal stage as the source of variation. Serum hormones were analyzed using the MIXED procedure of SAS for repeated measures with day of the estrous cycle as the source of variation. Does within treatment was used as the RANDOM effect in the mixed procedure to account for variation relative to animal. All values are represented as least squares means plus or minus the standard error of the mean (LSmeans $\pm$ SEM).

3. Results

3.1. Circulating concentrations of progesterone and leptin

As expected, serum progesterone increased ($P<0.001$; Fig. 1) relative to age of the CL. Progesterone concentrations were higher ($P<0.03$) in the mid stage group, beginning at D7 of the sampling period, than the late stage group of does; however, 6 out of 8 (75%) mid stage does had more than 2 CL upon sample collection compared to 7 out of 11 (63.6%) does in the late stage group. Circulating concentrations of leptin did not differ throughout the sampling period relative to CL stage group (early, mid, late) nor between groups.

3.2. Gene expression of angiogenic factors, leptin, and ObRb

Messenger RNA levels of all quantified angiogenic factors, leptin, and ObRb varied ($P = 0.003$, Fig. 2) in luteal tissue relative to stage of the luteal phase, i.e early, mid, and late stage CL. Expression of FGF2, Ang1, and VEGF mRNA was higher ($P<0.001$) in mid stage tissues compared to early tissues. However, in the late stage CL, expression of Ang1 and FGF2 was lower ($P<0.001$) than mid stage CL. Unlike Ang1 and FGF2, VEGF expression in the late stage CL was similar to mid stage CL. Interestingly, leptin and ObRb exhibited an inverse pattern of expression relative to Ang1 and FGF2, i.e. both leptin and its receptor were lower ($P = 0.003$) in mid stage luteal tissue compared to early stage luteal tissue. Furthermore, leptin and ObRb expression were higher ($P<0.001$) in late stage luteal tissue than in the mid stage CL; moreover, leptin expression appeared to be highest ($P<0.001$) in the late stage CL.

3.3. Localization of angiogenic factors, leptin, and ObRb

All angiogenic factors were expressed in the CL throughout the luteal phase; however, spatial expression varied relative to angiogenic factor and day. Fibroblast growth factor 2, Ang1, and VEGF were detected in vascular endothelial cells and luteal cells, both large and small (Fig. 3A). Angiopoietin 1 was primarily localized to developing luteal vasculature; however, in the mid and late stage CL Ang1 was primarily localized to the cytosolic region of luteal cells. Fibroblast growth factor 2 was predominantly localized to luteal vasculature and the surface (cell membrane) of luteal cells in early stage CL. In mid and late stage CL, FGF2 was predominantly cytosolic. Vascular endothelial growth factor was uniformly localized to vessels, perivascular cells, and the cell membranes and cytosol of luteal cells regardless of luteal stage. Leptin and its receptor were present in the membrane and cytosol of small and large luteal cells and in vascular endothelial cells in early luteal tissue (Fig. 3B). In mid and late CL, leptin and ObRb were predominantly localized to cellular cytosol of luteal cells.

3.4. The effect of leptin on angiogenic factor expression

Early and mid luteal cultures were responsive to leptin treatment resulting in decreased ($P<0.05$; Fig. 4) progesterone secretion. However, cellular responsiveness was attenuated and inconsistent in late luteal cultures. Leptin at the -11M dose stimulated ($P = 0.02$; Fig. 5) gene expression of FGF2, Ang1, and VEGF in the early stage luteal cultures only. There was no treatment effect on angiogenic factor expression in mid or late stage luteal cultures.

4. Discussion

Similar to previous studies, FGF2, Ang1, and VEGF expression increased as the CL matured (Stirling et al., 1991; Kawate et al., 2003; Sugino et al., 2000). However, some studies have reported that early, rather than mid stage CL, exhibit the highest expression of these factors (Berisha et al., 2000; Wulff et al., 2000). The discrepancy is attributed to the time at which CL were collected. In the study herein, early CL were collected on D3 of the estrous cycle, which is ~35-42 h post ovulation in the caprine species (Romano and Abella, 1997; Kawate et al., 2000). In the previous reports, CL were collected at least 24 h later than our collection time and over a range of 2-3 days (Berisha et al., 2000; Wulff et al., 2000). Hence CL collected later in development should have greater expression activity corresponding to greater overall tissue. In the early stage CL, FGF2 and VEGF were present in both small and large luteal cells as well as in vessels, which concurs with previous reports (Grazul-Bilska et al., 1993; Shams et al., 1994; Gabler et al., 2004). However, unlike VEGF, FGF2 was primarily localized to vascular endothelial cells and the surface of luteal cells, which implies that it is likely binding to a receptor (Derer et al., 2012). Interestingly, FGF2 has been reported to stimulate VEGF expression (Seghezzi et al., 1998); moreover, VEGF is expressed in both vascular endothelial cells and luteal cells of the early bovine and human CL (Berisha et al., 2000; Wulff et al., 2000; Gabler et al., 2004). Therefore, FGF2 may be upregulating VEGF expression in the caprine CL to promote vascular development. Unlike FGF2 and VEGF, Ang1 was almost exclusively detected in and around luteal vasculature in the early stage. This is largely attributed to the necessity of Ang1 in stabilizing nascent vessels through the recruitment of perivascular (pericytes) and smooth muscle cells to the abluminal vessel (Suri et al., 1998). Without Ang1 a deregulation in the number and size of inter-endothelial junctions would occur leading to vascular hyperdilation and hemorrhage (Suri et al., 1998; Baffert et al., 2006). As the CL matured, Ang1 became more localized to cellular cytosol of luteal cells, which is indicative of increased storage and reduced secretion (Cammisotto and Bendayan, 2012) coinciding with the overall stabilization of luteal vasculature. Similar to previous reports in the human and bovine CL, Ang1 decreased during the late stage of the luteal phase, signaling a reduction in angiogenic activity and vascular stabilization (Tanaka et al., 2004). However, in contrast to previous reports (Berisha et al., 2000; Zhang et al., 1993), VEGF expression remained elevated and FGF2 decreased in the late stage of the caprine CL. This discrepancy may also be attributed to day of tissue collection that occurred over a range of days rather than one specific day of the luteal phase. Day 15 of the caprine estrous cycle is considered the beginning of the late luteal stage (Camp et al., 1983; Kawate et al., 2000). Hence, functional regression of the CL has not progressed far enough to modify neither FGF2 nor VEGF, which have been implicated in the prevention of vascular endothelial cell apoptosis (Gerber et al., 1998) and a regression-induced inflammatory response (Neuvians et al., 2004), respectively. Circulating concentrations of progesterone support the supposition that functional regression in the late stage group had not advanced enough to reduce measurable concentrations. On average, the CL will significantly reduce progesterone production as the CL prepares and initiates functional and subsequent structural regression between days 15 and 17 of the average 21-day caprine cycle (Kawate et al., 2000; Kawate et al., 2003). Although the gene expression of FGF2 and VEGF did not exhibit a pattern in the late stage CL as previously reported, it is

highly probable that FGF2 and VEGF expression patterns would reflect previous studies over the next 48 h to allow vascular degeneration as the CL regresses.

In contrast to the angiogenic factor pattern of expression, leptin and ObRb expression was higher in early than in mid stage CL, which implies that the role of leptin in luteal function may be limited to early developmental processes. Moreover, there appeared to be heavier immunodetection of leptin in the luteal cells and developing vasculature of early tissue compared to the mid and late stages. Furthermore, leptin's influence on angiogenic hormone expression was limited to early stage luteal cells despite a treatment-induced decrease in progesterone secretion in the mid and late stage cultures. However, unlike the decrease in progesterone secretion, leptin stimulated an increase in expression of FGF2, Ang1, and VEGF at a single dose. The single dose response may be attributed to the signal transduction cascades [mitogen activated protein kinases (MAPK) vs. Janus activated kinases/signal transducer and activator of transcription (JAK/STAT)] of ObRb (Yamashita et al., 1998). Each angiogenic factor analyzed in the study herein can be regulated by leptin via the MAPK signaling pathway (Lee et al., 2005; Brown et al., 2004; Sabbieti et al., 2005); however, nanogram quantities of leptin decreases MAPK protein content in cultured granulosa cells (Sirotkin and Grossman, 2007). Hence, reduced MAPK availability could diminish leptin's ability to influence FGF2, Ang1, and VEGF expression at the higher doses of leptin in the treatments reported herein.

Similar to a previous report in the porcine CL (Smolinska et al., 2010), leptin expression increased in the late stage CL. Such an increase in mRNA content would imply that leptin may serve a purpose in the CL as it prepares to regress, even though there was a discernible reduction in immunodetection of leptin protein in late stage tissue. Hence, the increase in message may be attributed to a decrease and/or cessation in translated protein, a natural occurrence as the CL begins the process of functional regression (Musicki et al., 1994; Stocco et al., 2007). Although ObRb mRNA also increases in late stage tissue, it has been reported that a prominent increase in the expression and activity of Suppressors of Cytokine Signaling (SOCS), primary inhibitors of the ObRb signaling cascade, occurs in regressing lutea (Curlewis et al., 2002). Essentially, such increases in inhibitor activity may negate the necessity of translated leptin in late stage tissue and explicate the loss of leptin's ability to consistently regulate progesterone secretion in the late stage luteal cultures. Overall, these changes do not appear to be associated with circulating leptin considering the observation that serum concentrations of leptin did not differ among does between the different luteal stage groups. Collectively, the temporal and spatial pattern of leptin in luteal tissue and its effect on angiogenic hormones in vitro supports the hypothesis that leptin is involved in or associated with the vascularization of the developing CL.

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References

- Aleffi S, Petrai I, Bertolani C, Parola M, Colombatto S, Novo E, Vizzutti F, Anania FA, Milani S, Rombouts K, Laffi G, Pinzani M, Marra F. Upregulation of proinflammatory and proangiogenic cytokines by leptin in human hepatic stellate cells. *Hepatology*. 2005; 42:1339–1348. [PubMed: 16317688]
- Arosh JA, Parent J, Chapdelaine P, Sirois J, Fortier MA. Expression of cyclooxygenases 1 and 2 and prostaglandin E synthase in bovine endometrial tissue during the estrous cycle. *Biol. Reprod.* 2002; 67:161–169. [PubMed: 12080013]
- Baffert F, Le T, Thurston G, McDonald DM. Angiopoietin-1 decreases plasma leakage by reducing number and size of endothelial gaps in venules. *Am. J. Physiol. Heart Circ. Physiol.* 2006; 290:H107–H118. [PubMed: 16126815]
- Berisha B, Schams D, Kosmann M, Amselgruber W, Einspanier R. Expression and tissue concentration of vascular endothelial growth factor, its receptors and localization in the bovine corpus luteum during estrous cycle and pregnancy. *Biol. Reprod.* 2000; 63:1106–1114. [PubMed: 10993833]
- Brannian J, Zhao Y, McElroy M. Leptin inhibits gonadotropin-stimulated granulosa cell progesterone production by antagonizing insulin action. *Human Reprod.* 1999; 14:1445–1448.
- Brown C, Gaspar J, Pettit A, Lee R, Gu X, Wang H, Mannin C, Voland C, Goldring SR, Goldring MB, Livermann TA, Gravalles EM, Oettgen P. ESE-1 is a novel transcription mediator of angiopoietin-1 expression in the setting of inflammation. *J. Biol. Chem.* 2004; 279:12794–12803. [PubMed: 14715662]
- Bustin SA. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. *J. Mol. Endocrinol.* 2000; 25:169–193. [PubMed: 11013345]
- Cao R, Brakenhielm E, Wahlestedt C, Thyberg J, Cao Y. Leptin induces vascular permeability and synergistically stimulates angiogenesis with FGF-2 and VEGF. *Proc. Natl. Acad. Sci. U.S.A.* 2001; 98:6390–6395. [PubMed: 11344271]
- Cammisotto P, Bendayan M. A review on gastric leptin: the exocrine secretion of a gastric hormone. *Anat Cell Biol.* 2012; 45:1–16. [PubMed: 22536547]
- Camp JC, Wildt DE, Howard PK, Stuart LD, Chakraborty PK. Ovarian activity during normal and abnormal length estrous cycles in the goat. *Biol. Reprod.* 1983; 28:673–681. [PubMed: 6221766]
- Cardenas H, Burke KA, Bigsby RM, Pope WF, Nephew KP. Estrogen receptor β in the sheep ovary during the estrous cycle. *Biol. Reprod.* 2001; 65:128–134. [PubMed: 11420232]
- Curlewis JD, Tam SP, Lau P, Kusters DHL, Barclay JL, Anderson ST, Waters MJ. A prostaglandin F $_{2\alpha}$ analogue induces suppressors of cytokine signaling-3 expression in the corpus luteum of the pregnant rat: A potential new mechanism in luteolysis. *Endocrinology*. 2002; 143:3984–3993. [PubMed: 12239110]
- Delavaud C, Bocquier F, Chilliard Y, Keisler DH, Gertler A, Kann G. Plasma leptin determination in ruminants: effect of nutritional status and body fatness on plasma leptin concentration assessed by a specific RIA in sheep. *J. Endocrinol.* 2000; 165:519–526. [PubMed: 10810316]
- Derer S, Bauer P, Lohse S, Scheel AH, Berger S, Kellner C, Peipp M, Valerius T. Impact of epidermal growth factor receptor (EGFR) cell surface expression levels on effector mechanisms of EGFR antibodies. *J. Immunol.* 2012; 189:5230–5239. [PubMed: 23100515]
- Dozois CM, Oswald E, Gautier N, Serthelon JP, Fairbrother JM, Oswald IP. A reverse transcription-polymerase chain reaction method to analyze porcine cytokine gene expression. *Vet. Immunol. Immunopathol.* 1997; 58:287–300. [PubMed: 9436272]
- Ferrara N. Vascular endothelial growth factor: basic science and clinical progress. *Endo. Rev.* 2004; 25:581–611.
- Gabler C, Plath-Gabler A, Killian GJ, Schams D. Expression pattern of fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF) system members in bovine corpus luteum endothelial cells during treatment with FGF2, VEGF, or oestradiol. *Reprod. Domest. Anim.* 2004; 39:321–327. [PubMed: 15367264]
- Garcia MR, Amstalden M, Williams SW, Stanko RL, Morrison CD, Keisler DH, Nizielski SE, Williams GL. Serum leptin and its adipose gene expression during pubertal development, the

- estrous cycle, and different seasons in cattle. *J. Anim. Sci.* 2002; 80:2158–2167. [PubMed: 12211386]
- Gerber H-P, Dixit V, Ferrara N. Vascular endothelial growth factor induces expression of the antiapoptotic proteins Bcl-2 and A1 in vascular endothelial cells. *J. Biol. Chem.* 1998; 273:13313–13316. [PubMed: 9582377]
- Glock JL, Brumsted JR. Color flow pulsed Doppler ultrasound in diagnosing luteal phase defect. *Fertil. Steril.* 1995; 64:500–504. [PubMed: 7641901]
- Grazul-Bilska AT, Redmer DA, Killilea SD, Zheng J, Reynolds LP. Initial characterization of endothelial mitogens produced by bovine corpora lutea from the estrous cycle. *Biochem. Cell Biol.* 1993; 71:270–277. [PubMed: 7506042]
- Hazzard TM, Stouffer RL. Angiogenesis in ovarian follicular and luteal development. *Baillière's Best Pract. Res. Clin. Obstet. Gynecol.* 2000; 14:883–900.
- Kalender H, Arikan S. Size distribution of dispersed luteal cells during oestrous cycle in Angora goats. *Reprod. Domest. Anim.* 2007; 42:457–460. [PubMed: 17845599]
- Karlsson C, Lindell K, Svensson E, Bergh C, Lind P, Billig H, Carlsson LM, Carlsson B. Expression of functional leptin receptors in the human ovary. *J. Clin. Endocrinol. Metab.* 1997; 82:4144–4148. [PubMed: 9398729]
- Kawate N, Morita N, Tsuji M, Tamada H, Inaba T, Sawada T. Roles of pulsatile release of LH in the development and maintenance of corpus luteum function in the goat. *Theriogenology.* 2000; 54:1133–1143. [PubMed: 11131331]
- Kawate N, Tsuji M, Tamada H, Inaba T, Sawada T. Changes of messenger RNAs encoding vascular endothelial growth factor and its receptors during the development and maintenance of caprine corpora lutea. *Mol. Reprod. Develop.* 2003; 64:166–171.
- Kendall NR, Gutierrez CG, Scaramuzzi R.J, Baird, D.T. Webb R, Campbell BK. Direct in vivo effects of leptin on ovarian steroidogenesis in sheep. *Reproduction.* 2004; 128:757–765. [PubMed: 15579593]
- Lee CC, Chen SC, Tsai SC, Wang BW, Liu YC, Lee HM, Shyu KG. Hyperbaric oxygen induces VEGF expression through ERK, JNK, and c-Jun/AP-1 activation in human umbilical vein endothelial cells. *J. Biomed. Sci.* 2005; 22:1–14.
- Lin J, Barb CR, Kraeling RR, Rampacek GB. Developmental changes in the long form leptin receptor and related neuropeptide gene expression in the pig brain. *Biol. Reprod.* 1998; 64:1614–1618. [PubMed: 11369586]
- Loffler S, Aust G, Kohler U, Spaniel-Borowski K. Evidence of leptin expression in normal and polycystic human ovaries. *Mol. Human Reprod.* 2001; 7:1143–1149.
- Mao J, Treacy BK, Almeida FRCL, Novak S, Dixon WT, Foxcroft GR. Feed restriction and insulin treatment affect subsequent luteal function in the immediate postovulatory period in pigs: progesterone production in vitro and messenger ribonucleic acid expression for key steroidogenic enzymes. *Biol. Reprod.* 2001; 64:359–367. [PubMed: 11133694]
- Matsumoto Y, O'Gorman CW, Gonzales E, Keisler DH, Stanko RL, Garcia MR. Effects of development, the estrous cycle and 17 β -estradiol on adipocyte leptin, ER α and ER β gene expression in gilts. *J. Anim. Vet. Advan.* 2006; 5:762–770.
- Munoz-Gutierrez M, Findlay PA, Adam CL, Wax G, Campbell BK, Kendall NR, Khalid M, Forsberg M, Scaramuzzi RJ. The ovarian expression of mRNAs for aromatase, IGF-I receptor, IGF-binding protein-2, -4 and -5, leptin and leptin receptor in cycling ewes after three days of leptin infusion. *Reproduction.* 2005; 130:869–881. [PubMed: 16322546]
- Musicki B, Aten RF, Behrman HR. Inhibition of protein synthesis and hormone-sensitive steroidogenesis in response to hydrogen peroxide in rat luteal cells. *Endocrinology.* 1994; 134:588–595. [PubMed: 7507829]
- Neuvians TP, Berisha B, Schams D. Vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) expression during induced luteolysis in the bovine corpus luteum. *Mol. Reprod. Develop.* 2004; 67:389–395.
- Novak S, Almeida FRCL, Cosgrove JR, Dixon WT, Foxcroft GR. Effect of pre- and postmating nutritional manipulation on plasma progesterone, blastocyst development, and the oviductal

- environment during early pregnancy in gilts. *J. Anim. Sci.* 2003; 81:772–783. [PubMed: 12661658]
- Redmer DA, Dai Y, Li J, Charnock-Jones DS, Smith SK, Reynolds LP, Moor RM. Characterization and expression of vascular endothelial growth factor (VEGF) in the ovine corpus luteum. *J. Reprod. Fertil.* 1996; 108:157–165. [PubMed: 8958842]
- Reynolds LP, Redmer DA. Expression of the angiogenic factors, basic fibroblast growth factor and vascular endothelial growth factor, in the ovary. *Amer. Soc. Anim. Sci.* 1998; 76:1671–1681.
- Romano JE, Abella DF. Effect of service on duration of oestrus and ovulation in dairy goats. *Anim. Reprod. Sci.* 1997; 47:107–112. [PubMed: 9233510]
- Ruiz-Cortes ZT, Men T, Palin MF, Downey BR, Lacroix DA, Murphy B. Porcine leptin receptor: molecular structure and expression in the ovary. *Mol. Reprod. Develop.* 2000; 56:465–474.
- Ryan NK, Van Der Hoek KH, Robertson SA, Norman RJ. Leptin and leptin receptor expression in the rat ovary. *Endocrinology.* 2003; 144:5006–5013. [PubMed: 12959975]
- Sabbieti MG, Marchetti L, Babrielli MG, Menghi M, Materazzi S, Menghi G, Raisz LG, Hurley MM. Prostaglandins differently regulate FGF-2 and FGF receptor expression and induce nuclear translocation in osteoblasts via MAPK kinase. *Cell Tissue Res.* 2005; 319:267–278. [PubMed: 15654655]
- SAS.. SAS/STAT 9.1 User's Guide. SAS Inst. Inc.; Cary NC: 2004.
- Schams D, Amselgruber W, Einspanier R, Sinowatz F, Gospodarowicz D. Localization and tissue concentration of basic fibroblast growth factor in the bovine corpus luteum. *Endocrine.* 1994; 2:907–912.
- Seghezzi G, Patel S, Ren SJ, Gualandris A, Pintucci G, Robbins ES, Shapiro RL, Galloway AC, Rifkin DB, Mignatti P. Fibroblast growth factor-2 (FGF-2) induces vascular endothelial growth factor (VEGF) expression in the endothelial cells of forming capillaries: an autocrine mechanism contributing to angiogenesis. *J. Cell Biol.* 1998; 141:1659–1673. [PubMed: 9647657]
- Shalaby F, Rossant J, Yamaguchi TP, Gertsenstein M, Wu X-F, Breitman ML, Schuh AC. Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. *Nature.* 1995; 376:62–66. [PubMed: 7596435]
- Sierra-Honigsmann MR, Nath AK, Murakami C, Garcia-Cadena G, Papapetropoulos A, Sessa WC, Madge LA, Schechner JS, Schwabb MB, Polverini PJ, Flores-Riveros JR. Biological action of leptin as an angiogenic factor. *Science.* 1998; 281:1683–1686. [PubMed: 9733517]
- Sirotkin AV, Grossmann R. Leptin directly controls proliferation, apoptosis and secretory activity of cultured chicken ovarian cells. *Compar. Biochem. Physiol. Mol. Integ. Physiol.* 2007; 148:22–429.
- Smolinska N, Kaminski T, Siawrys G, Przala J. Leptin gene and protein expression in the ovary during the oestrous cycle and early pregnancy in pigs. *Reprod. Domest. Anim.* 2010; 45:e174–e183. [PubMed: 19930136]
- Spicer LJ, Francisco CC. The adipose obese gene product, leptin: evidence of a direct inhibitory role in ovarian function. *Endocrinology.* 1997; 138:3374–3379. [PubMed: 9231790]
- Spicer LJ, Francisco CC. Adipose obese gene product, leptin, inhibits bovine ovarian thecal cell steroidogenesis. *Biol. Reprod.* 1998; 58:207–212. [PubMed: 9472942]
- Stirling D, Waterman MR, Simpson ER. Expression of mRNA encoding basic fibroblast growth factor (bFGF) in bovine corpora lutea and cultured luteal cells. *J. Reprod. Fert.* 1991; 91:1–8.
- Stocco C, Telleria C, Gibori G. The molecular control of corpus luteum formation, function, and regression. *Endo. Rev.* 2007; 28:117–149.
- Sugino N, Kashida S, Takiguchi S, Karube A, Kato H. Expression of vascular endothelial growth factor and its receptors in the human corpus luteum during the menstrual cycle and in early pregnancy. *J. Clin. Endocrinol. Metab.* 2000; 85:3919–24. [PubMed: 11061557]
- Suri C, McClain J, Thurston G, McDonald DM, Zhou H, Oldmixon EH, Sato TN, Yancopoulos GD. Increased vascularization in mice overexpressing angiopoietin-1. *Science.* 1998; 282:468–471. [PubMed: 9774272]
- Suzuki T, Sasano H, Takaya R, Fukaya T, Yajima A, Nagura H. Cyclic changes of vasculature and vascular phenotypes in normal human ovaries. *Human Reprod.* 1998; 13:953–959.
- Tanaka J, Acosta TJ, Berisha B, Tetsuka M, Matsui M, Kobayashi S, Schams D, Miyamoto A. Relative changes in mRNA expression of angiopoietins and receptors tie in bovine corpus luteum

- during estrous cycle and prostaglandin $F_{2\alpha}$ -induced luteolysis: a possible mechanism for the initiation of luteal regression. *J. Reprod. Develop.* 2004; 50:619–626.
- Welter H, Wollenhaupt K, Einspanier R. Developmental and hormonal regulated gene expression of fibroblast growth factor 2 (FGF2) and its receptors in porcine endometrium. *J. Steroid Biochem. Mol. Biol.* 2004; 88:295–304. [PubMed: 15120423]
- Wulff C, Wilson H, Lague P, Duncan WC, Armstrong DG, Fraser HM. Angiogenesis in the human corpus luteum: localization and changes in angiopoietins, tie-2, and vascular endothelial growth factor messenger ribonucleic acid. *J. Clin. Endocrinol. Metab.* 2000; 85:4302–4309. [PubMed: 11095472]
- Yamashita T, Murakami T, Otani S, Kuwajima M, Shima K. Leptin receptor signal transduction: OBRa and OBRb of fa type. *Biochem. Biophys. Res. Comm.* 1998; 246:752–759. [PubMed: 9618284]
- Zachow RJ, Magoffin DA. Direct intraovarian effects of leptin: impairment of the synergistic action of insulin-like growth factor-I on follicle-stimulating hormone-dependent estradiol-17 beta production by rat ovarian granulosa cells. *Endocrinology.* 1997; 138:847–850. [PubMed: 9003026]
- Zhang J, Redmer DA, Reynolds LP. Vascular development and heparin-binding growth factors in the bovine corpus luteum at several stages of the estrous cycle. *Biol. Reprod.* 1993; 49:1177–1189. [PubMed: 7506941]

Fig 1.

Daily circulating concentrations of progesterone (top) and leptin (bottom) in cycling does from the early (n=12), mid (n=8), and late (n=11) luteal stage groups beginning at D0 of the estrous cycle. Progesterone concentrations increased ($P<0.001$) as luteal tissue developed and was higher ($P<0.05$) in the mid-luteal group of does compared to the late luteal group. No difference in circulating concentrations of leptin between luteal stage nor day of the estrous cycle was detected.

Fig 2.

Gene expression of VEGF, Ang1, FGF2, leptin, and ObRb in the early (n=12; D3), mid (n=8; D10), and late (n=11; D15) stage luteal tissue from mature does on D3, 10, and 15 of the estrous cycle. Total RNA was extracted and relatively quantitated using real-time PCR. Relative quantities of PCR amplicons were transformed to $\log_{10}$ and normalized with L19. ^{a,b,c}Means differ ($P < 0.02$) by luteal stage.

Fig 3.

Immunohistochemistry of FGF2, VEGF, Ang1, (Fig 3A), leptin and ObRb (Fig 3B) in CL tissue from early (n=6), mid (n=6), and late (n=6) stages of the luteal phase of the estrous cycle in does. Figure 3C and 3D are normal serum controls for secondary antibody activity and chromogen stain utilized in the immunodetection process of FGF2, VEGF, Ang1 (3C), leptin and ObRb (3D). Immunodetection of leptin in late stage tissue was scarce; therefore, the picture was photoshopped to create a contrast to distinguish the tissue from the background. Open triangles, black arrows, and open arrows represent large luteal cells, small luteal cells, and vessels, respectively. Tissue was observed at 20X magnification; scale bar, 50 μ m.

Fig 4.

Concentrations of progesterone in aspirated cultured media. Progesterone decreased in early (P 0.001; $n=6$) and mid (P 0.03; $n=6$) stage dispersed luteal tissue. Progesterone in late stage luteal cultures was attenuated and inconsistent. *Means differ from control dose (0M).

Fig 5.

FGF2, Ang1, and VEGF gene expression in early (n=6), mid (n=6), and late (n=6) stage dispersed luteal cultures incubated with leptin (0, 10^{-12} , 10^{-11} , 10^{-10} , 10^{-9} , 10^{-8} M) for 24 h. Leptin increased ($P = 0.02$) the expression of VEGF, FGF2, and Ang1 in early luteal cultures in the 10^{-11} M treatment dose. Leptin had no effect on angiogenic factor expression in mid and late stage luteal cultures. *Means differ from control dose (0M).

Table 1

Primer sequences for RT-PCR

Gene	Sense	Antisense	Annealing	Estimated Size (bp)	Gene Bank #	Reference
VEGF	5'-CCACGAAAGTGGTGAAGTTCA-3'	5'-GGAAGCTCATCTCTCCTATG-3'	52°C	257	NM_174216	
Ang1	5'-GAGCAGCCTGATCTTACATG-3'	5'-GCATTCTCTGTAGTCAAGCC-3'	52°C	482	NM_213959	
FGF2	5'-TCAAAAGGAGTGTGTGCGAAC-3'	5'-CAGGGGCCACATACCAACTG-3'	52°C	161	AJ577089	Welter et al. 2004
Ob-Rb	5'-GCCGGAAACATTGAGCATC-3'	5'-AGCATCTGCTGACCAAGCTA-3'	52°C	368	AF 092422	
Leptin	5'-ACAGAGGGTCACCGGTTTGG-3'	5'-TAGAGGGGAGGCTTCCAGGAC-3'	52°C	258	AF026976	Lin et al. 1998
L19	5'-AGCTGTGACTGTCCATTTC-3'	5'-CTTGGCAGCCTTGTCTGC-3'	52°C	299	AY158223	Cardenas et al. 2001
Cyclophilin	5'-TGCCATCCAACTCAG-3'	5'-TAAACCCACCGTCTTCTT-3'	52°C	369	AF14571	Dozoiser et al. 1997

bp = base pair