Abstract

ZORRILLA, LEAH M. Control of Luteolytic Sensitivity in the Porcine Corpus Luteum. (Under the direction of Dr. John E. Gadsby).

The porcine corpus luteum (CL) is unusual in that it does not show a luteolytic response to an exogenous dose of prostaglandin F-2α (PGF-2α) until after day 12 of an 18-21 day cycle, which is in marked contrast to other farm animal species in which luteolysis can be induced after about 6 days of the estrous cycle. The overall objective of these studies was to elucidate the mechanism by which the porcine CL acquires luteolytic sensitivity (LS=the ability to respond to PGF-2α), which we hypothesized occurs between days 7 and 13 of the estrous cycle, and about which little is known. We examined the temporal patterns of expression and cellular localization of the endothelin (ET)-1 system, protein kinase (PK)C isoforms, as well as genes associated with apoptosis at different stages of the estrous cycle to determine whether changes in expression correlated with the development of the acquisition of LS (i.e. between days 7-13). Additionally, we developed a cell culture system to investigate whether tumor necrosis factor-α (TNF-α) was capable of sensitizing porcine luteal cells to PGF-2α in vitro, and thus whether it may play a role in development of LS in vivo.

Our studies of expression of the ET-1 system components showed that endothelin converting enzyme -1 (ECE-1) expression (protein) was increased on day 10 of the cycle, which would likely result in an increase production of active ET-1 peptide in the CL. Additionally, the results from the PKC study demonstrated that PKC ε protein increased on day 13 (compared to day 10), suggesting that this particular PKC isoform may play a role in mediating the development of LS in the porcine CL. Furthermore, we showed that apoptosis-associated genes such as TNF-receptor I (TNFRI), p53 and iNOS/eNOS were expressed early (day 7) in the estrous cycle, suggesting the possibility that they may play also role in the acquisition of LS. Our in vitro data demonstrated that TNF-α sensitized porcine luteal cells to PGF-2α in a dose dependent manner. TNF-α also dose-dependently increased the expression of Endothelin Receptor A (ET_A) and PKC isoforms βII and ε in association with PGF-2α sensitivity.

Overall, our in vivo and in vitro data are consistent with a working model in which TNF-α (from infiltrating macrophages in the early CL) acts to increase ET-1 (via increased

ECE-1) and ET_A, resulting in increased expression of PKC ε, which enables luteal cells to become sensitive to the luteolytic effects of PGF-2α.

CONTROL OF LUTEOLYTIC SENSITIVITY IN THE PORCINE CORPUS LUTEUM

by

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Dedication

For my son

Biography

Leah is the daughter of Craig and Nancy Rose and has a younger brother Jason and a younger sister Allison. She grew up in Schenectady, New York and graduated from Mohonasen High School. She then pursued her B.S. in Animal Science at the University of New Hampshire, where she met her future husband Dan. They then moved to North Carolina so Leah could pursue her doctorate in Comparative Biomedical Sciences with a particular interest in Reproductive Endocrinology. She began the program in August of 2002 under the direction of Dr. John Gadsby. In September of 2005, Leah married her college sweetheart and in July 2006 found out they were expecting their first child. Their son Zachary was born on February 25th.

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Introduction

The delay in the development of luteolytic sensitivity (LS=ability to respond to PGF-2α) in the pig has long been recognized as a major problem in reproductive management of this agriculturally important species. Porcine corpora lutea (CL) do not become sensitive to the effects of prostaglandin F-2(PGF-2) α until after day 13 of an 18-21 day cycle, and therefore PGF-2α analogs are not effective or efficient in regulating estrous cycles in swine. It has been reported that macrophages and their primary cytokine, tumor necrosis factor (TNF)-α play a role in luteolysis [1-3] in the late stage of the cycle by inhibiting steroidogenesis [4, 5], stimulating luteal PGF-2α synthesis [6-8], and activating apoptosis [9, 10]. However, recently macrophages have been identified in the early and mid CL (pig-[11], cow-[12]), which suggests that TNF-α may play a role before the onset of luteolysis. Macrophages and their capacity to produce TNF-α, have been shown to dramatically increase (four-fold) between the early (day 4-6) and mid (day 8-12) stages of the porcine luteal cycle, further increasing an additional 1-2 fold in the late luteal stage [11]. Moreover, TNF-α receptors have been characterized in the porcine CL to be present and unchanged from days 4-12 of the cycle, but increased on day 15 [13, 14]. It has also been demonstrated TNF-α receptors are present on SLC, LLC and Endothelial cells in the porcine [13] and bovine CL [9]. TNF-α's actions during this time are not clearly identified, however understanding the role that it plays and how TNF-α mediates LS may lead to the development of new approaches to regulate estrous cycles in female pigs, which would benefit the swine industry.

PGF-2α is known as the physiological luteolysin [15-17], however the detailed cellular and biochemical events associated with luteolysis remain largely uncharacterized [18], particularly in the pig. In many species, including the pig, PGF-2α is produced by the uterus and acts on the CL to cause functional (decreasing steroidogenesis) and structural (regression of the CL) luteolysis [19]. PGF-2α is also produced intra-luteally, however this only occurs in CLs that show LS (i.e. PGF-2α responsive, after day 13 in the pig) [20, 21]. PGF-2α acts on its G protein-coupled receptor (FP receptor) present on LLCs (predominantly) and SLCs (which possess a lower affinity PGF-2α binding site [19]. The data on whether endothelial cells also possess PGF-2α receptors remains controversial [16, 22, 23]. The lack of sensitivity to PGF-2α in the porcine CL before

D13 is believed not to be due to the lack of FP receptors, as they are present and apparently function as early as Day 5; it is suggested that the lack of LS may to be attributable to the lack of downstream signaling molecules such as PKC [21].

Endothelin-1 (ET-1) has been shown to enhance PGF-2α production in the human CL [24], and there is evidence in the cow [25-29] and sheep [30], which suggests that ET-1 interacts with PGF-2α in the control of luteolysis. Endothelin-1 (ET-1) was originally isolated from porcine aortic endothelial cells [31], and is a 21-amino acid peptide when active [32] that exerts is effects by interacting with endothelin receptor A (ET_A) or endothelin receptor B (ET_B), which are both G-coupled transmembrane receptors [33, 34] and are expressed in the porcine CL [35, 36]. In the CL, the ET_A antagonist BQ123 blocks the PGF-2α induced inhibition of progesterone production, suggesting that ET_A plays a critical role in PGF-2α mediated luteolysis [26]. Hinckley and Milvae (2001) showed a sub-luteolytic dose of PGF-2α is effective in decreasing progesterone following ET-1 pretreatment [30]. This data strongly supports ET-1 mediating PGF-2α-induced luteolysis [30]. In addition, this study showed that an injection of ET-1 at the mid luteal stage decreased plasma progesterone concentrations, and a luteolytic dose of PGF-2α stimulated gene expression of ET-1, showing a synergism of ET-1 and PGF-2α. They also demonstrated that an injection with an ET_A antagonist (BQ123) at the mid-luteal phase in the ewe diminished the luteolytic effect of PGF-2α, suggesting a role for ET_A receptor.

It has been suggested that the lack of sensitivity of the porcine CL to PGF-2α before D13 is due to a deficiency in protein kinase C (PKC) signaling [21]. PKCs are a family of serine/threonine kinases that play a wide variety of roles in controlling cellular function [37]. It has been shown in the bovine and ovine CL that PGF-2α actions are mediated by PKC signaling [38, 39]. Sen and coworkers (2004) recently showed that PKC isoform expression in the bovine CL was stage-dependant, and more specifically that the *novel* (calcium independent) isoforms, particularly PKC ε, increased 11-fold after acquisition of LS [40]. Additionally, these authors reported that PKC ε specifically plays a mediatory role in PGF-2α induced inhibition of steroidogenesis in luteal cells, suggesting that this PKC isoform may be directly linked to LS [41].

In addition to inducing decreased progesterone production (i.e. functional regression), PGF-2α also leads to structural regression which is associated with apoptosis [42-46], and it has been suggested that TNF-α and Fas are important mediators of PGF-2α-induced luteolysis [5, 47, 48]. More recently it has been suggested that PGF-2α induces luteolysis, *in vivo,* in part by increasing cytokines such as FasL, which may play a role in activating Caspase-3 driven apoptosis (see below) [49]. Treatment of PGF-2α has been associated with the onset of apoptosis in the bovine CL [18, 46]. Apoptosis, or programmed cell death, is characterized by nuclear and cytoplasmic condensation, internucleosomal DNA fragmentation [50] and the formation of apoptotic bodies [51]. Luteal cell apoptosis thought to be a key process in CL regression [42-46] and is characterized by the loss of steroidogenic potential and luteal cell death [44, 52] and is believed to be stimulated by PGF-2α [42-46], FasL [49] and TNF-α [9]. In addition to TNF-α's role in LS, TNF-α has been shown to induce apoptosis in bovine endothelial cells, after the decline in progesterone production [9].

To understand acquisition of LS in the pig, the abovementioned factors must be studied in hope of providing important information on control of LS. Therefore our studies were designed to examine TNF-α's effect on luteal cells in vitro, expression (mRNA and protein) of both the ET-1 system and PKC isoform expression throughout the estrous cycle and expression (mRNA) of apoptosis-related genes to better elucidate the mechanism in which CLs acquire a response PGF-2α, and are thus reviewed in detail below.

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Corpus Luteum (CL)

The CL was first described in 1573 when Coiter characterized the CL as cavities filled with "yellow fluid", and Marcello Malpighi later named the cavities the corpus luteum meaning "yellow bodies" [1]. De Graaf suggested that the number of CL was related to the number of fetuses, and in 1901 Fraenkel demonstrated that CL were required for maintenance of pregnancy in the rabbit [2]. Prenant then suggested that the CL was a gland of internal secretion, and in 1929 this concept was proven by Corner and Allen when an alcoholic extract from a sows CL maintained pregnancy in the rabbit [1]. Wintersteiner and Allen isolated and purified a crystal structure, and in 1934 the hormone was named 'progesterone' by Slotta, and the compound was synthesized later that year by Butenandt and Westphal [1].

The corpus luteum is a transient endocrine gland whose primary role is to produce progesterone for the maintenance of pregnancy [2]. The CL develops from residual thecal and granulosa cells after ovulation [2]. The CL growth and development is under the control of lutenizing hormone (LH), as well as other hormones such as growth hormone (GH), prolactin (PRL) and estradiol (E2) [2]. The porcine ovary normally contains between 10-25 CL in cycling sows and normal pregnancy is 114 days [3]. The cells of the mature CL contain two steriodogenic cells types, 1) large luteal cells (LLCs) which are of granulosa cell origin and contain very few LH receptors, and therefore do not respond to LH. However LLCs contain Prostaglandin F-2α (PGF-2α) receptors and are responsible for the luteolytic actions of PGF-2α, and 2) small luteal cells (SLCs) which are of theca cell origin, contain LH receptors, and therefore respond to LH stimulation by increasing progesterone production via the protein kinase A (PKA) and cAMP pathways [2]. When pregnancy occurs, the CL is required to produce progesterone to maintain a pregnant uterus. If pregnancy does not occur, the CL undergoes regression (through PGF-2α action) to allow for follicular growth and ovulation [2]. CL development occurs only after folliculogenesis and ovulation, and thus is briefly reviewed below.

Folliculogenesis

Follicular growth and maturation (folliculogenesis) is a series of sequential subcellular and molecular transformations of the follicle, including the theca, granulosa and

oocyte [4]. Follicular development begins at conception, proceeds through gestation and postnatal development and culminates in the ability to ovulate or become atretic [5]. Oogonia are transformed into primary oocytes and somatic cells adjacent to the oocyte differentiate into primordial granulosa cells resulting in the formation of primordial follicles. [6]. A novel member of the transforming growth factor beta (TGF-β) superfamily, namely growth differentiation factor 9 (GDF-9), is expressed in oocytes during early folliculogenesis and appears to be involved in early follicular formation [7]. Other growth factors (e.g. TGFβ-1, TGFβ-2) are also proposed to play an important regulation of early folliculogenesis [8]. The primordial follicles are the resting stockpile of non-growing follicles, which continuously leave the non-growing pool by conversion into primary follicles. Within the primary follicle, the oocyte is surrounded by a single layer of cuboidal granulosa cells [9] (Fig.1). When a follicle is released, it continues to grow until ovulation or until the follicle degenerates (atresia). Multiplication of granulosa cells yields a secondary follicle with 2-3 layers of cuboidal granulosa cells. At the final stage of its development, the secondary follicle appears surrounded by differentiated epitheloid cells named theca interna. The secondary follicle with its theca interna is called a preantral follicle (Fig.1). The growth of the follicle up to the preantral stage appears gonadotropin independent [9]. However the presence of theca interna cells in the growing follicle causes further growth and maturation to become completely dependent on pituitary gonadotropins.

The onset of the steroidogenic activity of the preantral follicle is marked by an accumulation of follicular fluid whose confluence forms the cavity (antrum) in the follicle [9]. The tertiary stage of growing follicle, named the antral follicle, is characterized by the presence of an antrum in multiple layers of granulosa cells, and the presence of basement membrane, which separates the granulosa cells from theca cells [9](Fig. 1). The theca externa resembles a fibrous membrane around the theca interna [9]. Antral formation and final follicle growth up to the ovulatory stage is completely gonadotropin dependent. At the end of follicular development, the follicle becomes a preovulatory or mature follicle (Fig.2).

Follicular development and ovulation are dependant upon a functional microvascular network [10]. An elaborate vascular bed is a characteristic feature of the theca interna of mature ovarian follicles [11]. The capillaries surrounding the theca layer

develops so that it can reach the granulosa layer [12], and nutrients and hormones can be supplied through diffusion to the granulosa cells [11]. The increase in blood flow to the theca cells allows for gonadotrophins and other systemic biochemical and hormonal factors for follicular development [11]. Follicular development is dependant on angiogenesis [13], and which follicular atresia is associated with a decrease in vascularization. Angiogenesis is evident in the late pre-antral stage during thecal layer acquisition [14]. The vasculature then develops to form two connected capillary networks in the theca interna and externa layers, but does not penetrate the basement membrane, and consequently the granulosa cell layer remains avascular until ovulation [15]. There is an increase in both vascular area and blood flow in the pre-ovulatory follicle during spontaneous ovulation [16]. Berisha and collegues (2000) also showed there was an increase in blood flow and volume in the CL, which was closely associated with the increased plasma progesterone concentrations [18].

Vascular endothelial growth factor (VEGF) is the principal angiogenic factor controlling follicular angiogenesis that stimulates microvascular endothelial cell proliferation and migration, as well as promotes vascular permeability [14]. Granulosa cells are the major source of VEGF in the follicles [17, 18], with mRNA expression increasing as the follicle develops [18, 19]. There is a direct accumulation of VEGF in the follicular fluid, which is likely to direct blood vessel extension towards the granulosa cell layer [14]. VEGF is also expressed in the theca cell layer [17, 18]. VEGF mediates its action through two tyrosine kinase receptors named VEGF receptor (VEGFR) -1 and -2 [20]. VEGFR-1 and VEGFR-2 are localized to endothelial cells of the theca cell layer indicating that VEGF acts in a paracrine manner [19], and during selection, follicular concentrations of VEGF may play an important role in determining which follicle becomes dominant. The exact regulation of VEGF in the follicle is not known, however studies have shown that gonadotrophins can stimulate granulosa cell production of VEGF, both in vivo and in vitro [17, 18, 21], suggesting that inadequate gonadotrophin support could lead to a decreased follicular vascularization and impaired function [14]. Fibroblast growth factor (FGF)-2 is another endothelial cell mitogen found in the ovary. FGF-2 is located on the endothelial cells and pericytes of the theca cell layer with higher levels of expression in more developed follicles [18].

Hormones Controlling Follicular Growth

The mechanisms and factors involved in the development of preantral follicles and the role of gonadotrophins in the growth of preantral follicles are not clear [22]. The gonadotrophins follicle stimulating hormone (FSH) and LH play an important role in the preovulatory follicle, and metabolic hormones such as insulin, insulin-like growth factors (IGF's) and leptin are also involved either by directly influencing hypothalamic-pituitary function [23] or by affecting gonadotrophin action at the ovary [24]. Hunter and colleagues (1992) suggested that gonadotropins are not required for the recruitment of follicles in pigs, but lack of FSH may be associated with follicular atresia, and therefore, local paracrine and autocrine factors (IGF, epidermal growth factor, transforming growth factor -α) may be predominant regulators of early follicle growth [25].

Gonadotrophins

Several studies carried out in hypophysectomized rats showed that preantral follicular development in gonadotropin deprived animals was interrupted, and some follicles became atretic [26, 27]. It was suggested that growth and maturation of the follicle becomes completely dependent on pituitary gonadotrophin in the rodent [27]. In contrast, it appears that the initiation of porcine preantral follicle growth is not dependent on pituitary gonadotropins [9]. Oxender and co-workers (1979) showed that exogenous gonadotrophins were unable to stimulate follicular growth in 30-day old gilts whose ovaries contained mostly primordial and primary follicles [28]. However, the same treatment induced ovulation in pigs on day 70 of age, when a few antral follicles were evident [28]. With *in vitro* studies, Morbeck and collegues (1993) showed that FSH had no effect on proliferation of porcine granulosa cells (GCs) from primary follicles, stimulated proliferation of GC's from secondary follicles, but failed to stimulate progesterone secretion from granulosa cells obtained from both primary and secondary follicles [31]. It was suggested that species differences exist in the role of gonadotrophins during early follicle development [29].

The ability of gonadotrophins to act on ovarian follicles depends on the expression of gonadotrophin receptors on ovarian cells [24]. FSH is the main hormone controlling follicular growth in cattle, sheep and pigs, and its secretion is controlled by the main secretory products of large dominant follicles, E2 and inhibin [14]. At the start of the follicular phase in the pig, there is a proliferating pool of approximately 50 follicles from

which the ovulatory population is selected during the follicular phase [9]. The emergence of follicular waves is preceded by a transient increase in FSH [30, 31]. Around the time of follicular selection granulosa cells acquire LH receptors that are required for further development [31, 32]. The recruitment phase is followed by a decline in FSH due to the negative feedback by E2 and inhibin from the recruited follicles to below the threshold for further follicular selection, when the dominant follicle can use LH to support its growth [14]. In preovulatory follicles, the LH receptor is expressed in both granulosa and theca cell layers [33, 34], and the amount of LH receptor mRNA increases as the follicles develop from 2mm to 6mm in diameter. In 8 mm follicles, after the LH surge, LH receptor mRNA is not expressed [34]. This is in opposition to FSH receptor mRNA which is reduced in 4 mm follicles and absent in follicles greater than 4 mm [36]. The preovulatory follicle releases itself from its dependence on FSH, and small follicles on the ovary continue to express FSH receptors, but FSH concentrations are reduced as larger follicles suppress FSH secretion to basal values [36]. In gilts treated with FSH, Gutherie and colleagues (1988) found that the FSH-treated gilts had a higher number of 3-6 mm follicles, but the number of 7-8mm follicles and plasma concentrations of estradiol were not increased when compared to controls [35]. Esbenshade and coworkers (1990) reviewed studies that showed purified pig LH could induce estrous in sows and gilts, but only when higher doses were administered [36]. Lower doses of LH induced estrus when LH and FSH were used, therefore showing that follicles are dependant on both FSH and LH for follicular growth, and that the follicles switch from being FSH- to LH-dependant between the 2-4 mm stages [36].

Insulin-like Growth Factors (IGFs)

IGF-I and IGF-II are ligands related to insulin [24], and IGF-I, IGF-II, their receptors and IGF-binding proteins (IGFBPs) regulate growth, differentiation and apoptosis of ovarian cells [37-39] in the follicle or can originate from endocrine sources [24]. Normally, IGFs are localized to specific cell layers within the developing follicle and the IGF system corresponds to the location of LH and FSH receptors in the follicle [24]. Granulosa and theca cells express insulin receptors as well as type I and type II IGF receptors [34, 40], and therefore insulin, IGF-I and IGF-II all act directly on ovarian cells. The pig follicle expresses IGF-I on the granulosa cell layer and IGF-II on the thecal cell

layer, and when acting alone both cause growth, differentiation and survival of ovarian cells [37-39]. When the IGFs and insulin act synergistically with gonadotrophins, the number of gonadotrophin receptors increases, as well as an increase in the activity of the gonadotrophin receptor second messenger systems [24]. Concurrently, gonadotrophins increase the type I IGF receptor expression and may increase IGF-I synthesis in granulosa cells [27]. Both insulin and IGFs play a role in follicular development during periods of underfeeding or weight loss because undernutrition causes a decrease in plasma concentrations of IGF-I and insulin, which causes a decrease in the responsiveness of the ovary to gonadotropins and ultimately leads to a decrease in follicular growth [40, 41]. The co-localization of these genes indicates coordination of gonadotrophin and IGF action with in the ovary that may control growth, differentiation and steriodogenesis of theca and granulosa cells [24].

IGF-I and its receptor mRNA are expressed constitutively throughout follicular development, and therefore is not suggested to be involved with follicular growth [34, 37, 39]. However this does not mean it is not necessary for growth and development, and is suggested that IGF is important for all stages of follicular growth [24]. IGF-II mRNA expression is highest in the theca layer of 6mm follicles when concentrations of estradiol are highest in the follicular fluid [34]. The increase in IGF-II and increase in steriodogenic enzymes at this time, is associated with an increase in LH receptor in both granulosa and theca cells of 6mm follicles [34], and suggests that IGF-II is involved in ovulation and lutenization. In addition, IGFBPs play an important role in the availability of IGFs. The IGFBP's mediate the interactions of IGF-I and IGF-II with their receptors, and may also regulate cell growth and differentiation by titrating the trophic effects of IGF [37, 39]. IGFBP-2 mRNA was examined in the sow ovary and was decreased in large preovulatory follicles, and IGF-I and IGF-II action in large follicles may be enhanced when IGFBP-2 are low [42]. IGFBP-4 mRNA concentrations were similar in 2, 4, and 6 mm follicles and was increased in both granulosa and theca cells of 8mm follicles. LH also stimulates IGFBP-4 mRNA in vitro [43], and is suggested to be involved in luteinization and/or ovulation [24].

Transforming growth factors (TGFs)

TGFs are also suggested to play roles in follicular development. Interactions between theca cells and granulosa cells influence the progression of follicle development.

Theca cells produce TGF-α [44] and TGF-β [45] to regulate granulosa cell growth and function. Using RT-PCR and immunolocalization studies, Singh and Armstrong (1995) demonstrated that the expression of TGF-α mRNA and protein were in various components of medium sized porcine ovarian follicles, and were predominantly localized in cumulus and granulosa cells of all stages of follicular development [45]. In contrast, TGF-α signals in theca cells were weakly detectable, and no TGF-α mRNA was detected in the oocyte [45].

Evidence from *in vitro* studies support that TGFs also modulate ovarian steroidogenesis in the pig. TGF-α was shown to be a potent dose-dependent stimulator of IGF-I production [46], and TGF-α stimulated basal estrogen production in prepubertal porcine ovarian granulosa and theca cells in vitro [47]. In granulosa cells, TGF-α is more potent than FSH in stimulating estrogen production [49]. LH does not stimulate estrogen production in prepubertal porcine theca cells but rather attenuates that stimulated by TGF-α [49]. Treatment with genistein (an inhibitor of protein tyrosine kinase) attenuated TGF-α-induced estrogen biosynthesis, thereby suggesting that the action of TGF-α is mediated through protein tyrosine kinase [49].

Both porcine theca and granulosa cells secrete TGF-β, primarily TGF-β1, in vitro[45]. Using in situ hybridization and northern blot analysis, it was shown that expression of TGF-β1 mRNA was localized to both theca and granulosa cells. In contrast, immunoprecipitation with TGF-β antibody detected TGF-β peptides only from theca cells, and not from granulosa cells [47]. Since both theca and granulosa cells produce the TGF-β 1 mRNA, but only theca cells translate and secrete the actual growth factor, May and collegues (1996) suggested that the theca is likely the source of TGF-β 1 during follicle development [47]. TGF-β alone had no apparent influence on granulosa cell growth, but it inhibited EGF-stimulated granulosa cell proliferation in a dose-dependent manner [48, 49].

Epidermal growth factor (EGF)

EGF primarily is known as a potent mitogen for cultured granulosa cells of different species, including pigs [50]. It was shown that EGF consistently stimulated DNA synthesis in porcine granulosa cells and stimulated granulosa cell proliferation in a culture of platelet-poor plasma-derived serum [51]. Although IGF-I and TGFβ failed to induce the aforementioned effects, both IGF-I and TGFβ enhanced EGF-stimulated DNA synthesis

and proliferation of porcine granulosa cells [53]. Since EGF treatment increases FSH receptor number, EGF-mediated granulosa cell proliferation was associated with the expression of FSH-binding sites [52]. While EGF alone did not stimulate theca cell proliferation, it markedly enhanced the proliferative action of PDGF [53]. EGF increased DNA in granulosa cell cultures from primary or secondary follicles, while FSH increased DNA in granulosa cell cultures from secondary but not from primary follicles [29]. The results of *in vitro* studies suggested that the porcine ovary synthesizes EGF [54, 55].

Tumor necrosis factor-alpha (TNF-α)

TNF-α has pleiotropic effects on cultured porcine granulosa cells [56]. The specific, high affinity, low capacity binding sites for TNF-α were evident in untransformed porcine granulosa cells [58] Treatment with the combination of insulin and FSH increased the number of TNF-α binding sites [58]. In addition, TNF-α induced expression of its own receptor (TNF receptor type I), but downregulated the expression of progesterone receptor (PR) and TGFβ receptor type II in both unstimulated and insulin/forskolin-induced luteinized cells [57]. Since both PR and TGFβ receptor type II are markers of luteinization, Prange- Kiel and coworkers (2001) suggested that TNF-α exerts an inhibitory influence on luteinization [59]. One experiment showed that TNF-α inhibits steroidogenic activity of both granulosa and theca cells [56]. TNF-α suppressed insulin- and insulin- plus FSH-stimulated cAMP accumulation and progesterone secretion, but did not affect basal progesterone accumulation or DNA content [58]. Likewise, TNF-α did not affect basal accumulation of progesterone by theca cells, but caused a marked dosedependent inhibition of LH or LH+insulin-stimulated progesterone accumulation by theca cells [58]. An outstanding effect of TNF-α on the blockade of FSH-stimulated progesterone biosynthesis in granulosa cells is that TNF-α downregulates specific mRNA encoding the cytochrome P450 side chain cleavage (P450scc) enzyme [56]. Likewise, TNF-α inhibited IGF-I-stimulated P450scc mRNA concentrations by preventing IGF-I-stimulated binding of P2, a second transcription factor, to the IGF response element (IGFRE) and thus inhibits the IGF-mediated transcriptional activity of the P450scc gene [59].

Follicle Selection

Follicular growth and atresia occur continuously during the luteal phase yielding a proliferating pool of approximately 50 follicles of 2-6 mm in diameter, and from this proliferating pool, the development of a specific number of ovulatory follicles involves 2 processes, namely recruitment and selection [25]. Recruitment is characterized by rapid growth (or atresia) of the follicle from the proliferating pool combined with an inhibition of growth of additional follicles into the proliferating pool [25]. In the pig, recruitment starts between days 14 and 16 of the estrous cycle and produces a cohort of growing follicles from which preovulatory follicles are selected [25]. The number of recruited follicles usually is more than the number of preovulatory follicles selected for ovulation [25]. It is likely that all recruited follicles grow to a certain point (usually just before the final stages of follicular development), and they are unable to survive without survival signals (selection) at this critical time [25].

In pigs, Guthrie and colleagues (1993) reported that a decrease in plasma FSH and an increase in estradiol concentration are associated with an increased number of large follicles (> 5 mm) [62]. In addition, the number of small (1-2 mm) and medium (3-5 mm) follicles decreases at the time (day 18) when follicles first become estrogen-active (≥ 100 ng estradiol/ml of follicular fluid) [62]. The proportion of atretic small and medium folliclesincreased to 100 and 59 % respectively, around day 20 of the estrous cycle when serum estradiol reached its peak and FSH remained low [62]. Furthermore, FSH sensitive-adenylate cyclase activity (cAMP determination) was observed only in granulosa cells obtained from healthy small and medium but not large follicles. In contrast, LH sensitive-adenylate cyclase was found only in granulosa cells obtained from large follicles as early as day 16 and throughout preovulatory maturation. Overall, the authors concluded that selection of ovulatory follicles begins by day 18 of the porcine estrous cycle, and decreased secretion of FSH causes loss and atresia of non-ovulatory follicles [60].

Ovulation and Luteinization

Ovulation occurs as a result of the LH surge and other local factors including steroids, prostaglandins and peptides in a time-dependant manner [11]. The LH surge triggers structural and biochemical changes that causes the rupture of the graafian follicle,

causing oocyte expulsion and development of the CL from the remaining tissue [13]. Gonadotrophins produce factors such as hyaluronic acid to cause cumulus expansion [61]. The cumulus and oocyte separate from the granulosa cells. Gonadotropins induce the follicular production of local vasoactive factors, such as PGE2, histamine, and angiogenic factors, to increase blood flow to the follicle and vascular permeability to produce edema in tissue surrounding follicle [11]. Gonadotropins and local mediators activated by gonadotropins, trigger increase protein synthesis and cellular differentiation within follicle, and steroid biosynthesis shifts to production of progesterone. Progesterone triggers production of plasminogen activator from the granulosa and collagenase from the theca interna [62]. Concurrently, gonadotropins increase production of PGE2 and PGF2α, which triggers production of plasminogen activator from the granulosa cells [63]. Plasminogen is converted to plasmin by plasminogen activator [64]. Plasmin then converts inactive collagenase to the active form which weakens the collagen of the tunica albuginea and thecal layer. Granulosa cells also increase the production of follicular fluid. The stigma begins to push out and weaken so that the follicle ruptures without an increase in follicular pressure. Finally, PGF2α stimulates smooth muscle to contract and force the ruptured follicle to empty [64].

The preovulatory LH surge is the common stimulus for lutenization [65]. Corner (1919) established the sequence of lutenization in the pig, confirming that granulosa cells were an integral component of the CL [67]. Following the expulsion of the ovum, the granulosa layer is thrown into folds about the follicular antrum which contains blood and follicular fluid [67]. Theca cells develop in the developing CL by invasion of connective and vascular tissue at the folds [67]. The vascular components soon reach the cavity of the collapsed follicle and eventually extensive capillary branching completes vascularization of the CL [67]. This capillary network eventually carries theca cells that become dispersed throughout the CL. The granulosa cells undergo massive hypertrophy with eightfold volume increases relative to their preovulatory size [67]. Two cell populations with different functional characteristics are represented in the pig [66, 67] .

Lutenization and Cell Division

Lutenization is the final phase in differentiation of theca and granulosa cells, a process that begins with formation of the primordial follicle before birth [67]. Lutenization

represents exit from the cell cycle and terminal differentiation of the granulosa cell descendents in the CL [67]. Granulosa cells retain their capacity to proliferate in vitro [68] even after they have undergone lutenization [69]. Corner reported the occurrence of mitosis in the theca-like cells in the porcine CL (1919), and Niswender (1985) suggested mitosis occurred in SLCs in the sheep CL [70]. Much of the growth of the pig CL is the result of cellular hypertrophy [71] and little information is available on the contribution of cell proliferation [67]. Corner (1919) confirmed that cell division was occurring by observation of the mitotic spindle in the theca cells of the periovulatory follicle and in follicles undergoing lutenization [1]. Ricke and collegues (1999) showed extensive mitosis occurs in the CL during the first four days after ovulation, and about 25-40% of the mitotic cells are derived from the theca or granulosa, whereas other mitotic cells represent proliferation of vascular elements [71]. Co-localization of mitotic and steriodogenic enzyme signals indicated that luteal steriodogenesis and cell division occurs in proliferating cells, particularly in the early CL [73].

Differentiation of theca and granulosa cells

Granulosa and theca cells undergo functional differentiation during lutenization [67]. In most species, the theca responds to LH stimulation by synthesis of androgens, which diffuse across the basement membrane to the granulosa compartment and are aromatized to estrogens, under the influence of FSH [67]. In pigs, 3β-hydroxysteriod dehydrogenase (3β-HSD) which converts pregnenolone to progesterone and is found in the theca of the growing follicle [72]. Pig follicles are different from other species because the theca cells express aromatase and produce estrogens that complement the production of granulosa cells [73]. P450aromatase expression in the pig CL persists [74], and both LLCs and SLCs maintain their capability to secrete estrogen [66]. Luteinization in pigs involves alteration of the pattern of steroid synthesis in favor of progesterone, particularly by the granulosa cells [75], as indicated by the early acquisition of the expression of P450scc, which converts cholesterol to pregnenolone, and of 3β-HSD [74]. This change is accompanied by a large increase in total steroid output, requiring an increase in cholesterol, the substrate for steroid synthesis [67]. In the pig, luteinization is characterized by coordinated upregulation of cholesterol synthesis, importation and intracellular trafficking genes, including the LDL receptor and sterol carrier protein 2 [75], steriodogenic acute

regulatory protein (StAR), p450scc [69, 76, 77], and Niemann-Pick C1 protein [77]. Expression of StAR is a key event in granulosa cell remodeling and the factors that control this transition are essential to regulating lutenization [67].

The expression of gonadotrophin receptors changes as a follicle becomes the CL [67], and the FSH receptors present on granulosa cells of the follicle are lost during lutenization [78]. LH receptors are downregulated initially, relative to granulosa cells from periovulatory follicles, followed by recovery in the number of receptors as the cycle progresses [79]. Data is conflicting on whether or not both SLC and LLC retain LH receptors. Immunolocalization studies show LH is restricted to theca descendants (SLCs) [74], however in situ analyses indicates that both LLC and SLC contain LH receptor mRNA [33]. Kaminski and colleagues (2000) confirmed these findings showing by RT-PCR and immunolocalization that LH receptor and protein were localized to both LLC and SLC [80].

Markers of Lutenization

The theca of the follicle produces androgens that are aromatized to estrogens by cytochrome P450 aromatase in granulosa cells [81]. The loss of androgen production and aromatization mark luteinzation in some species [82]. The P450 17α enzyme complex is rate limiting and necessary for androgen formation in the follicle [83] and P450 17α is present in the theca, but not the granulosa of developing follicles in the pig [81]. P450 17α is dispersed throughout the vascular tracts throughout the mature porcine CL [83]. The disappearance of aromatase, the rate-limiting enzyme in estrogen synthesis, by both theca and granulosa compartments of the follicle [73] and by the mature CL [66], and an in vitro lutenization of pig granulosa cells engenders rapid loss of aromatase expression [69]. Changes in other steriodogenic enzymes mark lutenization, P450scc is acquired within 7 hours of the ovulatory stimulus in the rat CL, and its expression increases during in vitro lutenization of pig granulosa cells [52, 69, 83]. StAR imports cholesterol into the mitochondria and is essential for steriodogenesis [84]. It is present in theca but not granulosa cells of follicles and is acquired during in vitro and in vivo lutenization of granulosa cells in the pig [69]. StAR expression is an important marker of lutenization [67].

Cholesterol Trafficking and Luteinization

The principle source of substrate for progesterone is lipoprotein-borne cholesterol [88, 89]. Lutenization causes upregulation of the cholesterol-trafficking pathway including lipoprotein receptors, cholesterol transport proteins and the enzymes that catalyze cholesterol synthesis [89]. Dramatic increases in expression of the porcine low density lipoprotein (LDL) receptor in the follicle, after ovulatory stimulus and persisting through the luteal phase [75]. Expression of StAR has also been shown to undergo lutenization-dependent upregulation in the pig [69, 75, 76]. Following uptake LDL-derived cholesterol, it briefly resides in endosomes or lysosomes before it undergoes dispersion to membranes, esterification or metabolism [90].

Intracellular Signaling and Luteinization

LH stimulates cAMP formation and activation of the PKA pathway, and it has been suggested that cAMP serves as the initial signal for luteinization of granulosa cells, which then become refractory to cAMP stimulation [65]. Constitutive expression of P450scc and cAMP insensitivity of P450scc during the luteal phase occurs in the pig [91].

Activation of the protein kinase C (PKC) pathway by phorbol ester downregulates induction of aromatase expression in rat granulosa cells and with LH, induces P450scc, progesterone production, and morphological markers of lutenization [89]. Inhibition of PKC abolishes lutenization in rat granulosa cells [92], in contrast induction of PKC activity both prevents [93] and reverses [69] structural luteinization of porcine granulosa cells. It also eliminates cAMP-induced StAR expression in luteinized pig granulosa cells [69].

Ligand induction and maintenance of Lutenization

Lutenization and maintenance of the CL depends on members of three protein families: gonadotrophins, cytokine-prolactin family and the insulin and insulin-like growth factors [94]. The stimulus for ovulation is the preovulatory release of LH from the pituitary, as in other mammals [94]. In the pig, gonadotrophins provoke expression of inducible form of cyclo-oxygnease enzyme (COX-2) by the granulosa cells that catalyzes the first rate-limiting step in conversion of arachidonic acid to prostaglandins [95]. The sequence of COX-2 expression after ovulation has not been investigated in pigs, but is present and inducible in the CL at days 8 and 17 of the estrous cycle [96]. A second catalytic route for arachidonic acid is via the lipoxygenase enzymes, resulting in

hydroxyeicosinoids [94]. The LH surge is the stimulus for initiation of other events in lutenization, specifically changes in steriodogenesis. Hypophysectomy in pigs shows that the pituitary is necessary for luteal support throughout gestation and LH is essential in the early CL, and that prolactin plays a role later in pregnancy [97]. In pig granulosa cells, lutenization occurs in vitro only when serum is present in the medium [98], and insulin and IGFs also contribute to luteninzation and CL maintenance during the luteal phase [94]. Plasma membranes of pig luteal cells have receptors or IGF-I and IGFBPs are expressed differentially in the pig CL throughout the luteal phase [99]. Insulin and IGFs are essential for the steriodogenic changes that characterize luteal formation in vitro [69, 100]. An overall model of lutenization is demonstrated in Figure 3.

Domestic Animal Cycle

Ovulation and CL formation are under gonadotrophic control and repetitive cycles occur with a relatively short follicular phase and a relatively long luteal phase [1]. During the follicular phase, the rise of estrogen from the preovulatory follicle induces a short period of sexual receptivity (estrus) as well as inducing the preovulatory surge of LH [1]. Therefore mating is synchronized with ovulation. In the luteal phase, the CL undergoes luteolysis which terminates the luteal phase and a new ovarian cycle is initiated [1]. In event of mating the uterine luteolytic signal is subverted and the CL continues to secret progesterone for the maintenance of pregnancy [1]. In the pig, the CL is the sole source of progesterone throughout pregnancy [1].

Corpus luteum (CL)

The transitory organ forms from follicles as a consequence of ovulation and secretes the progesterone necessary for creating a uterine environment hospitable for survival of embryos and fetuses [94]. Pigs are different from other ruminants because the CL is required for the entire 16 weeks of gestation [94].

Formation of the CL

The period from the onset of the LH surge to first ovulation was estimated as a mean of 44 hours [101], and in eCG-hCG-treated gilts the mean interval ranges from 34-48 hours [102]. During the period between the ovulatory stimulus and ovum expulsion, the

follicle undergoes changes, including hyperemia and theca cell hypertrophy [94]. There is rearrangement of the layers of the granulosa cells to looser associations, characterized by reduced intercellular contract, and vascular remodeling begins before ovulation and invagination occurs at the sites of major vein and artery complexes [94]. Within 24 hours after ovulation, vascular and associated thecal tissues have not yet breached the follicle wall, but the regions of invagination are still substantially deeper and wider then in preovulatory follicles (Corner 1919, as reported by [1]). The follicle wall then decomposes and invasion of the vascular elements occurs into the granulosa cell compartment. Corner (1919) also described the invasion of the follicle compartment by the vascular elements and theca, and that the thecal cells were dispersed throughout the luteal parenchyma where they either dissociated into single cells, or into small groups of two or three cells [1].

Normal development of the CL depends on vascularization which allows for progesterone production, growth factors angiogenic factors and vasoactive substances [11]. The cells of the follicle undergo dramatic changes to become the CL, and the rate of cell growth associated with CL development from the follicles has been compared to that of tumors [103, 104]. CL development involves neo-vascularization, and once formed CL represent one of the most highly vascularized tissues in the body [105]. Following the expulsion of the mature ovum, the follicle wall collapses, and under the influence of angiogenic and mitogenic factors (FGF, PDGF, IGF-I, VEGF) capillaries invade the developing CL [1]. The angiogenic process within the CL reaches a peak 2-3 days after ovulation [106], and the majority of the steriodogenic cells of the mature CL are in contact with one or more capillaries [106].

LH is the primary luteotropin, promoting progesterone secretion and growth of the CL [1], but described below, LH only acts on the SLCs; large cell steroidogenesis under basal conditions is high, suggesting a degree of autonomy of this cell type from hormonal control, although as described below, hormones/factors such as PGE and IGF-1 have been shown to stimulate steroidogenesis by this cell type [1].

Steriodogenic Luteal Cells

The granulosa cells of the follicle undergo a massive hypertrophy in which their volume increases 30 fold compared to preovulatory size [65]. Granulosa cells develop into the steroid-producing, LLCs (>20μm) [107], which respond to the luteolytic effects of

PGF-2α [108-110]. The theca cells of the preovulatory follicle differentiate into steroid producing, SLCs (~10-20μm), [65] and respond to LH [108-110]. SLC and LLC are regulated differently; thus SLC have LH receptors and respond to LH with increased progesterone secretion, whereas LLC have very few LH receptors and do not show increased steroidogenesis in response to LH [111]. LLC do contain receptors for PGF-2α, PGE and insulin-like growth factor-1 (IGF-1), and can therefore be regulated by these factors [67, 110, 112-115]. In the bovine CL, morphologic analysis demonstrated that there is a fluctuation in the ratio of small and large cells, suggested to indicate that small cells may differentiate into LLCs [116].

Non-steroidogenic luteal cell types

In addition to the steroidogenic cells, the other luteal cell types are endothelial cells (which comprise approximately 50% of the CL), fibroblasts [106, 117] and immune cells, including macrophages, T lymphocytes and monocytes [103, 118-120].

Endothelial Cells

Following ovulation, as discussed above, neovascularization occurs during CL formation, at a rate which has been compared to tumor formation [104], as new vessel growth is required for the formation and function of the CL. The CL is one of the most highly vascular tissues in the body [121], and ECs represent over 50% of the total cells of the CL [106, 117]. Endothelial cell proliferation has been examined in many species including the pig [71], and it has been demonstrated that endothelial cell proliferation (in the bovine CL) is highest during formation of the CL, then decreases at mid cycle and remains low during regression [104]. The vasculature of the CL is essential for CL development, since inhibition of angiogenesis during CL formation is associated with inadequate luteal function [106, 122-125].

ECs have been isolated from the pig [126], as well as many other species, and the specific endothelial cell types found in the CL have provided important clues to understand the presence and role of these cells in CL function [104]. These specific and distinguishable EC sub-types have some common functional and morphological characteristics, but endothelial cells from small and large blood vessels from either the same or different organs differ in morphology, surface molecule expression and function [104].

For example in the bovine CL, five distinct subtypes of endothelial cells have been characterized by the above characteristics [127]. All five EC types (named Types 1-5) express von Willibrand Factor (Factor VIII); however staining varies among the cell types, some showing distinct granular patterns, others show a diffuse perinuclear pattern [104]. Each EC type shows a distinct and unique pattern of microtubules, actin and vimentin filaments, and fibronectin matrix [128-131]. It is interesting to note that type 5 EC of the bovine CL are thought to represent immature granulosa cells [132], as they show similar morphological features to granulosa cells in vitro, as well as exhibiting many different characteristics than the other ECs [104]. When compared, type 5 cells show morphological characteristics of granulosa cells (isolated from small antral follicles), produce small quantities of progesterone, and are unresponsive to LH treatment, and the authors suggest these cells may represent stem cells for the renewal of luteal cells [132].

One important product of endothelial cells is Endothelin-1 (ET-1) which is thought to mediate luteolysis in the CL in many species and is discussed below (rabbit- [133]; cow- [134-138]; sheep- [139]; pig- [115, 140]).

Immune cells

White blood cells were first described in the bovine CL in 1968 by Lobel and Levy [141]. They observed that lymphocytes were present in the connective tissue surrounding the luteal vasculature in day 14 CL [141]. On days 15-17 the lymphocytes migrated in among the luteal cells, and by day 19 macrophages were also present [141]. The macrophages were later shown to be involved in phagocytosis of cell remnants [142]. Subsequently both lymphocytes and macrophages were shown to be present throughout the luteal phase [143-145]. In addition to the above mentioned cell types, there is evidence in several species to show that macrophages infiltrate the CL [145-149], however most studies suggest infiltration occurs at the time of luteolysis [145, 148, 150, 151]. In the rabbit, withdrawal of estradiol results in an increase in macrophages [152] and in rats macrophage infiltration is associated with pro-estrous prolactin surge [151, 153, 154]. Recruitment of macrophages in rats, rabbits and cows is probably mediated by monocyte chemoattractant protein-1 (MCP-1) [120, 154-157]. Intercellular adhesion molecule -1 (ICAM-1) expression has also been shown to increase at the time of prolactin-induced luteal regression in the rat CL [158], at the same time as macrophage and monocyte recruitment, suggesting that there

are two molecules to attract, activate and promote adhesion of immune cells in the tissue [159]. There is also evidence of T lymphocytes migrating and proliferating in luteal tissue at the time of luteolysis [151]. In the pig CL, numbers of macrophages increase four-fold during the early (days 4-6) and mid (days 8-12) stages of the porcine cycle [147]. Macrophages are the primary source TNF-α and have been suggested to play a key role in inducing structural luteolysis at the end of the cycle [159].

Function of the CL

Steriodogenesis

The substrate for steroidogenesis is cholesterol, which is normally produced in the liver and transported to steriodogenic tissues such as the follicle, corpus luteum, adrenal cortex and testis in the form of lipoproteins [2]. Low-density lipoprotein (LDL) and high-density lipoprotein (HDL) are the most common sources of cholesterol for the corpus luteum [160, 161]. The uptake of LDL occurs by receptor-mediated endocytosis [162], which efficiently carries ~2,500 cholesterol molecules [2]. Once internalized the endosomes combine with lysosomes where LDL dissociates from its receptor making free cholesterol available to the cell [2]. The LDL receptor is then degraded or recycled [163]. Uptake of HDL occurs after receptor binding to a plasma-membrane bound HDL binding protein, and HDL is transported into the cell via an unknown mechanism [164]. Once cholesterol is in the cell it can be used for steroidogenesis or for formation of cell membranes, or can be esterified for fatty acid to form cholesterol esters and is then stored [165]. The cholesterol esters then form lipid droplets characteristic of steriodogenic cell types [2]. Cholesterol esterase (activated by PKA) hydrolyzes the lipid droplets to free cholesterol to use by the cell [2].

The steroidogenic pathway in luteal cells requires cholesterol either from de-novo synthesis, from stored lipid droplets or from cholesterol containing lipoproteins, which require specific membrane bound (i.e. lipoprotein) receptors [2]. Synthesis of steroids is dependant upon transport to the mitochondria and then from the outer to the inner mitochondrial membrane where cholesterol side chain cleavage enzyme complex cleaves the side chain from cholesterol to form pregnenolone [166]. Transport to the outer mitochondrial membrane requires intact cytoskeleton, as inhibitors of microtubule and

microfilament [167] function to prevent cholesterol accumulation in the mitochondria. The phosphorylation of the cytoskeleton probably influences the rate of transport [2]. Sterol proteins also appear to play a role in transport of cholesterol into the mitochondria [168, 169]. Stimulation of trophic hormones can also enhance transport of cholesterol into the mitochondria [2]. The rate limiting step in steroidogenesis is transport of cholesterol from the outer mitochondrial membrane to the inner mitochondrial membrane, which is done by StAR [2]. In addition, peripheral-type benzodiazepine receptor, present in the membranes of steriodogenic cells, has also been shown to play a role in transport of cholesterol [168]. Furthermore, endogenous ligand for this receptor are required for a normal steriodogenic response to human chorionic gonadotrophin (hCG) [170]. All three seem to be required for normal transport of cholesterol from the outer to inner mitochondrial membrane, the site of cholesterol side chain cleavage [2].

Luteotrophic hormones are those that support growth and/or function of the CL [2]. During a normal luteal phase, the CL increases and size and produces progesterone [2]. Concentrations of hormones (FSH/LH) in serum are dependant upon the amount of steriodogenic tissue, blood flow and the capacity to synthesize progesterone. Blood flow also increases as concentrations of progesterone in serum increases [2]. The action of trophic hormones (i.e. LH, IGF-I, GH, FSH) on steroidogenic luteal cells mobilizes and delivers cholesterol to the mitochondria, where it must be transported across the inner mitochondrial membrane [171].

Luteal Development and Function

The pituitary gland is required for normal luteal development and function, and if the pituitary is removed on day 5 of the estrous cycle (in ewes), then the CL does not increase in weight and concentrations of progesterone in serum remain at or below levels observed on day 5 [172, 173]. The loss of luteal weight, when compared with control ewes, is associated with a decrease with a number of SLC and fibroblasts and a decrease in the size of both LLC and SLC [172]. The decrease in progesterone does not seem to be associated with decreased uptake of lipoproteins, as mRNA of LDL receptor of HDL binding protein are not decreased [174]. However removal of the pituitary caused a decreased in StAR, P450-scc and 3β-HSD [173]. The decrease in the size and number of

steriodogenic cells and the decreased capacity for steroidogenic cells to secrete progesterone result in reduced ability of the CL to secrete progesterone [2].

After the CL is fully formed in the ewe, removal of the pituitary results in regression of the CL [175-177]. In ewes, cattle and in pregnant (but not cycling) sows, treatment with antisera against LH caused a decline in luteal weight and/or luteal content of progesterone [178, 179]. In ewes the removal of all pituitary hormones had a more severe effect on luteal functions than with treatment of the LH antisera [177]. Both treatments results in a decrease in mRNA encoding StAR, P450-scc and 3β-HSD [177], suggesting that LH is required to maintain normal expression of these enzymes.

Studies were designed to determine whether LH was required for normal luteal development and function. Use of a GnRH antagonist in primates prevents normal luteal development [180], and causes a rapid decline in secretion of progesterone from mature CL [181-183]. In cows, treatment with GnRH antagonist impaired normal function of the CL, indicating pulsatile release of LH is necessary for function, however treatment was not as dramatic as in the primate, and treatment after CL matured had no effect on function [184]. In sheep, treatment with GnRH antagonist showed very little effect on the secretion of progesterone [185]. Therefore LH pulses are required for normal luteal development in the primate and bovine CL, however LH pulses do not seem to be required in the ewe for normal luteal development.

In the pig, a luteotrophic role for estradiol is not well defined. Implantation of estradiol capsules into the CL results in growth and increased progesterone secretion [186]. Estradiol also reduces secretion of PGF-2α from the uterus, therefore preventing luteolysis, which may account for some of the effects of estradiol in the pig [187]. Control of progesterone secretion has been studied in many species [2]. SLC and LLC differ in the basal secretion of progesterone, as LLC secrete more than 2-40 times unstimulated SLC do [2]. In the pig, LH increases secretion from SLC, but not LLC [188]. In the bovine and ovine CL, binding of LH to its receptor activates adeylate cyclase, leading to increased expression of cAMP and PKA activation [189, 190]. Acute steroidogenic effects of LH are not modulated by the three steroidogenic enzymes involved in progesterone synthesis, however, it was postulated that LH increased steroid production by increasing StAR [173]. Addition of LH to SLC has also been demonstrated to activate phospholipase C (PLC), and

therefore PKC [189], however depending on culture conditions, some saw activation of PKC and others did not [2].

Both GH and IGF have been shown to increase secretion of progesterone [2]. GH could affect luteal function by binding its receptor and activating tyrosine kinase JAK2 [191]. Additionally, GH can influence luteal function by directly increasing IGF-I which phoshorylates insulin receptor substrate I and increases the activity of phosphoinositide 3-kinase, which can activate phosphoinositide phosphatidylinositol 3-phosphate, a second messenger [192]. This may act to prevent cell death.

In addition Prostaglandin E (PGE) and I (PGI) have also been shown to be expressed in the early stages of the luteal cycle, and show decreased levels in the late stages of the cycle, and thus are proposed to be involved in luteal development [193]. Addition of PGI2 was shown to increase secretion of progesterone in the cow, ewe and human [109, 194-196]. In the human, luteal cells treated with PGI2, showed an increase in cAMP accumulation, suggesting it might increase progesterone secretion through PKA activation [194]. However in the ewe, PGI2 sites were localized to both SLC and LLC [197, 198] and thus the exact mechanism in which it increases progesterone secretion is yet to be determined [2]. PGE2 has been shown to increase progesterone secretion in bovine and ovine luteal cells [109, 194-196]. In the sheep PGE2 receptors are on LLCs [195] and only LLC respond to PGE2 by increasing progesterone [194]. In the early bovine studies, PGE2 increased levels of cAMP and presumably PKA, however in purified ovine LLCs, PGE2 did not increase levels of cAMP or adenlyate cyclase [194], therefore suggesting that PGE2 may act to mediate progesterone secretion by more than one signaling pathway [2].

In summary, in SLC of most species and in LLC of nonhuman primates and rats, luteotrophic hormones such as LH and PGI2 act to mediate progesterone secretion through activation of PKA, which increases transport of cholesterol to the P450-scc enzyme complex [199]. In LLC of pigs, humans, cows and sheep, hormones such as PGE2, GH, PGI2 and IGF-I is not mediated by increase in PKA, possibly because PKA is constitutively activated [190], and thus stimulation in these cell types is limited [2].

Luteolysis

Progesterone secretion from the CL is required for normal pregnancy in all domestic animals [200], however if no pregnancy occurs, progesterone secretion from the CL must cease to allow for the reproductive cycle to begin again. PGF-2α of uterine origin is the primary luteolytic factor in domestic livestock, including the pig; however there are many interactions that occur with other endocrine hormones as well as the different cell types within the ovary [200]. The physiological and morphological process in which the CL responds to uterine PGF-2α, ceases production of progesterone and then undergoes apoptosis is called luteolysis [201], and in the pig the porcine CL doesn't not respond to PGF-2α until after day 13 of an 18-21 day cycle. Therefore, the period in which the CL acquires the ability to respond to PGF-2α can be described as luteolytic sensitivity (LS), and during this time various endocrine and signaling events occur to "prime" the CL for PGF-2α actions. Once the CL responds to PGF-2α, luteolysis occurs. Luteolysis can be described by both functional luteolysis, characterized by inhibition of progesterone production [201], followed by structural luteolysis and regression where the cells of the CL undergo apoptosis [202].

Uterine Involvement in Luteolysis

Uterine involvement in luteolysis was first recognized when removal of the uterus in guinea pigs resulted in extended lifespan of the CL [203]. This has also been shown in many other animals including the pig [204]. It was also shown in pigs that the uterus had a local effect versus a systemic effect when the removal of the uterine horn adjacent to the ovary with CL prevented regression [206]. In most species, removal of one uterine horn causes maintenance of CL in the ovary on the side on which hemihysterectomy is performed [205-208]. The animals showed persistence of the CL in the ovary on the side where the uterine horn was absent, provided vascular connections with the remaining uterine horn were also absent [209]. Therefore, it was suggested that the luteolysin traveled from the uterus to the ovary with a CL via a local route, later identified to be through the ovarian venous and artery supplies [210]. This occurred as the uterine venous supply drains in the ovarian arterial supply known as the counter current mechanism [206, 211]. In the sow, bilateral regression of CL in both ovaries occurred after hemihysterectomy [212], however if the amount of tissue in the remaining horn was 25%, the CL in the opposite

ovary no longer regressed [214]. Delcamp and Ginther (1973) showed that a crossover of the venous drainage between both uterine horns occurred [210]. PGF-2α was identified and measured by radioimmunoassay (RIA) in the uterine vein blood of the sow at luteolysis, and a local effect of PGF-2α was shown when PGF-2α was infused into the adjacent uterine vein [213]. However, there was also evidence that the opposite ovary was also affected. This was suggested to be caused by either PGF-2α reaching the opposite ovary either systemically [214] or by lymphatic connections with the opposite uterine horn [215]. It is also likely that PGF-2α can act in part via the systemic circulation, since ~40% of tritium-labeled PGF-2α infused into the pulmonary artery traverses the lungs unchanged [216]. This indicated that there might be both a local and systemic effect of the uterus on the CL in the pig. Bazer and colleagues (1977) have also shown that PGF-2α is the luteolysin in the sow and suggested that the reduction in uterine PGF-2α in early pregnancy is caused by a change in the uterus from an endocrine function to an exocrine function so that PGF-2α is secreted into the uterine lumen [217]. This group also proposed that endogenous estrogen secreted during early pregnancy may be responsible for inhibition of luteolysis by switching the secretion of PGF-2α from the venous side of the uterine circulation to the uterine lumen [218]. In peripheral blood of sows, PGFM is elevated as a series of pulses at the time of luteolysis, confirming the role of PGF-2α as a luteolytic agent in the pig [219].

Neuroendocrine Control of Luteolysis

Hormonal factors which control the pulsatile secretion of uterine PGF-2α has been studied, particularly in the sheep, and a model has been developed to explain how ovarian steroid hormones control the process (as described by McCracken [1]). Towards the end of the luteal phase, loss of progesterone action occurs due to down regulation of its own receptor, in the hypothalamus and in the endometrium, resulting in the return of estrogen action in both tissues. Estrogen action will stimulate hypothalamic oxytocin pulse generator to secrete high-frequency bursts of low levels of oxytocin intermittently and simultaneously up regulate endometrial oxytocin receptors in the uterus. Low levels of PGF-2α (sub-luteolytic) will be released from the uterus due to the interaction of pituitary oxytocin and endometrial oxytocin receptors. Low levels of PGF-2α will initiate release of

luteal oxytocin, acting via the PGF-2α receptor in the CL to initiate a supplemental secretion of luteal oxytocin, which will amplify PGF-2α release. PGF-2α levels will now be high enough to active the low sensitivity receptors and will inhibit progesterone secretion (luteolysis) and release additional oxytocin in a positive feedback manner. In the sow, which synthesize large quantities of endometrial oxytocin, uterine PGF-2α is also released in a pulsatile manner and may be regulated by the central oxytocin pulse generator.

Functional Regression

Uterine PGF-2α is well recognized as the primary initiator of luteolysis, and decreases progesterone production in cultured luteal cells stimulated by gonadotrophins in the pig [220] as well as other species. PGF-2α also increases ET-1, which is also suggested to mediate PGF-2α induced inhibition of progesterone synthesis [11]. TNF-α binding sites are also present in ECs derived from the bovine CL [11]and ET-1 secretion is significantly stimulated by treatment with TNF-α [11].

A key process in steroidogenesis is the cleavage of cholesterol to pregnenolone by P-450scc, located on the inner mitochondrial membrane [221]. A potential mechanism of action for the acute anti-steriodogenic effect of PGF-2α could involve disruption of cholesterol transport into the mitochondria or by an effect on P-450scc [1]. Multiple studies have shown that steroidogenesis is more dependant on cholesterol delivery to the steriodogenic enzymes than the actual levels of enzymes, which suggests that PGF-2α may elicit an anti-steriodogenic effect before and after cholesterol transport to the mitochondria [1].

PGF-2α also causes a reduction in luteal blood flow [222], and morphological changes in luteal cell type. In early luteolysis, lipid droplet accumulation increases in the cytoplasm of the CL in pigs [223], and the number of protein-containing secretory granuoles declines [224-226]. Capillaries begin to change, showing protrusion of ECs into the lumen, increases in size and number of membrane junctions between ECs, EC fragmentation (apoptosis) followed by complete disintegration of the capillary [209, 227-229]. Lysosomes and autophagasomes increase as luteolysis progresses, as well as the number of steriodogenic cells decrease [228] and intracellular organelles become

disorganized [200]. Specifically, LLCs which contain PGF-2α receptors (FP), respond both physiologically and morphologically to PGF-2α, whereas SLCs do not respond as dramatically to PGF-2α [200].

ET-1 in Regression

ET-1 has been implicated as a mediator of functional regression in the rat [230], sheep [231], bovine [232] and human [233] luteal tissue. ET-1 has no effect on angiotensin II, oxytocin, progesterone or PGF-2α in the early luteal phase, however ET-1 seems to play a role during PGF-2α induced regression of CL during the mid luteal stage, as sub-luteolytic dose of PGF-2α during the mid luteal phase, followed by an intraluteal injection of ET-1 resulted in rapid decrease in progesterone [232]. Additionally intraluteal injections of ET-1 receptor antagonist BQ123 (ET_A specific) before PGF-2α dosing results in a decreased response to PGF-2α in sheep [139]. Furthermore, ET-1 peptide and ET_B mRNA levels are elevated during spontaneous regression [234], and administration of PGF-2α at midcycle increases mRNA for ECE-1, ET-1, ET_A and ET_B, as well as increasing ET-1 peptide expression in the bovine CL [230, 231, 234-237]. In the bovine CL, ET-1 increases occur in the LLCs and ECs (via immunocytochemistry) [235]. This data suggests that ET-1 has a functional role in blood vessel changes in the regressing CL, perhaps to coordinate the regression of capillaries and the degenerative process in the arterioles [151]. There is no evidence, to date, that shows a direct relationship of ET-1 affecting the viability of either ECs or steriodogenic cells [238].

NO in Regression

Nitric Oxide, a local vasodilator, may also play a direct role in luteolysis in the regressing CL [239]. In the bovine CL, infusion of L-NAME, a NOS inhibitor, caused a significant increase in PGF-2α in days 17 and 18 of the cycle, suggesting NO plays an important role in initiation of luteolysis [239]. In the rabbit, it has been demonstrated that prostaglandins increase luteal NOS activity and progesterone production, depending on the stage of the CL, as PGF-2α caused a 2.5 fold increase in NOS activity and a decrease in progesterone production on day 9 CL [240]. NO inhibitors also acutely inhibit progesterone release from day 9 CL of pseudopregnant rabbits [241].

Structural Regression

Structural regression, which follows functional regression, is required to allow for new follicular development. It has been shown that TNF-α as well as Fas/FasL and other cytokines are important in structural luteolysis. Bovine luteal cells are sensitive to FasL-induced cell death in the presences of interferon-γ (IFN-γ) and IFN-γ with TNF-α [242]. Both cytokines have also been shown to stimulate Fas mRNA expression in mouse [243] and bovine CL [242]. Additionally FasL sensitivity in bovine luteal cells was correlated with an increase in Fas mRNA expression induced by cytokines, suggesting that FasL induces cell death of bovine luteal cells mediated via the Fas/FasL system [242].

Decreases in progesterone levels and interruption of growth factor signaling in the CL may also promote activation of inflammatory cells, resulting in increases in TNF-α and IFN-γ [152]. TNF-α increases expression of major histocompatibility (MHC) class I glycoproteins in cultured bovine luteal cells [244]. It is hypothesized that these glycoproteins are recognized by cytotoxic T lymphocytes in order for the T lymphocytes to phagocytize luteal cells [244]. IFN-γ and TNF-α also reduce the viability of bovine and murine luteal cells [242, 243].

Onset of structural regression in the CL also involves disruption in vasculature, as the endothelial cells loose tight junctions and the permeability barrier are disrupted [104]. Subsequently ECs detach from the basement membrane and occlude small blood vessels [104]. Capillaries will then disappear, followed by an increase in arterioles with a thickened wall, possibly due to the presence of an increased number of smooth muscle cells [245-247]. This thickening is suggested to be a degenerative event [151], and demonstrates the vascular changes that occur during the CL lifespan, and correlate with CL degeneration [104].

Tumor Necrosis Factor-alpha (TNF-α)

TNF-α, produced by macrophages, is a 17 kDa non-glycolsolyated protein [201], which when physiologically active circulates as a homotrimer [248]. Depending upon the cell type, TNF-α has a variety of actions such as proliferation, differentiation, eliciting inflammatory responses, mediating steroidogenesis, and apoptotic or necrotic cell death

[57]. TNF-α is a member of the TNF super family, which consists of 18 members [201]. All of the family members have a conserved C-terminal domain coined the TNF homology domain, which is a trimeric domain and is responsible for receptor binding, and has approximately 20-30% homology between [201].

The release of TNF-α is associated with the expression of **t**umor necrosis factor alpha convertase (TACE) which cleaves pro-TNF-α in response to extracellular stimuli [201]. TACE is a member of the A disintegrin and metalloprotease (ADAM) family, and is a multidomain, type 1 transmembrane protein that includes a zinc-dependant catalytic domain [249]. Reactive oxygen species (ROS) are suggested to mediate TACE activation [249]. When active TNF-α is released, it trimerizes and must bind to one of two receptors, TNFRI or TNFRII [249]. The receptors for the TNF super family also have a family of receptors, of which TNFRI and TNFRII are two [201]. The TNF receptor super family has distinguishing cysteine rich domains made up of disulfide bonds [201]. Binding of TNF-α induces trimerization of the receptors, and causes recruitment of several proteins to form a signaling complex, of which is dependant upon cell type. This allows for its array of cellular responses.

TNF-α effects are mediated by two distinct receptor types, with molecular sizes 55 (TNFRI) and 75 (TNFRII) kDa [250] (Figure 4). The extracellular domains of both are conserved and cysteine-rich, typical of the TNF-α receptor super family of receptors [201]. However the intracellular domains are different, and therefore ligand binding to these two receptors lead to pleiotrophic actions depending on cell type [248]. Activation of the 55kDa TNF-α receptor contains an intracellular death domain, which is required for apoptotic signaling pathways [251], and in the CL TNFRI been associated with luteolytic actions [252-255], as well as increasing intracellular ceramide levels causing apoptosis [256].

TNFRII can induce gene transcription for cell survival, growth and differentiation [201]. When TNF-α binds to TNFRII (75 kDa protein), activated TNFRII interacts with TRAF2 (a member of a family of signal transduction proteins that associates with the cytoplasmic tail of the TNF receptor family), nuclear factor κB (NFκB, a transcription factor) is activated, and expression of survival genes is up-regulated and apoptosis induced by TNF-α is prevented [257-259]. Thus, TRAF2 expression is considered to be a good

indicator of TNF-α dependent cell proliferation in both TNFRI and TNFRII signaling cascades [259].

TNF-α Signaling

Binding of TNF-α to each receptor activates different intracellular pathways [251] (Figure 5). TNF-α binding to TNFRI has been shown to activate the PL-A2 pathway [260], the PLC and PKC pathways [261] and the PKA pathway in many tissues. Sakumoto and colleagues (2000) showed that TNF-α binding to TNFRI activated Raf/Ras and the MAPK cascade causing PLA2 activation and PGF-2α secretion [254] Additionally, TNF-α can activate NFκB which has been shown to control transcription of cyclooxygenase (COX)-2 [262], which is a rate-limiting enzyme for converting arachidonic acid to prostaglandins [263]. TNF-α can also activate NFκB which then activates IKK signaling that phosphorylates IKB and NFκB protein, leading to ubiquitation and degradation of IKB and translocation of free NFκB dimmers to the nucleus [264], causing cell survival, and this pathway may be how TNF−α acts in the early CL to prevent premature luteolysis.

Additionally, TNF-α signaling in luteal cells increases phorphorylated p38 and JNK [265]. Increases in JNK activity has been shown to induce phosphorylation and inactivation of Bcl-2 protein (anti-apoptotic) [265]. Human granulosa cells show a decrease in Bcl-2 levels upon treatment with TNF-α [266]. This data suggests that TNF-α may increase FasL or interferon-γ to induce apoptosis through inactivation of Bcl-2 [267]. Additionally, TNF-α has activated sphingomyelin-ceramide pathways, resulting in apoptosis [267]. In the bovine CL, TNFRI mRNA was present throughout the estrous cycle, and therefore at least in the bovine CL, these receptors mediate TNF-α effects.

TNFRII is expressed by cells of the immune system and endothelial cells, and its exact role is less clear [268]. However, TNFRII mediates part of TNF-α effects, including proliferation of T cells and B cells, NFκB activation, and cytotoxicity, and may potentate the effects of TNFRI by ligand passing to the lower-affinity TNFRI [269]. Analyses with TNF-α and TNF-α receptor-specific neutralizing antibodies showed that stimulation of TNFRII does not directly engage the apoptotic program, but relies on the induction of endogenous, membrane-bound TNF-α, which subsequently activates TNFRI to induce apoptosis [270]. TNFRII is expressed in porcine granulosa cells and is suggested to play a

role in follicular atresia [271], however whether or not TNFRII is expressed or has any functional significance in the porcine CL is yet to be determined [201].

TNF-α in the CL

It has been suggested that TNF-α is involved in the later stages of luteolysis by inhibiting steroidogenesis [159, 272] stimulating luteal PGF-2α synthesis [156, 272, 273] and activating apoptosis [253, 274, 275]. However, recently macrophages have been identified in the early and mid CL in the pig [147] and cow [149], which suggests that TNF-α may play a role before the onset of luteolysis. Macrophages and their capacity to produce TNF-α, have been shown to dramatically increase (four-fold) between the early (day 4-6) and mid (day 8-12) stages of the porcine luteal cycle, further increasing an additional 1-2 fold in the late luteal stage [147]. Moreover, TNF-α receptors have been found to be present and unchanged in the porcine CL from days 4-12 of the cycle, but were increased on day 15 [276, 277]. TNF-α receptors have been located on SLC, LLC and ECs in the porcine [276] and bovine CL [253]. In the bovine CL, TNF-α presumably acting via TNFR, acts to increase the production of both ET-1 and PGE2, by ECs in vitro [252] and therefore TNF-induced ET-1 secretion by EC may serve to influence the steriodogenic capacity of the luteal (i.e. SLC, LLC) cells [104]. The levels of TNFR mRNA were also elevated in physiologic or PGF-2α induced regression of the CL [253].

TNF-α mRNA is present in LLCs [278] and ECs [279], and it has been suggested that because of the high affinity binding sites for TNF-α on ECs [276], TNF-α may control EC function [279]. Friedman and colleagues (2000) showed there is more TNF-RI present on bovine ECs then in steroidogenic cell types [253]. Recently, Okano and colleagues (2006) show porcine luteal cells (days 10-14) showed a dose and time dependant response to increasing TNF-α levels [275].

TNF-α in Apoptosis

TNF-α is known to stimulate apoptosis in many different cell types via death-domain receptor (as discussed above). In addition it stimulates the sphingomyelin (SM) pathway to produce ceramide. Treatment of cells with ceramide, mirrors the actions of TNF-α and induces apoptosis (via the activation of caspases) in various mammalian cell lines [280, 281] and in ovarian follicles [282]. Rueda and co-workers (2000) demonstrated that TNF-α induced an increase in phosphorylated p38 MAPK and jun-n-terminal kinase

(JNK), which have been shown to induce phosphorylation of Bcl-2, and thus caused inactivation of this protein [283]. Bcl-2, an anti-apoptotic factor, is reduced when stimulated with TNF-α in human granulosa cells [266]. These data indicate that TNF-α induces apoptosis by activating ceramide and inactivating the Bcl-2 protein [267]. Collectively, TNF-α is implicated in having an important role in mediating both acquisition of LS and inducing apoptosis in the CL, however the exact signaling mechanisms are yet to be fully elucidated.

Prostaglandin F2- alpha (PGF-2α)

PGF-2α is known as the physiological luteolysin [1, 2, 200], however the detailed cellular and biochemical events associated with luteolysis remain largely uncharacterized [284], particularly in the pig. In many species, including the pig, PGF-2α is produced by the uterus and acts on the CL to cause functional (decreasing steroidogenesis) and structural (regression of the CL) luteolysis [110]. PGF-2α is also produced intra-luteally, however this only occurs in CL that show LS (i.e. PGF-2α responsive, after day 12-13 in the pig) [96, 285]. The CL has been recognized as a site of PGF-2α production in the cow [193] and PGF-2α secretion by the bovine CL increases during PGF-2α induced luteolysis [286-288] and therefore may play a role as an amplifier of uterine PGF-2α during luteolysis in this species as well [289].

Prostaglandin F-2α Receptor (FP)

The FP receptor is a seven transmembrane receptor [207]. There have been two isoforms of the FP receptor characterized, FPA and FPB, which differ only in the carboxyl terminus [290]. FPB lacks the 46 carboxyl-terminal amino acids, including the sites that are responsible for PKC phosphorylation [290]. The carboxy terminus of the FPA is a substrate for PKC and PKC phosphorylation is responsible for regulation of second messenger pathways by FPA. FPA has also been shown to be subject to rapid negative feedback from PKC, via phosphorylation on one or more of the PKC phosphorylation consensus sites present on FPA [290]. In addition, PGF-2α binding to either receptor has been shown to activate a Rho signaling pathway, resulting in tyrosine phosphorylation of p125 focal adhesion kinase, formation of stress actin fibers and cell rounding [291].

PGF-2α acts on its G protein-coupled receptor (FP receptor) present on LLCs and SLCs, which possess a lower affinity PGF-2α binding site, which may represent a different binding moiety [110]. It has been reported that endothelial cells also possess PGF-2α receptors although this remains controversial issue [1, 292, 293].

PGF-2α Signaling

PGF-2α binding to the FP receptor activates the phospholipase C (PLC) system and causes the accumulation of inositol 1, 4, 5-triphosphate (IP3) and 1, 2-diacylglycerol (DAG) [294]. IP3 binds to its receptor in the endoplasmic reticulum which stimulates an increase in cytoplasmic Ca^{2+} [295, 296]. Ca^{2+} and DAG subsequently activate the PKC pathway. It was also shown in bovine luteal cells that PGF-2α binding to FP also activates each component of the Raf/Mek1/ERK signaling pathway [297], increases levels of c-jun and c-fos mRNA and activates the AP-1 transcription factors [297].

Hormonal Regulation of PGF-2α Synthesis

Release of arachidonic acid from phospholipids by phospholipase A2 (PLA2) activation is the rate limiting step in prostaglandin synthesis [298]. Released arachidonic acid is then converted to PGF-2α by prostaglandin synthase, also called cyclooxygenase (COX), which has a constitutive (COX-1) and an inducible (COX-2) form [299]. The site of PGF-2α synthesis is the uterine endometrium [1], and it is suggested that estrogen increases uterine PGF-2α production by stimulating the activity of enzymes controlling prostaglandin synthesis [300]. Estradiol has been shown to increase the activity of PLA2 [301, 302]. The priming effect of progesterone on endometrial PGF-2α synthesis is considered to be due to the accumulation of lipids in the endometrium since progesterone increases lipid accumulation in rats [303]. Progesterone may also enhance PGF-2α synthesis by increasing the concentration and activity of endometrial prostaglandin synthase [304].

PGF-2α actions in the CL

The lack of sensitivity to PGF-2α of the porcine CL before D13 is believed not to be due to the lack of FP receptors, as they are present and apparently function as early as Day 5; it is suggested that the lack of LS may to be attributable to the lack of downstream signaling molecules such as PKC [96]. Recently Diaz and Wiltbank (2005) demonstrated

that the acquisition of LS in the porcine CL was also associated with the ability of PGF-2α to inhibit two critical components of the cholesterol transport pathway (i.e. StAR and LDL receptor) [305].

In addition to decreasing progesterone production (i.e. functional regression), PGF-2α also leads to structural regression which is associated with apoptosis [202, 306-309], and as described above, it has been suggested that TNF-α and Fas are important mediators of PGF-2α-induced luteolysis [159, 243, 310]. More recently it has been suggested that PGF-2α induces luteolysis, *in vivo,* in part by increasing cytokines such as FasL, which (as described below) may play a role in activating Caspase-3 driven apoptosis [311].

Endothelin (ET)

Endothelins are a family of three peptides (Endothelin-1, -2, and -3) which share a common structure of 21 amino acids with four cystine residues at positions 1, 3, 11, and 15, which link to form two intrachain disulphide bridges between 1 and 15 and 3 and 11 [312]. ET-1 was originally isolated from porcine aortic endothelial cells [313], and is a member of a structurally related peptide family that includes ET-2, ET-3 and sarafotoxins [312]. Sarafotoxins have been found in the cardiotoxic venom of snakes, and it is suggested that the sarafotoxins and endothelins evolved from the same ancestor, however in snakes sarafotoxins are used as an exocrine poison, in mammals ETs for an endocrine signal [314].

ET-1 is produced by a variety of cells including endothelial cells, vascular smooth muscle cells and various epithelial tissues, while ET-2 is expressed mainly in the intestine and kidney and ET-3 is expressed mainly in the brain [313]. ET-1 is produced by endothelial cells which line blood vessels, but also human macrophages [315] and polymorphonuclear leukocytes [316], suggesting a role for ET-1 in inflammation. ET-1 also stimulates the release of autacoids (i.e. NO) and hormones, decreases glomerular filtration rate, and increases cell growth and division [317].

Endothelins, specifically ET-1, are synthesized in luteal endothelial cells from a 212-amino acid precursor, pre-proendothelin-1 (ppET-1) (Figure 6). ppET-1 is then cleaved into a biologically inactive, 38-amino acid peptide, Big-Endothelin (Big ET-1) by an endopeptidase [137], and then is secreted from the cells. Endothelin-converting enzyme-1 (ECE-1) cleaves Big ET-1 into the biologically active ET-1 at the target cells (i.e. LLC)

[232]. ET-1 exerts its effects by interacting with endothelin receptor A (ET_A) or endothelin receptor B (ET_B), which are both G-coupled transmembrane receptors [318, 319], and are expressed in the porcine CL [320] . ET-1 preferentially binds to ET_A (versus ET-2 or ET-3) [321], although ET_B binds all ET's (ET-1, ET-2, ET-3) with equal affinity [318, 319].

Pre-pro-Endothelin-1 (ppET-1)

ppET-1 mRNA expression is regulated by a mechanism involving receptor-mediated mobilization of intracellular Ca^{2+} and activation of PKC in ECs [322]. Activation of PKC results in c-jun protein synthesis, after which the binding of c-jun to the TPA-response element increases, and induces ppET-1 mRNA [322]. ppET-1 may also be contained in secretory vesicles and constitutively secreted, and its processing might occur during vesicle transport [314].

Big Endothelin (Big ET)

Porcine Big ET-1 is a thirty nine amino acid peptide cleaved from ppET-1 by an endopeptidase and is biologically inactive [323]. Porcine Big ET-1 is hydrolyzed at the Trp21-Val22 bond, by ECE, to generate ET-1 and a C-terminal fragment to produce the biologically active ET-1 [323].

Endothelin-1 (ET-1)

The human ET-1 gene is located on chromosome 6 [322]. ET-1 is a mitogenic peptide for many cells types- endothelial cells, vascular and bronchial smooth muscle cells, fibroblasts, keratinocytes, mesangial cells, osteoblast, melanocytes and endometrial stromal cells [324], and ET-1 activity can be amplified by interactions with other growth factors (i.e., EGF, FGF, IGF, PDGF, TGF, and IL-6) [325].

The microtubular system plays a role in ET-1 secretion by transferring the synthesized ET-1 to the cell surface of ECs [322]. ET-1 secretion is also regulated by intracellular Ca^{2+} release from $Ca2^{+}$ stores and by Ca^{2+} calmondulin ([322]. Tasaka and Kitazumi (1994) have suggested that this occurs by phosphorylation of the myosin light chain, by myosin light chain kinase and its activation by the Ca^{2+}-calmodulin complex [322]. This process facilitates the formation of filamentous myosin and actin which probably participate in ET-1 secretion, by transporting the ET-1 containing vesicles towards the cell membrane in the ECs. The combination of a short ET-1 mRNA half-life (~15 minutes) [312], and a limited intracellular storage of ET-1 suggests that there should be a

close parallel between mRNA levels and peptide secretion [326]. Therefore the release of active peptide must be controlled via 1) regulation of gene transcription; 2) mRNA stabilization and/or 3) regulation of ECE activity [326]. Data from rat smooth muscle cells suggests that ET-1 is primarily controlled at the transcriptional level [327], and limited data is available on the regulation of ECE expression [328].

ET-1 has many effects depending on cell and tissue type, ranging from gene expression, cell contraction, and proliferation to survival in cardiac myocytes, vascular smooth muscle cells and smooth muscle cells [329]. ET-1 induces the activation of MAPK, ERK1 and ERK2 [330, 331] and is believed to promote cell growth (in smooth muscle cells), as well as suppresses apoptosis in cancer cells (human smooth muscle cells, ovarian carcinomas, and prostatic smooth muscle cells) [331-333]. The ET system also plays an important role in the early development of the neural crest and in the new formation of organs [314]. ET-1 levels in plasma of normal patients are very low (approximately 0.26-5 pg/ml), indicating that it is considered mainly to be an autocrine/paracrine factor; however blood levels of ET-1 (and Big ET-1) are elevated in shock, myocardial infraction, vasospastic angina kidney failure and other connective tissue disorders [334]. Patients undergoing hemodialysis or kidney transplantation or those undergoing cardiogenic shock, myocardial infarction or pulmonary hypertension levels are as high as 35 pg/ml [334]. Since there are many important diseases such as hypertension, heart disease, arteriosclerosis, asthma, renal failure and many other associated diseases, in which ET-1 levels are elevated, much research has been carried out using ET-1 antagonists in attempts to treat them [335-338].

ET-1 expression also correlates with vascularization of tumors [339] and malignancy in ovarian cancer [340]. In colon cancer, ET-1 inhibits apoptosis mediated by Fas ligand [341] or triggers anti-apoptotic signaling through Bcl-2-dependant and PI3K mediated pathways [333]. In ovarian cell lines (OVCA 433) ET-1 induces calcium signaling, activates MAPK p125 and enhances the expression of the early response genes [340]. In ovarian cancer cells, ET-1 mRNA is significantly increased compared to normal tissue, and ET_A is the predominant receptor found in these cell lines [340]. Using BQ123, a selective ET_A inhibitor, ET-1 stimulated growth was inhibited and growth rates of unstimulated cells were strongly reduced, while BQ788 (ET_B inhibitor) had no effect on

cellular growth rates [342, 343]. Similar roles for ET-1 and ET_A have been seen in both breast and cervical cancers. Over expression of ET-1, ET_B and specifically ET_A, correlated with aggressive types of human breast carcinoma [344]. They also demonstrated an association between increased ET_A expression and resistance to chemotherapy in advanced breast carcinomas [344]. Similarly, BQ123 inhibits the ET-1 induced proliferative effect in cervical cancers, whereas an ET_B antagonist had no effect [345]. Therefore targeting ET_A is a potential target for anticancer therapy. ET-1 metabolism/clearance (from plasma) has been proposed to occur in one of two ways: 1) ET_B receptor-mediated uptake followed by lysosomal degradation [346, 347], or 2) Catabolism by extracellular neutral endopeptidase(s) [348].

Endothelin-2 (ET-2)

ET-2 is two amino acids different than ET-1, whereas ET-3 is six amino acids different than ET-1. In normal plasma, ET-2 circulates at less than 20%, ET-3 less than 60% of levels of ET-1. Porcine ppET-2 is made up of 214 amino acids with a 26-residue signal sequence, Big ET-2, mature ET-2 and ET-2 like peptide [349]. Porcine ppET-2 and human ppET-2 have approximately 70% sequence homology [349]. In the mouse, ET-2 (also called vasoactive intestinal factor or VIC) is highly expressed in intestinal epithelia and skin [350], tissues that are in contact with microorganisms in the environment. Studies have shown that ET-2 gene is strongly expressed in and limited to the gastrointestinal tract, gonads and the pituitary [351, 352]. Receptor selectivity for ET-2 is similar to ET-1, and many biological activities of ET-2 overlap with ET-1 [353].

In the mouse intestinal tract, ET-2 immunoreactivity was localized to the epithelial cell of the mucosal layer in the intestinal tract, and intracellularly ET-2 was concentrated near the basement membrane [353]. These authors suggested that ET-2 may be secreted from the basement membrane of epithelial cells and may act as a chemoattractant for macrophages to modulate function of immune cells for mucosal defense, and therefore ET-2 may be a therapeutic target for intestinal diseases such as irritable bowel disease [353]. Studies have also shown that microorganisms produce molecules that antagonize the ET-1 receptor system [354, 355] and produce an ET-converting enzyme-like activity [356]. However the exact function of ET-2 in the pig is yet to be elucidated. Hypoxia upregulated ET-2 mRNA in breast tumor cell lines and ET-2 acts as a hypoxia-induced autocrine

survival factor for breast tumor cells [357]. ET-2 is also a chemoattractant for macrophages and may modulate distribution of them in tumors [358], as well as be involved in the invasion of tumor cells [357]. ET-2 is also one of four candidate tumor markers in uveal melanoma cell lines and may be involved in uveal melanoma development in the eye [359].

Recently, it has been suggested that ET-2 acts as a last moment trigger of follicle rupture; Ko and collegues (2006) found that ET-2 was exclusively and transiently expressed in the granulosa cells immediately before ovulation in the rat [360]. In addition, administration of ET-2 to ovarian tissue induced rapid contractions, whereas tezosentan, a non-selective receptor antagonist, diminished the ET-2 effect [360]. This study suggested that ET-2, produced by granulosa cells, diffuses through a weakened follicular wall to reach the theca externa where smooth muscle cells are located, and induces muscle cell contraction [360]. Contraction of smooth muscle cells results in follicular constriction, increasing follicular pressure creating tension in the follicle wall [360]. This causes follicle wall rupture where structural integrity is lowest [360, 361]. These authors also suggest that ET-2 smooth muscle constriction of one follicle, may lead to simultaneous constriction of other follicles, as smooth muscle layers are interconnected, and may cause multiple rupturing follicles, explaining why in super-ovulated animals, multiple oocytes are released within a short time and travel together down the oviducts [360]. The presence of ET's in follicular fluid of women undergoing in vitro fertilization also suggest a similar role for ET-2 in women [360].

Endothelin-3 (ET-3)

ET-3 has been shown to cause contractile responses in prostatic smooth muscle and/or mediate mitogenic activity in human prostatic smooth muscle cells [362] and in canine prostatic epithelial cells [363].

Endothelin Converting Enzyme (ECE)

Two ECEs, ECE-1 and ECE-2, transcribed from different genes, have been cloned and characterized [364, 365]. Both belong to the thermolysin subfamily of proteases, which also comprises neutral endopeptidase and Kell blood group protein [366]. Both ECEs represent zinc-binding metalloproteases with large extracytoplasmic domains (i.e. outside cell associated with the membrane) [367]. Comparing bovine sequences of ECE-1 and 2, reveal an overall sequence similarity with 59% amino acids being identical [367]. ECE-1

has been shown to be expressed in a very broad range of tissues, whereas ECE-2 is mainly expressed in the nervous system [367].

Endothelin Converting Enzyme-1 (ECE-1)

ECE-1 is a type II membrane protease that belongs to the neprilysin family of zinc metallopeptidases [364, 368]. Expression is primarily located to vascular endothelial cells of all tissues, but ECE-1 is also found in nonvascular cells [236, 369-371]. ECE-1 has intracellular and membrane bound isoforms [364], and has a single transmembrane, a short N-terminal cytosolic tail and a large C-terminal extracellular domain that contains the enzymatic active site [372]. ECE-1 also has 10 N-linked glycosylation sites which aid in protein folding [373]. ECE-1 hydrolyzes the Trp-21-Val/Ile 22 bands of the Big-ETs to produce the biologically active ETs [364, 374]. ECE-1 can also hydrolyze other substrates, and therefore it has been suggested that ECE-1 may be a useful target for therapeutic purposes, for several diseases, particularly Alzheimer's [375-377]. There have been four protein isoforms of ECE-1 identified in the human, rat and cow [373, 378-380]. These isoforms named ECE-1a, ECE-1b, ECE-1c, and ECE-1d, are encoded by one gene, and the expression of each isoform is regulated via a distinct promoter, that results in each isoform having a unique amino terminus [373, 378, 379]. The amino terminal sequences determine different subcellular localization of each of these isoforms while the extracellular domain containing the active site is identical for each isoform [381-383].

ECE-1a is most abundant in endothelial cells, resides within the intracellular secretory vesicles and is constitutively transported to the cell surface, whereas ECE-1b is located intracellularly close to the trans-Golgi networks [314]. ECE-1c and ECE-1d are both localized at the cell surface and thus probably act as ectoenzymes [314]. Most recently Klipper and coworkers (2006) found splice variants of ECE-1b, -1c and -1d all which lack the 3' exon and shortens the protein from approximately 120 kDa to approximately a 75 kDa protein [384]. These splice variants lack the domain that contains the signal peptide portion and therefore are thought to remain in the cytosol and are nonglycosylated [384]. These authors suggest that the splice variants of ECE-1 may function to degrade small peptides such as angiotensin I, bradykinin, neurotensin and substance P by internalizing their receptors [384]. ECE-1a mRNA expression may be induced by 1) Activation of PKC by DAG which is released from PIP-3 by activation of

phospholipase C via G-protein coupled receptors, 2) Activation of the Raf-MEK1/2-p44/p42 by RTKs or 3) Ras-Raf interaction related to the activation of G-protein coupled receptors [367].

Granulosa and theca interna cells of preovulatory follicles express ECE-1 weakly or moderately, whereas ECE-1 is strongly expressed in lutenizing granulosa and theca interna cells during CL formation [385]. In the human CL, ECE-1 is highly expressed on both LLC and SLC, and ECE-1 protein was more strongly expressed on the CL in early and midluteal phases than in late phase, perhaps indicating a role for ECE-1 expression during lutenization [385]. ECE-1 activity in human CL was also significantly enhanced by the treatment with hCG and IL-1 during 4 days of culture [385]. These authors suggested that in the human CL, ECE-1 is a cell surface differentiation-related molecule of human granulosa and theca interna cells, with unknown functions [385].

Endothelin Converting Enzyme-2 (ECE-2)

It has been reported that ECE-2 was expressed in human vascular endothelial cells of the uterus, ovary, heart, lung, and liver [386]. There are also four isoforms of ECE-2 that differ in their N-terminal cytoplasmic tails [387]. These isoforms are termed ECE-2a-1, ECE-2a-2, ECE-2b-1 and ECE-2b-2 [387]. In the bovine, expression of these isoforms was found to be very different, ECE-2a-1 and ECE-2a-2 were expressed in many tissues including liver, kidney, adrenal gland, testis and endothelial cells, whereas ECE-2b-1 and ECE-2b-2 was expressed in the brain and adrenal gland [387]. In the human, 3 isoforms have been found, ECE-2A and ECE-2B (which has two splice variants) [388]. The largest difference between ECE-1 and ECE-2 (other than possessing only 59% homology), is that ECE-2 has a pH optimum of 5.5, suggesting ECE-2 functions to increase ET synthesis in acidic environments [365].

Endothelin Receptors

Two distinct receptors, ET_A and ET_B have been identified and DNA clones encoding each have been isolated [318]. Both receptors belong to G-protein coupled receptor family, mediating all of the effects of the ETs, and are distributed in many different tissues, and in different proportions, suggesting the potential of opposing regulatory activities [314, 389]. A third ET receptor named ETC has been discovered that binds ET-3 with greater affinity than ET-1 or ET-2 [314]. Cloning of the ETC receptor was

first reported in amphibians, but has yet to be cloned from mammalian tissues [314]. Each receptor (ET_A/ET_B) contains seven transmembrane spanning domains and exhibits structural similarity to G-protein coupled membrane proteins. mRNA encoding these receptors have been detected in many tissues including heart, lung, kidney and the brain, however the distribution of receptor subtypes is tissue specific [390]. Two endothelin receptors (ET_A and ET_B) both activate PI hydrolysis and arachidonic acid release, but exhibit distinct effects on the cyclic AMP cascade when expressed in the same CHO cell type [391]. ET_A showed stimulatory effects on cAMP formation in response to agonist interaction, whereas ET_B displayed considerable inhibition of the forskolin-stimulated cAMP accumulation [391]. ET_A receptors and a subpopulation of ET_B receptors mediate vasoconstriction and are located on vascular smooth muscle cells [392]. A second subset of ET_B receptors mediate vasodilation and are located on vascular endothelial cells [319], [393].

Endothelin Receptor A (ET_A)

ET_A receptors are selective for ET-1, bind ET-1 with highest affinity [348], and are predominant (versus ET_B) in cardiovascular tissues [319]. ET_A receptors on vascular smooth muscle are linked to vasoconstriction and have been associated with cardiovascular, renal and CNS diseases [394]. In addition, ET_A is the predominant receptor in cancer cells (prostate, bladder, ovarian and breast tumors), and is involved in tumor progression through activation of tumor cell proliferation, inhibition of apoptosis, effects on endothelial cell proliferation and vascular permeability via vascular endothelial growth factor (VEGF) [324]. ET-1, acting via ET_A, causes activation of PLC, tyrosine kinase and Ras [395], and ultimately activation of RAF/MEK/MAPK pathway leading to transcription of protocogenes (c-fos, c-myc, c-jun) and to increased cell growth and proliferation [396]. ET-1 also can signal through PLC, PKC, p38 MAPK, ERK1/2, and JNK in certain cell types (rabbit CL-[397], adrenal cortex- [398], porcine cerebral microvascular endothelium [399] as well as through PKA, particularly in breast cancers [400, 401].

Endothelin Receptor B (ET_B)

Binding of ETs to ET_B located on endothelial cells results in the release of NO and prostacyclin (via PLC and PKC pathway) and therefore causes vasodilation [402]. Although evidence for ET_B actions is scarce, ET-1 actions via ET_B can mediate endothelial

cell proliferation and migration [344, 403].The main effect of ET_B activation is the regulation of cellular apoptosis, angiogenesis and clearance of ET-1 [238]. ET-1 may induce endothelial cell apoptosis through ET_B receptor binding [238], as ET_B levels are elevated around the time of spontaneous CL regression in the cow [133, 235, 385]. ET_B receptors are predominant (versus other tissues) in non-cardiovascular tissues, including CNS and kidney, and interact with the ET-1, ET-2, ET-3, which bind with equal affinity [319, 348].

ET Signaling

Upon binding to ET_A, ET-1 stimulates phospholipase C activity and increases intracellular Ca^{2+} concentrations, activates PKC and Mitogen Activated Protein kinase (MAPK) (Figure 7). In ovarian cancer cells, ET-1 also activates EGF receptor activation that will also activate MAPK via a non-receptor tyrosine kinase such as Src. Activation of Src forms Shc/Grb-2 complexes to activate Ras/MAPK [333, 340, 395]. Additionally, ET-1 also signals through PKA, as described above. ET-1 also triggers anti-apoptotic signaling through phosphatidylinositol 3-kinase (PI3K)- via the AKT pathways [404].

ET's binding to ET_B triggers several intracellular signaling pathways including activation of phopholipase C and increases in cytosolic free calcium and inositol phosphates [391, 405, 406]. ET_B activation is also associated with tyrosine phosphorylation of several cellular proteins [407] and with activation of both ERK and JNK [408-410].

ET-1 in the CL

ET-1 has been shown to play a role in luteolysis [411], and it inhibits progesterone release in both granulosa and luteal cells in many species [135, 139, 233]. ET-1 has also been shown to enhance PGF-2α production in the human and rabbit CL [412], and there is evidence in the cow [135, 411, 413] and sheep [139], which suggests that ET-1 interacts with PGF-2α in the control of luteolysis. Hinckley and Milvae (2001) showed that a sub-luteolytic dose of PGF-2α is effective in decreasing progesterone and causing luteolysis following ET-1 pretreatment [139]. In addition, this study also showed that an injection of ET-1 at the mid luteal stage decreased plasma progesterone concentrations, and that a luteolytic dose of PGF-2α stimulated gene expression of ET-1. These authors also demonstrated that an injection with an ET_A antagonist (BQ123) at the mid-luteal phase in the ewe diminished the luteolytic effect of PGF-2α, suggesting a role for the ET_A receptor.

These data strongly supports a role for ET-1 in synergizing, facilitating, and possibly mediating, PGF-2α-induced luteolysis [139].

In the bovine CL, the ET_A antagonist BQ123 blocked PGF-2α induced inhibition of progesterone production, suggesting that ET_A plays a critical role in PGF-2α mediated luteolysis also in the cow [135]. ET-1 induces endothelial cell apoptosis through ET_B receptor binding [238]. Thus since luteal ET_B levels are elevated around the time of spontaneous CL regression in many species, it is possible that ET_B plays a similar apoptotic role during lutolysis [137].

ETs in Follicles

ETs are present in follicular fluid of porcine ovaries, at levels about half of that found in plasma [414, 415]. These authors also found a higher concentration of ETs in medium and large follicles versus small follicles [414, 415]. ET-1 inhibited LH-induced progesterone accumulation and cAMP accumulation in cultured porcine granulosa cells in a dose-dependant manner [415]. Since ETs inhibited the secretion on progesterone from cultured granulosa cells stimulated by relatively low doses of LH, these authors suggested that ETs have a physiological role in vivo, which could be the inhibition of luteinization of granulosa cells until ovulation occurs [415]. ETs also inhibited LH-stimulated cAMP accumulation in a dose-dependant manner, which they suggest is by one of two mechanisms 1) ETs inhibit the LH receptor formation or action by either inhibiting receptor synthesis, changing receptor conformation, or completely blocking the ligand-receptor including of LH or 2) ETs directly or indirectly inhibit adenylate cyclase. One mechanism involving adenyalate cyclase is that the ET receptor in granulose cells activated by ET mediates inhibition of adenylate cyclase through Gi [414]. These data suggest that ET acts as a modulator of steroid metabolism in preovulatory follicles.

In porcine granulosa cells, ET-1 activates a pathway that interrupts gonadotrophin-stimulated steroidogenesis [414, 416-418], and involves accumulation of IP_3, as well as a transient rise in Ca^{2+} [416]. These actions suggest a coupling of the ET-1 receptor to the PLC-PKC signaling pathway [419]. In rat granulosa cells, ET-1 reduces cytochrome P450-scc and 3β-hydroxystariod dehydrogenase (3β-HSD) [417].

Protein Kinase C (PKC)

PKCs are a family of serine/threonine kinases that play a wide variety of roles in controlling cellular function from cell proliferation to cell death [420], and distribution of PKC isoforms show tissue and cell- specificity [421]. There are 3 families of PKC isoforms that are divided by their regulatory domains which determines physiological response [422]. The three categories (and activators) are 1) ***Conventional***, activated by calcium, diacylglycerol (DAG) and phosphatidylserine (PS) [PKC- alpha (α), Beta I (βI), Beta II (βII), Gamma (γ)]; 2) ***Novel,*** activated by DAG and PS, *not* calcium [PKC-Delta (δ), Epsilon (ε), Eta (η), Theta (θ), and Mu (μ- also known as PKD, but classified here with the novel isoforms because of novel-like C1 domains and ability to bind DAG and PS]; 3) ***Atypical***, activated by PS [PKC-Iota (ι) mouse)/ Lamda (λ) human, and Zeta (ζ)] [422].

PKC Structures

All PKC isoforms have both regulatory and catalytic domains, the regulatory domains contain motifs which control substrate binding and the catalytic domains contain binding sites for ATP [423] (Figure 8). PKC isoforms have conserved (C1-C4) and variable regions (V1-V5) [423]. C1 domains in all PKCs have the following characteristic motif-HZ12-CX2CXnCX2CX4HX2CX7C, where H is histidine, C is cysteine, X is any other amino acid and n is either 13 or 14 [422]. This motif is repeated in both conventional and novel PKCs, whereas the atypical PKCs have only a single copy (Colon-Gonzalez 2006). Diacylglycerol kinases (DGKs) also have two copies of this motif, and many other molecules contain single C1 domains (c-Raf, Kinase suppressor of Ras, regulators of small G-proteins- RASGRP GEFs, Vav, and molecules involved in vesicle release from synaptic terminals- Munc13) [422]. These C1 domains resemble DNA binding regions of transcription factors, and are called “zinc fingers” in early studies, although they do not bind DNA [422]. Now it is widely accepted that these regions are designated C1A and C1B if they occur in the same molecule [424]. At the N-terminus, two cysteine-rich sequences are found in the conventional and novel isoforms and are present for DAG and phorbol ester binding [423]. The C2 region in conventional isoforms is present for Ca^{2+} binding, and is absent in all novel and atypical isoforms [423]. C-terminal regions C3-V5 are present in all PKC isoforms and are the catalytic domains [423]. The C3 region contains the ATP biding site, and only PKC ζ is slightly different than the others [423]. The most potent PKC

inhibitor drugs are designed to inhibit the ATP binding site [423]. The C4 region can reach the pseudosubstrate binding site, which is important for interaction with substrates, and with synthetic peptide analogues which have been demonstrated to act as potent inhibitors of PKC isoforms bind here [425].

The structures of PKC regulatory domains are important in that they determine the cellular localization and regulation of its activity for each isoform. Structure also determines whether PKC isoforms may be involved in cancers, cardiac and lung disease or diabetes among others, which make PKC isoforms molecular targets for disease therapies [422]. The regulatory domains of the conventional and novel isoforms have double C1 domains (C1a, C1b) and C2 domains. Conventional PKCs have C2 following the C1 domains, which allows Ca^{2+} and DAG binding. The novel isoforms, have C2 domain preceding the C1 domains, which does not allow for Ca^{2+} binding, but still allows DAG to activate these isoforms [422]. Atypical isoforms have no C2 domain and a single C1 domain, and there for are not Ca^{2+} or DAG sensitive. These isoforms contain PB1 domains which can only interact with other PB1 domain-containing proteins. The catalytic domains of all of the PKC families are closely related and contain the ATP binding sites, and are responsible for phosphotransferase activity [422]. Therefore, cellular localization and milieu will determine the physiological response of PKC activation.

PKC Activation

Activation and translocation of the PKC isoforms is facilitated by binding of activated PKC to anchoring proteins called Receptor for Activated C Kinases (RACKs). RACKs act as a scaffolding protein that brings the activated PKC into contact with its substrates [426]. Binding is selective and there may be more than one RACK for each isoform [423]. RACKs are not G proteins but resemble the Gb-subunit and share 50% homology with them [426]. RACKs also bind to IP_3 and IP_3 receptors and are critical for IP_3-mediated Ca^{2+} release [426]. Conventional isoforms bind via the C2-domain, and the C2-like region of the novel isoforms is where the PKC-RACK interaction occurs [423]. RACKs play an important role in translocating the activated PKCs from the cytosol to the membrane, and can modulate the stability and activity of PKCs by altering phosphorylation at the regulatory sites [427, 428].

There are 3 common ways of PKC activation; 1) Tyrosine phosphorylation on the activation loop [429], 2) DAG-mediated activation via translocation from the cytosol to the membrane and 3) cleavage in the V3 region to generate an active catalytic fragment that is no longer inhibited by the regulatory domain [430]. The phosphorylated form (activation method #1*)* is released into the cytosol and the pseudosubstrate can now access the active site [431]. The PKC then positions (via translocation) near the membrane to access DAG [249]. All inactive PKCs do not stay in the cytosol, some have been shown to reside in or near the nucleus, golgi, mitochondria or on actin [249] and upon activation are moved to the membrane. Translocation of PKC is isoform-, cell type- and activator-specific, and is tightly regulated by various cofactors (including RACKs) [432, 433].

PKC Signaling

PKCs are involved in many signal transduction pathways, and can be induced by phosphatidylinositol phospholipases (PI-PLCs), Phosphoinositide-3 kinase (PI3Ks) and Phospholipase D (PLD) [434]. Hormone/factor-receptor mediated activation of PI-PLCs catalyzes the formation of DAG and inositol triphosphate (IP_3) from Phosphatidylinositol 4,5-Bisphosphate (PIP_2); IP_3 promotes Ca^{2+} release from the endoplasmic reticulum, and the Ca^{2+} and DAG then promotes the activation of conventional and novel PKCs via translocation to the membrane [434]. PLD can also produce DAG through hydrolysis of phosphatidylcholine [435]. Upon activation, PKC can transmit signals through various signaling cascades, including the MAPK cascade, which involves Raf-1, MEKs and ERKs [421]. PKCs are able to reversibly activate downstream signaling molecules such as Raf-1 [436-438] and the Bcl-2 protein (anti-apoptotic) cascade [439]. The Raf-1 signaling cascade is involved in transduction through the cytoplasm to the nucleus, and overstimulation by PKC may contribute to overexpression of many genes, leading to excess cellular proliferation [421]. Additionally it has been suggested that PKC activates the Bcl-2 proteins which are believed to play an important role as a cytoprotective device against lethal stimuli [440], causing cells to undergo apoptosis. Figure 9 demonstrates the signaling pathways known to be associated with PKC ε expression.

PKC in the CL

It has been suggested that the lack of sensitivity of the porcine CL to PGF-2α before day 13 is due to a deficiency in PKC signaling [96]. The role of PKC as a mediator of PGF-

2α action during luteolysis has been studied in many species [189, 441-445]. PKC involvement in luteolysis was first demonstrated in the sheep by Wiltbank and colleagues This study showed a dose-dependant inhibition of progesterone secretion when treated with PGF-2α, however with pretreatment of PMA, the PGF-2α induced reduction of progesterone effect was abolished, suggesting that PGF-2α acts through PKC to decrease progesterone secretion during luteolysis [441]. When human luteal cells from the mid-late luteal phase were treated with cloprostenol, a PGF-2α analogue, a dose-dependant increase in PKC expression and activation was detected (measured by translocation of protein from cytosol to membrane, western blot analysis), suggesting PGF-2α acts in a similar manner in the human CL [446]. Sen and colleagues (2004) recently showed that PKC isoform expression in the bovine CL was stage-dependant, and more specifically that the *novel* (calcium independent) isoforms, particularly PKC ε, increased 11-fold after acquisition of LS, suggesting that this PKC isoform may be directly linked to LS [445]. In cell culture studies using a PKC ε isoform specific inhibitor, these authors also reported that PKC ε specifically plays a mediatory role in PGF-2α induced inhibition of steroidogenesis in luteal cells [447]. More recently, it was shown that PKC ε was exclusively expressed in luteal steroidogenic cells, and not endothelial cells, and that PKC ε was stimulated by Endothelin-1 (ET-1) in day 10 bovine CL [448]. Therefore these authors concluded that PKC ε plays a regulatory role by decreasing progesterone during regression [448]. In another series of studies in the rat CL, it was demonstrated that PKC δ, another novel isoform, was required for maintenance and function of the CL [449]. Additionally, it has been demonstrated that PGF-2α and PKCs induce cyclooxyengase-2 (COX-2) which acts in a positive feedback mechanism and functions to up regulate PGF-2α in the CL [199]. Tatsukawa and colleagues (2006) also suggest that intracellular pathways acting via PKC are related to regulation of luteal apoptosis and regression [450]. Therefore the expression and localization of PKC isoforms in the porcine CL could aid in understanding the control of LS as well as both functional and structural regression.

Conventional Isoforms (α, βI, βII, γ)

PKC Alpha

PKC α is involved in cell maturation and proliferation, and its activation can result in multilayer cell proliferation, as well as modulation of lateral cell-to-cell connections and transepithelial resistance (human intestinal epithelium) [451]. PKC α is known to be an upstream activator of the IκB kinase complex (IκK) [452], which leads to phosphorylation and subsequent degradation of IκB, which releases NFκB to migrate into the nucleus to bind to its response elements [453]. PKC α also mediates the actions of TNF-α in the stimulation of IκK and NFκB by epithelial cells [443], which suggests that PKC α is important in activation of other signaling pathways involved in induction of ubiquitin-proteasome proteolysis pathway and protein degradation [453].

Expression of PKC α in the epidermis of transgenic mice induces the expression of COX-2 and TNF-α and increases neutrophil accumulation [454]. PKC α also regulates cPLA2 activity and COX-2 expression to increase AA and PGE2 synthesis in skin [454]. PKC α expression is increased in breast cancers [455], and malignant gliomas [456], however PKC α is under-expressed in colon cancer cells [457]. These observations suggest that PKC α can act as an oncogene in some cells and can block tumor cell growth in others.

Naor and co-workers (1989) showed that activation of PKC α stimulated LH release from pituitary cells in vitro, and that PKC α may be overexpressed in the pituitary to facilitate exocytosis of peptide or protein hormones [458]. Orwig and colleagues (1994) observed PKC α expression in the bovine CL (day 8) mostly distributed in the cytosol versus the membrane [459]. These and other authors suggest that a mechanism similar to that observed in the pituitary [458], constitutive PKC α, may exist in the bovine CL to mediate the actions of PGF-2α to cause exocytosis of oxytocin-secretory granules from bovine CL (LLCs) [460].

PKC Beta I

It has been suggested that PKC βI protects barrier integrity by stabilizing microtubules and actin cytoskeletal through EGF or TGF-α signaling (in the human intestine) [461, 462]. It has also been reported that PKC βI is involved in stabilization of IκBα, which prevents NFκB activation, suggesting a possible role in apoptosis [463].

PKC Beta II

PKC βII has been demonstrated to be expressed (protein by western blot) in the bovine CL in both the early and mid CL [445]. Expression was significantly higher on day 10 versus day 4 of the cycle, and when luteal cells were treated with PGF-2α, PKC βII was responded to treatment (shown by increase in membrane protein) [445]. More recently, the same authors showed PKC βII expression on LLCs, SLCs and ECs of the cow, and they suggest that expression of this isoform may be responsible for PGF-2α's inability to elicit response in the early cycle [448].

PKC Gamma

It has been reported that PKC γ is expressed mainly in the brain and spinal cord [464], and that it is characteristic of normal CNS tissue [465]. Data has also been generated which shows that PKC γ is involved in insulin-like growth factor-induced colonic epithelial cell migration, suggesting a mediatory role in tissue repair [466].

Atypical Isoforms (δ, ε, η, μ, θ)

PKC Delta

PKC δ is known to promote both cell differentiation and apoptosis [467]. The activation of PKC δ and its associated apoptotic effects may occur via three separate mechanisms: 1) ERK and c-jun signaling induce PKC δ to translocate from the cytosol to the membrane where it mediates ultraviolet induced signaling transduction and apoptosis in JB6 cells [468], 2) Ceramide can induce PKC δ translocation to the golgi resulting in PKC δ activation through tyrosine phosphorylation [469], and 3) PKC δ is activated via nitration and then plays an important role in apoptosis by phophorylating and activating p53, causing degradation of MDM2 [469]. These authors suggest that nitration-induced PKC δ activation is a novel mode of cell death in Parkinson's disease [470].

PKC δ may mediate TNF-α induced cytotoxicity and apoptosis in intestinal barrier repair during the inflammation process [451]. Exposure to antioxidants (i.e. H_20_2) caused activation and translocation of PKC δ causing disruption of intestinal barrier [471], and PKC δ activation is tightly associated with iNOS upregulation leading to cytoskeletal oxidation and disfunction [472]. PKC δ has been shown to accumulate in the mitochondria where it induces cytochrome c release to induce apoptosis in human leukemia cells [473].

PKC Epsilon

PKC ε is involved in gene expression, neoplastic transformation, cell adhesion, mitogenicity and cellular motility [249]. It has also been associated with the nervous, exocrine, endocrine, and inflammatory and immune systems, and possibly may play a role in the etiology of Alzheimer's [474]. PKC ε is also oncogenic and may do so by activating the mitogenic signaling pathway involving Ras and Raf-1 kinase [249]. Decreased levels of PKC ε in breast cancer cells sensitize them to the apoptotic effects TNF-α, which suggests that PKC ε may normally block apoptosis [475]. Activation of PKC ε has been shown to protect myocytes from ischemia and reperfusion injuries [476], as well as protect against ischemia damage induced by glucose deprivation in neuronal cell lines [477].

Orwig (1994) was the first to report PKC ε expression in the bovine CL, and they showed that its expression was highly localized to the membrane (western blot analysis, versus PKC α in cytosol in same cells) [459]. However no cellular localization was performed to determine the luteal cells in which PKC ε was present, nor did they suggest a specific function for PKC ε in the bovine CL. More recently, Sen and coworkers (2004) showed that PKC ε was differentially expressed and increased 11-fold on day 10 versus day 4 in the bovine CL and they hypothesize that this specific isoform is responsible for the inability for PGF-2α to act on the CL prior to LS [445]. In cell culture studies using a PKC ε isoform specific inhibitor, these authors also reported that PKC ε specifically plays a mediatory role in PGF-2α induced inhibition of steroidogenesis in luteal cells [447]. They also demonstrated that PKC ε was exclusively expressed in luteal steroidogenic cells, and not endothelial cells, and PKC ε was stimulated by ET-1 in day 10 bovine CL, strongly suggesting that PKC ε plays a mediatory role in luteolysis [448]. A study in cardiac smooth muscle cells showed that chronic elevated ET-1 concentrations infused in vivo increased PKC ε (protein by western blot analysis) after 18 hours of infusion [478].

Nitric oxide-induced injury (in rat colonic mucosal cells) is associated with increased levels of PKC ε [479]. Aksoy and colleagues (2002) showed that PKC ε activation in human monocyte-derived dendritic cells is essential for IL-12 synthesis in response to LPS [423]. PKC ε is a critical component of TLR-4 signaling and therefore plays a key role in regulating macrophage and dendritic cell activation in the response of

the innate immune to pathogens [423]. Additionally macrophages from PKC ε null mice have a severely attenuated response to LPS, secrete reduced amounts of cytokines, and display a severe defect in NFκB activation [480].

Furthermore, PKC ε has been implicated in regulation of cell survival, as overexpression of PKC ε protected MCF-7 cells from TNF-α induced apoptosis [481] and promoted the survival of lung cancer cells [482]. More recently, it was shown that PKC ε expression is essential for the survival of glioma cells [483].

PKC Eta

PKC η is highly expressed in the epithelia, particularly squamous epithelium, where it associates with the cyclinE/cdk2/p21 complex to inhibit cdk-2 kinase activity, leading to G1 arrest [484]. PKC η knockout mice also show a high sensitivity to carcinogenesis, suggesting it is negatively involved in tumor promotion through stimulation of keratinocyte differentiation [484].

PKC Mu

PKC μ, also known as PKD, contains a pleckstrin homology domain (PH), is highly expressed in thymus and hematopoetic cells, and is the most novel of the PKC isoforms. However, PKC μ has a similar structure to the novel PKC isoforms, the novel-like conserved 1 (C1) domains and ability to bind DAG and PS and thus is grouped with them [422]. PKC μ has been implicated in signaling mechanisms controlling cell proliferation and apoptosis [485]. It additionally has been implicated in metastasis, immune response and golgi restructuring and function [485].

PKC Theta

PKC θ is expressed during T lymphocyte activation [486]. In addition, PKC θ acts to ensure the survival of activated CD4+ T cells that mediate immune responses by upregulating Bcl-xL [487].

Atypical Isoforms (ι, λ, ζ)

PKC Iota

IGF-I treatment of a porcine granulosa cell line (JC-410; which lack expression of gonadotropin receptors, but can still respond to forskolin and cholera toxin) significantly

increases PKC ι in nuclear extracts, which followed the same time course of activation of P-450scc by IGF-I [488]. Urban and collegues (2004) determined that PKC ι modulates the transcriptional activity of porcine P-450scc IGFRE through a mechanism independent of kinase catalytic activity [488].

In human ovarian cancer, both PKC ι and θ mRNA expression was significantly up-regulated compared with the normal ovary [489]. PKC ι is highly expressed in ovarian carcinomas compared with other human tumors, although even higher expression of PKC ι was found in some lung and prostate cancers [489]. PKC ι was significantly increased and associated with progression-free survival in ovarian cancer, and high expression of PKC ι was associated with increased cyclin E expression and proliferation in vivo [490]. In lung cancer, PKC ι is also increased, and its overexpression in lung cancer cells is required for their transformation [491]. Therefore, PKC ι is a potential oncogene in human cancers.

PKC Lamda

PKC λ can induce oxidant-like injury including cytoskeletal depolymerization and instability in intestinal cells [492]. Banan and colleagues (2004) showed that oxidants induce disruption of epithelial barrier integrity through activation of PKC λ [492]. Dominant-negative animals that did not express PKC λ were protected against all measures of oxidant-induced disruption, suggesting that PKC λ mediates oxidant (i.e. H_2O_2) damage in the intestine [492].

PKC Zeta

PKC ζ modulates intestinal barrier function, by protecting the intestinal barrier against oxidant-induced damage [493]. PKC ζ is an essential part of EGF-induced protection of intestinal barrier as when cells overexpress PKC ζ, they are protected against oxidants even without EGF [494]. Inhibition of PKC ζ causes loss of EGF protection against oxidants and loss of barrier integrity [494]. mRNA levels for PKC ζ have also been used as a marker for neoplastic activity of the colon [495], and low levels of PKC ζ mRNA were found in fecal samples in a animal model of colon cancer. ET-1 induces PKC ζ translocation to actin-rich microfilaments of the cytoskeleton in myometrial cells [496], and thus PKC ζ is thought to have a role in actin reorganization in ET-1 stimulated cells. PKC

ζ also mediates ET-1 induced myometrial contractions at the end of pregnancy in women [496].

Additionally, it has been suggested that PKC ζ is a direct target of both TNF-α and ceramide in U937 cancer cells [497]. PKC ζ is not regulated by DAG and phorbol esters, but is responsive to ceramide [497]. PKC ζ moves from the cytoplasm to the nucleus (not the membrane, as with other isoforms) in response to ceramide activation, which suggests that it may mediate transcriptional regulation induced by ceramide [498].

ET-1 and PKC

Incubation of rat smooth muscle cells with ET-1 increased PKC α (protein, measured by Western analysis) 3-fold within 48 hours in vivo, and in vitro concentrations of PKC ε protein were elevated after 18 hours of incubation with ET-1, with maximum expression at 72 hours [478]. PKC ζ was not changed after ET-1 treatment. PKC isoforms (α, δ, ε) were also shown to be translocated into the nucleus after 30 and 60 minutes of ET-1 incubation showing activation of several PKC isoforms by ET-1. After 6 hours of stimulation, no isoforms were translocated and all isoforms were in the cytoplasm, showing that long incubations periods do not increase PKC activation. Therefore in smooth muscle cells elevated ET-1 increased expression of PKC isoforms [478].

Apoptosis

Apoptosis, or programmed cell death, is characterized by nuclear and cytoplasmic condensation, internucleosomal DNA fragmentation [499] and the formation of apoptotic bodies [500]. Apoptosis is an evolutionarily conserved mechanism orchestrated by the genome-encoded proteins that form part of two distinct signaling cascades, the intrinsic and extrinsic cascades [284] (Fig. 10). The intrinsic cascade is thought to be activated by stimuli such as drugs, radiation, or growth factor withdrawal, causing an increase in mitochondrial membrane permeability; this in turn is caused by an increase in the ratio of pro-apoptotic to anti-apoptotic Bcl-2 family members [501]. The increase in mitochondrial permeability results in the release of proteins [i.e. apoptosis initiating factor (AIF-1), cytochrome c, procaspases, and heat shock proteins] from the intermembrane space into the

cytoplasm [502-504]. The release of these proteins activates caspases, a family of aspartic-acid specific cysteine proteases (-3,-7,-9) [505].

The extrinsic cascade is activated by extracellular signals (i.e. FasL, TNF-α) by ligation of specific receptors of the TNF family such as CD95/Fas/Apo-1, TNFR and the receptors for TRAIL (Death Domain containing proteins, DR4, DR5) [506]. Ligand binding of the trimerized receptor recruits adaptor molecular such as FADD and TRADD to the death-inducing complex (DISC) and activates Caspase-8. Activated Caspase-8 is responsible for the activation of further downstream signaling [506] including the effector Caspases -3, -7 and -9 to cleave various cellular substances such as actin, poly(ADP-ribose) polymerase (PARP), fodrin and lamin that contribute to the morphological changes seen in apoptotic cells [507].

The final cellular event in apoptosis, whether apoptosis is initiated by the intrinsic or extrinsic pathway, is DNA fragmentation. This is mediated by internucleosomal cleavage of DNA by endonucleases (Caspase-activated DNase, Nuc70, endonuclease G) activated by Caspase 3, to yield small DNA (180-bp) fragments which are considered to be the hallmark of cellular apoptosis [284].

Apoptosis in the CL

Apoptosis in the CL is characterized by the loss of steroidogenic potential and luteal cell death [202, 508] and is believed to be initiated by PGF-2α [202, 306-308], FasL [242, 311], TNF-α [253] IFN-γ [242] and IFN-γ and TNF-α in combination [509]. Luteal cell apoptosis thought to be a key process in structural regression of the CL [202, 306-308]. In the CL, the intrinsic cascade is thought to play an important role in controlling the rate of apoptosis [501]. Apoptotic family members such as Bcl-2 (anti-apoptotic), Bcl-x (anti-apoptotic) and Bax (pro-apoptotic) have all been found to be expressed in the human [510-512], canine [513], rat [514], and bovine [515, 516] CL . An apoptosis-inducing member of the Bcl-2 family is Bax, which forms homodimers with itself and then heterodimerizes with Bcl-2; this complex subsequently acts on the mitochondrial membrane to form pores which allow mitochondrial proteins (e.g. cytochrome C, APAF-1) to escape [517]. Increased expression of Bax (which counteracts Bcl-2 death repressor activity; [517]) relative to Bcl-2, has been correlated with the occurrence of apoptosis in granulosa cells during atresia, and in bovine and human luteal cells during regression [518-520].

In addition to TNF-α's role in the control of the acquisition of LS, TNF-α has been shown to induce apoptosis in bovine endothelial cells, after the decline in progesterone production [253]. This was shown by both morphological and biochemical measures of apoptosis including shrunken nuclei, DNA fragmentation and activation of caspase-3. However neither Fas ligand nor PGF-2α altered the viability of these cells [274]. TNF-induced cell death was mediated by the MAPK family (ERK, p38 and JNK), as well as via activation of sphinogmyelinase and ceramide [274]. Treatment with ceramide itself increased JNK and induced EC apoptosis [274]. Therefore this data suggests that TNF-α, or a member of this family may mediate the actions of PGF-2α-induced regression of EC during structural regression of the bovine CL. In the pig, Wuttke and colleagues (1993) demonstrated TNF-α stimulated apoptosis in luteal cells, *in vitro* [521]. However, the exact mechanism or which cell type TNF-α acts to induce apoptosis in porcine luteal cells has not been determined.

In addition to PGF-2α's effect on steroidogenesis, PGF-2α is also thought to stimulate apoptotic pathways in many species (pig- [202], cow- [309], [306], ewe- [307]). Treatment of the bovine CL (in vivo) with PGF-2α has been associated with the onset of apoptosis [284, 309]. Mice treated with PGF-2α 48 hours post ovulation displayed a 22-fold increase in caspase-8 activity (initiator Caspase), even though the PGF-2α receptor is not known to be coupled with caspase-8 activity [311]. Carambula and colleagues (2002) showed that activation of caspase-3 (effector caspase) is required for structural involution of the mouse CL [522]. More recently, the same authors demonstrated that caspase-3 deficient mice were resistant to apoptosis induced by PGF-2α *in vivo*, and that caspase-3 was a required mediator of PGF-2α induced luteolysis [311]. They additionally suggested that PGF-2α initiated luteolysis at least in part, involved increased bioactivity and bioavailability of cytokines such as FasL to activate caspase-3 driven apoptosis [311]. In the bovine CL, treatment of luteal cells with sFasL, *in vitro*, activated the SM pathway, as measured by ceramide production, whereas treatment with PGF-2α had no effect on ceramide [523]. These data suggest that PGF-2α increases FasL which in turn activates the SM pathway, producing ceramide to drive luteal apoptosis.

Fas and FasL

Fas ligand (FasL), produced by T lymphocytes, is a member of the TNF super family and is recognized to have apoptotic actions in the CL [201]. Fas antigen (Fas) is a cell surface receptor that when bound to FasL, induces apoptosis [243, 524] and structural luteolysis [201]. The Fas signaling pathway involves protein-protein interactions that result in a cascade of protease activities, cleavage of cellular substrates and cell death [525]. The apoptotic signaling mechanism involves FasL binding to Fas, and the recruitment of FADD, an adaptor protein which then recruits the initiator caspase-8 [526]. The caspase cascade (discussed above) continues, leading to DNA fragmentation and cell death [527].

Fas and FasL in the follicle

Fas and FasL are expressed in granulosa and thecal cells of the follicle in the cow [528] and rat [529], and activation of Fas/FasL is implicated as a key event in follicular atresia [530, 531]. Mice carrying the mutation of lymphoproliferation (*lpr*), in which Fas is extremely reduced, or generalized lymphoproliferative disease (*gld*), which lacks functional FasL, develop lymphadenopathy and splenomegaly, and can suffer from autoimmune diseases such as nephritis and vasculitis [532]. In addition, mice which carry mutations in Fas (*lpr*) or FasL (*gld*) show abnormal follicular development and irregular CL regression [530]. Furthermore, some of the *lpr* and *gld* mice show mono- and polycystic ovaries. These data suggest that Fas/FasL plays an important role in follicular apoptosis, and therefore may control luteal apoptosis in a similar manner.

Fas and FasL in the CL

Fas and FasL are expressed during the luteal phase in rat and mouse CL [530], and activation of Fas occurs around the time that luteolysis [533, 534] and atresia occur [243, 528, 535-537]. Additionally, Fas is expressed in human [538] and bovine CL [242] and at higher levels in the regressed CL (mouse [530], human [539]). FasL in rat was also elevated in the regressing rat CL [534]. In the bovine CL, *in vitro* treatment with sFasL activates the SM pathway, as measured by increased ceramide production, whereas treatment with PGF-2α had no effect on ceramide [523]. Treatment with sFasL decreased the numbers of apoptotic cells by 35%, and treatment with C-8 ceramide resulted in 90% of cells showing apoptosis within 24 hours of treatment. These authors suggested that C-8 ceramide affected all cell types in culture, whereas only those cells which express Fas were responsive to

sFasL. Thus they proposed that Fas/FasL and ceramide play a critical role in luteal apoptosis [523]. The detailed mechanisms by which Fas/FasL mediate regression of the CL has not yet been determined in many species [201, 523], nor has Fas/FasL expression and its affects on SM signaling been examined in the porcine CL.

Nitric Oxide (NO)

NO is a short-lived (a few seconds) free-radical gas that has the ability to diffuse freely through biological membranes [540]. NO is involved in vasodilation, reducing smooth muscle cell proliferation, controlling apoptosis [506, 541] acting as a neurotransmitter [540], among other functions. NO, generated by immune cells, is a functional part of the hypothalamus-pituitary-gonadal axis, and plays a role in luteal development and function [240, 542-547]. Endogenous NO is synthesized and regulated from L-arginine by a family of NO synthase (NOS) isozymes [endothelial NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS)] [548]. nNOS and eNOS are constitutive and are responsible for basal release of NO, whereas iNOS is expressed in response to cytokines (i.e. TNF-α) and lipopolysaccarides [549]. eNOS and nNOS are activated by increases in Ca^{2+}, and therefore release of NO is within several minutes, whereas iNOS is calcium-independent, responds to immune or inflammatory signals and produces large amounts of iNOS for up to several days [550].

The activity of NO can be divided into cGMP dependant and cGMP independent pathways [372]. NO can affect cellular functions through both posttranslational modifications of proteins directly (nitration, nitrosylation), and indirectly (methylation and ribosylation), the main physiological signaling pathway of NO is activation of guanylate cyclase, formation of cGMP, and concomitant protein phosphorylation [551].

NO can induce apoptosis in several cell types, including the ovary, and most of the four pathways by which this occurs are independent of cGMP [506], but all of which result in apoptosis via the mitochondrial pathway. The four ways in which NO can act as a pro-apoptotic inducer are 1) Directly inducing cytochrome c release through the loss of mitochondrial membrane potential [506]. Cytochrome c activates the caspase-dependant signal cascade, via generation of superoxide which reacts with NO to form peroxynitrate to induce cytochrome c release. This tends to occur in abnormal cells (cancer and leukemia

cell lines) rather than in normal cells [552], 2) Activation of caspase through NO-induced p53 expression due to DNA damage [553], NO induces p53 accumulation through p21 upregulation or by inducing apoptosis by increasing the ratio of Bax/Bcl-xL, cytochrome c and caspase activation [553]. 3) Activation via NO donors or NOS of JNK/SAPK and p38MAPK which activates caspase 3 [554-556], and 4) NO increasing ceramide levels through sphingomyelinase (SMase) and caspase-3 activity [557, 558].

NO can also act as an anti-apoptotic factor by protecting cells from apoptosis induced by TNF-α, oxidative stress and serum or glucose deprivation [506]. These effects depend upon cell type. NO/cGMP can prevent apoptosis in hepatocytes, neuronal cells, embryonic motor neurons, B lymphocytes, eosinophils, and ovarian follicles [506]. Additionally, NO and cGMP protect lymphocytes from increasing Bcl-2 [559] and Akt/PKC activation, as well as gene expression through NFκB activation [506]. In some of these cell types, NO suppresses cytochrome c release, ceramide generation and caspase activation by cGMP production [506]. NO can also act in a anti-apoptotic manner by modifying a cystine at the enzyme catalytic site on all capases by nitrosylation [560], and therefore capases can not be activated. NO can also induce expression of several cytoprotective genes such as HSP70 and HSP32 which protects cells from apoptosis [506]. This occurs via HSP70 inhibiting Apaf-1 by associating with the caspase recruitment domain (CARD) of Apaf-1 and thus preventing apoptosome formation [561].

NO in the Ovary

Data suggests that NO plays an important role in ovarian physiology including roles during follicle growth, ovulation and CL function [540]. NO is generated by several ovarian cell types, including granulosa cells (reviewed in Tamanini [559]), as well as luteal endothelial cells [139] and in macrophages [562]. NO is known to enhance the activity of cyclooxygenase (COX), stimulate prostaglandin production [563], and NO donors decreased progesterone secretion by human granulosa cells *in vitro* [564]. eNOS is the constitutively expressed calcium-dependent endothelial isoform of NOS, and NO it produces is the most important relaxing factor to blood vessels and is essential for maintaining normal vascular resistance [565]. In the ovary, NO data is often in conflict depending on species and cell type.

In pig granulosa cells, eNOS expression has been confirmed, although iNOS has not been detected and little is known of the exact functions of the NO synthases in the pig ovary [565-567]. For example studies have been carried out in the pig to determine NO effects on steriodogenesis, and most data shows that NO exerts negative effects, possibly through a direct action on steroid-secreting cells [566-568].

NO in the CL

There are conflicting reports of NO regulation and action in the CL, depending on stage of development and species. In a study by Jo and collegues (1995), IFN-γ and TNF-α synergistically increased NO production in mouse luteal cells, and they suggest that NO produced from multiple cytokines play a role in decreasing progesterone in luteal cells [569]. In mid-stage rat CL, NO stimulates progesterone production via iNOS mediated-NO [543, 544, 570]. NO produced by luteal endothelial cells increases blood flood and increases VEGF production in the sheep [106], rabbit [241, 571] and rat [572, 573].

NO has also been suggested to play a role in luteolysis, as NO stimulates PGF-2α synthesis (human, [546], bovine [509]), this in turn increases NOS activity and acts via a positive feedback mechanism (rabbit [240], rat [547]), to further increase PGF-2α, resulting in further decreases in progesterone production in the rat [573] , rabbit [240, 241] and bovine [545] CL. Inhibition of NO counteracts spontaneous and PGF-2α-induced luteolysis [509], which suggests NO plays a significant role in LS. Skarzynski and colleagues (2005) also showed that TNF-α and NO induce apoptosis in by increasing expression of bcl-2 family genes and stimulate expression and activity of caspase-3 [509].

However, NO can also play an anti-apoptotic role in response to TNF-α [574] by blocking caspase and bid activation as shown in human alveolar epithelial cells. NO can also suppress NFκB action by inducing and stabilizing IκB in human ECs [575]. These actions may explain some of the conflicting reports in the CL, as NO can inhibit TNF-α's apoptotic actions in the early CL, and therefore ceramide and other pro-apoptotic pathways are not activated, but as the CL ages, it may acquire the ability to respond to NO and act on steriodogenic cells (i.e. by increasing PGF-2α) and promotes apoptosis during the late stage, suggesting a "switch" in TNF-α mediated actions from the early to the late luteal stage.

In a recent study in the bovine CL, iNOS and eNOS expression (mRNA, eNOS protein) were high in the early luteal stage and were progressively reduced later in the cycle [576]. PGF-2α further reduced the levels of NOS expression. These authors suggest that the elevated levels of NO, suggested by the elevated NOS levels, is likely to play a role in the varied stages of angiogenesis by modulating vascular hyperpermeability, migration, proliferation, and organization of ECs into a network structure [576]. Grazul-Bilska and colleagues (2006) hypothesize that a decreased level of eNOS may be required for luteolysis, whereas greater eNOS expression may be necessary for angiogenesis and maintenance of blood vessel function during the development of the CL [577], a hypothesis which is also shared by Motta and colleagues (2001), who also observed similar trends in the rat CL [542].

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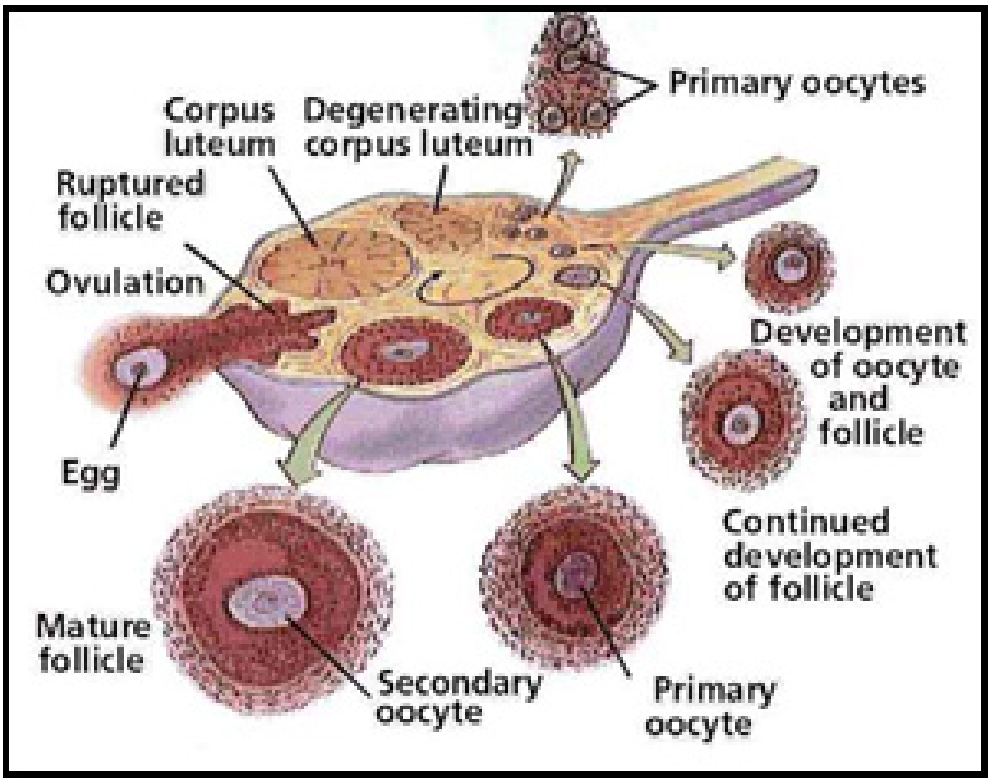

Figure 1. Follicular Development

http://www.infertilityfriends.org/images/13a_ovarianfollicle.jpg

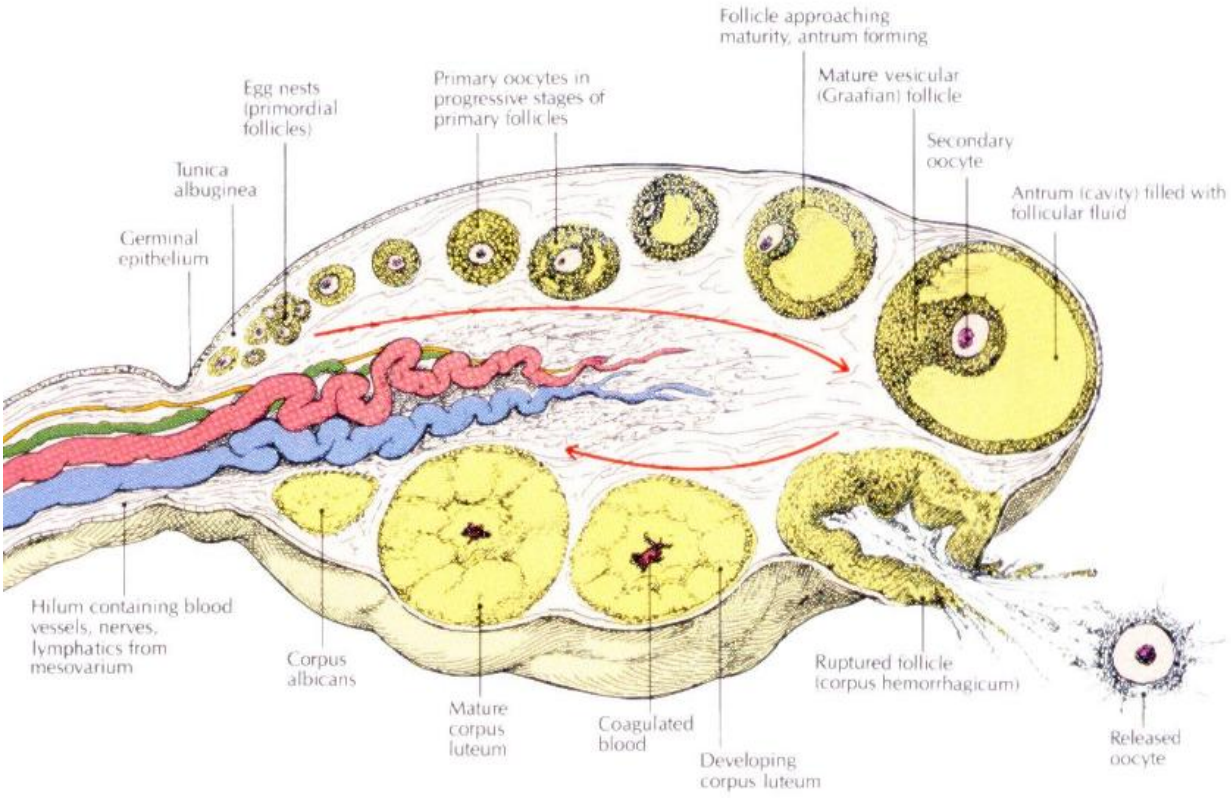

Figure 2: The ovarian cycle

http://faculty.etsu.edu/currie/images/follicle3.jpg

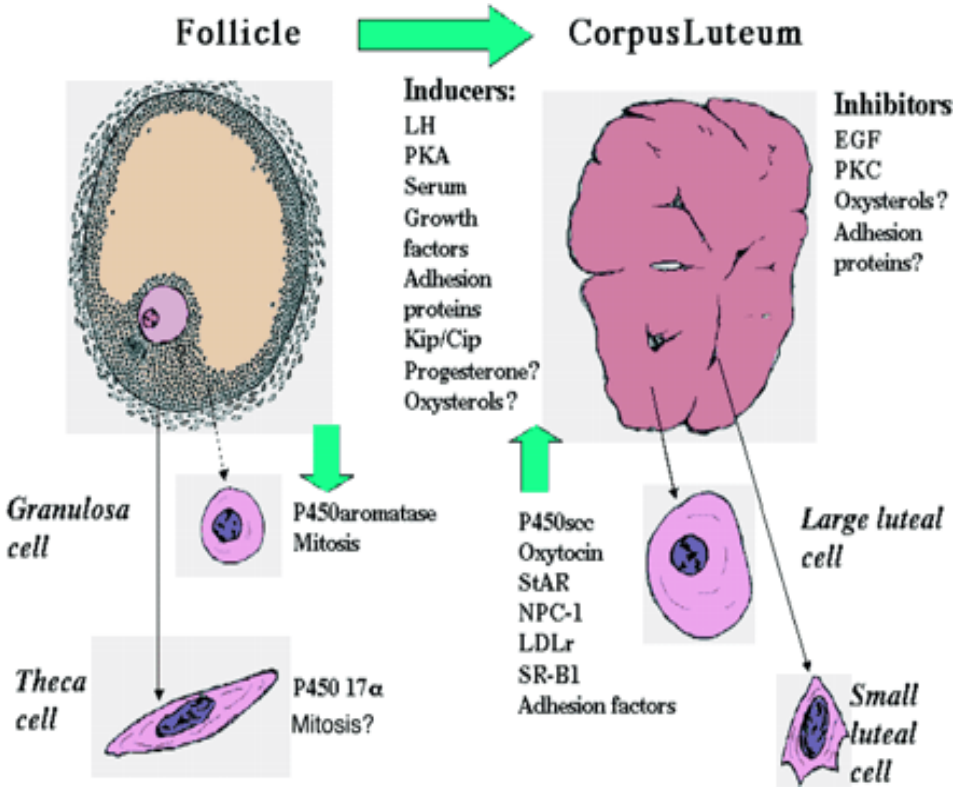

Figure 3: Model of Lutenization and potential regulatory mechanisms, Murphy, 2001

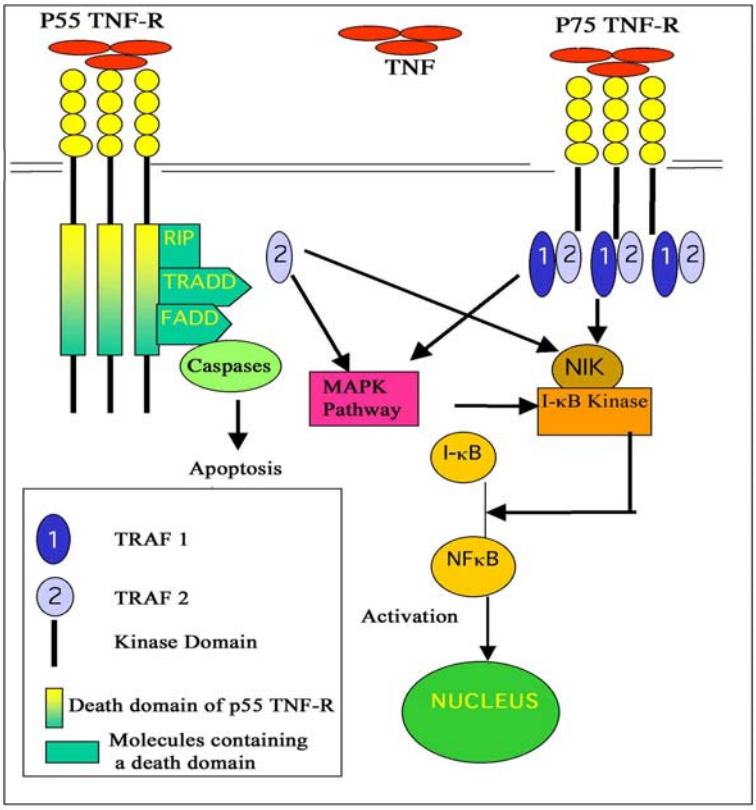

Figure 4: TNF-α Receptor Structures and Signaling Pathways
http://www.cancer-therapy.org/volume2_2004/html/17_Waterston/Image1.jpg

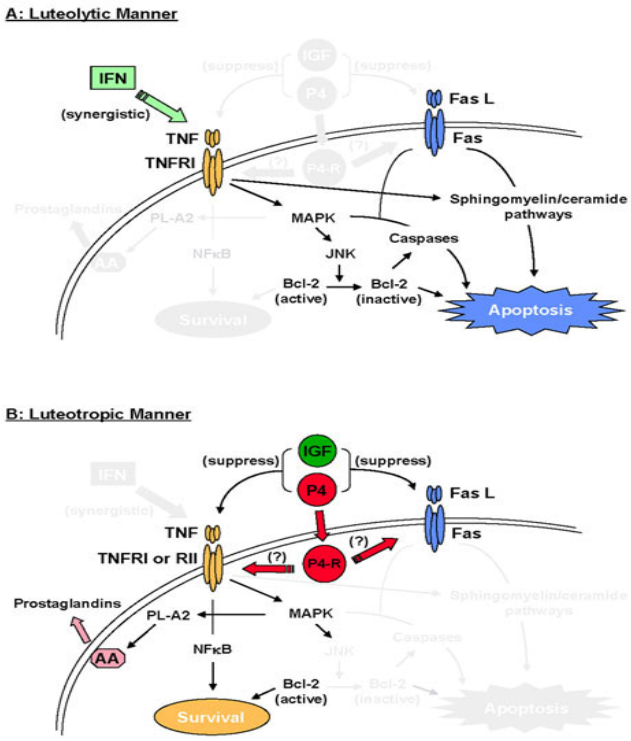

Figure 5: TNF-α signaling mechanisms: A) TNF-α acts in a luteolytic manner; B) TNF-α acts in a luteotrophic manner, Okuda et al, 2003

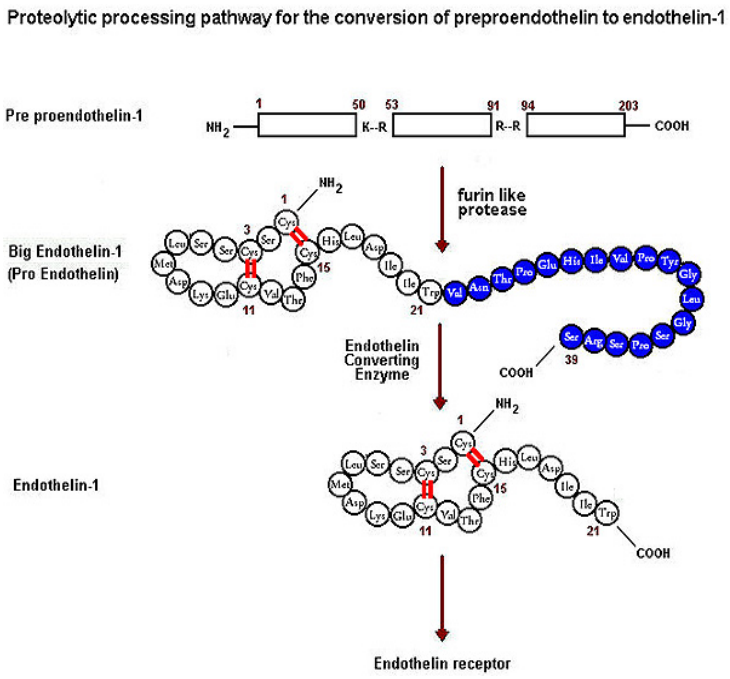

Figure 6: Endothelin Processing Pathway
http://www.bioscience.org/2003/v8/e/1103/figures.htm

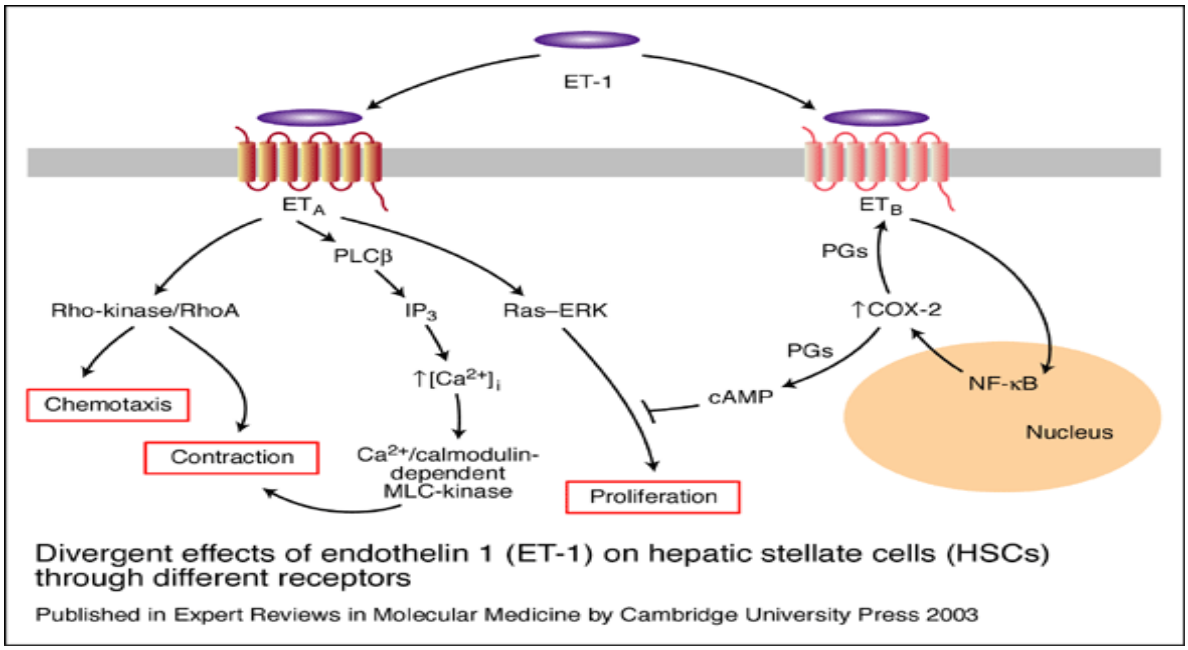

Figure 7: Endothelin Signaling Pathway

http://www-ermm.cbcu.cam.ac.uk/03005738h.htm

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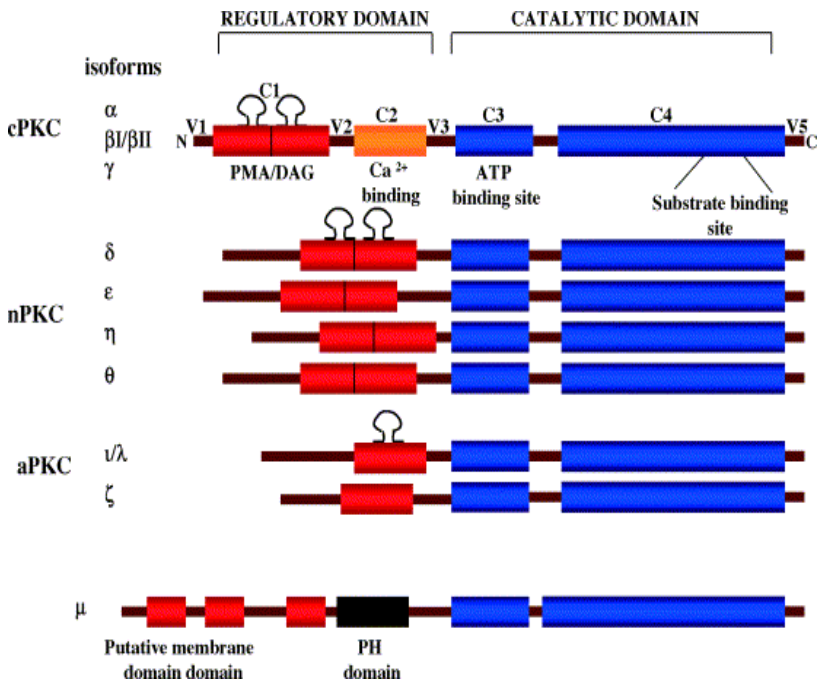


Figure 8: PKC isoform Structures, Mochly-Rosen & Gordon, 1998

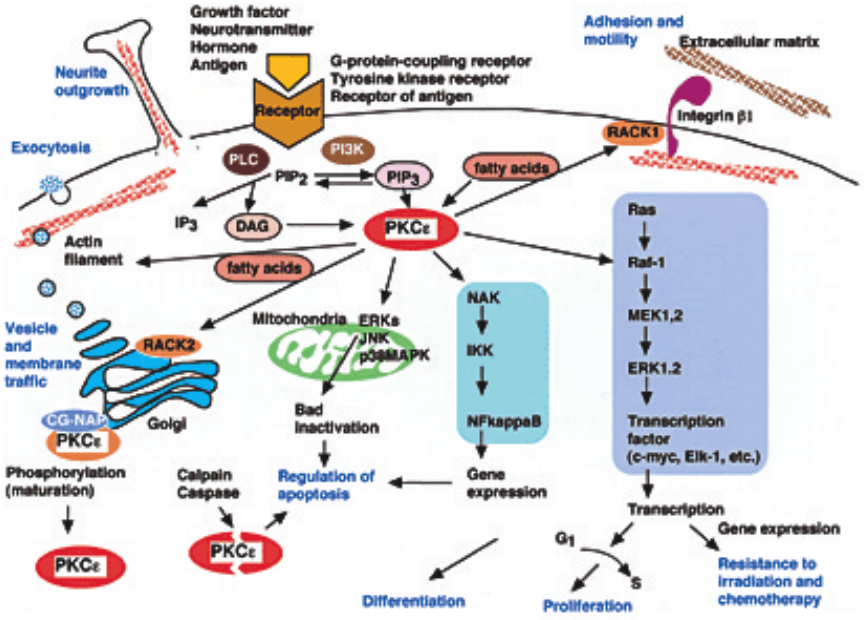
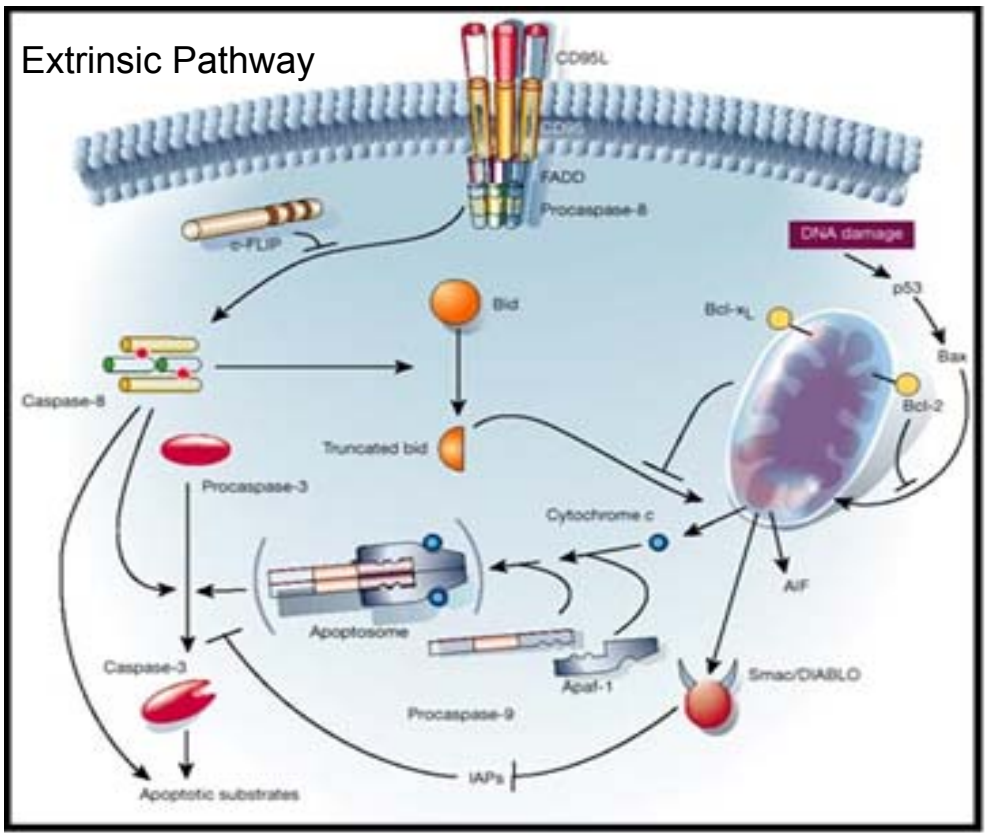


Figure 9: PKC ε Signaling Pathway

http://wwwsoc.nii.ac.jp/jbiochem/jb/132-6/6fcbgpf2.htm



Intrinsic Pathway

Figure 10: Apoptosis Signaling - Extrinsic and Intrinsic Pathways

http://people.bath.ac.uk/pr1cemb/Apoptosis.htm

Overall Rationale

Based upon the data discussed above, we hypothesized that the presence of TNF-α (due to an influx of macrophages) in the early to mid cycle porcine CL may play a critical role in regulating LS in the pig. We propose that TNF-α plays a role much earlier in the cycle than previously suggested, and stimulates the Endothelin "system", by increasing expression of ET-1 and upregulating ET_A. We further propose that ET-1 action (possibly on LLCs) increased PKC isoform expression (particularly PKC ε) to mediate LS in the porcine CL. Additionally we propose that genes associated with apoptosis (Bax, Bcl-x, Fas, FasL) also may play a role in LS by being regulated during the early stage of the estrous cycle. Therefore we designed several experiments to test these hypotheses and propose the cell model in Fig.1:

1. Does TNF-α sensitize porcine luteal cells to PGF-2α in vitro?
2. Does the Endothelin-system (ET-1, ECE-1, ET_A) play a role in mediating LS in the porcine CL?
3. Does activation of particular PKC isoforms (specifically PKC ε) mediate LS in the porcine CL?
4. How does the regulation of apoptotic genes play a role in mediating LS in the porcine CL?

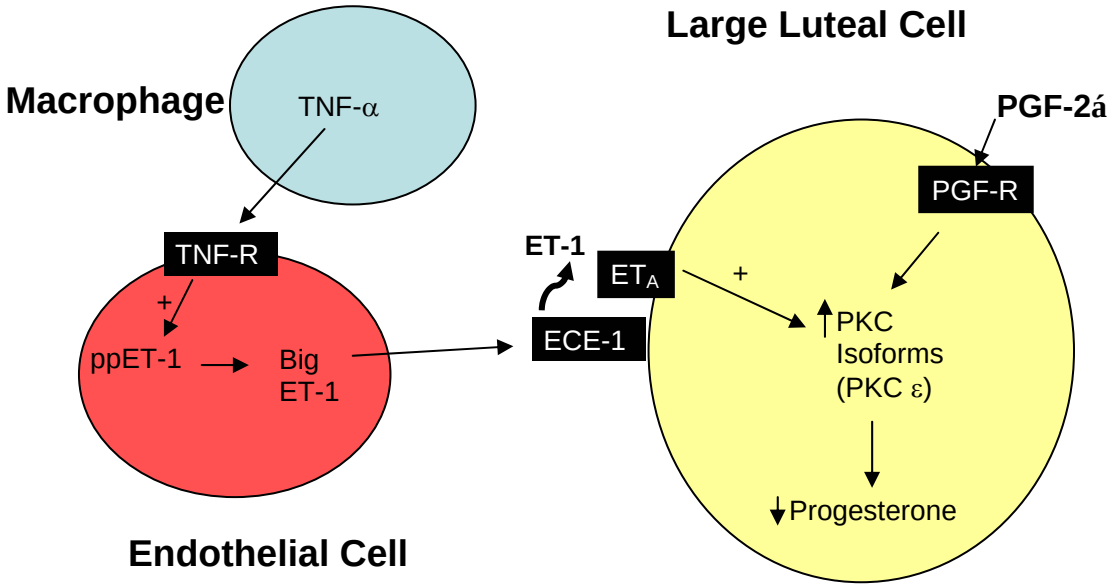


Figure 1: Proposed Cell Model: Acquisition of Luteolytic Sensitivity in the Porcine Corpus Luteum

In the early-mid CL, macrophages infiltrate the CL, secreting TNF-α. TNF-α acts on its receptor (presumably on ECs) to increase ppET-1 expression. ppET-1 protein is produced and ECE-1 cleaves Big ET-1 at the LLC membrane, producing bioactive ET-1. ET-1 then acts on its ET_A receptor (on LLCs) to increase PKC expression and activation. PKC then mediates PGF-2α response causing a decrease in Progesterone secretion and initiation of luteolysis.

Tumor Necrosis Factor - α Sensitizes Porcine Luteal Cells to Prostaglandin F-2α[1]

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ABSTRACT

Prostaglandin F-2α (PGF-2α) has long been recognized as the physiological luteolysin in the pig, although the porcine corpus luteum (CL) lacks sensitivity to the luteolytic actions of PGF-2α until days 12-13 of the estrous cycle. However the mechanism by which the CL acquires luteolytic sensitivity to PGF-2α (LS) is not understood. Tumor necrosis factor (TNF)-α is well known to play a role in luteolysis, however recent studies showed a significant (4-fold) increase in the influx of luteal macrophages and their capacity to secrete TNF-α, between days 4-6 and 8-12 of the estrous cycle in the pig. Thus we hypothesized that TNF-α may play a role in the development of LS which occurs between days 7 and 13 of the estrous cycle. Therefore in this study, we proposed to determine whether treatment of day 4 luteal cells in culture with TNF-α would sensitize them to the luteolytic effects of PGF-2α in vitro. CL collected on day 4 of the estrous cycle (day 0 of culture) were dissociated and the luteal cells obtained were placed in culture for 2 days to facilitate attachment. On day 2 of culture, TNF-α (0, 0.1, 1, 10 ng/ml) was added (TNF-α "priming"), and on day 4 of culture luteal cells were "challenged" with PGF-2α (0, 0.1, 1, 10 ng/ml); media and cells were collected on day 4 for examination of Endothelin (ET)-1 (media), and/or ET_A and protein kinase C isoforms (cells; protein by Western blots), and on day 6 for determination of progesterone (media), and/or ET_A and protein kinase C isoforms (cells). Our results showed that priming with as little as 0.1 ng/ml of TNF-α, sensitized luteal cells to PGF-2α at levels as low as 0.1 ng/ml, as determined by a significant decrease in medium progesterone concentrations, measured by RIA on day 6. In addition, TNF-α dose-dependently increased ET-1 secretion, Endothelin Receptor A (ET_A), PKC-βII and PKC-ε expression (Western blots) on day 4. TNF-α priming (followed by control challenge), also showed a dose dependent (based on TNF-α dose) stimulation of ET_A, but decreased PKC βII and had no effect on PKC-ε (protein) expression, as measured on day 6. ET-1 itself was not capable of inducing LS, and neither TNF-α nor ET-1 had sensitizing actions on purified large luteal cells. These studies suggest that TNF-α plays a role in the acquisition of LS in porcine luteal cells, and that ET-1/ET_A may be involved, although this mechanism appears to involve the interaction of large luteal cells with one

or more other luteal cell types. Furthermore, TNF-α's sensitizing actions were associated with an up-regulation of PKC-βII and -ε, suggesting a role for these PKC isoforms. Overall, these findings support data from the bovine CL in which both ET-1/ET_A and PKC-ε are believed to be involved in control of LS in that species. Finally, these studies demonstrate an important and very sensitive porcine luteal cell culture system, which can be utilized to test the signaling pathways and mechanisms involved in the acquisition of luteolytic sensitivity (LS) in the pig.

INTRODUCTION

The porcine corpus luteum (CL) is unusual in that it does not show a complete luteolytic response to an exogenous dose of PGF-2α until after day 12 of an 18-21 day cycle [1-3]. This is in marked contrast to other farm animal species in which luteolysis can be induced after about 6 days of the estrous cycle [4, 5]. It has been suggested that the acquisition of luteolytic sensitivity (LS; ability to respond to the luteolytic effects of PGF-2α) in the pig is not due to a lack of PGF-2α receptors, as they are present and functional as early as day 5 [6, 7], but may be due to a lack of post-receptor signaling (i.e. Protein Kinase C) [8, 9]. Recent data has shown that there is a 4-fold increase in the influx of macrophages into the porcine corpus luteum and in the capacity of macrophages to secrete TNF-α, between the days 4-7 and 8-12 luteal phase [10]. Macrophages are the primary source TNF-α and have been suggested to play a key role in inducing structural luteolysis at the end of the cycle [11], however the presence of macrophages and TNF-α secretion during the early estrous cycle suggests that TNF-α may play a role much earlier than has been previously realized. In particular, we hypothesized that the acquisition of LS occurs between days 7 and 13, and in view of the temporal association between the appearance in elevated macrophages/TNF-α and the onset of LS, we further hypothesized that TNF-α may play a critical physiological role in promoting LS in the porcine corpus luteum. Thus in the present study we proposed to examine whether TNF-α was capable of inducing the sensitivity of porcine luteal cells to PGF-2α in an in vitro cell culture model, to begin to address this hypothesis.

TNF-α, produced by macrophages, is a 17 kDa non-glycosylated protein [12], and depending upon the cell type, TNF-α can have a wide variety of effects such as stimulating cell proliferation (marmoset luteal cells) [14] and cell differentiation, eliciting inflammatory responses, and promoting apoptotic or necrotic cell death [15]. In the CL, there is an abundant literature supporting the view that TNF-α has key roles in both functional and structural luteolysis by inhibiting steroidogenesis [11, 16], stimulating luteal PGF-2α synthesis [16-18], and activating apoptosis [19-21]. However, as described above, the numbers of luteal macrophages, and their capacity to produce TNF-α, have been shown to dramatically increase (four-fold) between the early (day 4-6) and mid (day 8-12) stages of the porcine estrous cycle, further increasing an additional 1-2 fold in the late luteal stage [10]. These data indicate the macrophage secreted TNF may play a role much earlier in the luteal phase that previously believed. Moreover, TNF-α receptors have been found to be present as early as day 4, remain unchanged in the porcine CL until day 12 of the cycle, but were increased on day 15 [22, 23]. TNF-α receptors have been located on small luteal cells (SLC), large luteal cells (LLC) and endothelial cells (EC) in the porcine [21] and bovine CL [19]. TNF-α presumably acting via its receptor (TNFR), acts to increase the production of both ET-1 and PGE-2, by bovine luteal ECs in vitro [24]. Therefore TNF-induced ET-1 secretion by EC may serve to influence the steriodogenic capacity of the luteal (i.e. SLC, LLC) cells [25]. In view of this association between TNF-α and ET-1, we also examined ET-1 and its receptor ET_A as possible mediators of the luteolytic sensitizing actions of TNF-α, in our current studies, especially as ET-1/ET_A has also been implicated in the control of LS in the cow (see below).

Endothelin-1 (ET-1) is synthesized in endothelial cells from a 212-amino acid precursor, pre-proendothelin-1 (ppET-1). ppET-1 is then cleaved into a biologically inactive, 38-amino acid peptide, Big-Endothelin (Big ET-1) by an endopeptidase [26], and then is secreted from the cells. Endothelin-converting Enzyme-1 (ECE-1) cleaves Big ET-1 into the biologically active ET-1 at the target cells (i.e. via a membrane associated ECE-1 isoform (ECE-1b) in LLC) [27]. ET-1 exerts its effects by interacting with Endothelin receptor A (ET_A) or Endothelin receptor B (ET_B), which are both G-coupled transmembrane receptors [28] and are expressed in the porcine CL [29, 30]. ET-1 has

been shown to play a role in luteolysis [31], and it inhibits progesterone release in both granulosa and luteal cells in many species [32-35]. ET-1 has also been shown to enhance PGF-2α production in the human and rabbit CL [36], and there is evidence in the cow [31, 33, 37-39] and sheep [35], which suggests that ET-1 interacts with PGF-2α in the control of luteolysis. In addition, Meidan and colleagues (1999) have proposed that ET-1 acting via $ET_{A,}$ may play a role in control of LS in the cow, providing an additional rationale for studying the ET-system in our current study [39].

PGF-2α is known as the physiological luteolysin [40-42], however the detailed cellular and biochemical events associated with luteolysis remain largely uncharacterized [43] particularly in the pig. In many species, including the pig, PGF-2α is produced by the uterus and acts on the CL to cause functional (decreasing steroidogenesis) and structural (regression of the CL) luteolysis [6]. PGF-2α is also produced intra-luteally, however this only occurs in CL that show LS (i.e. PGF-2α responsive, after day 12-13 in the pig) [8, 44].

Sen and colleagues (2004) recently showed that PKC isoform expression in the bovine CL was stage-dependant, and more specifically that the *novel* (calcium independent) isoforms, particularly PKC ε, increased 11-fold after acquisition of LS, suggesting that this PKC isoform may be directly linked to LS [9]. In cell culture studies using a PKC-ε isoform specific inhibitor, these authors also reported that PKC ε specifically plays a mediatory role in PGF-2α induced inhibition of steroidogenesis in luteal cells [45]. More recently, it was shown that PKC ε was exclusively expressed in luteal steroidogenic cells, and not endothelial cells, and that PKC ε was stimulated by ET-1 in day 10 bovine CL [46]. Therefore these authors conclude that PKC ε plays a regulatory role by decreasing progesterone (P_4) during regression.

Although the control of luteolytic sensitivity has been studied in other species (guinea pig [47], pig-[48], sheep-[49], cow-[50-52]), it is not well understood in the porcine CL. As discussed above, both ET-1/ET_A as well as PKC ε have been implicated in control of LS in the cow. Thus in the present study we examined the sensitizing effects of both TNF-α and ET-1 in cultured porcine luteal cells to the luteolytic effects PGF-2α. We provide evidence that suggests that TNF-α sensitizes cells to PGF-2α, and increases ET-1 production, expression of ET_A and increases PKC expression. Therefore, the

presence of macrophages and TNF-α in the early (day 4-6) to mid (day 8-12) cycle may be critical for controlling acquisition of luteolytic sensitivity in the pig. Thus these studies were undertaken with a view to improving our understanding of the control of LS which is critical for the development of new approaches to estrous cycle regulation in this species.

MATERIALS AND METHODS

Chemicals and Reagents

Ketamine, xylazine and halothane were purchased from Webster Veterinary Supply (Sterling, MA). Collagen I plates, M199, Penstrep, Gentamycin Sulphate (Biotech Research Grade) and Fungizone were purchased from Fisher Scientific (Fairlawn, NJ). ITS was purchased from BD BioSciences (San Jose, CA). TNF-α was purchased from R&D Systems (Minneapolis, MN). Collagenase (Type IV) was purchased from Worthington Biochemical Corp. (Lakewood, NJ). Trypsin was purchased from CellGro (Herdon, VA). Endothelin EIA was purchased from Assay Designs (Ann Arbor, MI). Progesterone RIA kits were purchased from Diagnostic Product Corp. (Los Angeles, CA). 4-12% Bis-Tris gels, molecular weight markers, Fetal Calf Serum and Alexafluor secondary antibodies for immunofluorescence were purchased from Invitrogen (Carlsbad, CA). PVDF membrane was purchased from Pall Company (Pensacola, FL). Antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) (PKC isoforms) or Chemicon (Temecula, CA) (ET_A). ECL kits were purchased from GE Healthcare (Piscataway, NJ). Unless noted all other chemicals and reagents were purchased from Sigma (St. Louis, MO).

Animals

Cycling gilts (White Landrace X Black line hybrid) were obtained from the herd at 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 day 4 of the cycle. On the day of surgery, animals were anesthetized with i.v. injection of Ketamine (2.2 mg/kg) and Xylazine (0.4 mg/kg), and gilts were then maintained on halothane. Ovaries were collected via midventral

laparotomy. All animal use protocols were approved by NCSU Institutional Animal Care and Use Committee (IACUC).

Luteal Cell Dissociation

CL were either collected from day 4 cycling animals (as described above, 7 cultures) or from local abattoir (2 cultures) and transported in M199 with antibiotics and antimycotics on ice. CL from abattoir were visually examined and were chosen based on appearance (structures were either corpora hemorrhagica or young CL with clear ovulation papillae) and on the lack of medium and large follicles on ovary, and were estimated to be approximately day 4. CL were dissociated as previously described [6]. Briefly, CL were dissociated in collagenase for 30 minutes at 37°C. Dissociated cells were removed and remaining tissue was incubated for another 30 minutes. This was repeated until all cells were completely dissociated. Cells were washed and placed in Percoll to remove red blood cells. Large luteal cells (LLC) were then counted and viability was determined by Trypan blue exclusion. Cell viability was determined to be over 90%.

Luteal Cell Elutriation

In a single experiment, a mixed luteal cell preparation was elutriated as described previously [52, 53]. Through centrifugal elutriation, a mixed small luteal cell preparation (containing small steroidogenic cells, endothelial cells and various other small cells $\leq$ 20 μm) and an enriched LLC fraction ($\geq$70% LLC) were obtained [52, 53], and LLC were cultured as described below.

Lipoprotein preparation

The LDL fraction from pig plasma was obtained by differential ultracentrifugation, using blood obtained from barrows as previously described [54].

Cell Culture

Day 4 luteal cells were cultured as follows. Briefly, the mixed (or enriched LLC) luteal cell preparations were resuspended in culture medium (M199; containing 22 mmol

Hepes, 4mmol sodium bicarbonate, 100 000 iu/ml penicillin, 100 μg/ml streptomycin, 50μg/ml gentamicin, 0.1% BSA, ITS, 2% FCS, 110 nmol hydrocortisone and 50 μg cholesterol/ml pig LDL). Costar 24-well tissue-culture treated plates coated with Collagen type I (Corning Inc., Corning, NY) were seeded with 100,000 large cells (mixed and LLC cell preparations; Day 0 of culture). The plates were then incubated in a humidified incubator at 37◦C with 5% CO2 in air for 2 days to facilitate cellular attachment, (see Fig. 1 for culture treatment time-line). On day 2 of culture, media and unattached cells were removed from plates, and M199 containing various concentrations of the following treatments were added to each well (in 1ml): TNF-α (0, 0.1, 1, 10, 100 ng/ml; n =3-6 wells per dose per culture, 8 cultures) or ET-1 (0, 0.1, 1, 10, 100 ng/ml; n=3-6 wells per dose per culture, 3 cultures). Each treatment was diluted in the M199 described above. On day 4 of culture, media and unattached cells were again removed and control medium containing PGF-2α (0, 1, 10, 100 ng/ml; n= 2-6 wells per treatment) was added and incubated for 2 further days. On the last day of culture (day 6), media were removed and saved for progesterone analysis. Additionally, luteal cells from selected wells on day 4 and 6, were either lysed using protein lysis buffer (1M Tris-HCl, 1M NaCl, 0.1M ETDA, 0.1M EGTA, 0.1% TritonX100, NP-40 and protease inhibitors) for subsequent protein analysis or lysed with Tri-Reagent for RNA analysis and both were frozen at -20 until future use (see Figure 1 for culture time-line). Finally, in one selected culture, cells were removed from the culture wells by trypsinization (250 μl 0.1% trypsin added per well for 10 minutes on ice) on day 4 or 6, to monitor cell viability. One well per treatment was sacrificed and a minimum of 100 cells (LLC and SLC) were counted. As shown in Table 1 no significant changes were observed in the viability of luteal cells over the course of the culture period between treatments.

Progesterone radioimmunoassay

Media were collected from luteal cell cultures and immediately stored at –20◦C. Samples from all cell cultures were thawed and a trial RIA was run to determine media dilutions. Samples from the highest, lowest and some in between treatment groups (i.e. 0 TNF, 0 PGF; 100 TNF, 10 PGF) were diluted at 1:100, 1:250 and 1:500, to determine the appropriate dilution (i.e. that dilution which gave showed between 20 and 80% binding)

for the culture media. Samples were then all diluted with PBS gel to the appropriate dilutions. Progesterone concentrations were measured using the Coat-a-Count radioimmunoassay kits as described and validated [54].

Endothelin (ET-1) EIA

Culture media from day 4 of culture were diluted (1:500) in EIA buffer and ET-1 concentrations were measured using the Endothelin-1 EIA as described in the assay manual.

Western Blotting

Total protein was extracted from luteal cells collected on day 4 of culture using lysis buffer (20 mM HEPES buffer containing 2mM EDTA, 2mM EGTA, 1% Triton-X100, 20 μg/ml apoprotinin, 20 μg/ml leupeptin, 4 μg/ml PMSF*),* followed by centrifugation at 14,000 x g to remove cellular debris. Protein concentrations were determined using the Micro-BCA protein assay (Pierce, Rockford, IL) according to manufacturer's instructions. Western blots were performed as previously reported [55]. Briefly, 20 μg of protein was loaded on to 4-12% Bis-Tris gels and subjected to electrophoresis at 120 volts at room temperature for 50 minutes. Following electrophoresis, proteins were blotted on to PVDF membranes at room temperature for 1 hour. Membranes were blocked in 5% milk TBS-Tween for 1 hour at room temperature and then incubated with primary antibody (1:200: PKC isoforms, ET_A) overnight at 4°C. Membranes were washed in TBS-Tween three times for 5 minutes, followed by incubation in secondary antibody (anti-rabbit, 1:2000) for 1 hour at room temperature, and washed another three times. Chemiluminescence (using ECL kit) was used to visualize protein bands and bands were quantitated with Lumi-Imager software (Roche, Indianapolis, IN). The specificity of protein bands was determined in separate gels using antibody which has been previously mixed with an excess of blocking peptide (used for generation of the antibody) for each antibody.

Statistical Analysis

Progesterone values were calculated by normalizing all data (within each culture) to controls, which were standardized to 100%, (Figs. 2A, 3A) and were then averaged across different cultures (n=3 or 4 cultures per treatment Fig. 2A; n=3 cultures per treatment- Fig. 3A, number of cultures equals number of animals). For Figures 2B-2F and Figures 3B-D (data from individual cultures/animals), progesterone values were calculated expressed as average data for each treatment (n=3-6 wells/treatment) as ng/ml for each separate culture. Figure 6 progesterone values were calculated by averaging wells across treatment (n=6) and then normalizing data to controls, which were standardized to 100%. Figure 7 progesterone values were calculated by averaging wells across treatment (n=6) and expressed as ng/ml. All statistical analyses were carried out using one-way analysis of variance, followed by Duncan's test for significance using Statistical Analysis Software (SAS, Cary, NC). Western blot values were averaged across treatments (n=2 or 3 from one culture) and were analyzed using one-way ANOVA (samples with n=3 per treatment) followed by Duncan's test for significant was determined using SAS. All means are reported as least-squares means +/- SEM. Differences were considered significant at $p < 0.05$, unless otherwise noted.

RESULTS

In these studies 2 distinct patterns of response to TNF-α and PGF-2α were observed, as described below:

Pattern 1: TNF-α sensitizes porcine luteal cells to PGF-2α in vitro (Fig. 2)

As shown in Fig. 2 exposure of porcine (day 4 cycle) luteal cells to TNF-α (0, 0.1, 1, 10 ng/ml) between days 2-4 of culture (TNF-α "priming"), sensitized them to the luteolytic effects of PGF-2α added on day 4 (PGF-2α "challenge") and as measured by progesterone concentrations in spent culture medium collected on day 6 of culture, as detailed below (Fig. 1; Fig. 2).

Fig. 2 A represents the average data expressed as % control (to normalize data for presentation and statistical purposes) of 3-4 cultures (animals), while Figs. 2B-2F illustrate the data from individual cultures showing data expressed as progesterone on ng/ml (i.e. not normalized to control).

As shown in Fig. 2A, TNF-α priming followed by control (0 ng/ml PGF-2α) challenge, did not result in significantly decreased progesterone levels, indicating that TNF-α itself had no detrimental effects on luteal cell steroidogenesis. However, in response to PGF-2α challenge at the 1 ng/ml dose, a decrease in progesterone production was observed with TNF-α (priming) culminating in a significant decrease (compared with 0 TNF-α) observed at 0.1, 1 and 10 ng/ml of TNF-α.

Additional experiments were done with various doses of TNF-α and PGF-2α, and these individual culture (= 1 animal) results are presented below. Individual cultures also showed clear evidence of TNF-priming, inducing sensitivity to PGF-2α.

Culture 1 (Expt. 1, Fig. 2B): (cells from abbattoir, n=3 per treatment) Although there was a significant increase in progesterone from 0 TNF-α and 1 ng/ml of TNF-α, both 10 and 100 ng/ml of TNF-α decreased progesterone compared with 0 TNF-α (Fig. 2B). Also PGF-2α treatment (100 ng/ml) of control (0 TNF-α) cells did not cause a decrease in progesterone, indicating that these luteal cells were not sensitive to PGF-2α. However, priming with 10 and 100 ng/ml of TNF-α, showed a significant decrease in P4 as compared to these TNF-α doses without PGF-2α treatment, indicating TNF-induced sensitization.

Culture 2 (Expt. 2, Fig. 2C): (cells from D4 of cycle, n=5 wells per treatment) In this experiment TNF-α priming (all doses) with 0 PGF-2α challenge had no significant effects on progesterone. Also PGF-2α challenge (10 ng/ml) on control cells also had no effects on progesterone secretion. However, when TNF-primed cells (all doses) were challenged with PGF-2α, there was a significant decrease in progesterone.

Culture 3 (Expt. 3, Fig. 2D): (cells from D4 of cycle, n=4 per treatment). In this culture, TNF-α priming, as well as PGF-2α treatment (1 ng/ml) on its own, had an effect on progesterone secretion. However, TNF-α priming (10 ng/ml) followed by PGF-2α challenge (1 ng/ml) showed the most significant decrease in progesterone, showing TNF-α's sensitizing effect on the luteal cells.

Culture 4 (Expt. 4, Fig. 2E): (cells from D4 surgery, n=4 per treatment). A similar response was observed to that seen in Culture 2, in that TNF-α priming (without PGF-2α challenge) had no effects on progesterone secretion, PGF-2α challenge (1 ng/ml) without

TNF-α priming had no effects on progesterone, and PGF-2α challenge of luteal cells primed with 0.1 and 1 ng/ml TNF-2α induced a significant decrease in progesterone, indicative of "sensitization".

Culture 5 (*Expt. 5; Fig. 2F*). (cells from day 4 of cycle, n=4 per treatment). In this culture, although TNF-α priming (0 PGF-2α) had some inhibitory effects on progesterone, PGF-2α challenge (1 ng/ml PGF-2α) of control cells did not significantly decrease progesterone, and 1 ng/ml TNF-α priming followed by PGF-2α challenge resulted in significantly decreased progesterone. At a higher dose (10 ng/ml), PGF-2α challenge on its own induced a decrease in progesterone, however priming with both 0.1 and 1 ng/ml TNF-α followed by PGF-2α treatment (10 ng/ml) showed a more dramatic decrease in progesterone production, again indicative of TNF-α inducing sensitization to PGF-2α.

Pattern 2: Luteolytic response to PGF-2α "rescued" by TNF-α (Fig. 3)

In contrast to the pattern described above, in several cultures we observed a different pattern of response to TNF-α priming, followed by PGF-2α challenge, as shown in Fig. 3A. This response was characterized by luteal cells showing sensitivity to PGF-2α challenge without requiring TNF-α priming, but which displayed a "rescue" of luteal steroidogenesis provoked by PGF-2α challenge, in response to increased TNF-priming, in a (TNF-α) dose dependent manner. Thus, increasing doses of TNF-α "priming" treatment (0, 1, 10 ng/ml) followed by control (0 ng/ml PGF-2α) challenge had no significant effect on progesterone production, similar to that described above (Fig. 2A). However in these cultures, luteal cells that received no priming with TNF-α (i.e. TNF-α 0 ng/ml) showed a luteolytic response to PGF-2α challenge at 1 ng/ml, as indicated by significantly decreased progesterone production ($p<0.01$; vs. 0 TNF-α and 0 PGF-2α, suggesting that in these cultures luteal cells acquired luteolytic sensitivity without the requirement for TNF-α priming (perhaps these were from more mature corpora lutea which were responsive to PGF-2α in vivo or had acquired this sensitivity in response to culture conditions e.g. plating between day 0 and 2 of culture). Interestingly, the luteolytic response to 1 ng/ml PGF-2α was overcome with increasing doses of TNF-α (1

and 10 ng/ml), as indicated by the restoration of progesterone secretion (Fig. 3A), suggestive of a "rescue" response to TNF-α –priming of luteal cells between days 2 and 4 of culture.

Figures 3B-3D show individual culture experiments used to obtain the results presented in Figure 3A.

Culture 1 (Expt. 6, Fig. 3B): (Cells from day 4 of cycle, n=6 wells per treatment): TNF-α priming followed by control PGF-2α challenge (0 ng/ml) had no effect on progesterone secretion, but PGF-2α (at 1 and 10 ng/ml) challenge on control cells (0 TNF-α) did cause a significant decrease in progesterone, indicating that luteal cells acquired PGF-2α sensitivity without a need for TNF-α. However, TNF-α priming (0.1-10 ng/ml) appeared to "rescue" luteal cell steroidogenesis (inhibited by PGF-2α alone, 1 ng/ml) and restored P_4 levels back to those seen in control and TNF-α (no PGF-2α challenge) cultures. A similar trend was shown in cells treated with 10 ng/ml of PGF-2α when primed with 10 ng/ml of TNF-α as compared to controls.

Culture 2 (Expt. 7, Fig. 3C): (cells from day 4 of cycle, n=4 wells per treatment). In this culture a similar rescue effect of TNF-α pretreatment to that observed in Culture 1, was seen with similar doses of TNF-α and PGF-2α (Fig. 3C). No effect of TNF-α was seen on its own, but PGF-2α (0 TNF-α) induced a significant decrease in progesterone at 1 ng/ml. Progesterone concentrations were increased again with TNF-α priming at 1 and 10 ng/ml.

Culture 3 (Expt. 8, Fig. 3D): (cells from day 4 of cycle, n=6 wells per treatment). This culture also showed a similar pattern of responses to TNF-α priming and PGF-2α challenge. TNF-α priming with no PGF-2α challenge did not significantly effect progesterone concentrations. Both doses of PGF-2α challenge of control (0 TNF-α) cells decreased progesterone significantly. However 0.1 and 1 ng/ml of TNF-α priming followed by 1 ng/ml of PGF-2α treatment progesterone levels were increased back to those seen in non PGF-2α treated cultures. In response treatment with 10 ng/ml of PGF-2α following TNF-α priming, there was a dose-dependant restoration in P_4, such that in 10 ng/ml of TNF-α and 10 ng/ml of PGF-2α, treated cells progesterone was not

significantly different from controls (TNF-10 ng/ml), again showing the "rescue" effect by TNF-α.

TNF-α increases ET-1 secretion in porcine luteal cells in vitro (Fig. 4)

In a single experiment (n=1 animal) we measured ET-1 concentrations (as measured by EIA) in the medium on day 4 (after TNF-α priming) and observed that TNF-α at 10 and 100 ng/ml increased ET-1 secretion approximately 40% in day 4 cultured luteal cells, n=2 wells per treatment (Fig.4).

TNF-α dose dependently increased PKC βII, ε and ET_A – day 4 of culture *(Fig. 5A)*

In one culture experiment (n=1 animal, culture showing Pattern 1 response - TNF-α sensitizing effect; Fig. 2E) we measured the expression of PKC isoforms βII and ε, as well as ET_A receptor by western blot analysis, in luteal cells collected on day 4 following TNF-α priming (and thus before PGF-2α challenge). This was done to determine whether TNF-α priming treatment would increase the expression of these signal transduction components in luteal cells, which we hypothesized may be critical in the "sensitizing" actions of TNF-α. As seen in Figure 5A, 1 ng/ml TNF-α treatment significantly increased PKC βII and ET_A expression (n=3). Similarly, TNF-α appeared to dose-dependently increase PKC ε (n=2), although no statistics could be performed since only 2 wells per treatment were examined.

TNF-α increased ET_A, decreased PKC βII, but had no effect on PKC ε – day 6 of culture *(Fig. 5B)*

In another culture experiment (culture showing Pattern 2 response - TNF-α rescue; Fig. 3B), we also measured the expression of PKC ε and ET_A and the phosphorylated (and thus activated) and non-phosphorylated forms of PKC βII collected on day 6 following TNF-α priming, to determine if TNF-α treatment would increase the expression of these components long term (i.e. 96 hours after initial treatment). These cultures did not receive PGF-2α challenge. Similar to that seen on day 4 of culture (Fig. 5A), TNF-α treatment dose-dependently increase ET_A receptor expression (solid black

bars, $p<0.05$; Fig. 5B). However, in contrast to that seen on day 4, TNF-α dose-dependently decreased both the phosphorylated (dotted bars, $p<0.05$) and non-phosphorylated (solid white bars, $p<0.05$) PKC βII expression and had no significant effect on PKC ε expression (striped bars; Fig. 5B).

TNF-α or PGF-2α treatment does not induce cell death in cultured porcine luteal cells (Table 1, Culture 2, Fig. 2C)

The viability of porcine luteal cells on day 4 after TNF-α "priming" (0.1-1 ng/ml), and on day 6 following PGF-2α "challenge" (1 and 10 ng/ml) of TNF-α primed cells (0.1-10 ng/ml) (Table 1) was determined by trypan blue exclusion. At least 100 LLC and SLC for each treatment were counted. After 4 days of culture with either 0, 0.1 or 1 ng/ml of TNF-α, cell viability ranged from 81 -94% (LLCs) and 86 -94% (SLC), with no indication of a TNF-α effect, suggesting that contrary to its effects at later stages of the estrous cycle, TNF-α did not cause luteal cell death in young (day 4 of cycle) luteal cells in cultured for 2 days (day 2-4 culture). Cell viability on 6 day of culture with TNF-α priming (0-10 ng/ml) followed with either 1 or 10 ng/ml of PGF-2α challenge, was then determined. In LLCs cell viability ranged from 77-100% and in SLC, viability was 80-96%, with no clear evidence of TNF-α and/or PGF-2α treatment effects. Ironically, the lowest viability was seen in LLCs with no treatment at all (77.3%). Therefore we conclude that neither TNF-α nor PGF-2α treatment had deletrious effects on luteal cell viability for up to 6 days in culture, and that decreased P4 concentrations seen in response to either TNF-α or PGF-2α treatments cannot be explained by increased levels of cell death. This has been confirmed recently in preliminary studies examining DNA laddering (typical of apoptotic cells) patterns on agarose gels. We did not see any evidence of DNA laddering in response to either TNF-α or PGF-2α (data not shown) on day 6 of culture.

ET-1 Does Not Sensitize Porcine luteal cells to PGF-2α (Fig. 6)

In view of preliminary observation of the increased level of ET-1 secretion and the up-regulation of the ET_A receptor in luteal cells, in response to TNF-α, we attempted to determine whether ET-1 treatment (0, 0.1, 1, 10 ng/ml) given on day 2 of culture (in

place of TNF-α) would, like TNF-α, also "sensitize" luteal cells to the luteolytic effects of followed by PGF-2α (added as a challenge on day 4 of culture).

In a single experiment (1 animal, n=6 wells per treatment), Progesterone concentrations were not significantly changed in response to control (0 ng/ml PGF-2α) challenge, following ET-1 priming, which was similar to that seen with TNF-α priming (Fig. 2 and 3). However, none of the PGF-2α challenge doses (0.1 or 1 ng/ml) had any significant luteolytic effects at any ET-1 treatment doses. In fact, the highest dose of ET-1 (with 1 ng/ml of PGF-2α) had a tendency (p=0.06) for increased P4 production (compared with ET-1 treatments on its own), although this tendency was not statistically significant from any other treatments.

Effects of TNF-α, ET-1 and PGF-2α on Large Luteal cells in vitro (Fig. 7)

There were no significant differences in medium progesterone concentrations in response to either 0 or 1 ng/ml PGF-2α at any dose of TNF-α. Thus, although MLC from the same culture showed the sensitization response to TNF-α similar to that seen in Fig. 2 (data not shown), no such response was observed with an enriched preparation of LLC (Fig. 8), suggesting that TNF's priming actions must be mediated via another luteal cell type. Future follow up studies are in progress to determine the identity of the luteal cell sub-type which is critical to the TNF-induced acquisition of luteolytic sensitivity to PGF-2α.

DISCUSSION

Understanding the control of the acquisition of LS in the porcine CL is of major importance, since the lack of LS for most of the estrous cycle in pigs render PGF-2α analogs ineffective for estrous cycle regulation. Improved understanding of the control of LS would eventually lead to novel approaches or new drug therapies to facilitate estrous cycle regulation, which could have a significant economic impact to the swine industry.

Thus in these studies, the aim was to develop a luteal cell culture method to study the control of LS development in the porcine CL in vitro. Specifically we investigated whether macrophage secreted TNF-α plays a role within the CL before luteolysis, and to

test the hypothesis that TNF-α acts to sensitize luteal cells to PGF-2α, and thus is critically involved in the acquisition of LS in vivo during mid cycle (between days 7 and 13).

The approach to these studies was to dissociate corpora lutea and establish culture conditions that would keep luteal cells viable and functional for 4-6 days. Additionally it was important to establish appropriate doses and times of treatments that would also maintain cell viability. We chose to use day 4 early luteal stage copora lutea for these studies since these cells were still differentiating in vivo, they would probably continue to differentiate and function in vitro (Note: luteal cells from fully differentiated CLs are notoriously difficult to maintain in long term cell culture). In addition, examining how TNF-α may act on porcine luteal cells earlier than previously reported [11,16-21] has yet to be studied in the pig, and thus forms the rationale for this study.

The major finding of this study was the observation that, in several cultures, TNF-α "priming" of porcine luteal cells had a sensitizing effect on luteal cells to the luteolytic (anti-steroidogenic) actions of PGF-2α (Fig. 2). In these studies, TNF-α priming (with as little as 0.1 ng/ml) followed by PGF-2α challenge (1 ng/ml) showed a significant decrease in progesterone production (as compared to control, 0 TNF-α and 0 PGF-2α, Fig. 2A), and thus luteal cell steroidogenesis. This finding that TNF-α can sensitize luteal cells to PGF-2α in vitro, suggests that TNF-α may have similar actions within the porcine CL during the mid-cycle (days 7-13) in vivo (earlier than previously demonstrated), when macrophages infiltrate the CL in increasing numbers, and their secretory capacity for TNF-α increases 4-fold [10]. Thus these data support our hypothesis that TNF-α plays a critical role in control of acquisition of LS in the pig.

Because TNF-α has been shown to play an important role in apoptosis in the CL [19-21], we also wanted to determine if TNF-α and PGF-2α were inducing apoptosis. Therefore, we wanted to determine the viability of the cells after exposure to the various doses. Therefore we cultured day 4 luteal cells as described, and after 48 hours of TNF-α treatment sacrificed wells and cell counts on both LLCs and SLCs (all small cells were included). Additionally we determined viability of cells treated with PGF-2α as well as combinations of the two (Table 1). In day 4 TNF-α treated cells, SLC populations

treated with TNF-α had higher viability than the control group (92% or higher versus 86%). Of the LLC populations, 0.1 TNF-α treatment viability was slightly higher than control, and 1 ng/ml of TNF-α viability was 81%, still considered a healthy population of cells. In the day 6 cells, the lowest viability in the LLC populations was with control treatments, so we concluded that even the highest doses of TNF-α and PGF-2α (both 10ng/ml) were not contributing to cellular death, and suggested that the P_4 effect we see in the cultures is real and not because of a decrease in viable cells producing P_4. Of the SLC population, day 6 control viability was 87%, and treatment with the highest doses of TNF-α and PGF-2α yielded a viability of 95%, higher than control, suggesting that these doses of TNF-α and PGF-2α are not contributing to cell death. Also, in preliminary experiments we did not observe any evidence of DNA laddering, which is regarded as an indicator of cellular apoptosis (data not shown). Nevertheless, even though we observed no evidence of cell death or apparent apoptosis in our cultures, we proposed to examine the role of apoptosis-associated genes (including TNF-R, p53, iNOS/eNOS, Bax and Bcl-x) during acquisition of LS, to determine whether the expression of these genes may influence PGF-2α response in vivo (See Chapter 4 for complete rationale).

Next we wanted to test the hypothesis that TNF-α sensitizing mechanism may involve increasing ET-1 production from ECs, since TNF-α receptors have been located on SLC, LLC and ECs in the porcine [22] and bovine CL [18]. Friedman and colleagues showed there is more TNF-RI present on bovine ECs than in steroidogenic cell types [19]. In addition, in the bovine CL, it has been proposed that TNF-α, presumably acting via TNFR, acts to increase the production of both ET-1 and PGE2, by luteal ECs in vitro [24]. Therefore since ET-1 is believed to influence luteolytic responsiveness of the bovine CL to PGF-2α [56], TNF-induced ET-1 secretion by EC may play a similar role in the porcine CL [25]. Therefore, in one culture, we measured ET-1 secretion in day 4 cultured luteal cells in response to TNF-α on day 6 (48 hours after TNF-α treatment). In this preliminary experiment, we saw an increase in ET-1 secretion in cells treated with both 10 and 100 ng/ml of TNF-α (Fig. 4), suggesting that perhaps in the porcine CL, TNF-α, acting through its receptors located on ECs, increases expression of ET-1. We then wanted to determine whether TNF-α increased expression of ET-1 receptor,

particularly ET_A, which has been suggested to be the receptor involved in luteolytic actions in the CL [31, 35]. Our data showed that TNF-α significantly increased expression of ET_A with as little as 1 ng/ml of TNF-α (on both D4 and D6) and further increased ET_A expression with 10 ng/ml of TNF-α in D6 treated cells (Fig. 5A and 5B, black solid bars). We provide preliminary evidence that TNF-α dose-dependantly increases ET-1 production and increases ET_A receptor, perhaps located on LLCs to mediate LS in the pig.

Additionally, it is well known that the lack of sensitivity to PGF-2α of the porcine CL before D13 is believed not to be due to the lack of FP receptors, it is suggested that the lack of LS may to be attributable to the lack of downstream signaling molecules such as PKC [8]. Recently Sen and collegues (2004) suggested that it was the conventional isoforms (PKC α, βI, βII and γ) that was responsible for the lack of LS in the early bovine CL, however it was the novel isoform PKC ε that was increased 11-fold between the early and late CL [9]. In addition they reported that it was PKC ε that specifically plays a mediatory role in PGF-2α induced inhibition of steroidogenesis in bovine luteal cells, suggesting that this PKC isoform may be directly linked to LS [45]. Most recently, it was shown that PKC ε was exclusively expressed in luteal steroidogenic cells, and not endothelial cells, and PKC ε was stimulated by Endothelin-1 (ET-1) in day 10 bovine CL. Therefore these authors concluded that PKC ε plays a regulatory role by decreasing Progesterone (P4) during regression [46]. Furthermore, TNF-α binding to its receptor has also been shown to activate the PLC and PKC pathways [57]. Therefore we also wanted to determine whether or not TNF-α had any effect on PKC expression on day 4 and 6 of culture. We examined the expression of PKC βII, PKC ε and phosphorylated (and thus activated) PKC βII (day 6 only) in response to various TNF-α doses (Fig. 5A). The findings of this preliminary study suggest that TNF-α directly can increase PKC βII and ε isoform expression with as little as 1 ng/ml and 2 days after treatment (as compared to control, 0 TNF-α, in day 4 cells). In day 6 luteal cells (96 hours after TNF-α treatment) however, PKC βII expression was significantly decreased with TNF-α treatment (all doses, Fig.5B, open bars), as was pPKC βII expression. PKC ε, the isoforms which has been highly suggested to be responsible for LS in the bovine CL [45, 46], showed no

significant differences with any TNF-α treatment on day 6 of culture. Since in these cultures examined on day 6, luteal cells had TNF-removed for the previous 48h (day 4-6), these data suggest that continuous exposure to TNF-α may be key to maintaining or further up-regulating PKC isoform levels. Taken together, these findings support the hypothesis that PKC isoforms, particularly PKC βII and ε, may play a role in the sensitizing actions of TNF-α on porcine luteal cells in vitro (and possibly in vivo).

Because we determined that TNF-α sensitized porcine luteal cells to PGF-2α, and our preliminary data showed that TNF-α increased ET-1 peptide and ET_A receptor expression, we wanted to determine if ET-1 directly sensitized luteal cells to PGF-2α. The same doses of ET-1 were used as for TNF-α treatments in the previous cultures (0, 0.1, 1 and 10 ng/ml). Our results of this one culture showed no significant differences in P_4 secretion with ET-1 alone, nor ET-1 followed by PGF-2α (either 0.1 or 1 ng/ml), doses in which TNF-α and PGF-2α showed a significant decrease in P_4 production (Fig. 6). There was a slight trend (p=0.6) towards increasing expression with the highest ET-1 and PGF-2α dose versus control, albeit non-significant. Therefore, these data suggest that ET-1 may not be the sole mediator of TNF-α's sensitizing actions in vitro, (and presumably in vivo). Alternatively, although we observed increased ET_A in these cultures, we cannot guarantee that they were expressed on LLC, which could account for why ET-1 may not affect progesterone secretion directly. Our studies described in Chapter 3 addressed the expression and localization of ET system components in vivo, and show clear LLC localization on day 13. Thus, from these preliminary culture studies, our data do not support a role for ET-1 as the only mediator of the sensitizing actions in vitro, although we cannot rule out a joint role of both TNF-α and ET-1 in this process. Further studies to examine the roles of ET-1 and TNF-α on the sensitization of luteal cells in vitro will confirm their roles.

Finally, we wanted to examine the role that the LLCs played in LS, as LLCs are the site for primary P_4 production, and are believed to be the cells which respond to both ET-1 [32, 39] and PGF-2α [58]. Elutriation of our samples provided us with an enriched LLC population which we examined along with a mixed luteal cell preparation for TNF-α and ET-1 – mediated sensitization in vitro. Our results showed no significant effect of

either TNF-α or ET-1 on LLC, although TNF-α did cause sensitization of the mixed luteal cells preparation suggesting that a small luteal cell sub-type (as yet undetermined), together with LLC is required for TNF-α to cause sensitization of these cells to PGF-2α's actions. This finding may suggest that release of a TNF-α induced hormone or factor, from small luteal cells that mediate this response on LLC. This implicates the other small luteal cell types (ECs, SLCs, and immune cells) as critical components in the control of LS. Follow up studies are being planned to determine the importance of the roles of these other luteal cell types in acquisition of LS in the porcine CL.

In contrast to the sensitization effects of TNF-α we described above, we also observed a different pattern (Pattern 2) of response in some cultures (3 out of 8 total). These data summarized in Figure 3 demonstrated that luteal cells were sensitive to PGF-2α without requiring TNF-priming. We suggest that this response may be due to the age of the CL at the time of culture. All three of the animals in which we collected CLs for these cultures were from day 4 surgery animals. Although the stage of the cycle is "known," the actual age of the CL can vary as much as 24-48 hours, based on once a day heat checking and the fact that ovulation which normally take about 40-48h can also be delayed in some animals. However, in each of these cultures, luteal cells also demonstrated that TNF-priming (prior to PGF-2α) caused a restoration in progesterone secretion which was decreased by PGF-2α, suggestive of a "rescue" effect. Although we have no explanation for this response pattern or even why it differed from Pattern 1, there is an observation from some animals studies which may represent a similar response. In studies carried out in the pig [59], the authors administered PGF-2α on day 9, or on day 9 and 10, of the estrous cycle and demonstrated a transient decline in P_4 production, followed within 72 hours by a return of P_4 levels were back to those prior to treatment [61]. However, when the same dose of PGF-2α was given on day 12 of the cycle, P_4 decreased and remained low, indicating that CLs are responsive to exogenous PGF-2α, on day 12 [59]. These findings suggest a "luteotropic" or at least "anti-luteolytic" effect of TNF-α at the level of the porcine CL, although the mechanism by which this occurs is not clear at this time. TNF-α has been shown to have a luteotrophic role in the CL [12], and TNF-R (type II), a non DD containing receptor, is known to induce anti-apoptotic

gene expression and is involved in the anti-apoptotic effect of TNF-α [60, 61]. TNF−α binds to the extracellular domain of TNF-RII, which causes activation of NFκB and expression of survival genes are up-regulated and thereby apoptosis induced by TNF−α is prevented [61-64]. Thus TNF-RII may be the dominant receptor expressed in the CLs used in cultures showing Pattern 2, and leading to anti-apoptotic effects of TNF-α, which may explain the effect seen in Fig. 3. This may also help explain why we saw no evidence of cell death or apoptosis in our cultures. However, it does not explain why TNF-α had sensitizing actions but no rescue response in Pattern 1 cultures. The explanation could reside in subtle but critically important differences in age or maturity of the CLs used in these cultures.

Overall these studies have given us an important method to study acquisition of LS in the porcine CL, as we have been able to develop a culture method in which the cells remain healthy and viable, as well as respond to TNF-α and PGF-2α doses well within physiological range. Our results suggest that TNF-α does play a role much earlier in the cycle than previously demonstrated, and that TNF-α can sensitize porcine luteal cells to the luteolytic actions of PGF-2α. Although as yet defined, the mechanism by which TNF-α sensitizes porcine luteal cells may involve the ET-1 system and PKC isoforms PKC βII and PKC ε. Our studies also suggested that the TNF-α sensitizing mechanism involves interactions between LLC and one or more other luteal cell sub-types. Further studies are warranted to improve our understanding of the TNF-sensitizing mechanism and to gain a much clearer and better understanding of control of acquisition of LS in the pig.

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Table 1. Cell Viability in Cultured Luteal Cells[1]

	Large Luteal Cells	Small Luteal Cells
Day 4		
0 TNF	89.2%	86.3%
0.1 TNF	93.8%	92.0%
1 TNF	81.1%	93.6%
Day 6		
0 TNF 0 PGF	77.3%	87.1%
0.1 TNF 1 PGF	100.0%	93.1%
1 TNF 1 PGF	85.7%	80.4%
10 TNF 1 PGF	100.0%	89.1%
0 TNF 10 PGF	95.5%	90.7%
0.1 TNF 10 PGF	90.3%	90.1%
1 TNF 10 PGF	85.7%	90.6%
10 TNF 10 PGF	100.0%	95.5%

[1] n=1 culture

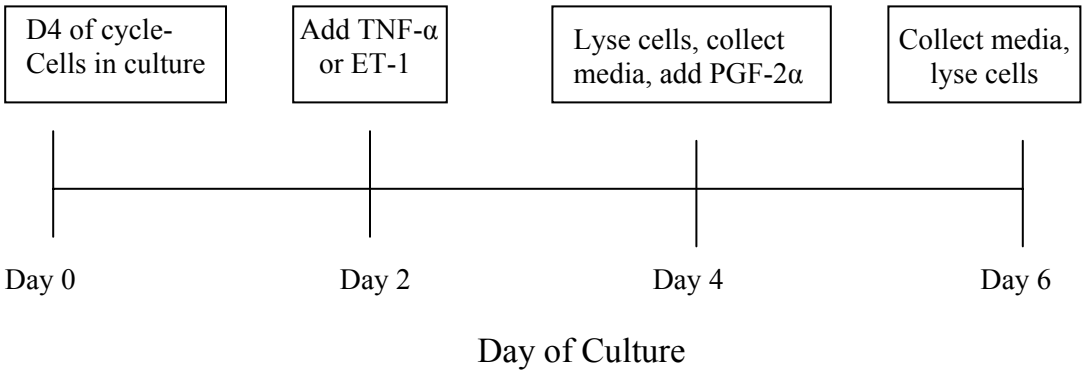

Fig. 1: Cell culture protocol. Day 0 of culture (day 4 of cycle) cells are plated. On day 2 of culture, TNF-α or ET-1 was added. Day 4 of culture media was removed, cells lysed and PGF-2α treatments were added. On the final day of culture (day 6) culture media was removed and cells were lysed for future analysis.

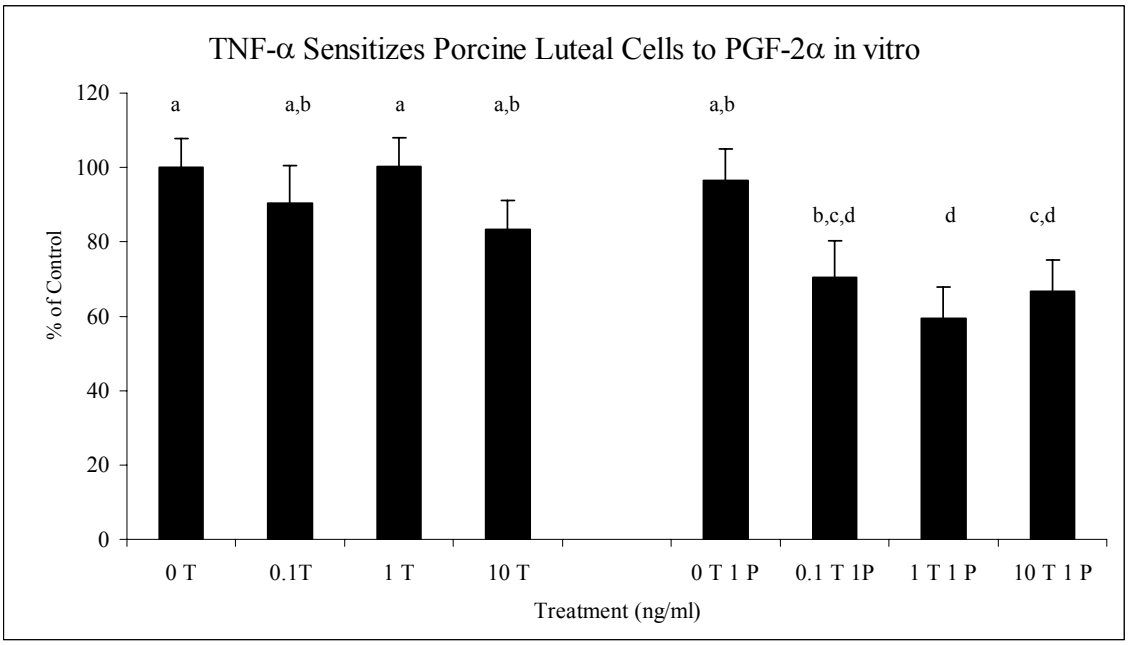

Fig 2A: TNF-α sensitizes porcine luteal cells to PGF-2α. Progesterone was determined in day 6 cultured luteal cells in response to TNF-α and PGF-2α, n=3-4 cultures per treatment. T= TNF-α, P= PGF-2α. Bars represent % of control +/- SEM of the P_4 values. Different letters denote significant differences between groups ($p<0.05$).

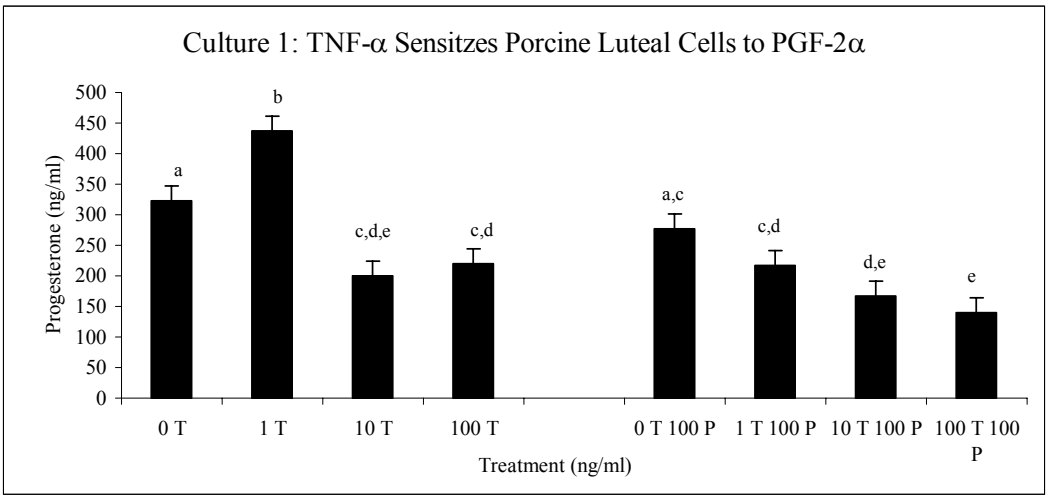

Figure 2B. TNF-α sensitizes porcine luteal cells to PGF-2α, experiment 1. Progesterone (P_4) was determined in day 6 cultured luteal cells in response to TNF-α and PGF-2α, n=3 wells per treatment. T= TNF-α, P= PGF-2α. Bars represent P_4 levels (ng/ml) +/- SEM. Different letters denote significant differences between groups ($p<0.05$).

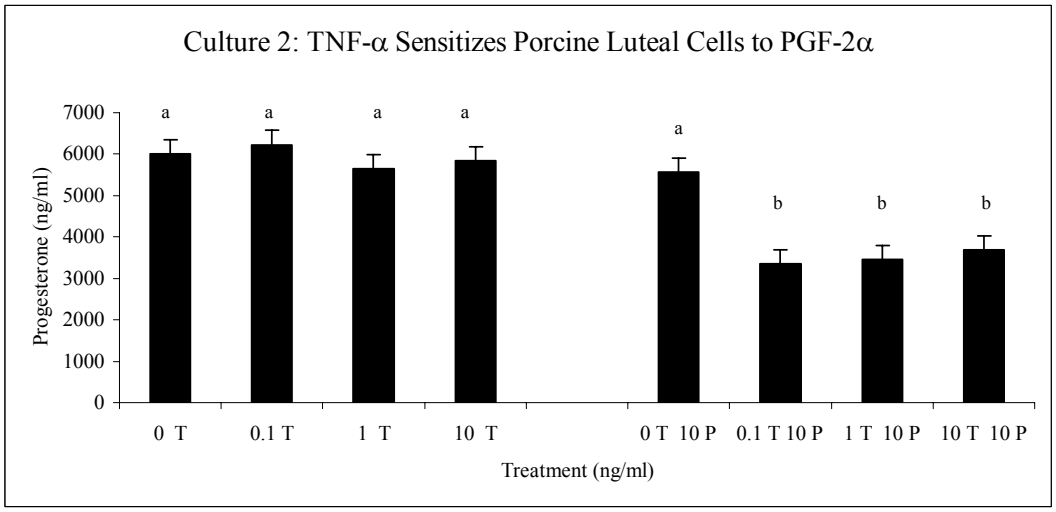

Figure 2C. TNF-α sensitizes porcine luteal cells to PGF-2α, experiment 2. Progesterone (P_4) was determined in day 6 cultured luteal cells in response to TNF-α and PGF-2α, n=5 wells per treatment. T= TNF-α, P= PGF-2α. Bars represent P_4 values +/- SEM. Different letters denote significant differences between groups ($p<0.05$).

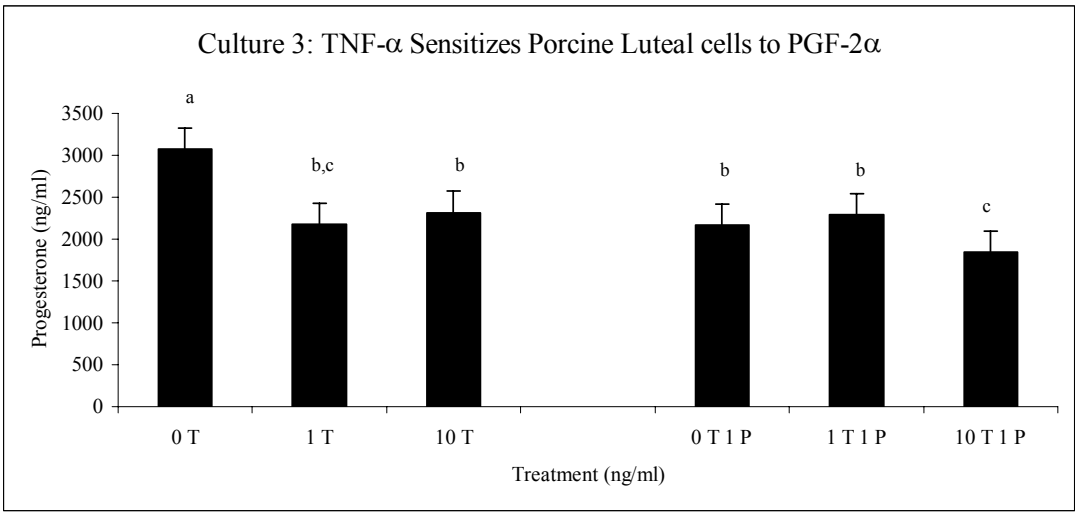

Figure 2D. TNF-α sensitizes porcine luteal cells to PGF-2α, experiment 3. Progesterone (P_4) was determined in day 6 cultured luteal cells in response to TNF-α and PGF-2α, n=4 wells per treatment. T= TNF-α, P= PGF-2α. Bars represent P4 levels (ng/ml) +/- SEM. Different letters denote significant differences between groups ($p<0.05$).

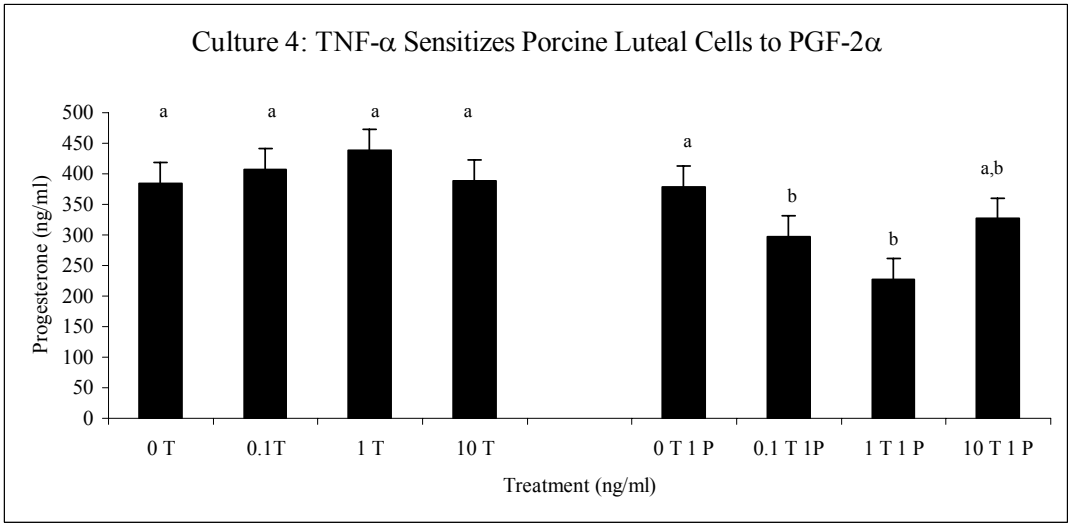

Figure 2E. TNF-α sensitizes porcine luteal cells to PGF-2α, experiment 4. Progesterone was determined in day 6 cultured luteal cells in response to TNF-α and PGF-2α, n=4 wells per treatment. T= TNF-α, P= PGF-2α. Bars represent P4 levels (ng/ml) +/- SEM. Different letters denote significant differences between groups ($p<0.05$).

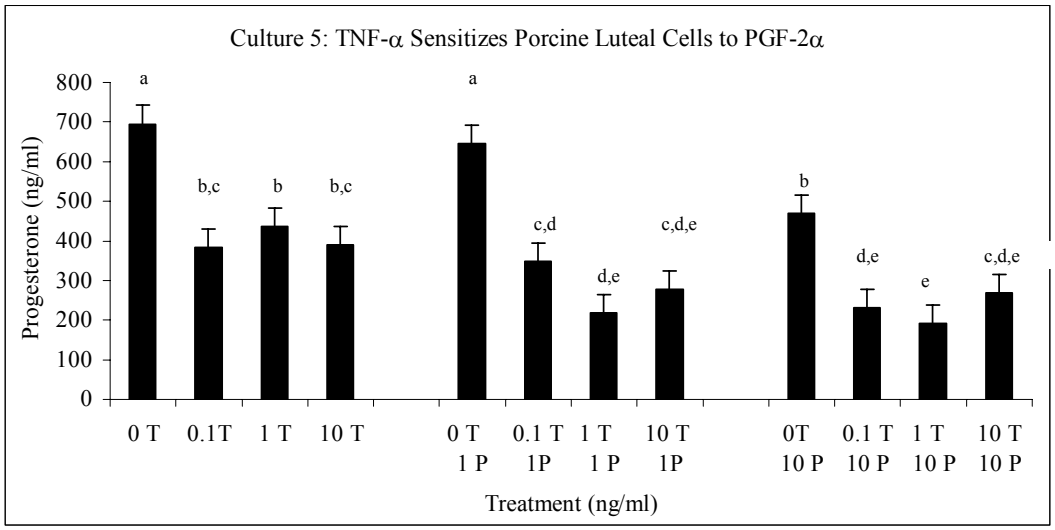

Figure 2F. TNF-α sensitizes porcine luteal cells to PGF-2α, experiment 5. Progesterone (P_4) was determined in day 6 cultured luteal cells in response to TNF-α and PGF-2α, n=6 wells per treatment. T= TNF-α, P= PGF-2α. Bars represent P4 levels (ng/ml) +/- SEM. Different letters denote significant differences between groups ($p<0.05$).

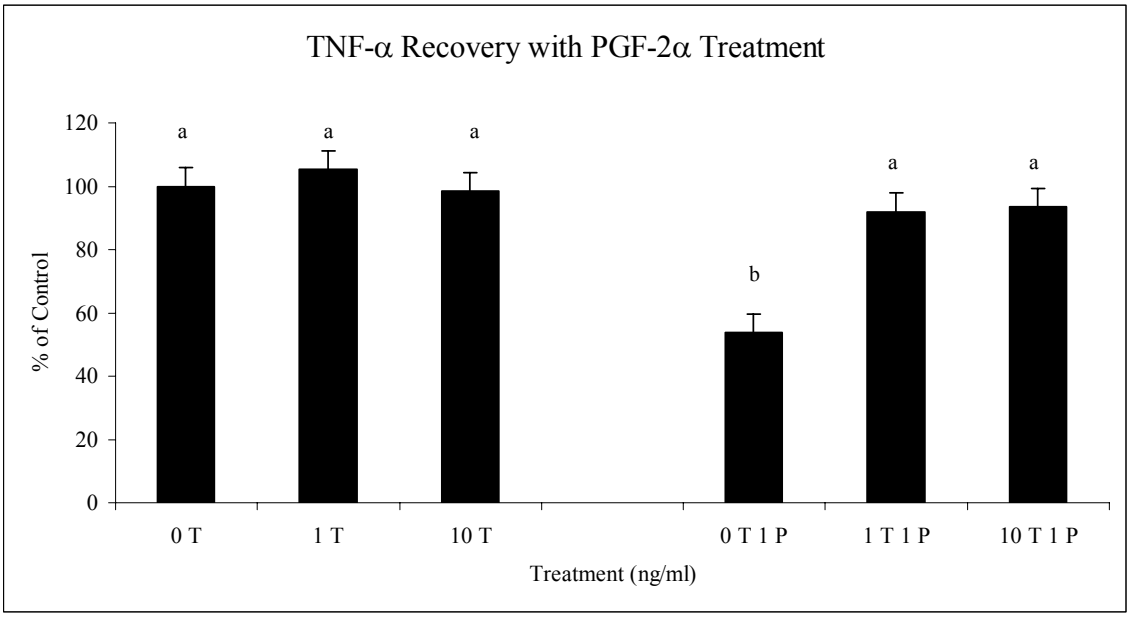

Fig. 3A: Luteolytic Response to PGF-2α: Rescued by TNF-α. Progesterone (P_4) was determined in day 6 cultured luteal cells in response to TNF-α and PGF-2α, n=3 cultures. T= TNF-α, P= PGF-2α. Bars represent % of control +/- SEM of the P_4 values. Different letters denote significant differences between groups ($p<0.05$).

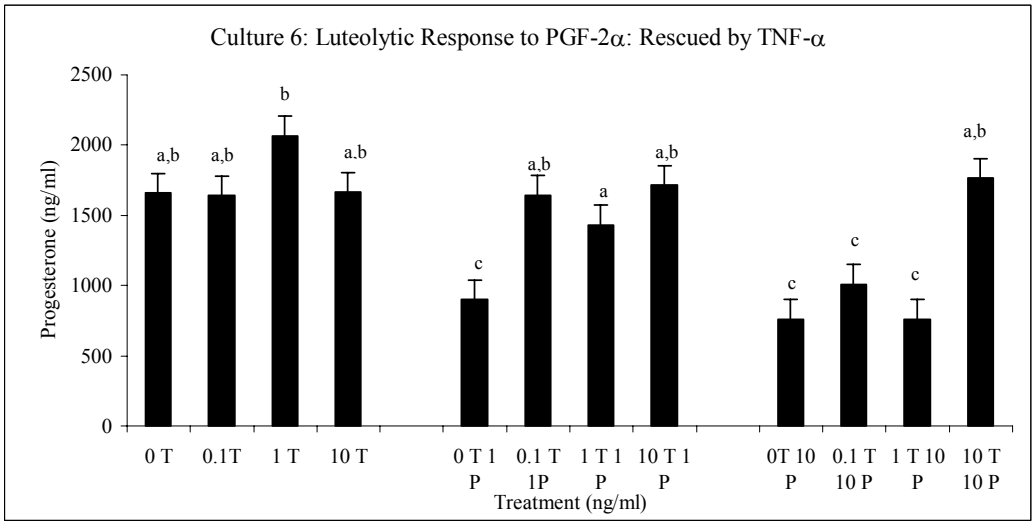

Fig. 3B: Luteolytic Response to PGF-2α: Rescued by TNF-α, Experiment 6. Progesterone (P_4) was determined in day 6 cultured luteal cells in response to TNF-α and PGF-2α, n=6 wells per treatment. T= TNF-α, P= PGF-2α. Bars represent P4 levels (ng/ml) +/- SEM. Different letters denote significant differences between groups ($p<0.05$).

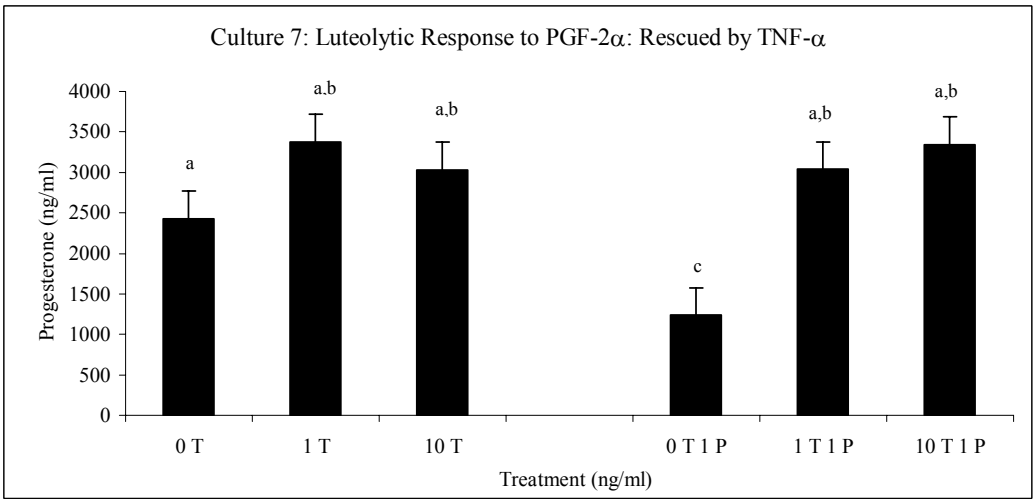

Fig. 3C: Luteolytic Response to PGF-2α: Rescued by TNF-α, Experiment 7. Progesterone (P_4) was determined in day 6 cultured luteal cells in response to TNF-α and PGF-2α, n=4 wells per treatment. T= TNF-α, P= PGF-2α. Bars represent P4 levels (ng/ml) +/- SEM. Different letters denote significant differences between groups ($p<0.05$).

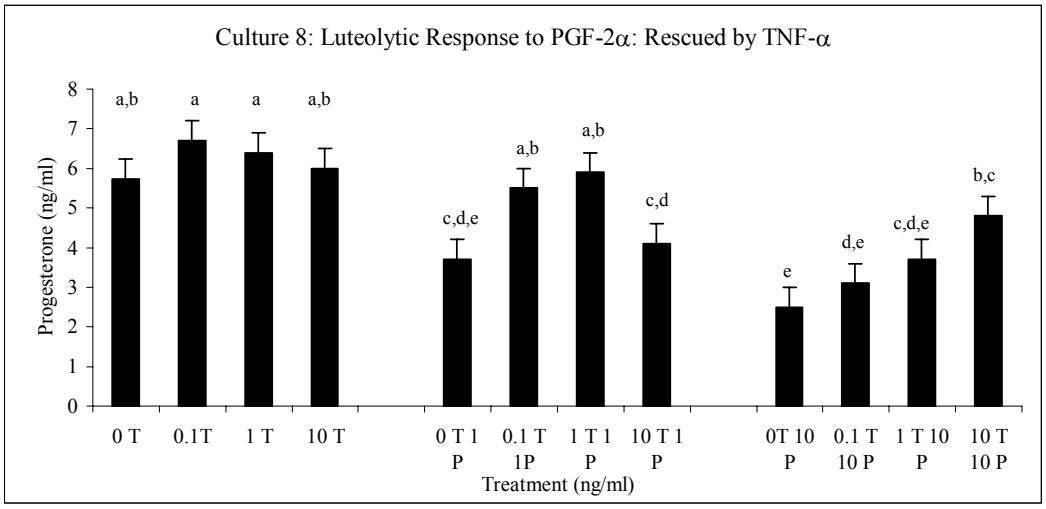

Fig. 3D: Luteolytic Response to PGF-2α: Rescued by TNF-α, Experiment 8. Progesterone (P_4) was determined in day 6 cultured luteal cells in response to TNF-α and PGF-2α, n=6 wells per treatment. T= TNF-α, P= PGF-2α .Bars represent P4 levels (ng/ml) +/- SEM. Different letters denote significant differences between groups ($p<0.05$).

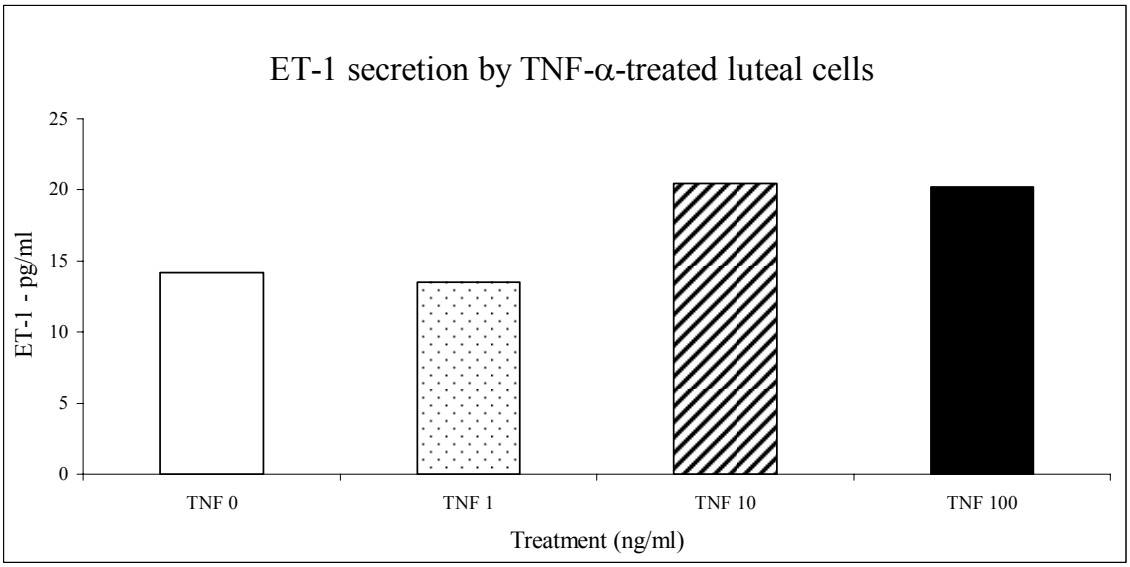

Fig. 4: TNF-α increases ET-1 secretion in a dose-dependant manner in cultured luteal cells, n=2 wells per treatment. ET-1 secretion was measured by EIA, and shows TNF-α stimulated secretion with 10 and 100 ng/ml of TNF-α.

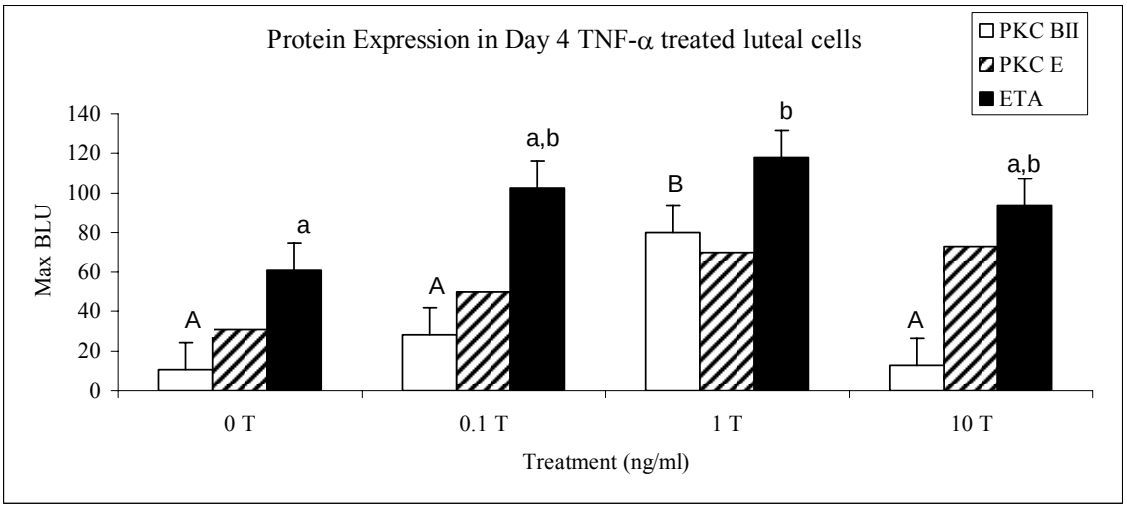

Fig. 5A: Protein expression in TNF-α treated luteal cells. Protein expression was determined by Western Blot analysis, all values represent protein of interest, n=2-3 wells per treatment. White bars represent PKC βII expression in response to TNF-α. Striped bars represent PKC ε expression in response to TNF-α. Solid black bars represent ET_A expression in response to TNF-α. T= TNF-α. Bars represent the least-squared means +/- SEM of the densitometric analysis. Different letters denote significant differences between treatments ($p<0.05$).

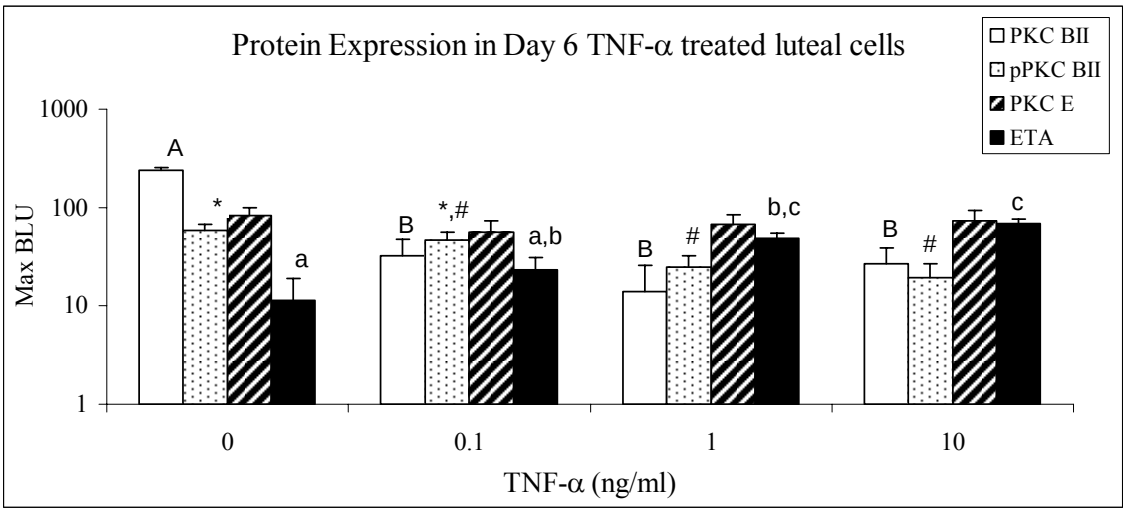

Fig. 5B: Protein expression in TNF-α treated luteal cells. Protein expression was determined by Western Blot analysis, all values represent protein of interest, n=3-4 wells per treatment. White bars represent PKC βII expression in response to TNF-α. Dotted bars represent pPKC βII expression in response to TNF-α. Striped bars represent PKC ε expression in response to TNF-α. Solid black bars represent ET_A expression in response to TNF-α. T= TNF-α. Bars represent the least-squared means +/- SEM of the densitometric analysis. Different letters or symbols denote significant differences between treatments ($p<0.05$).

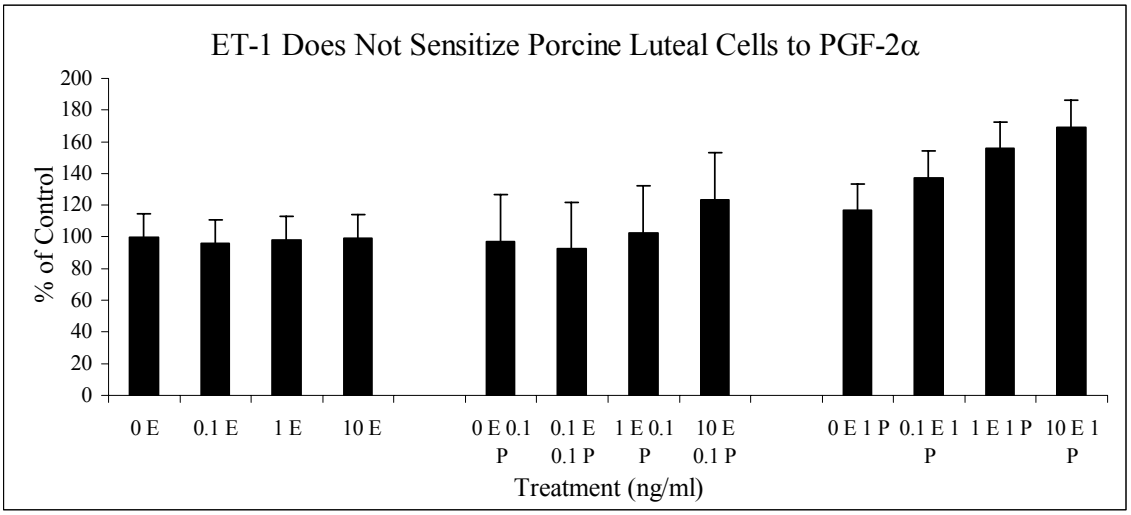

Fig 6: ET-1 does not sensitize porcine luteal cells to PGF-2α in vitro. Progesterone (P_4) was determined in day 6 cultured luteal cells in response to ET-1 and PGF-2α, n=6 wells per treatment. E= ET-1, P= PGF-2α. Bars represent % of control +/- SEM of the P_4 level. No statistical significances were detected.

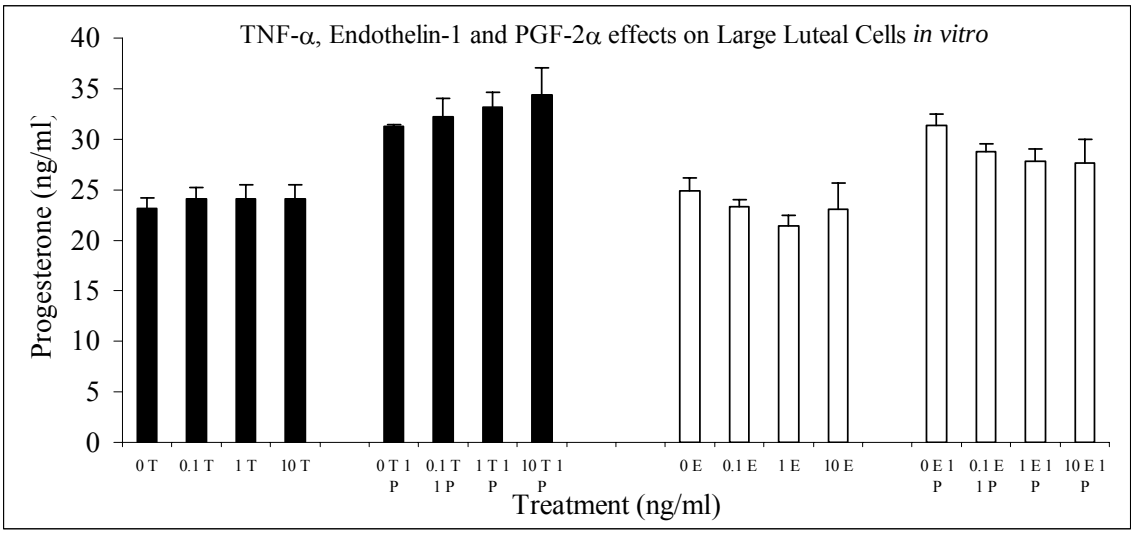

Fig. 7: TNF-α, ET-1 and PGF-2α effects on porcine LLCs in vitro. Progesterone (P_4) was determined in day 6 cultured luteal cells in response to TNF-α or ET-1 and PGF-2α, n=6 wells per treatment. T= TNF-α, E= ET-1, P= PGF-2α. Bars represent P4 levels (ng/ml) +/- SEM. No significant differences between treatments were found.

Spatial and Temporal Expression Patterns of Endothelin (ET) -1, Endothelin-Converting Enzyme-1 and ET- Receptors in the Porcine Corpus Luteum[1]

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ABSTRACT

Prostaglandin-F-2α (PGF-2α) has long been recognized as the physiological luteolysin in the porcine corpus luteum (CL); however the mechanism by which the CL acquires sensitivity to PGF-2α is not understood. It has been suggested that the endothelin (ET-1) "system", [pre-pro-ET-1 (ppET-1), endothelin-converting enzyme (ECE)-1, endothelin receptor A, (ET_A) and endothelin receptor B, (ET_B)] plays a mediatory role in luteolysis in ruminants. It was also suggested that ET-1 may be limiting to luteal sensitivity to PGF-2α during the bovine estrous cycle. Therefore, we investigated the expression (mRNA by RT-PCR and protein by Western blotting) of ET-1, ECE-1, ET_A and ET_B in porcine CLs collected throughout the estrous cycle (days 4, 7, 10, 13 and 15). Additionally, we determined the cellular localization of ET-1 system components using immunofluorescent labeling. Our results indicate that although ppET-1 mRNA expression was highest on D7 of the cycle, ppET-1 protein expression remained unchanged throughout the cycle. Immunofluorescence labeling for ppET-1 showed that it was localized exclusively to endothelial cells (EC). ECE-1 mRNA expression was highest on day 7 of the cycle, and ECE-1 protein expression was highest on days 10 and 15. ECE-1 protein was immunolocalized to ECs and large luteal cells (LLC). ET_A mRNA expression was highest on day 7 of the cycle, but showed no significant change in protein expression in the cycle, and thus displayed a similar pattern of expression to that of ppET-1. Immunofluorescence labeling confirmed ET_A expression in both endothelial cells (ECs) and LLCs. Both ET_B mRNA and protein were expressed consistently throughout the cycle and ET_B receptors were also localized to both ECs and small luteal cells (SLCs). The expression and localization of ET-1 and its system components shown in this study reveal a complex pattern of regulation in the porcine CL during the estrous cycle. The increased ECE-1 on day 10 may represent a key control point in the acquisition of LS. Further measurement of luteal concentrations of ET-1 should enable us to confirm the importance of ET-1 in the onset of LS.

INTRODUCTION

The porcine CL is unusual in that it does not show a luteolytic response to an exogenous dose of PGF-2α until after day 12-13 of an 18-21 day cycle [1,2,3]. This is in marked contrast to other farm animal species in which luteolysis can be induced after about 6 days of the estrous cycle [4,5]. It has been suggested that acquisition of luteolytic sensitivity (LS, ability to respond to PGF-2α) in the pig is not due to a lack of PGF-2α receptors, as they are present and functional as early as day 5 [6,7], but due to a lack of post-receptor signaling (i.e. Protein Kinase C) [8,9]. Therefore understanding the lack of sensitivity to PGF-2α earlier in the cycle is critical for the discovery of new approaches to estrous cycle regulation in this species. It has been suggested that the ET-1 system (ppET-1, ECE-1, ET_A, ET_B) plays an important role in luteolysis in many species (cow-[10,11,12,13,14]; sheep-[15]). More importantly, in the cow it has been suggested that a lack of ET-1 synthesis from its precursor (Big-ET-1), is due to the relative deficiency of ECE-1, which may account for the lack of luteolytic sensitivity (prior to day 6). Thus in the present study, we examined the expression patterns and localization of ET-1 system components in the porcine CL throughout the estrous cycle to investigate whether changes in ET-system expression may underlie control of luteolytic sensitivity in the pig.

ET-1, originally isolated from porcine aortic endothelial cells [16], is a 21-amino acid peptide that is a member of a structurally related peptide family that includes ET-2, ET-3 and sarafotoxins [17]. ET-1 is synthesized in endothelial cells from a 212-amino acid precursor, ppET-1. ppET-1 is then cleaved into a biologically inactive, 38-amino acid peptide, big-endothelin (Big ET-1) by an endopeptidase [13], and then is secreted from the cells. ECE-1 then cleaves Big ET-1 into the biologically active 21-amino acid ET-1 peptide at the target cells (e.g. LLC) [18]. ET-1 exerts is effects by interacting with endothelin receptor A (ET_A) or endothelin receptor B (ET_B), both of which are G-protein coupled transmembrane receptors [19,20] and are expressed in the CL of several species (bovine-[21,22], human- [23,24], primate- [25], ovine-[15], rat- [26]). ET-1 preferentially binds to ET_A (versus ET-2 or ET-3) [27], although ET_B binds all ET's (ET-1, ET-2, ET-3) with equal affinity [19,20].

ET-1 has been shown to play a role in luteolysis [28] and inhibits progesterone release in both granulosa and luteal cells in many species [11,15,19,29,30]. ET-1 has

also been shown to enhance PGF-2α production in the human and rabbit CL [31], and there is evidence in the cow [11, 28, 32, 33] and sheep [15], which suggests that ET-1 interacts with PGF-2α in the control of luteolysis. Hinckley and Milvae (2001) showed a sub-luteolytic dose of PGF-2α is effective in decreasing progesterone following ET-1 pretreatment, suggesting that ET-1 acted to sensitize ovine CL to PGF-2α [9]. In addition, this study showed that an injection of ET-1 at the mid luteal stage decreased plasma progesterone concentrations, and a luteolytic dose of PGF-2α stimulated gene expression of ET-1, showing a synergism of ET-1 and PGF-2α [9]. They also demonstrated that an injection with an ET_A antagonist (BQ123) at the mid-luteal phase in the ewe diminished the luteolytic effect of PGF-2α, suggesting a role specifically for the ET_A receptor [9]. In the bovine CL, the ET_A antagonist BQ123 blocked the PGF-2α induced inhibition of progesterone production, suggesting that ET_A plays a critical role in PGF-2α mediated luteolysis also in the cow [11]. Therefore the presence of ET_A receptors in the porcine CL may suggest they play a similar role in luteolysis. Additionally, ET-1 may induce endothelial cell apoptosis through ET_B receptor binding [34], as ET_B levels are elevated around the time of spontaneous CL regression in many species [13]. Thus the presence of ET_B receptor, particularly in the late stages of the cycle could implicate a role for ET_B in structural luteolysis in the pig.

Although the ET-1 system has been well characterized in the bovine CL [18], it has not been examined in the porcine CL. Thus in the present study we examined the expression patterns and localization of the ET-1 system components (ppET-1, ECE-1, ET_A, ET_B) in the porcine CL throughout the estrous cycle, to investigate whether changes in ET-system expression may underlie control of acquisition of luteolytic sensitivity in the pig.

MATERIALS AND METHODS

Chemicals and Reagents

Ketamine, xylazine and halothane were purchased from Webster Veterinary Supply (Sterling, MA). Tri-reagent and DNAse kit were purchased from Ambion (Austin, TX). Omniscript kit and MasterMix Kit were purchased from Qiagen (Valencia, CA). Primers were synthesized by Sigma-Genosys (Woodlands, TX). 4-12% Bis-Tris

gels, molecular weight markers and Alexa Fluor secondary antibodies for immunofluorescence were purchased from Invitrogen (Carlsbad, CA). PVDF membrane was purchased from Pall Company (Pensacola, FL). Antibodies were purchased from Chemicon (ET_A, ET_B), SantaCruz Biotechnology (ppET-1, ECE-1) or ABCAM (β-actin, von Willibrand Factor). ECL kit was purchased from GE Healthcare (Piscataway, NJ). Unless noted all other chemicals and reagents were purchased from Sigma (St. Louis, MO).

Animals

30 crossbred (females- Landrace and Yorkshire, males- Durco and Hamshir) cycling gilts were obtained from 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. On the day of surgery (days 4, 7, 10, 13 and 15 of the cycle; n = 6 per day), animals were anesthetized with i.v. injection of Ketamine (2.2 mg/kg) and Xylazine (0.4 mg/kg). Gilts were subsequently maintained on halothane and oxygen/nitrous oxide for the remainder of the procedure. Ovaries were collected via midventral laparotomy and CL were dissected from the ovary. CL were snap frozen in liquid nitrogen and stored at -80°C until further use. All animals were housed at NCSU Swine Unit and all protocols were approved by NCSU Institutional Animal Care and Use Committee (IACUC).

RNA Isolation

Total RNA was isolated from CL using Tri-Reagent® according to the manufacturer's protocol. Briefly, CL were homogenized in 1ml Tri-Reagent®/mg tissue. Chloroform was added, mixed and incubated for 15 minutes. Samples were centrifuged at 12,000g for 15 minutes and the aqueous phase containing RNA was removed. Ethanol was subsequently added and mixed. Samples were then transferred into an RNAqueous filter cartridge (Ambion) and centrifuged at 12,000g. RNA in collection tube was washed and eluted. RNA quality and concentration were determined by agarose gel electrophoresis and by NanoDrop spectrophotometry at 280 nm (Wilmington, DE),

respectively. Samples were then DNAase treated using the DNAse treatment kit (Ambion). RNA was subsequently quantified using the NanoDrop Spectrophotometer.

Reverse Transcription- Polymerase Chain Reaction

As a measure of mRNA expression, steady state levels of mRNA for PKCs were determined by semi-quantitative (sq) RT-PCR, as described below. DNAse-treated RNA was used to synthesis cDNA using the Omniscript® kit (Qiagen). Negative controls (no reverse transcriptase) were also run on each sample to ensure that DNAse treatment was successful in removing any genomic DNA contamination. Primers were designed porcine sequences when available (all except ECE-1 and H2A, bovine and human sequences respectively), and other primers were designed based on the sequence availability and expected homology to porcine sequences using Mac Vector (Accelrys Inc.). Primers were synthesized based upon the published GenBank sequences to produce the gene products (see Table 1 for primer sequences and bp sizes). All primers were validated for semi-quantitative (sq) RT-PCR by running each primer at 20, 25, 30, 35 and 40 cycles and choosing a cycle number that corresponded to the linear range of amplification (all 33 cycles except H2A, 28 cycles). Once primers were validated, sq RT-PCR was run using 1 ug of cDNA and the Taq PCR Mastermix® Kit (Qiagen). Thermocycler conditions were as follows: 3 minute initial denaturation 94°C, followed by 28 or 33 cycles of denaturation, 30 seconds 94°C; annealing, 1 minute 50°C; extension, 1 minute 72°C; followed by a 5 minute 72°C final extension. All gene products were separated on 2% agarose gels and stained with ethidium bromide. Products were quantified using Lab-Works imaging system (UVP Imagining Company, Upland, CA). Values were calculated as the ratio of gene product band intensity/H2A band intensity; H2A was used as a housekeeping gene since its levels of expression did not vary with stage of estrous cycle. All gene products were cleaned using MultiScreen $PCR_{\mu 96}$ PCR cleanup plates (Millipore, Billrica, MA), following the manufacturer's recommended protocol. RT-PCR amplicons were sequenced following the recommended protocol with the ABI BigDye v. 3.1 sequencing kit (Applied Biosystems, Foster City, CA). Sequencing reactions were purified using an ethanol/ammonium acetate precipitation protocol [35] and visualized using an ABI 3130XL Automated Sequencer

(Applied Biosystems, Foster City, CA). Sequences were compiled in Sequencher version 4.5 (Gene Codes Corp., Ann Arbor, MI) and NCBI BLAST searches were performed to verify sequence identities.

Western Blotting

Total protein was extracted from CL by homogenization in a 20 mM HEPES buffer containing 2mM EDTA, 2mM EGTA, 1% Triton-X, 20 μg/ml aprotinin, 20 ug/ml leupeptin, 4 ug/ml PMSF, followed by centrifugation at 14,000 x g to remove cellular debris. Protein concentrations were determined using the Micro-BCA protein assay (Pierce, Rockford, IL) according to manufacturer's instructions. Western blots were performed as previously reported [36]. Briefly, 20 ug of protein was loaded on to 4-12% Bis-Tris gels and subjected to electrophoresis at 120 volts at room temperature for 50 minutes. Following electrophoresis, proteins were transferred to PVDF membrane for 60 minutes at 30 volts. Membranes were blocked in 5% milk TBS-Tween for 1 hour at room temperature and then incubated with primary antibody (1:200: ET_A, ET_B, ECE-1, ppET-1; 1:2000: β-actin) overnight at 4°C. Membranes were washed in TBS-Tween three times for 5 minutes, followed by incubation in secondary antibody (anti-rabbit-ET_A, ET_B, ppET-1, ECE-1; anti-mouse- β-actin, 1:2000) for 1 hour at room temperature, and washed another three times with TBS-Tween. Chemi-illuminescence (ECL kit) was used to visualize protein bands and bands were quantitated with Lumi-Imager software (Roche, Indianapolis, IN). The specificity of protein bands was determined in separate gels using antibody which has been previously mixed with an excess of blocking peptide (used for generation of the antibody, data not shown). The quantity of chemiluminescence in specific protein bands were normalized to that obtained with the β-actin antibody.

Cell Dissociation and Immunofluorescence

CL were collected from day 13 cycling animals (as described above) and transported in M199 with antibiotics and antimycotics on ice. CL were dissociated as previously described [6]. Briefly, CL were dissociated in collagenase for 30 minutes at 37°C. Dissociated cells were removed and remaining tissue was incubated for another 30

minutes, which was repeated until all cells were completely dissociated. Cells were washed and placed in Percoll to remove red blood cells. LLCs were then counted and viability determined by trypan blue exclusion. Cell viability was determined to be over 90%. Cells were placed in chamber slides (Fisher Scientific, Waltham, MA) that were previously coated in 0.1% Gelatin. 25,000 cells (based on LLC count) were plated Medium 199 and incubated at room temperature for 1 hour. Medium was then removed and the plated cells were fixed in ice cold acetone for 10 minutes. Cells were rinsed with phosphate buffered saline (PBS) and blocked with 1% Normal Goat Serum in PBS for 30 minutes at room temperature. Primary antibodies (1:40; using antibodies described for Western blotting plus von Willibrand factor (EC marker) antibody to double stain cells) in 1% BSA in PBS (1:40; using antibodies described for Western blotting) were added and incubated overnight at 4°C. Slides were then washed with PBS, and Alexafluor secondary antibodies (1:800, anti-rabbit- ppET-1, ECE-1, ET_A, ET_B; 1:800 anti-mouse-von Willibrand Factor) were added and incubated for 2 hours at room temperature. LH receptor was used as a SLC marker (Fig. 4B), PGF-2α receptor was used as a LLC marker (Fig. 4E) and von Willibrand Factor was used as a EC marker (Fig. 4H). DAPI (Vector Laboratories, Burlingame, CA) were added to visualize nuclei and slides were viewed at 40x magnification using a Nikon Eclipse E400 Microscope.

Statistical Analysis

Values were calculated by dividing densitometric values from gene/protein of interest divided by the housekeeping gene/protein (H2A/β-actin). Values from day of cycle (n=6) were then averaged. All mRNA statistical analysis was carried out using Repeated Measures followed by one-way analysis of variance and Duncan's test for significance using Statistical Analysis Software (SAS, Cary, NC). Repeated measures for mRNA analysis was performed because each RT-PCR reaction was performed twice for each sample and gene. All protein statistical analysis was performed using one-way analysis of variance, followed by Duncan's test for significance using SAS. All housekeeping genes (H2A for mRNA and β-actin for protein) were also analyzed across the days of the cycle. All means are reported as least-squares means +/- SEM. Differences were considered significant at $p < 0.05$, unless otherwise noted.

RESULTS

Expression of mRNA levels throughout the estrous cycle

The expression of ppET-1, ECE-1, ET_A and ET_B mRNAs throughout the estrous cycle was determined (Figure 1). H2A values were analyzed across all days of the cycle and no differences in days were found (data not shown). ppET-1 mRNA expression was low on day 4, increased 7-fold ($p<0.01$) on day 7, declined ~ 2-fold ($p<0.01$, versus day 7) on day 10 and then remained low on days 13 and 15 (Fig. 1A). A similar pattern was seen in ECE-1 mRNA expression (Fig. 1B), as expression on day 7 was 7-fold higher than on day 4 ($p<0.01$), 3.5-fold higher than on day 10 ($p<0.01$), and approximately 50 fold higher than on days 13 and 15 ($p<0.01$; versus day 7). Again this pattern was demonstrated in ET_A mRNA expression (Fig. 1C). On Day 7, ET_A mRNA expression was almost 90-fold higher than on days 4, 13 or 15 ($p<0.01$), and 45-fold higher than on day 10 ($p <0.01$). In contrast, ET_B mRNA expression (Fig. 1D) showed no significant changes over the estrous cycle, although there was a tendency ($p=0.9$) for a decrease in expression on day 13 versus day 7. Representative RT-PCR products are shown in Figure 5A.

Protein expression throughout the estrous cycle

Protein expression of the ET-1 system was also examined throughout the estrous cycle (Figure 2). Expression of β-actin was analyzed across all days of the cycle and no differences were found (data not shown). ppET-1 (Fig. 2A), ET_A (Fig. 2C) and ET_B (Fig. 2D) showed no significant differences in protein expression throughout the estrous cycle. However, ECE-1 protein showed a significant (2-fold, $p<0.05$) increase on day 10 versus day 4, 7 and 13 (Fig.2B). Day 15 was not different from any other days. Representative Western blots are shown in Figure 5B.

Immunofluorescence in Day 13 CL

The cellular localization of ET-1 system (ppET-1, ECE-1, ET_A and ET_B protein) components was localized in day 13 CL by immunofluorescence. As shown in Figure 3, ppET-1 was expressed exclusively in ECs (Fig 3B), since they also stained positively von

Willibrand factor (Fig. 3C, double stained Fig. 3D). No large luteal cells were found to stain positively for ppET-1. However, ECE-1, although predominantly expressed in ECs (Fig. 3F), some LLCs (~10%) were found to be positive for ECE-1 (not shown), suggesting ET- peptide may be cleaved by both of these cell types in the porcine CL. ET_A receptors were primarily localized to LLCs (Fig. 3J), with a few (<5%) ECs and SLCs staining positive (not shown). ET_B receptors were shown to be expressed primarily on ECs and SLCs (Fig. 3N), however a few LLCs (< 5%) also stained positive (not shown). Positive controls for double labeling are shown in Figure 4.

DISCUSSION

The present study characterized and quantified the expression and cellular localization of the ET-1 system in porcine CL throughout the estrous cycle, and provided some evidence that elevated ET-1 (via increased ECE-1 protein on day 10) may be involved in acquisition of LS, which is hypothesized [37] to occur between days 7 and 13.

At the mRNA level, ppET-1, ECE-1 and ET_A all showed the same temporal pattern of expression; low levels throughout most of the cycle, but significantly increased expression on day 7. At the protein level, however, of these 3 genes only ECE-1 expression showed any significant temporal changes, with a 2-fold elevation observed on day 10. No significant temporal changes were seen in the expression of either mRNA or protein for ET_B. It is not known why differences were observed between mRNA and protein for each gene, but it is quite possible that control at both transcriptional and translational levels exist for these ET-1 system components. Nevertheless, we recognize the limitations of the technique (RT-PCR) used to measure mRNA expression which only monitors the steady state levels of mRNA, and cannot provide a definitive measure of transcriptional activity.

In the bovine CL, ppET-1 mRNA was found to be expressed at similar levels in both the early and mid luteal phases in one study [38]. In another study however, ppET-1 expression was highest in the regressing bovine CL, leading these authors to suggest that PGF-2α may play a role in increasing ppET-1 expression at that time [28]. A more recent report supports this latter publication [11], showing that ppET-1 mRNA was high after

ovulation, decreased in the mid-late cycle and then increased again after regression (>day 18) [13]. In the rat, ppET-1 was also seen to be expressed at its highest levels during luteal regression [39]. Our immunofluorescence study shows the localization of ppET-1 exclusively to ECs, which agrees with other localization studies showing ppET-1 exclusively to ECs in the bovine CL [11, 40].

Other studies have also shown that administration of PGF-2α at mid-cycle increases ET-1 expression (7-fold), as early as 2 hours after treatment, and at 24 hours after PGF-2α treatment [28]. In addition, Choudhary and colleagues (2004) also found that PGF-2α treatment increased ppET-1 mRNA on day 10 of the cycle, and increased ET-1 protein on day 10 in the bovine CL [41]. Confirmation of these data comes from another study showing that ET-1 mRNA increased at mid-cycle after treatment with cloprostenol, but not on day 4 of the cycle [42]. Finally, PGF-2α infused via microdialysis into the bovine CL on day 7, increased ET-1 release over a 12 hour period [43]. Our preliminary data showed that TNF-α increases ET-1 secretion (measured by EIA) and ET_A protein expression (measured by Western blot) in day 6 luteal cells *in vitro*, suggesting that increasing levels of ET-1 acting through the ET_A receptors (present on the LLCs) may play a critical role in LS in the pig.

As discussed above, ECE-1 mRNA expression was significantly higher on day 7 versus other days of the cycle ($p < 0.01$); however protein expression was significantly higher (2-fold) on day 10 than days 4, 7 and 13. This pattern of ECE-1 protein expression was similar to that seen in the bovine CL; these authors observed a four fold increase in both mRNA and protein expression between the early and mid luteal phase of the bovine cycle [25]. They further suggested that the low levels of ECE-1 expression in the early luteal phase may restrict the production of active ET-1 at this time, and thus render the CL insensitive to PGF-2α (CLs lack LS) at this stage [18, 38]. In ECE-1 half life studies, ECE-1 mRNA has been shown to remain stable for up to 38 hours in injured liver cells [44]. Therefore we suggest, similar to injured tissue (where there is tissue reorganization and an increase in angiogenesis, similar to angiogenesis in the CL), the CL may provide an environment such that the ECE-1 mRNA (elevated on day 7) remains stable until the specific time in LS (day 10) when ECE-1 protein levels are elevated, as required to cleave Big ET-1 into active ET-1 peptide resulting in LS. In addition, both steroidogenic

and endothelial cells express ECE-1 [38], and this study confirmed ECE-1 protein expression to both LLCs and ECs (although to much less of an extent in LLCs) in the porcine CL by immunofluoresence (Fig. 3H). Therefore, since ECE-1 is located on LLC, we suggest that ET-1 may be locally produced for action on the LLCs in the porcine CL, similar to what has been shown in the bovine CL [38].

In the cow, PGF-2α increases ECE-1 mRNA expression on day 10 (compared to day 1 and 4); however a reduction in ECE-1 protein expression was seen on day 10 and 17 in response to PGF-2α [41]. Furthermore, this study showed no change in ECE-1 activity throughout the cycle, and these authors have suggested that ECE-1 is not the limiting factor in ET-1 production. In our study, we determined mRNA and protein expression of ECE-1 during the normal cycle and did not examine the effects of PGF-2α treatment; however, although ECE-1 mRNA levels decreased with luteal regression (day 15), ECE-1 protein showed a secondary trend by increasing on day 15, which is consistent with a stimulatory effect of PGF-2α in the pig CL.

ET_A mRNA expression, similar to ppET-1 and ECE-1 was significantly higher in day 7 CL that on the other days of the cycle ($p < 0.01$). However, no significant difference was seen in protein expression throughout the cycle. This data suggests that it is not a lack of ET_A receptors early in the cycle that is responsible for the lack of LS, but it is due to some other component (i.e. ECE-1). Studies have suggested that ET_A is the receptor by which ET-1 acts in the CL, as ET_A selective antagonists, particularly BQ-123 blocked the ET-1 luteolytic effects in the rabbit [45]. Choudhary and colleagues (2004) saw an increase in ET_A mRNA, but not ET_B with PGF-2α treatment [41], as did Meidan and Levy (2002), both in the mid-cycle in the bovine CL [18]. In this study, ET_A was found to be localized on LLCs (Fig. 3J), as well as some ECs (data not shown) in the porcine CL. This suggests that the LLCs of the porcine CL, are not only the site of PGF-2α action [6, 7], but also a site of ET-1 action via ET_A receptors. Therefore ET-1 may act (via ET_A) either to sensitize luteal cells to PGF-2α (hypothesis; [37]) or in concert with PGF-2α to cause luteolysis. The precise mechanism by which they work together in the porcine CL must still be elucidated.

In contrast to ET_A mRNA, ET_B mRNA expression was not significantly different over the stages of the cycle, however there was a trend ($p=0.09$) towards a decrease on

day 13 versus day 7. Protein expression also remained constitutively expressed throughout the cycle, suggesting that ET_B receptors are present and function if activated, and therefore are not a limiting factor, either in acquisition of LS or in any other capacity. In the bovine CL, Berisha and colleagues (2002) found that ET_B mRNA increased in the late luteal phase and even more so after regression [12]. Additionally, an increase in ET_B mRNA was seen on days 13-18 the rabbit estrous cycle [45]. More recently a study in the bovine CL in which PGF-2α treatment was followed by an ET_A antagonist, an increase in ET_B mRNA by 24 hours after treatment was shown [46]. However no localization studies have been carried out in the bovine CL on ET_B to show what cell types ET_B is expressed on, and therefore the physiological role is not understood in the CL. ET-1 may induce endothelial cell apoptosis through ET_B receptor binding [34], as ET_B levels are elevated around the time of spontaneous CL regression in the cow [21], thus the presence of ET_B receptor, particularly in the late stages of the cycle could implicate a role for ET_B in structural luteolysis in the pig. Our immunofluorescence studies indicate that ET_B are expressed on ECs and SLCs (Fig. 3N), and a few LLCs (data not shown). ET-1, via ET_B, has been shown to increase vasodilation through NO production [47], and thought to be involved in ET-1 clearance as well as promote apoptosis particularly in prostate tissue [48]. Therefore, ET_B may be present in the porcine CL on more than one cell type to induce apoptosis to control some aspect of structural luteolysis in the late stage of the porcine estrous cycle.

Understanding the acquisition of LS in the porcine CL is of major economic interest for estrus cycle regulation in the pig. This data suggests that the ET-1 system plays a role in LS by increasing ECE-1, and therefore bioactive ET-1, around the time of acquisition of LS in the pig (acting through ET_A), and possibly at the end of the cycle to induce apoptosis through ET_B. Therefore, further studies are warranted to determine the causes of the refractoriness of the early CL to PGF-2α, and how the ET-1 system is playing a physiological role.

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Table 1. Primer sequences used for PCR[a]

Gene	Accession Number	Product Size			Cycles	Annealing Temp.
H2A	X52318	209	F	AGGACGACTAGCCATGGACGTGTG	28	50
			R	CCACCACCAGCAATTGTAGCCTTG		
ECE-1	NM_181009	421	F	GATGGTGATGTTGGCCAGCG	33	50
			R	GGGAACACAACCAGGCCATC		
ET_A	NM_214229	561	F	GCACAAAACCTGTATGCTCAATGC	33	50
			R	TGGCTGCTCCTCTCAGTCAGTGTTATGG		
ET_B	AY583500	147	F	TGCGAATCTGCTTGCTCCATCC	33	50
			R	GCATTTCACAGGTCATCGGG		
ppET-1	X07383	351	F	GCCATCAGCAACAGCATCAAAAC	33	50
			R	ATCACCGCAAAGGAGGAGAGAC		

[a] All primers are listed 5'-3'

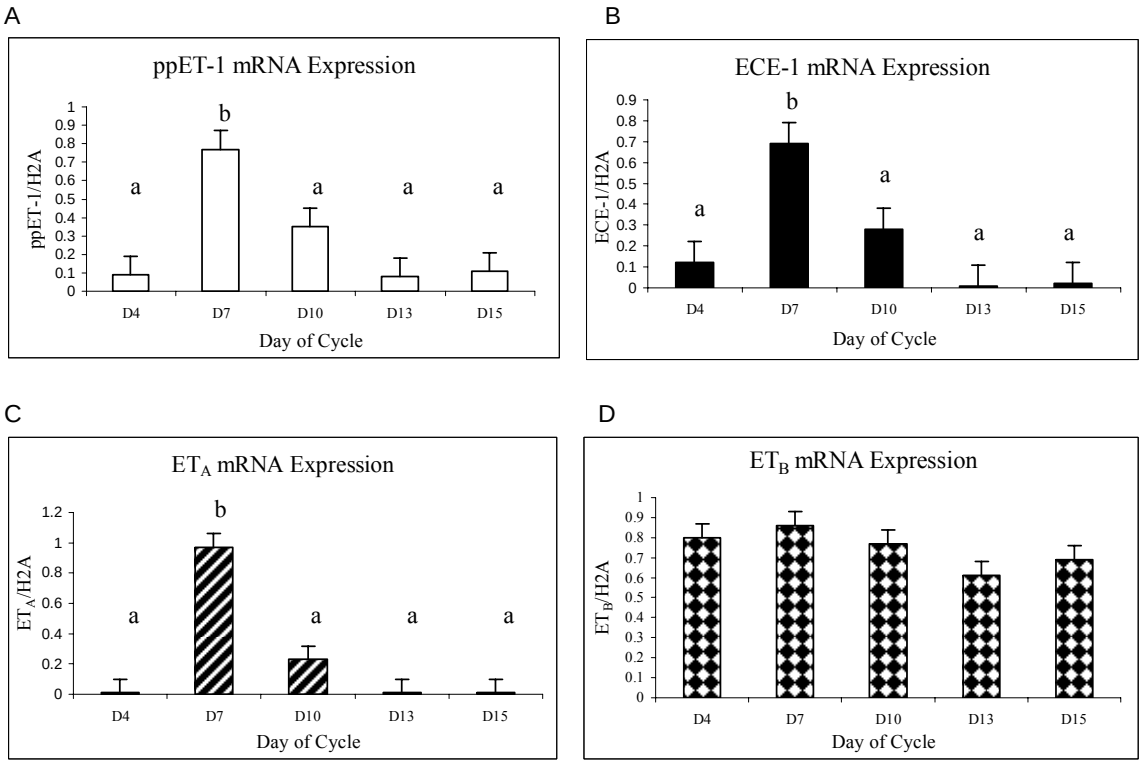

Fig. 1. mRNA expression in porcine CL throughout the estrous cycle. mRNA expression on days 4, 7, 10, 13 and 15 of the cycle was determined by semi-quantitative RT-PCR using H2A as a housekeeping gene. All values represent gene of interest/H2A. A) ppET-1, B) ECE-1, C) ET_A and D) ET_B. Bars represent the least-squared means +/- SEM of the densitometric analysis (n=6). Different letters denote significant differences between groups ($p<0.01$).

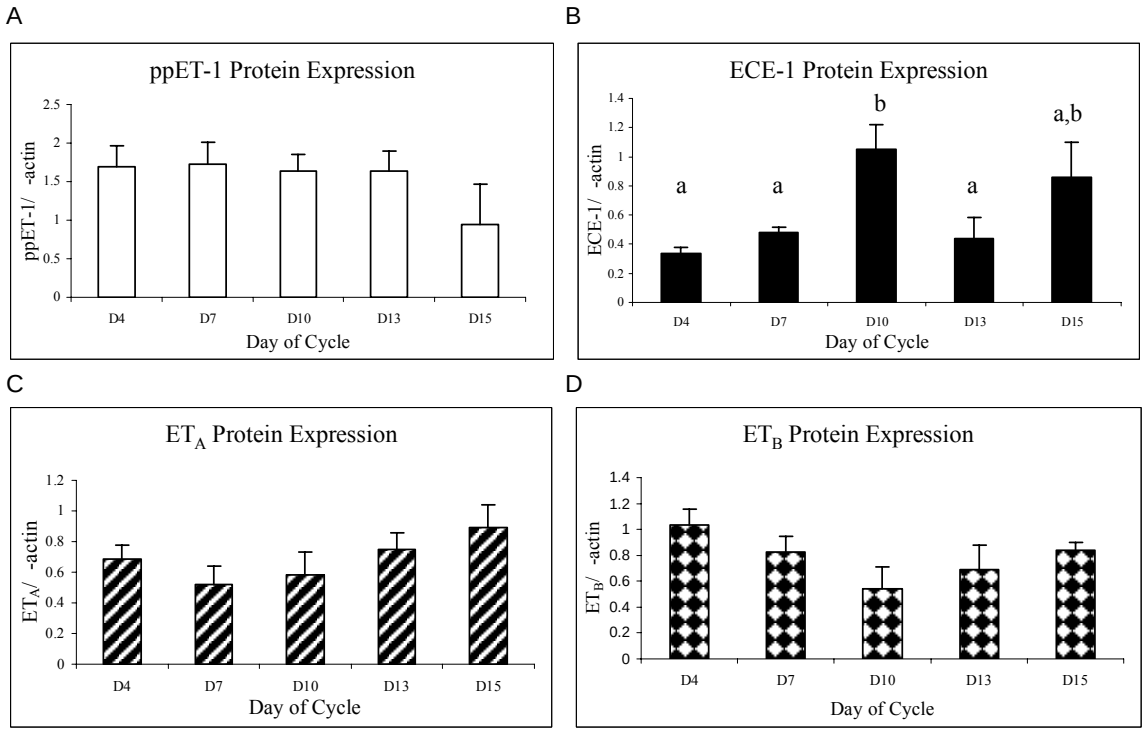

Fig 2.: Protein expression in porcine CL throughout the estrous cycle. Protein expression was determined on days 4, 7, 10, 13 and 15 of the cycle by Western Blot analysis using β-actin as a housekeeping gene. All values represent protein of interest/ β-actin. A) ppET-1, B) ECE-1, C) ET_A and D) ET_B. Bars represent the least-squared means +/- SEM of the densitometric analysis (n=6). Different letters denote significant differences between groups ($p<0.05$).

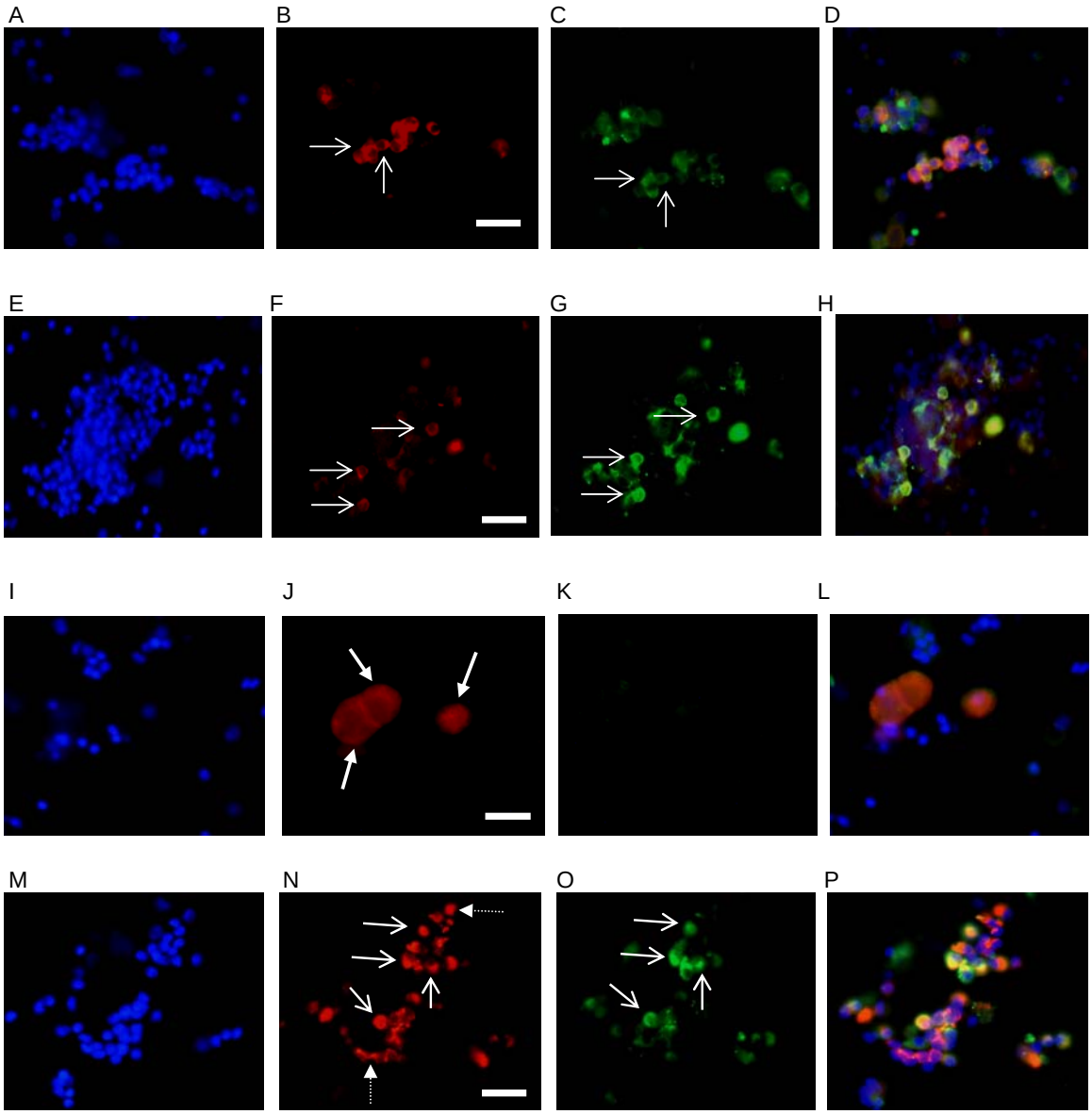

Fig. 3: Localization of ET-1 system components in day 13 mixed luteal cell preparations by immunofluorescence. Panels A, E, I, and M represent DAPI staining of nuclei. Panels B, F, J and N represent immuno-positive staining of ET-1 components. Panels C, G, K and O represent von Willibrand positive cells (EC marker). Panels D, H, L and P show DAPI/Immunostaining overlays. B shows ppET-1 is localized to ECs. F shows ECE-1 is localized to ECs. J shows ET_A is localized to LLCs. N demonstrates ET_B positive localization in ECs and SLCs. Large thick arrows represent LLCs, small thin arrows represent ECs. All panels are at 40x magnification. Bar represents 60 μm.

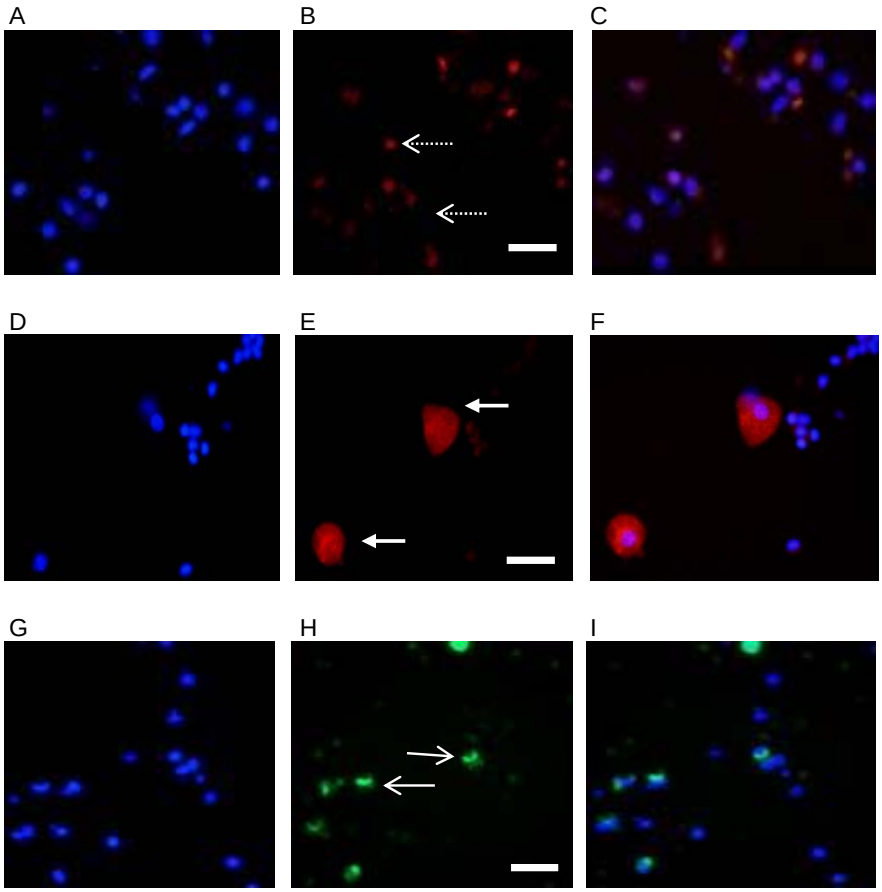

Fig. 4: Detection of luteal cell markers (controls) in day 13 mixed luteal cell preparations by immunofluorescence. Panels A, D and G represent DAPI staining of nuclei. Panel B represents LH receptor immunostaining of SLCs. Panel E represents Prostaglandin F-2α immunostaining of LLCs. Panel H represents immunostaining of ECs by von Willibrand Factor. Panels C, F and I show DAPI/Immunostaining overlays. Large thick arrows represent LLCs, small dotted arrows represent SLCs, small thin arrows represent ECs. All panels are at 40x magnification. Bar represents 60 μm.

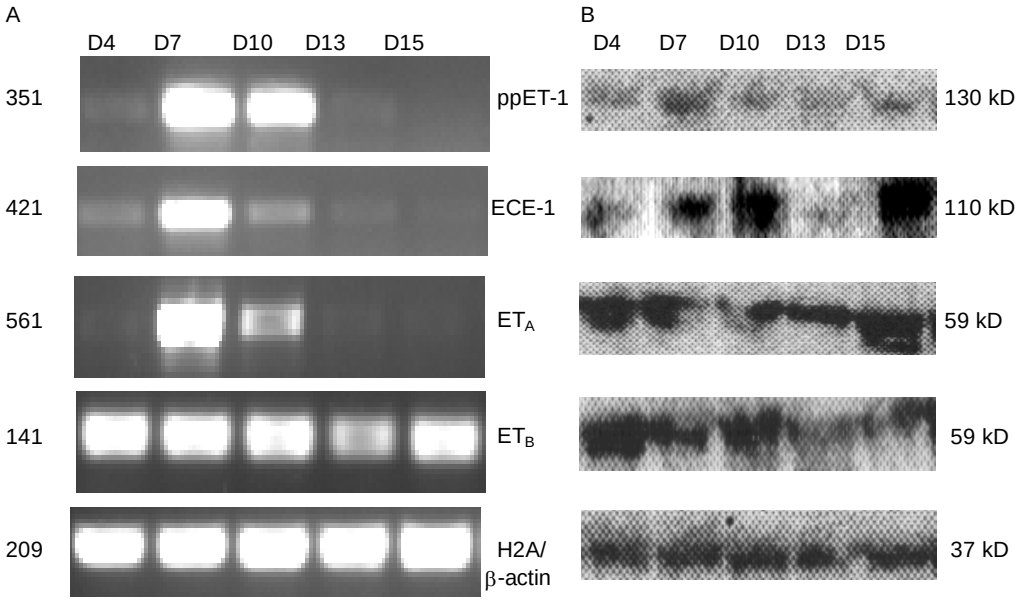

Fig. 5: Representative mRNA (A) and protein (B) analysis by RT-PCR and Western blotting of porcine luteal cells throughout the estrous cycle. Panels A show mRNA bands (H2A as housekeeper) and bp sizes. Panels B show protein bands (β-actin as housekeeper) and molecular weight.

Protein Kinase C (PKC) Expression and Cellular Localization in the Porcine Corpus Luteum and its implication in Luteolytic Sensitivity[1]

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ABSTRACT

Understanding the acquisition of luteolytic sensitivity (LS, ability to respond to Prostaglandin F-2α, PGF-2α) is critical for estrous cycle regulation in the pig. It has been well documented that porcine corpora lutea (CL) do not respond to exogenous PGF-2α before day 13 of an 18-21 day cycle, however the cellular or biochemical events that lead to the response to PGF-2α are poorly understood and have not been thoroughly studied. It has been suggested that it is not the lack of PGF-2α receptors present in luteal cells, but downstream signaling components, specifically protein kinase C (PKC), that explain the lack of LS before day 13. Therefore we examined the mRNA and protein expression patterns of the 12 PKC isoforms throughout the porcine estrous cycle, to determine whether PKC expression correlates with, and thus may be associated with the control of, the acquisition of LS in the pig**.** Semi-quantitative RT-PCR and Western blots were carried out to determine mRNA and protein expression patterns throughout the estrous cycle, and immunofluoresence was performed to determine the cellular localization of PKC isoforms in D13 CL. Our results showed that most PKC isoforms (i.e. α, βI, βII, δ, ε, η, λ, θ, ι and ζ) mRNA were maximally expressed on day 7 and day 10 (μ only) of the cycle, whereas PKCs γ and λ were unchanged, compared with other stages of the estrous cycle. In contrast, PKCη was expressed at it lowest level on day 7. At the protein level, only PKCε was significantly different (highest on day 13 versus day 10; $p<0.01$) during the estrous cycle. Immunofluoresence data also shows most PKC isoforms are localized to both large luteal cells (LLC) and small luteal cells (SLC), as is PKCε. LLCs are the cell type that PGF-2α acts upon to induce structural and functional luteolysis, and the presence of PKCε in porcine LLCs is consistent with data in the bovine CL suggesting a role specifically for PKCε, in acquisition of LS in the pig.

INTRODUCTION

The porcine corpus luteum (CL) is unusual among the other farm animal species in that it does not show a luteolytic response to exogenous Prostaglandin F-2α (PGF-2α) until ~day 13 of an 18-21 day estrous cycle. Estrous cycle regulation in the pig is of major economic interest for the swine industry, and understanding why porcine CL lack the ability to respond to PGF-2α earlier than day 13 of the cycle is of critical importance. It has been suggested that lack of luteolytic sensitivity (LS, ability to respond to PGF-2α) before day 13 is not due to a lack of PGF-2α receptors (FP), as they are present and functional as early as day 5 [1] [2], but more likely due to a lack of post-receptor signaling (i.e. Protein Kinase C, PKC) [3, 4]. The pattern of PKC isoform expression (mRNA/protein) and their cellular localization has not been previously investigated in the porcine CL throughout the estrous cycle, and therefore forms the basis of the current study. By examining the expression patterns and cellular localization of PKC isoforms, at different stages of the estrous cycle (both before and after acquisition of LS), it was expected that the data generated would provide evidence supporting the view that one or more isoforms may play a significant role in acquisition of LS in the pig.

PKCs are a family of serine/threonine kinases that play a wide variety of roles by controlling cellular function from cell proliferation to cell death [5]. There are 3 families of PKC isoforms that are categorized based on their intracellular regulation (and thus the structure of their regulatory domains) [6]. The three categories (with the activators) are 1) *Conventional*, activated by calcium, diacylglycerol (DAG) and phosphatidylserine (PS) [PKC- alpha (α), Beta I (βI), Beta II (βII), Gamma (γ)]; 2) *Novel,* activated by DAG and PS, *not* calcium [PKC-Delta (δ), Epsilon (ε), Eta (η), Theta (θ), and Mu (μ- also known as PKD, classified here with the novel isoforms because of novel-like conserved (C) 1 domains and ability to bind DAG and PS]; and 3) *Atypical*, activated by PS [PKC-Iota (ι in the mouse)/ Lamda (λ in the human), and Zeta (ζ)] [7]. All PKC isoforms are comprised of regulatory and catalytic domains; the regulatory domains contain motifs which control substrate binding while the catalytic domains contain binding sites for ATP [7]. The catalytic domains of all of the PKC families are closely related, contain the ATP binding sites, and are responsible for phosphotransferase activity [7]. Therefore, cellular localization and milieu determines the physiological response of PKC activation.

It has been suggested that the lack of sensitivity to PGF-2α in the porcine CL before D13 is believed not to be due to the deficiency of FP receptors, as they are present and apparently function as early as Day 5; it is suggested that the lack of LS may to be attributable to the lack of downstream signaling molecules such as PKC [3]. It has been shown in both the bovine and ovine CL that PGF-2α actions are mediated by PKC signaling [8], however little is known of the expression of PKC isoforms in the porcine CL during the estrous cycle. Studies on the porcine CL have shown that in day 10 cultured LLC and SLC, PMA stimulated PKC activity in both cell types, and increased progesterone production, suggesting that the refractoriness to PGF-2α on day 10 in LLC might be due to PKC activity in these cells [9]. Sen and colleagues (2004) suggested that the conventional isoforms that are expressed early in the cycle may be responsible for PGF-2α's inability to elicit response in the bovine CL, and recently showed that PKC isoform expression in the bovine CL was stage-dependant [4]. More specifically, that the *novel* (calcium independent) isoform, PKC ε, increased 11-fold after acquisition of LS [4]. These authors additionally reported that PKC ε specifically plays a mediatory role in PGF-2α induced inhibition of steroidogenesis in luteal cells, suggesting that this PKC isoform may be directly linked to LS [10]. More recently, it was shown that PKC ε was exclusively expressed in luteal steroidogenic cells, and not endothelial cells, and PKC ε was stimulated by Endothelin-1 (ET-1) in day 10 bovine CL. Therefore these authors concluded that PKC ε plays a regulatory role by decreasing progesterone (P4) during regression [11]. Additionally, it has been demonstrated that PGF-2α acting via PKC, induces cyclooxyengase-2 (COX-2) which acts in a positive feedback mechanism and functions to up regulate PGF-2α in the CL to cause luteal regression [12]. Tatsukawa and colleagues (2006) also suggest that intracellular pathways via PKC are related to regulation of luteal apoptosis and regression [13]. Therefore studies on the expression and localization of PKC isoforms in the porcine CL could aid in understanding both luteolytic sensitivity as well as the processes of functional and structural regression.

Although PKC isoform expression in the CL has been characterized in other species [4], PKC expression and localization has not been examined in the porcine CL. Therefore in the present study, porcine CL from different stages of the estrous cycle were

examined to determine the levels of 1) PKC isoform mRNA expression, 2) PKC isoform protein levels and 3) PKC protein localization to luteal cell subtypes. Overall these studies were designed to determine whether one or more of the PKC isoforms (with a particular emphasis on PKC ε) may play a critical role in acquisition of LS and luteolysis in the pig. The long term goal of these studies is to increase our understanding the control of LS so that new approaches or treatments to induce premature sensitivity to PGF-2α can be developed, which may enable effective estrous cycle regulation in this species.

MATERIALS AND METHODS

Chemicals and Reagents

Ketamine, xylazine and halothane were purchased from Webster Veterinary Supply (Sterling, MA). Tri-reagent® and DNAse kit were purchased from Ambion (Austin, TX). Omniscript® and MasterMix® kits were purchased from Qiagen (Valencia, CA). Primers were synthesized by Sigma-Genosys (Woodlands, TX). 4-12% Bis-Tris gels, molecular weight markers and secondary antibodies for immunofluorescence (Alexa Fluor) were purchased from Invitrogen (Carlsbad, CA). PVDF membrane was purchased from Pall Company (Pensacola, FL). Antibodies were purchased from SantaCruz Biotechnology (Santa Cruz, CA; All PKC Isoforms and blocking peptides) and ABCAM (Cambridge, MA; β-actin, von Willibrand Factor-endothelial cell marker). ECL kits were purchased from GE Healthcare (Piscataway, NJ). Unless otherwise noted, all other chemicals and reagents were purchased from Sigma (St. Louis, MO).

Animals

30 crossbred (females- Landrace and Yorkshire, males- Durco and Hamshir) cycling gilts were obtained from 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. On the day of surgery (days 4, 7, 10, 13 and 15 of the cycle; n = 6 per day), animals were anesthetized with i.v. injection of Ketamine (2.2 mg/kg) and Xylazine (0.4 mg/kg). Gilts were subsequently maintained on halothane and oxygen/nitrous oxide for the remainder of the procedure. Ovaries were collected via

midventral laparotomy and CL were dissected from the ovary. CL were snap frozen in liquid nitrogen and stored at -80°C until further use. All animals were housed at NCSU Swine Unit and all protocols were approved by NCSU Institutional Animal Care and Use Committee (IACUC).

RNA Isolation

Total RNA was isolated from CL using Tri-Reagent® according to the manufacturer's protocol. Briefly, CL were homogenized in 1ml Tri-Reagent®/mg tissue. Chloroform was added, mixed and incubated for 15 minutes. Samples were centrifuged at 12,000g for 15 minutes and the aqueous phase containing RNA was removed. Ethanol was subsequently added and mixed. Samples were then transferred into an RNAqueous filter cartridge (Ambion) and centrifuged at 12,000g. RNA in collection tube was washed and eluted. RNA quality and concentration were determined by agarose gel electrophoresis and by NanoDrop spectrophotometry at 280 nm (Wilmington, DE), respectively. Samples were then DNAase treated using the DNAse treatment kit (Ambion). RNA was subsequently quantified using the NanoDrop Spectrophotometer.

Reverse Transcription- Polymerase Chain Reaction (RT-PCR)

As a measure of mRNA expression, steady state levels of mRNA for PKCs were determined by semi-quantitative (sq) RT-PCR, as described below. DNAse-treated RNA was used to synthesis cDNA using the Omniscript® kit (Qiagen). Negative controls (no reverse transcriptase) were also run on each sample to ensure that DNAse treatment was successful in removing any genomic DNA contamination. Primers were designed porcine sequences when available, and other primers were designed based on the sequence availability and expected homology to porcine sequences. PKC Alpha, Beta I, Beta II (porcine sequences); PKC Delta, Epsilon, Eta, Lamda, Mu, Theta and H2A (human sequences); and PKC Iota and Zeta (mouse) primers were designed using Mac Vector (Accelrys Inc.). Primers were synthesized based upon the published GenBank sequences to produce the gene products and were designed from the following species (see Table 1 for primer sequences and bp sizes). All primers were validated for semi-quantitative (sq) RT-PCR by running each primer at 20, 25, 30, 35 and 40 cycles and

choosing a cycle number that corresponded to the linear range of amplification (all 33 cycles except H2A, 28 cycles). Once primers were validated, sq RT-PCR was run using 1 ug of cDNA and the Taq PCR Mastermix® Kit (Qiagen). Thermocycler conditions were as follows: 3 minute initial denaturation 94°C, followed by 28 or 33 cycles of denaturation, 30 seconds 94°C; annealing, 1 minute 50°C; extension, 1 minute 72°C; followed by a 5 minute 72°C final extension. All gene products were separated on 2% agarose gels and stained with ethidium bromide. Products were quantified using Lab-Works imaging system (UVP Imagining Company, Upland, CA). Values were calculated as the ratio of gene product band intensity/H2A band intensity; H2A was used as a housekeeping gene since its levels of expression did not vary with stage of estrous cycle (Fig. 11D). All gene products were cleaned using MultiScreen $PCR_{\mu 96}$ PCR cleanup plates (Millipore, Billrica, MA), following the manufacturer's recommended protocol. RT-PCR amplicons were sequenced following the recommended protocol with the ABI BigDye v. 3.1 sequencing kit (Applied Biosystems, Foster City, CA). Sequencing reactions were purified using an ethanol/ammonium acetate precipitation protocol [14] and visualized using an ABI 3130XL Automated Sequencer (Applied Biosystems, Foster City, CA). Sequences were compiled in Sequencher version 4.5 (Gene Codes Corp., Ann Arbor, MI) and NCBI BLAST searches were performed to verify sequence identities.

Western Blotting

PKC protein expression levels were determined by semi-quantitative western blots as described below. Total protein was extracted from CL by homogenization in 20 mM HEPES buffer containing 2mM EDTA, 2mM EGTA, 1% Triton-X, 20 μg/ml aprotinin, 20 μg/ml leupeptin, 4 ug/ml PMSF, followed by centrifugation twice at 14,000g to remove cellular debris. For cytosolic and membrane fractions, CL were homogenized in 20mM Tris-HCl, pH 7.5 containing 10mM EDTA, 2mM EGTA, 2mM PMSF and 0.1mg/ml leupeptin as previously described [15]. Samples were then centrifuged at 100,000xg for 1 hour at 4°C. Cytosolic fraction was removed and stored at -20°C, and remaining membrane pellet was resuspended in the buffer described above, plus 0.2% Triton-X100 and centrifuged at 100,000xg for 1 hour at 4°C**.** Membrane fraction was removed from remaining cell debris and stored at -20°C until future use. All

protein concentrations were determined using the Micro-BCA protein assay (Pierce, Rockford, IL) according to manufacturer's instructions. Western blots were performed as previously reported [16]. Briefly, 20 ug of protein was loaded on to 4-12% Bis-Tris gels and subjected to electrophoresis at 120 volts at room temperature for 50 minutes. Proteins were transferred to PVDF membranes at 30 volts for 60 minutes. Membranes were then blocked in 5% milk Tris-buffered Saline (TBS)-Tween for 1 hour at room temperature, followed by incubation with primary antibody (1:200, all PKC isoforms; 1:2000, β-actin) overnight at 4°C. Membranes were then washed in TBS-Tween three times for 5 minutes, followed by incubation in secondary antibody (all PKCs- anti-rabbit, 1:2000; β-actin- anti-mouse, 1:2000) for 1 hour at room temperature, and washed another three times in TBS-Tween. Chemi-illuminescence (using ECL kit) was used to visualize protein bands and bands were quantitated with Lumi-Imager software (Roche, Indianapolis, IN). The specificity of protein bands was determined in separate gels using antibody which had been previously mixed with an excess of blocking peptide (used for generation of the antibody, data not shown). The quantity of chemiluminescence in specific protein bands for whole CL and cytosolic fractions were normalized to that obtained with the β-actin antibody (Fig. 11H) using a Lumi-Imager software (Roche, Indianapolis, IN). Membrane densitometry is not corrected for β-actin, as it is not expressed in high enough levels, if at all, in this cellular fraction, and therefore is represented by raw densitometric values, Boehringer light units (maximum BLU).

Cell Dissociation

Day 13 CL were collected from cycling gilts and transported in M199 with antibiotics and antimycotics on ice to the laboratory. CL were dissociated as previously described [1]. Briefly, CL were dissociated in a collagenase containing enzyme preparation for 30 minutes at 37°C. Dissociated cells were removed and remaining tissue was incubated for another 30 minutes, which was repeated until all cells were completely dissociated. Cells were washed and placed in Percoll to remove red blood cells. Large luteal cells (LLC) were then counted by Trypan blue exclusion. Cell viability was determined to be over 90%.

Immunofluorescence

Chamber slides (Fisher Scientific, Waltham, MA) were coated with 0.1% Gelatin. 25,000 cells (based on LLC count) were plated Medium 199 and incubated at room temperature for 1 hour. Medium was then removed and the plated cells were fixed in ice cold acetone for 10 minutes. Cells were rinsed with phosphate buffered saline (PBS) and blocked with 1% Normal Goat Serum in PBS for 30 minutes at room temperature. Primary antibodies (PKC isoforms antibody plus von Willibrand factor antibody to double stain cells) in 1% BSA in PBS (1:40; using antibodies described for Western blotting) were added and incubated overnight at 4°C. Slides were then washed with PBS, and Alexa Fluor secondary antibodies [1:800, anti-rabbit- All PKC Isoforms; 1:800 anti-mouse- von Willibrand Factor (EC marker)] were added and incubated for 2 hours at room temperature. DAPI (Vector Laboratories, Burlingame, CA) was added to visualize nuclei and slides were viewed at 40X magnification using a Nikon Eclipse E400 Microscope.

Statistical Analysis

All statistical analysis of mRNA expression were carried out using Repeated Measures followed by Duncan's test for significance using Statistical Analysis Software (SAS, Cary, NC). Repeated measures for mRNA analysis was performed because each RT-PCR reaction was performed twice for each sample and gene. Protein levels were statistically analyzed using one-way analysis of variance, followed by Duncan's test for significance using SAS. All housekeeping gene (H2A, β-actin) expression levels were analyzed across the days of the cycle. All means are reported as least-squared means +/- SEM. Differences were considered significant at $p < 0.05$, and stated at $p < 0.01$ when differences were such.

RESULTS

Expression of mRNA levels throughout the estrous cycle

Conventional Isoforms (Fig. 1): The expression of conventional PKC mRNAs throughout the estrous cycle was determined (Figure 1). H2A values were analyzed across all days of the cycle and no differences in days were found (data not shown). PKC α, PKC βI

and PKC βII showed a similar mRNA expression pattern, in which levels were significantly increased on day 7 versus all other days (4, 10, 13 and 15). PKC α expression was ~5-fold greater on day 7 than on days 4, 13 and 15 and ~3-fold higher on day 7 compared with day 10 ($p<0.01$, Fig. 1A). PKC β I (Fig. 1B) and PKC β II (Fig. 1C) expression was increased on day 7, approximately 2-fold over all other days (4, 10, 13 and 15) ($p<0.01$). In contrast, PKC γ (Fig. 1D) expression was not significantly different throughout the cycle. PKC βII mRNA levels were ~3-fold higher than PKCβI, ~50-fold higher than PKCγ and over 100-fold greater than PKCα. Figure 11A shows representative PCR products for the conventional isoforms.

Novel Isoforms (Fig. 2): PKC δ (Fig. 2A) and ε (Fig.2B) mRNA expression showed similar expression patterns, with a significant increase on day 7 versus all other stages of the cycle (4, 10, 13 and 15). There was approximately a 5-fold increase in mRNA expression of PKC δ on day 7 versus day 4, 13 and 15, and a 2-fold increase versus day 10 ($p<0.01$, Fig. 2A). PKC ε expression was approximately 2 fold higher on day 7 versus all other days ($p<0.01$, Fig. 2B). PKC η expression was opposite of the other novel isoforms, as mRNA expression was highest on day 4 and 10, and lowest on day 7 ($p<0.01$, Fig. 2C). No difference in expression was seen on days 7, 13 or 15. PKC μ expression was significantly higher on day 7 and 10 (increased ~ 3-fold) versus the other days of the cycle, and reached lowest levels on day 15 ($p<0.01$, Fig. 2D). Day 4 expression was also significantly higher than day 15, and day 13 was not different from either day 4 or 15. PKC θ expression was highest on day 7 and was 3-fold higher than day 4, 13 and 15 ($p<0.01$, Fig. 2E). On day 10 PKC θ expression was not significantly different that any of the other days. PKC ε and μ mRNA levels were ~5-fold higher than levels of δ and η, and ~10-20-fold higher than PKC θ expression. Figure 11B shows representative PCR products for novel isoforms.

Atypical isoforms (Fig. 3): Both PKC ι (Fig. 3A) and ζ (Fig. 3B) expression followed similar patterns with highest mRNA expression on day 7 versus all other days. PKC ι was 5-fold higher on day 7 than on days 4, 13 and 15 and 2.5-fold higher on day 7 than day 10 ($p<0.05$, Fig. 3A). PKC ζ mRNA expression was 8-fold higher on day 7 versus days 4, 13 and 15 and 3-fold higher on day 7 than on day 10 ($p<0.01$, Fig 3C). In

contrast, PKC λ mRNA expression was not significantly different throughout the cycle (Fig. 3B). PKC ι mRNA expression levels were ~5-fold higher than levels of λ and ~10-fold higher than ζ. Figure 11C shows representative PCR products for atypical isoforms.

Protein expression throughout the estrous cycle in whole CL

Conventional isoforms (Fig. 4): The expression of conventional PKC protein throughout the estrous cycle was determined. Expression of β-actin was analyzed across all days of the cycle and no differences were found (data not shown). PKC α expression was not significantly different throughout the cycle (80 kD, Fig. 4A) as represented by the authentic 80kD band. However, there was a tendency ($p=0.1$) for the 40kD immunoreactive protein band, (believed to be the catalytic domain of PKC α; 40 kD, since it disappeared when using antibody preincubated with immunizing peptide) to show increased expression on day 13 versus all other days. PKC β I protein expression was not significantly different throughout the estrous cycle (Fig. 4B). PKC β II protein expression was also not significantly different throughout the cycle, however there was a tendency for increased expression on day 15 ($p=0.09$, Fig. 4C). PKC γ expression, neither intact protein (75 kD) nor presumptive catalytic domain (40 kD) was significantly different throughout the cycle (Fig. 4D). Figure 11E shows representative blots for each of the conventional isoforms.

Novel isoforms: PKC δ (Fig. 4E), η (Fig. 4G) or μ (Fig. 4H) protein expression was not significantly different during the estrous cycle. However, PKC ε (Fig. 4F) was significantly higher on day 13 versus the other stages of the cycle ($p<0.01$). PKC θ protein was not detected in any samples. Figure 11F shows representative blots for each of the novel isoforms.

Atypical isoforms: PKC λ (Fig. 4I) or ζ (Fig. 4J) protein expression was not significantly different throughout the cycle. PKC ι protein was not detected in any samples from any days. Figure 11G shows representative blots for each of the atypical isoforms.

Protein expression throughout the estrous cycle-Cytosol and Membrane Expression

Conventional isoforms (Fig. 5): Protein levels of the conventional isoform PKC α in luteal cytosol were not significantly different across days of the cycle, either the 80 kD (presumptive intact PKC α) or the 40kD (presumed catalytic domain) protein bands (Fig. 5A). PKC α membrane expression (80 kD) was also not significantly different throughout the cycle (Fig. 5B). PKC β I levels were also not significantly different in the cytosol (Fig. 5C), and PKC β I was not detected in the membrane fraction. PKC β II expression in the cytosol was not significantly different over days of the estrous cycle, although there was a tendency ($p=0.07$) for expression levels to increase on days 10 and 13 versus day 7 (Fig. 5D). PKC β II levels in membrane fractions did not vary significantly during the cycle (Fig. 5E). PKC γ protein was not detected in either cellular fractions throughout the cycle.

Novel Isoforms: PKC ε protein levels were not significantly different at any stages of the cycle in the cytosolic fraction (Fig. 6A), however, in the membrane fraction, PKC ε expression was significantly higher on days 4 and 13 versus day 10 of the cycle ($p<0.05$; Fig. 6B). Protein expression on day 7 and 15 were not different from any other days. PKC μ protein appeared as 2 distinct protein bands in both cytosol and membrane fractions, 100kD (believed to be the intact protein) and 40kD (thought to represent the catalytic domain). As seen in Fig. 6C, the 40kD component was the most highly expressed band and the levels of expression did not vary during the cycle. The presumptive intact protein (100 kD) represented only 20-30% of the total PKCμ immunoreactivity, and also did not vary significantly with stage of the cycle. Similarly in the membrane fraction, the presumptive catalytic protein band predominated (70-95%) of the total and showed not significant changes over days. The lesser expressed 100 kD band displayed a gradual increase in expression from days 4-13 although this pattern was not significantly different (Fig. 6D). There was no PKC δ, PKC η or PKC θ protein detected in either the cytosol or the membrane fraction in any fractions.

Atypical Isoforms: PKC ζ expression in the cytosol was significantly higher on day 4 versus day 7 and 15 ($p<0.05$; Fig. 6E). Protein expression on days 10 and 13 were not different than any other day of the cycle. PKC ζ membrane expression was not different

throughout the cycle (Fig. 6F). PKC ι and PKC λ were not detected in the cytosolic or membrane fractions at any stages of the cycle.

Immunofluorescence in D13 CL

The cellular localization of all PKC isoforms were localized in day 13 CL by immunofluorescence. Figure 7 shows immunolocalization of the conventional PKC isoforms. PKC α expression is localized to steriodogenic cells, both LLCs and SLCs (Fig. 7B). Staining with von Willibrand Factor did not show any positive PKC α staining in ECs (Figs. 7C and 7D). Similar to PKC α, PKC βI showed immunolocalization to both LLCs and SLCs (Fig. 7F), however few ECs also stained positive for PKC βI as denoted by the arrowheads (Figs. 7F/G) and yellow double staining in Fig. 7H. PKC βII also showed immunopositive localization to both LLCs and SLCs (Fig. 7J), in addition to a few positive ECs (Fig. 7K). PKC γ also showed positive staining in a few LLCs, SLCs and ECs (Fig. 7N). There were much fewer cells staining positive for PKC γ than any other conventional isoforms. Figure 7S represents the positive control von Willibrand factor localized to ECs.

Immunolocalization studies in the novel PKC isoforms are depicted in Figure 8. PKC δ expression was localized to steriodogenic cells, LLCs and SLCs (Fig. 8B). No ECs stained positive for this isoform. PKC ε showed expression in all three cell types, both steriodogenic (LLCs and SLCs) and ECs, although to a much lesser extent (Fig. 8F). PKC η expression was also localized to all three cells types, with SLCs showing the greatest number of positive cells (Fig. 8J). PKC μ also showed expression in all cell types, but was primarily localized to steriodogenic cells (LLCs and SLCs, Fig. 8N). PKC θ was not detected in any cell types (Fig. 8R).

Of the atypical isoforms, PKC λ showed limited expression in the LLCs, but no other cell type (Fig. 9F). PKC ζ was expressed in both LLCs and SLCs (Fig. 9J), but no ECs. PKC ι was not detected in any type (Fig. 9B).

DISCUSSION

In this paper, data have been presented to characterize and quantify the expression (mRNA and protein) and localization (protein) of twelve of the known PKC isoforms in the porcine CL throughout the estrous cycle. The present studies have demonstrated that all PKC isoforms are expressed at the mRNA level in the whole CL, and all PKC isoforms except PKC ι and θ are expressed at the protein level (by Western blot analysis, Figure 2). These studies also provide evidence of the cellular localization of each isoform at the protein level by immunofluorescence, either in LLCs, SLCs and/or ECs.

Overall the data presented here suggest that PKC ε which showed a temporal pattern of expression both mRNA and protein (increased mRNA on day 7, and protein on day 13) consistent with a possible involvement in control of the acquisition of LS in the pig, which we have hypothesized occurs between days 7 and 13 [17]. In this regard our data are in support of the data of Flores and colleagues working in the cow, who have also suggested a role for PKC ε in control of LS in the bovine CL [4]. Our data on cellular localization of this isoform also confirmed the findings from the bovine CL with predominant expression of PKC ε in steriodogenic cells [11], however some porcine ECs stained positive (Fig. 8H). In the bovine CL, PKC ε (protein [4], mRNA [11]) was significantly higher on day 10 versus the day 4 of the cycle. In our studies mRNA expression was ~2-fold greater on day 7 versus other days (Fig. 2B) and protein levels were increased approximately 30% on day 13 over day 10 (Fig. 4F). There was no difference in cytosol protein expression of PKC ε (Fig. 6A); however there was a significant decrease in membrane expression on day 10 versus days 4 and 7, and a significant increase on days 13 and 15 (back to levels seen on days 4 and 7, Fig. 6B). These data taken together, suggest that PKC ε could be playing a regulatory role by in regulating luteal sensitivity to PGF-2α (i.e. acquisition of LS), by mediating PGF-2α's actions in the porcine CL. In other studies in the cow, both the temporal patterns of PKC ε expression, as well as evidence showing that a specific PKC ε inhibitor blocked the anti-steroidogenic response of luteal cells to PGF-2α, strongly suggest that this PKC isoform specifically, plays a critical role in PGF-2α actions in the cow [4, 10]. Furthermore, PKC ε has been demonstrated to be translocated from cytoplasm to the

nucleus in response to endothelin (ET)-1 binding to its receptor ET_A in cardiac myocytes and causes an increase in endothelin converting enzyme (ECE-1) [18]. In smooth muscle cells, incubation with ET-1 also increased PKC ε protein expression (measured by western blot analysis) [19], and ET-1 also activates PKC ε in esophageal muscle cells [20] and translocates PKC ε in arterial smooth muscle cells [21]. In recent experiments examining the endothelin system in the porcine CL in our laboratory, we have demonstrated the expression of mRNA and protein expression of ppET-1, ECE-1 and its receptors. Most importantly, we showed an increase in ECE-1 protein expression on days 10 and 15 of the cycle, suggesting an increase in active ET-1 peptide, therefore suggesting a role for ET-1 in acquisition of LS in the pig (Chapter 2). These studies also showed that ET_A receptors were localized on LLCs, similar to the location of PKC ε (in the present study), and based on the aforementioned data, we propose a working model to describe a potential mechanism for the acquisition of LS in the porcine CL (Fig. 10). ET-1, whose levels are likely to increase between on day 10 (due to elevated ECE-1 levels on that day), acting on the ET_A receptor in LLC, increases PKC ε expression (which increase at the protein level on between day 10 and day 13), translocation and activation on day 13. PKC ε may also further increase ECE-1 expression to generate additional ET-1 (ECE-1 protein levels are also increased on day 15 of the cycle, Chapter 2), which is believed to play an important role in PGF-2α induced luteolysis [22], (including increasing vasoconstriction to aid in luteolysis), but now also allows PGF-2α to act to decrease progesterone and begin functional luteolysis in the porcine CL, which is a critical component of LS.

Of the other PKC isoforms, only PKC μ displayed an upregulation of mRNA and a pattern of increased protein levels in the membrane fraction (indicative of translocation/activation) on days 10 and 13, albeit non significantly. Such a temporal pattern of expression is certainly consistent with a possible role in regulating LS, and the localization of this isoform appeared in EC and LLC, although much higher levels were seen in LLCs. Thus, this isoform may a play a direct (LLC) or indirect (EC) role in the pathways involved in acquisition of LS by actions on either of these cell types. Since ET-1 precursor originating from endothelial cells is a critical component of our working

model (Fig. 10), we speculate that PKC μ in endothelial cells may be involved in this part of the pathway.

This study demonstrated that conventional PKC isoforms, α, βI and βII, novel isoforms δ and θ and atypical isoforms ι and ζ all showed mRNA expression that was significantly higher on day 7 versus all other days of the cycle, however protein expression patterns did not show this same trend. PKC α, βI, δ and ζ protein showed no significant differences over the stages of the cycle, which suggests that PKC mRNA expression levels do not necessarily predict PKC protein levels in porcine CL. It also clearly demonstrates the importance of verifying PKC levels by Western blot analysis to confirm both the presence and levels of protein in the CL, before suggesting a particular gene has an important role in a cellular or physiological process. Although in most cases, protein expression is shown to mirror mRNA expression, at least in our tissue, the expression patterns depicted with several of these PKC isoforms suggest that either not all mRNA is processed into protein or may be degraded faster than protein expression occurs.

PKC βII protein levels in the whole CL, although not statistically different ($p = 0.09$), showed a trend towards increasing on day 15 of the cycle (Fig. 4C), suggesting that PKC βII may be involved in apoptosis signaling or other late stage events. However this isoforms expression pattern is not suggestive of a role in LS in the pig. PKC βII protein expression was localized to all three cell types (LLCs, SLCs, ECs, Fig. 4F). PKC δ whose protein levels did not change over the cycle was localized to SLCs and ECs (Fig. 8B), and is probably not involved in LS in the pig.

PKC ι and θ protein was not detected in any of the Western blots nor with immunofluroscence. This data suggests that the RT-PCR data is much more sensitive than the Western blots analysis, as we were able to pick up these particular isoforms in mRNA, but it is likely that that more protein should be loaded (>20ug) in the gels to find expression in our samples. If PKC ι and θ is translated, the levels of PKC ι and θ present are likely to be in such low amounts that these PKC isoforms are not important in LS in the porcine CL.

PKC η showed an interesting mRNA expression pattern, the opposite from the other PKC isoforms, with lowest expression on day 7 (no different than day 13 or 15) (Fig. 2C). However, PKC η showed no significant difference in whole CL protein expression throughout the cycle, although compared to other days protein expression is lower, but non significant, on day 7 (Fig. 4G). PKC η could potentially be involved in LS in the porcine CL, not only because of these interesting patterns, but because immunofluroscence showed expression in all three cell types (Fig. 8L), with particular strength in SLCs. Further investigation is needed on this particular isoform before such conclusions can be made.

Both PKC γ mRNA (Fig. 1D) and protein (Fig. 4D) expression were not significantly different throughout the cycle, and therefore we suggest not involved in LS in the pig. PKC γ was detected in all three luteal cell types (Fig. 7N), however compared to some of the other isoforms, in much lower quantities. PKC λ mRNA was not significantly different throughout the cycle (Fig. 3B), nor was protein expression in the whole CL (Fig. 4I). Immunofluorscence data showed expression localized to LLCs (Fig. 9F), however very few cells were positive.

We also determined PKC protein levels specifically in cytosol and membrane fractions, as an increased level in the membrane suggests the presence of active PKC for most isoforms [23]. PKC α, βI, βII and ζ have been shown to translocate to the nucleus upon activation, to induce early response genes [20], depending on cell type [21]. Thus, we measured the levels of PKC isoforms in both cytosol and membrane fractions to look for evidence of translocation to the membrane during in the cycle, particularly between days 7 and 13, which would suggest that PKC activation, and therefore may play a role in LS. PKC α protein levels in either cytosol or membrane fractions were not different throughout the cycle (Fig. 5A, Fig. 5B). It is interesting to note that the cytosol fraction contained both the intact protein (~95%) as well as the catalytic domain (~5 %) while the membrane fraction contained only the intact protein. This could be due to damage to the cells during cell fractionation, which could have occurred to the cytoplasm fraction and the 40 kD protein is degradation. PKC α may be an important signaling molecule earlier in the cycle (by day 4) as expression of protein is highest on day 4, and does not change throughout the rest of the cycle, as suggested by Sen and collegues who suggested

conventional isoforms are responsible for PGF-2α refractoriness in the early bovine CL [4]. PKC α protein was detected in all luteal cell types (Fig. 4D) by immunofluroescence. PKC β I protein also showed no difference in expression in the cytosol (Fig.5B), was not detected in the membrane fraction and was localized to all of the luteal cell types (Fig. 4H). This suggests either there is no activation of PKC β I protein or that there is such little amount of PKC β I protein translocated, that it could not be picked up by Western blot analysis. PKC β II expression was also not significantly different in the cytosol, although there was a tendency for expression to be higher on days 10 and 13 of the cycle versus day 7 ($p=0.09$, Fig. 5D). PKC β II expression was not different in the membrane fraction (Fig.5E), and because there is no increase in translocation to the membrane, PKC β II is probably not involved in LS in the pig either.

PKC ζ protein expression in the whole CL also showed no significant differences (Fig.4J), and protein was localized to all of the luteal cell types (Fig. 9J). Protein levels in the cytosol were significantly lower on day 7 and 15 than on day 4 (day 10 and 13 were not different from any other day) (Fig. 6E). PKC ζ expression in the membrane was also not different on any days (Fig. 6F), therefore suggesting that PKC ζ probably does not regulate PGF-2α sensitization, and we can speculate that PKC ζ may be an isoform that prevents PGF-2α action in the early stages of CL development, similar to the conventional isoforms suggested by Sen and colleagues in the bovine CL [4]. PKC γ, δ, η, θ, ι, and λ were not detected in either the cytosol or membrane fractions suggesting that although present in the whole CL fractions, since they are not translocated and thus not activated, are probably not playing a significant role in LS in the porcine CL.

It is interesting to note that some isoforms showed two immunoreactive bands when probed with PKC antibodies, specificity of which was confirmed by preincubation of antibody with blocking peptide. It has been shown in other studies that the protein associated with the band at approximately 45 kD (40 kD in our samples), is the catalytic domain of the protein of PKC α, δ and ε [17-19], and because all of the PKC isoforms share a similar catalytic domain, all are approximately the same size [7]. Therefore, we also quantified the levels of both the intact protein and the assumed catalytic domain of the protein to determine if this gave us additional information about possible expression

or activation. PKC α, γ and μ were the three proteins whose expression showed 2 distinct bands, PKC α at 80 and 40 kD, PKC γ at 75 and 40 kD and PKC μ at 110 and 40 kD. In all of these cases, the expression of either the intact protein or the catalytic domain of PKC α and γ showed no significant difference in expression in the whole CL (Fig. 4A and Fig. 4D). The PKC α catalytic domain was only present in the whole CL (Fig.4A) and cytoplasmic (Fig.5A) samples, and no immunoreactive band was present in the membrane fractions. PKC γ protein was not detected in either the cytosolic or membrane fractions, as discussed above. PKC μ was expressed in both cell fractions, however neither the 100 kD intact protein nor the 40 kD catalytic domain increased over the cycle (cytosol Fig. 6C, and membrane Fig. 6D). We suggest that we detected the catalytic domain in these three proteins, and not all of the PKC isoforms, because of the antibody specificity to these regions, whether the "catalytic" domain fractions are breakdown products or activated PKC domains is unclear and requires further examination.

In summary, our data have revealed increased levels of expression of PKC ε in the porcine CL around the time when the porcine CL is developing the capacity to respond to the luteolytic actions of PGF-2α (LS), suggesting a critical role for this isoform in the porcine CL, as illustrated in the working model (Fig.10). Further studies are warranted to elucidate the exact mechanism by which PKC ε is involved in LS. In addition, this is the first study to provide a comprehensive characterization of the expression and cellular localization patterns of all of the known PKC isoforms in the porcine CL.

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Table 1. Primer sequences used for PCR[a]

Gene	Accession Number	Product Size		Sequence	Cycles	Annealing Temp.
H2A	X52318	209	F	AGGACGACTAGCCATGGACGTGTG	28	50
			R	CCACCACCAGCAATTGTAGCCTTG		
PKC Alpha	AY093442	356	F	AATCGTTTCGCCCGCAAAG	33	50
			R	TCACAGGTGTCACATTTCATCCC		
PKC Beta I	AY093443	256	F	TGCTGTATGAAATGTTGGCTGG	33	50
			R	CGTGAATCTCTTTGCGTTGC		
PKC Beta II	AY093444	254	F	CTGTATGAAATGTTGGCTGGGC	33	50
			R	GGCTGAATCTCTTTGCGTTCG		
PKC Gamma	AW360087	101	F	AAAGGCAGTTTTGGGAAGG	33	50
			R	CGTCATCCTGGACAATCACG		
PKC Delta	NM_212539	391	F	ACATCAAGATTGCCGACTTTGG	33	50
			R	AACCTCCGCTTTTCCAGCAG		
PKC Epsilon	NM_005400	227	F	TTTTGGAACGAGCAGAATGAGG	33	50
			R	GCCACAGAGAACCCTTGGAAATC		
PKC Eta	BP149822	453	F	GGGGATAATGCGACAAGGACTTC	33	50
			R	TGGGTGAGGAAAGGGTGATTGC		
PKC Mu	X75756	206	F	TGTGACTTTGGTTTTGCCCG	33	50
			R	TGGATTTGGTCGTGGATGTCC		
PKC Theta	NM_006257	470	F	TCACTCAGTTCTTCTGCTGCTTTC	33	50
			R	GACCACCTCATCATTCAGTATGGC		
PKC Iota	CK456623	408	F	GCAAATCAAAATCCTGAAGCCC	33	50
			R	ATAAGTCCATCTACCGCCGAGGTG		
PKC Lamda	AJ656326	222	F	ACAAGGACCCAAAGGAACGC	33	50
			R	TCACAATGTCATCGTCATCTGGAG		
PKC Zeta	NM_008860	491	F	TGCCAGATTCTACGCTGCTGAG	33	50
			R	CGTGAGACTTGATGTCGGAAAACC		

[a] All primers are listed 5'-3'

Table 2. PKC Isoform mRNA and Protein Expression in the Porcine CL[a]

Isoforms	mRNA	Protein- Whole CL	Protein-Cytosol	Protein- Membrane
Conventional				
Alpha	+	+	+	+
Beta II	+	+	+	-
Beta II	+	+	+	+
Gamma	+	+	-	-
Novel				
Delta	+	+	-	-
Epsilon	+	+	+	+
Eta	+	+	-	-
Mu	+	+	+	+
Theta	+	-	-	-
Atypical				
Iota	+	-	-	-
Lamda	+	+	-	-
Zeta	+	+	+	+

[a] + denotes mRNA/protein detected, - denotes no mRNA/protein detected

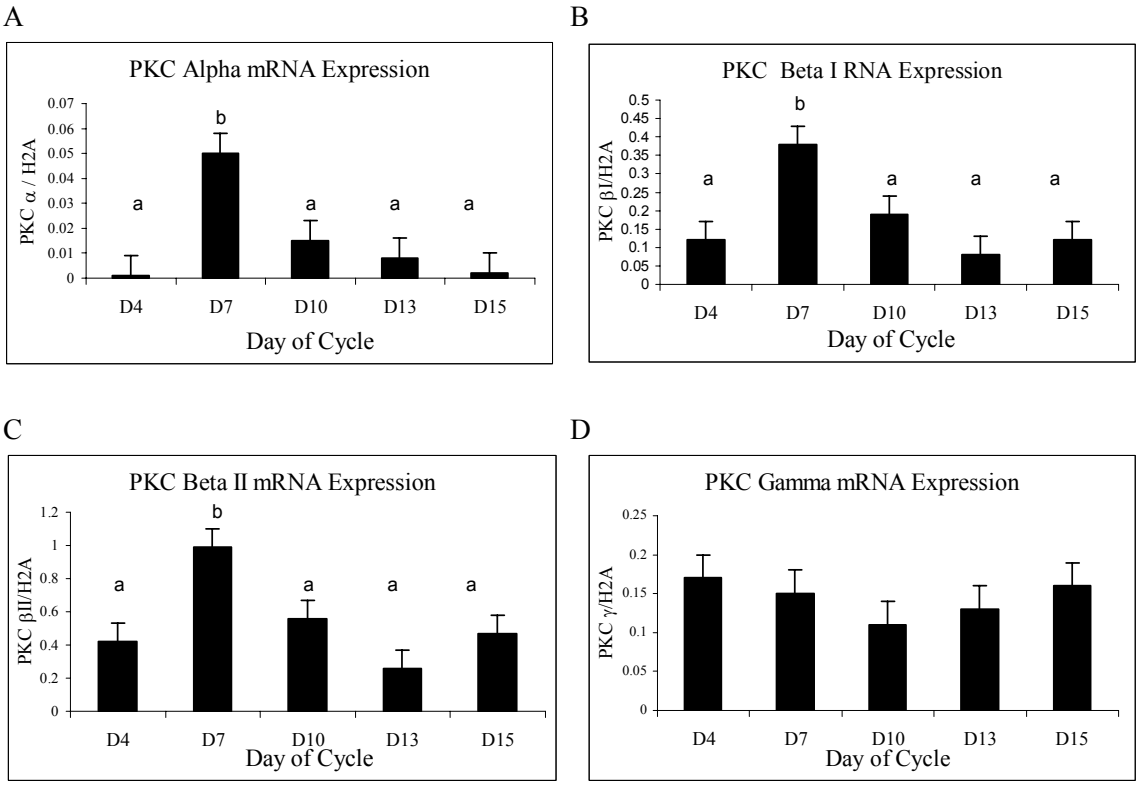


Fig. 1. Detection of PKC (conventional isoforms) mRNA in porcine CL throughout the estrous cycle. mRNA expression on days 4, 7, 10, 13 and 15 of the cycle were determined by semi-quantitative RT-PCR using H2A as a housekeeping gene. All values represent gene of interest expressed a ratio to H2A. A) PKC Alpha (α), B) PKC Beta I (β I), C) PKC Beta II (β II), D) PKC Gamma (γ). Data represent the least-squared means +/- SEM of the densitometric analysis. Number of animals per day = 6. Different letters denote significant differences between groups ($p<0.05$).

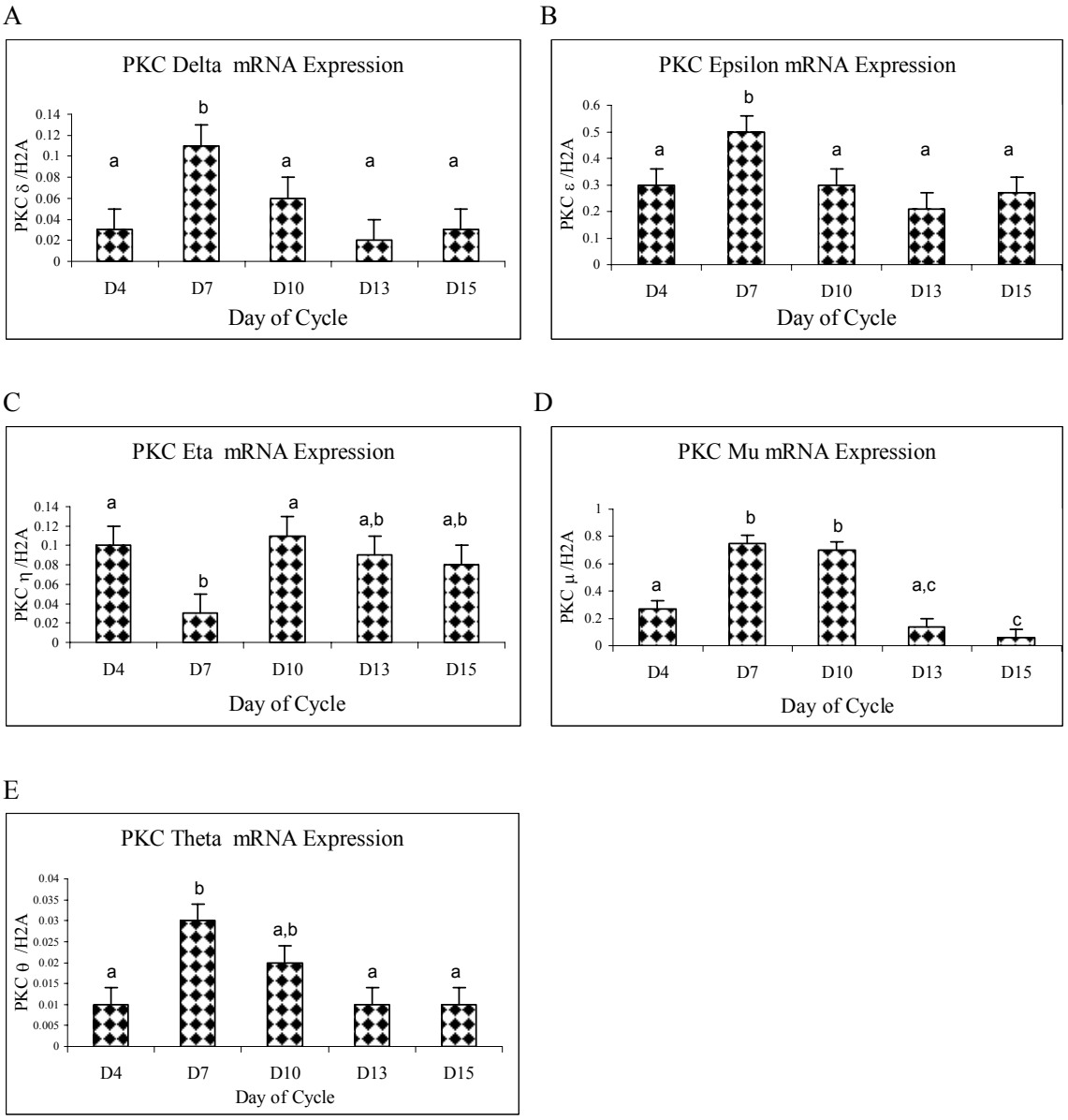


Fig. 2. Detection of PKC (novel isoforms) mRNA in porcine CL throughout the estrous cycle. mRNA expression on days 4, 7, 10, 13 and 15 of the cycle were determined by semi-quantitative RT-PCR using H2A as a housekeeping gene. All values represent gene of interest expressed as a ratio to H2A. A) PKC Delta (δ), B) PKC Epsilon (ε), C) PKC Eta (η), D) PKC Mu (μ), E) PKC Theta (θ). Bars Data represent the least-squared means +/- SEM of the densitometric analysis. Number of animals per day = 6. Different letters denote significant differences between groups ($p<0.05$).

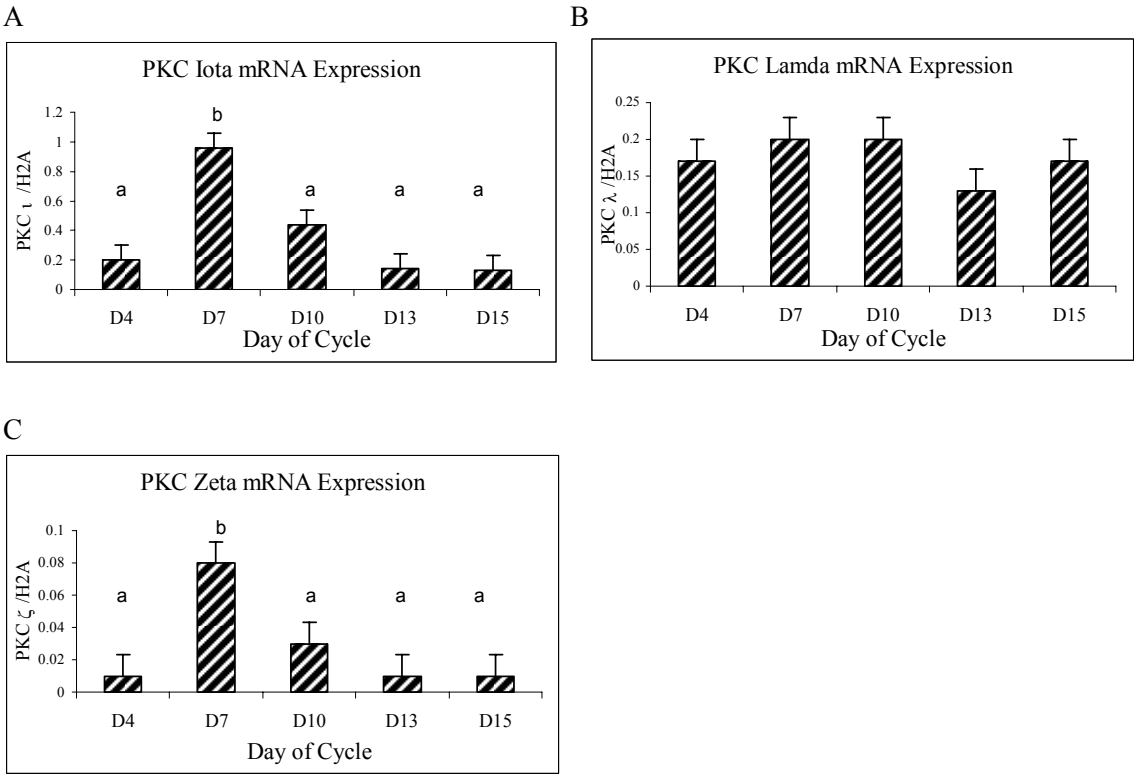
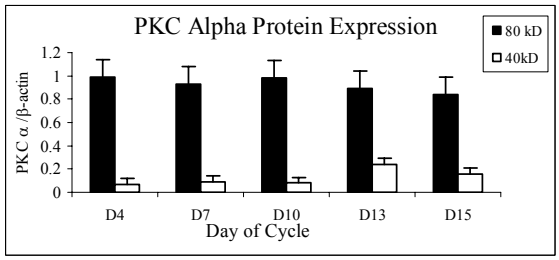


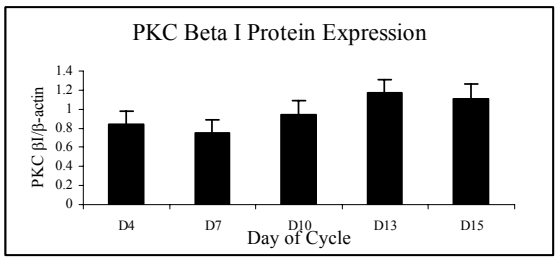
Fig. 3. Detection of PKC (atypical isoforms) mRNA in porcine CL throughout the estrous cycle. mRNA expression on days 4, 7, 10, 13 and 15 of the cycle were determined by semi-quantitative RT-PCR using H2A as a housekeeping gene. All values represent gene of interest/H2A. A) PKC Iota (ι), B) PKC Lamda (λ), C) PKC Zeta (ζ). Data represent the least-squared means +/- SEM of the densitometric analysis. Number of animals per day = 6. Different letters denote significant differences between groups ($p<0.05$).

Fig 4. PKC Protein concentrations in porcine CL (whole tissue) throughout the estrous cycle. Protein levels were determined on days 4, 7, 10, 13 and 15 of the cycle by Western Blot analysis using β-actin as a housekeeping gene. All values represent protein of interest expressed as a ration to β-actin. A) PKC Alpha (α) B) PKC Beta I (βI), C) PKC Beta II (βII), D) PKC Gamma (γ), E) PKC Delta (δ), F) PKC Epsilon (ε), G) PKC Eta (η), H) PKC Mu (μ), I) PKC Lamda (λ) and J) PKC Zeta (ζ). Data represent the least-squared means +/- SEM of the densitometric analysis. Number of animals per day = 6. Different letters denote significant differences between groups.

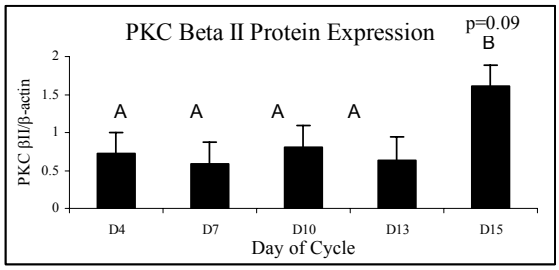
A


PKC Alpha Protein Expression
80 kD
40kD
PKC α /β-actin
1.2
1
0.8
0.6
0.4
0.2
0
D4
D7
D10
D13
D15
Day of Cycle

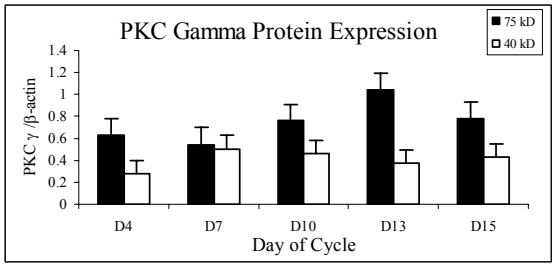
B


PKC Beta I Protein Expression
PKC βI/β-actin
1.4
1.2
1
0.8
0.6
0.4
0.2
0
D4
D7
D10
D13
D15
Day of Cycle

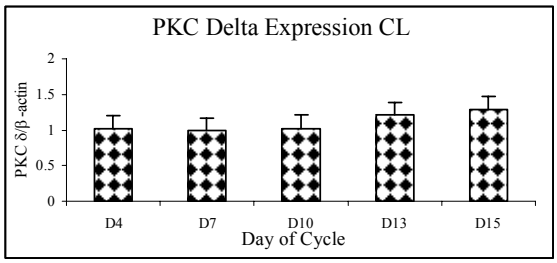
C


PKC Beta II Protein Expression
p=0.09
B
A
A
A
A
PKC βII/β-actin
2
1.5
1
0.5
0
D4
D7
D10
D13
D15
Day of Cycle

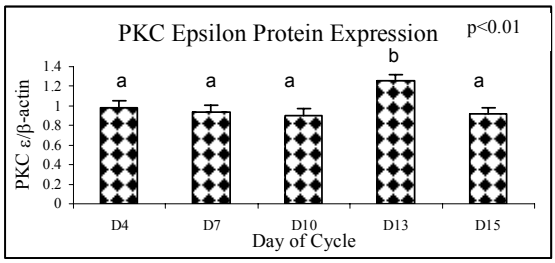
D


PKC Gamma Protein Expression
75 kD
40 kD
PKC γ /β-actin
1.4
1.2
1
0.8
0.6
0.4
0.2
0
D4
D7
D10
D13
D15
Day of Cycle

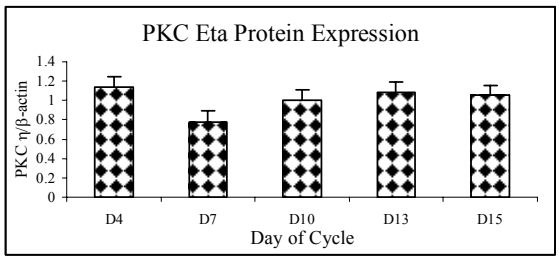
E


PKC Delta Expression CL
PKC δ/β-actin
2
1.5
1
0.5
0
D4
D7
D10
D13
D15
Day of Cycle

F


PKC Epsilon Protein Expression
p<0.01
a
a
a
b
a
PKC ε/β-actin
1.4
1.2
1
0.8
0.6
0.4
0.2
0
D4
D7
D10
D13
D15
Day of Cycle

G


PKC Eta Protein Expression
PKC η/β-actin
1.4
1.2
1
0.8
0.6
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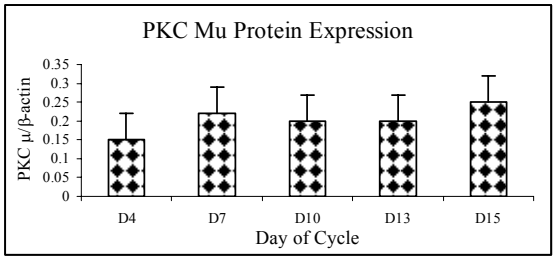
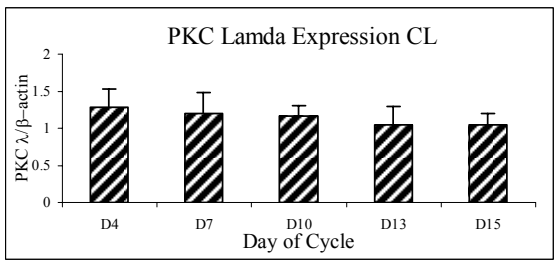
0.2
0
D4
D7
D10
D13
D15
Day of Cycle

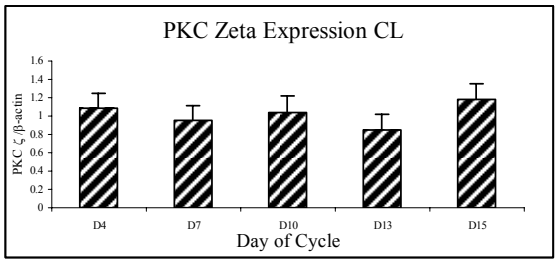
H


PKC Mu Protein Expression
PKC μ/β-actin
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
D4
D7
D10
D13
D15
Day of Cycle

I


PKC Lamda Expression CL
PKC λ/β-actin
2
1.5
1
0.5
0
D4
D7
D10
D13
D15
Day of Cycle

J


PKC Zeta Expression CL
PKC ζ /β-actin
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
D4
D7
D10
D13
D15
Day of Cycle

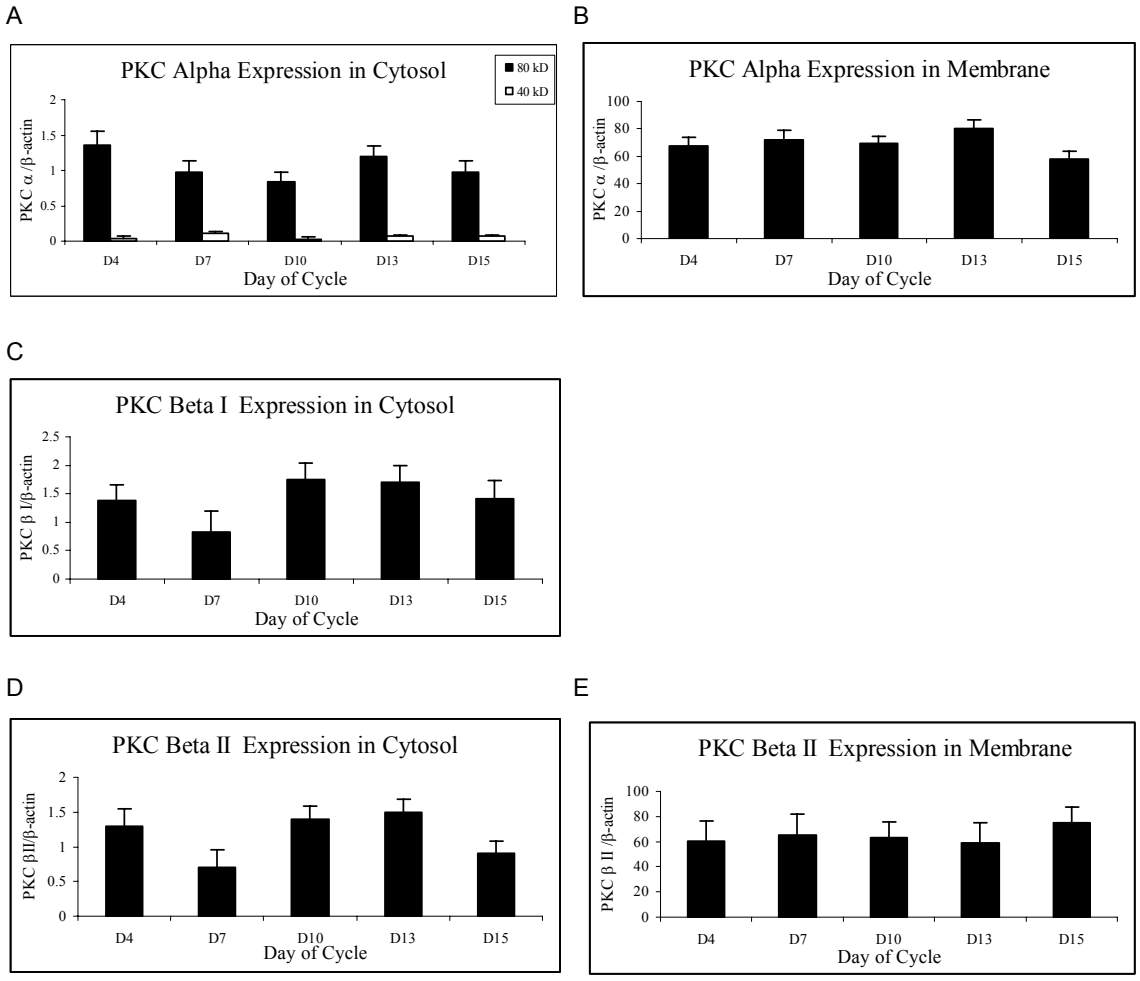


Fig 5. PKC (conventional isoforms) protein in cytosol and membrane fractions of the porcine CL throughout the estrous cycle. Protein levels were determined on days 4, 7, 10, 13 and 15 of the cycle by Western Blot analysis using β-actin as a housekeeping gene. All cytosol values represent protein of interest expressed as a ratio to β-actin. A) PKC Alpha (α) cytosol, B) PKC Alpha membrane, C) PKC Beta I (βI) cytosol, D) PKC Beta II (βII) cytosol, E) PKC Beta II membrane. Data represent the least-squared means +/- SEM of the densitometric analysis. Number of animals per day = 6.

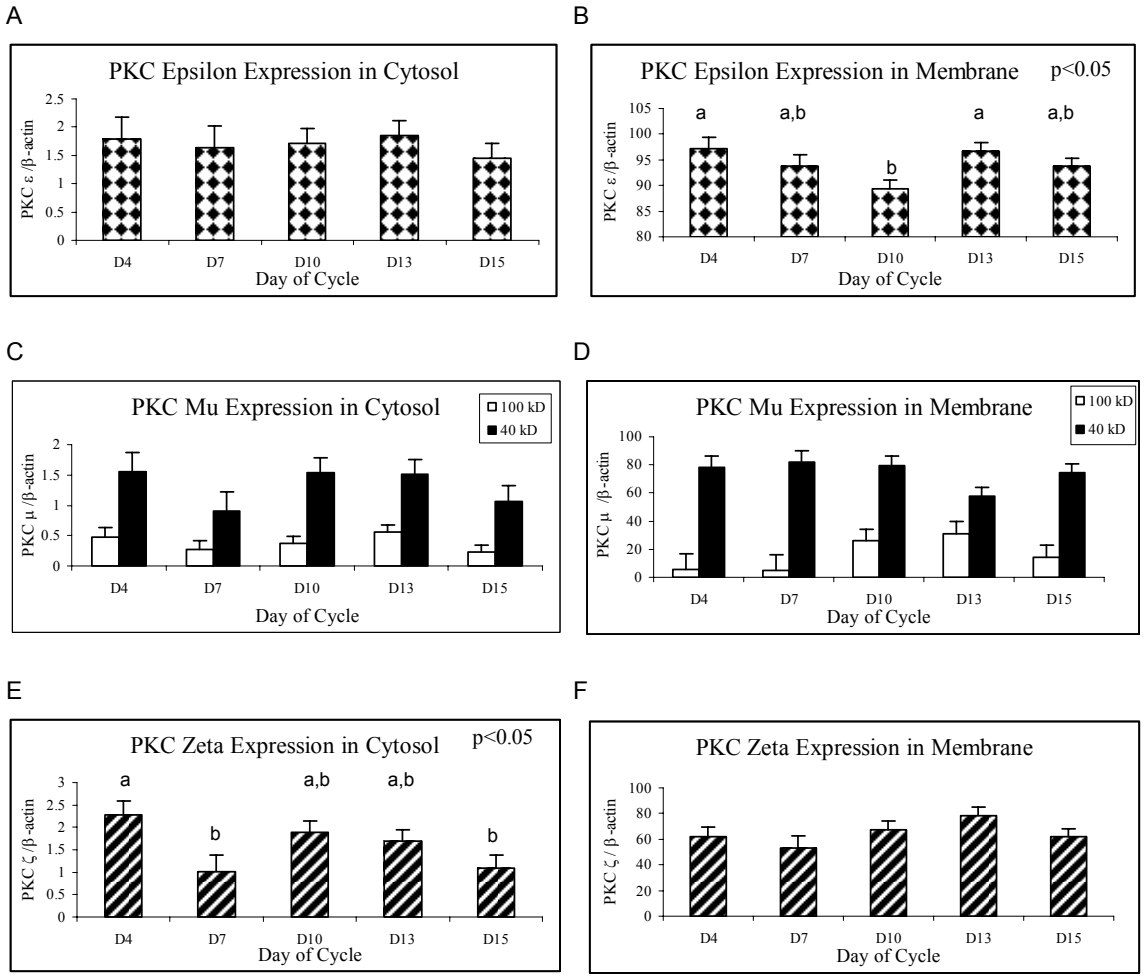
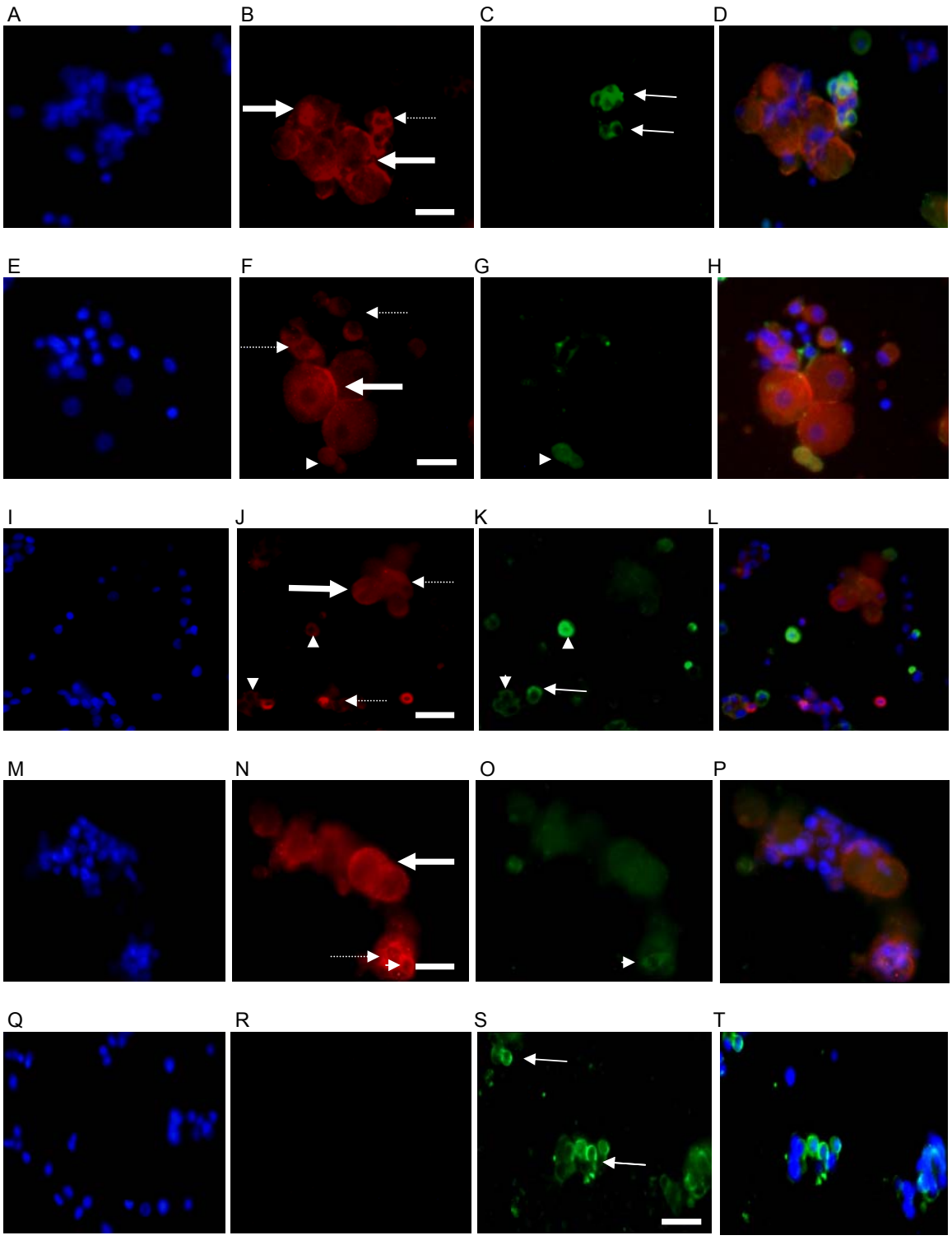


Fig 6. Protein (novel and atypical) concentrations in cytosol and membrane fractions of the porcine CL throughout the estrous cycle. Protein levels were determined on days 4, 7, 10, 13 and 15 of the cycle by Western Blot analysis using β-actin as a housekeeping gene. All cytosol values represent protein of interest expressed as a ratio to β-actin. A) PKC Epsilon (ε) cytosol, B) PKC Epsilon membrane, C) PKC Mu (μ) and D) PKC Mu membrane, E) PKC Zeta (ζ) cytosol, F) PKC Zeta membrane. Data represent the least-squared means +/- SEM of the densitometric analysis. Number of animals per day = 6. Different letters denote significant differences between groups.

Fig 7. Localization of PKC isoforms (conventional) in porcine luteal cells by double-labeling immunofluorescence. Protein localization was determined in day 13 porcine luteal cells. Panels A, E, I, M and Q represent DAPI staining of nuclei. Panels B, F, J and N represent immuno-positive staining of PKC isoforms. Panels C, G, K and O represent von Willibrand Factor (EC marker). Panels D, H, L and P represent triple staining of PKC antibody, von Willibrand Factor and DAPI overlay. Panel R represents negative control (no antibody), panel S represents positive control (von Willibrand Factor), and panel T represents double staining of DAPI and von Willibrand Factor. Large thick arrows denote positive LLCs, small dotted arrows denote positive SLCs, and small thin arrows denote positive ECs. Arrow heads show positive staining in ECs, shown in yellow in triple stained panels. All panels are at 40x magnification. Bar represents 60 μm.


A
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C
D
E
F
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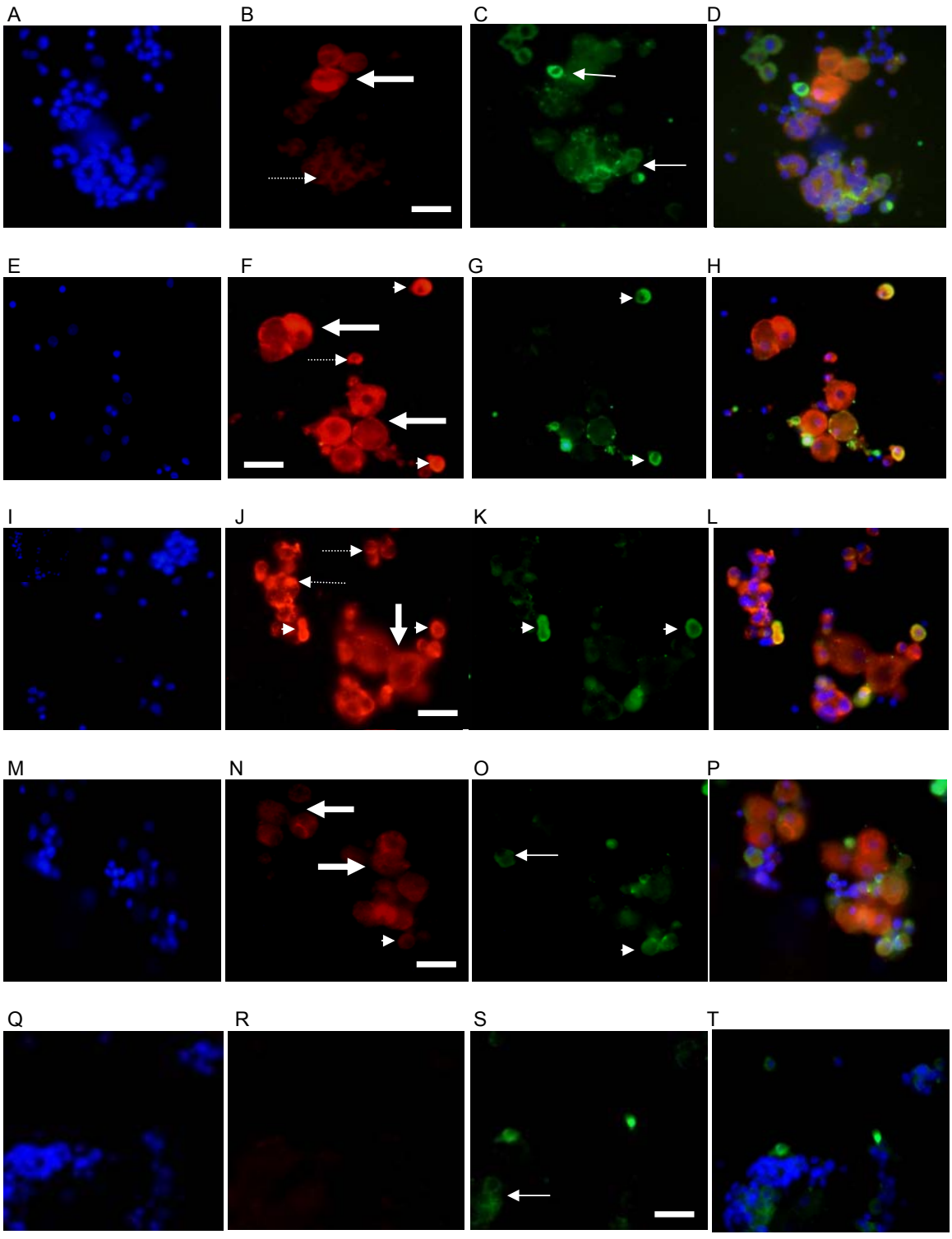
I
J
K
L
M
N
O
P
Q
R
S
T

Fig 8. Localization of PKC isoforms (novel) in porcine luteal cells by Immunofluorescence. Protein localization was determined in day 13 porcine luteal cells. Panels A, E, I and M represent DAPI staining of nuclei. Panels B, F, J and N represent immuno-positive staining of PKC isoforms. Panels C, G, K and O represent von Willibrand Factor (EC marker). Panels D, H, L and P represent triple staining of PKC antibody, von Willibrand Factor and DAPI overlay. Large thick arrows denote positive LLCs, small dotted arrows denote positive SLCs, and small thin arrows denote positive ECs. Arrow heads show positive staining in ECs, shown in yellow in triple stained panels. All panels are at 40x magnification. Bar represents 60 μm.


A
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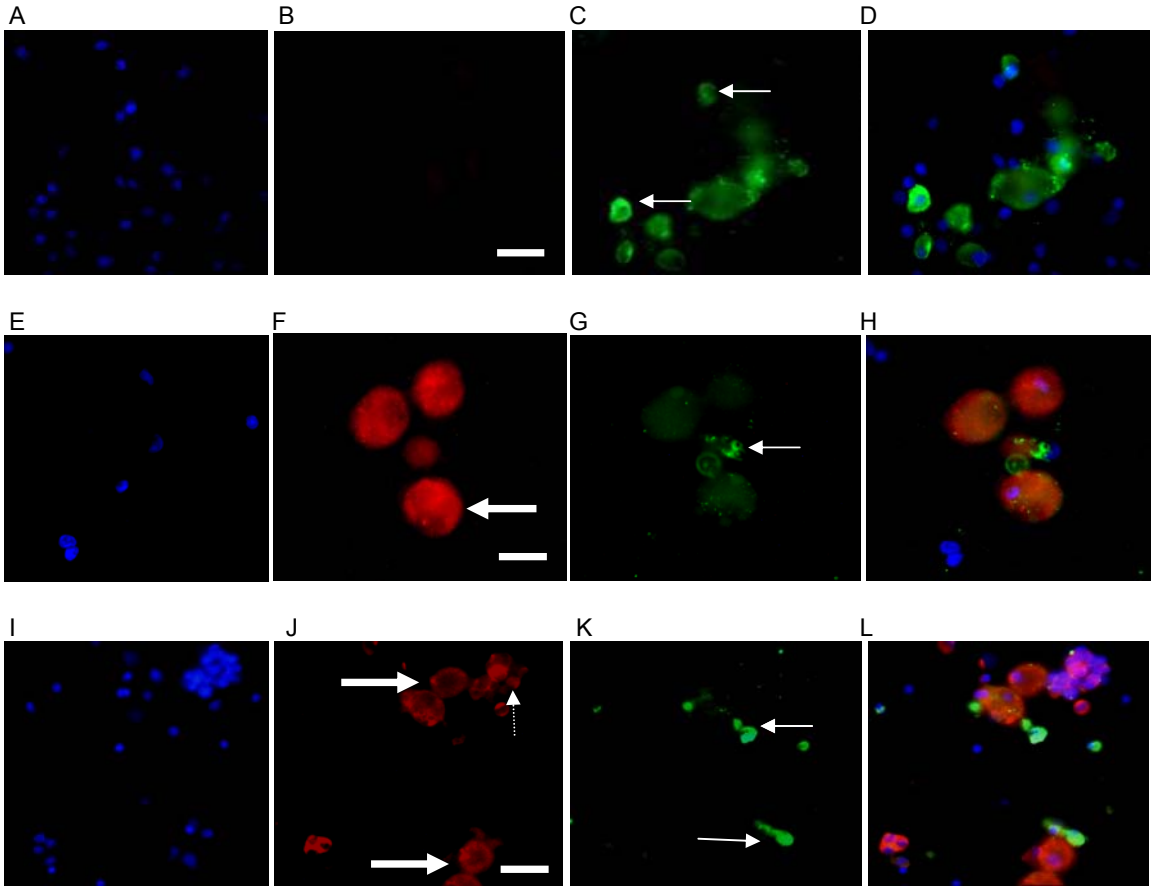


Fig 9. Localization of PKC isoforms (atypical) in porcine luteal cells by Immunofluorescence. Protein localization was determined in day 13 porcine luteal cells. Panels A, E, and I represent DAPI staining of nuclei. Panels B, F and J represent immuno-positive staining of PKC isoforms. Panels C, G and K represent von Willibrand Factor (EC marker). Panels D, H and L represent triple PKC antibody, von Willibrand Factor and DAPI overlay. Large thick arrows denote positive LLCs, small dotted arrows denote positive SLCs, and small thin arrows denote positive ECs. All panels are at 40x magnification. Bar represents 60 μm.

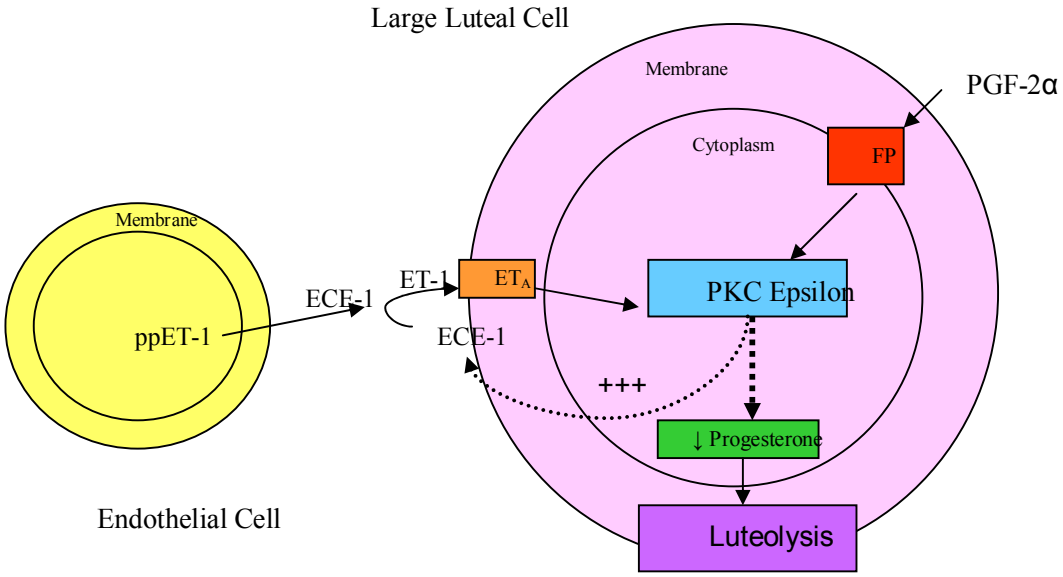
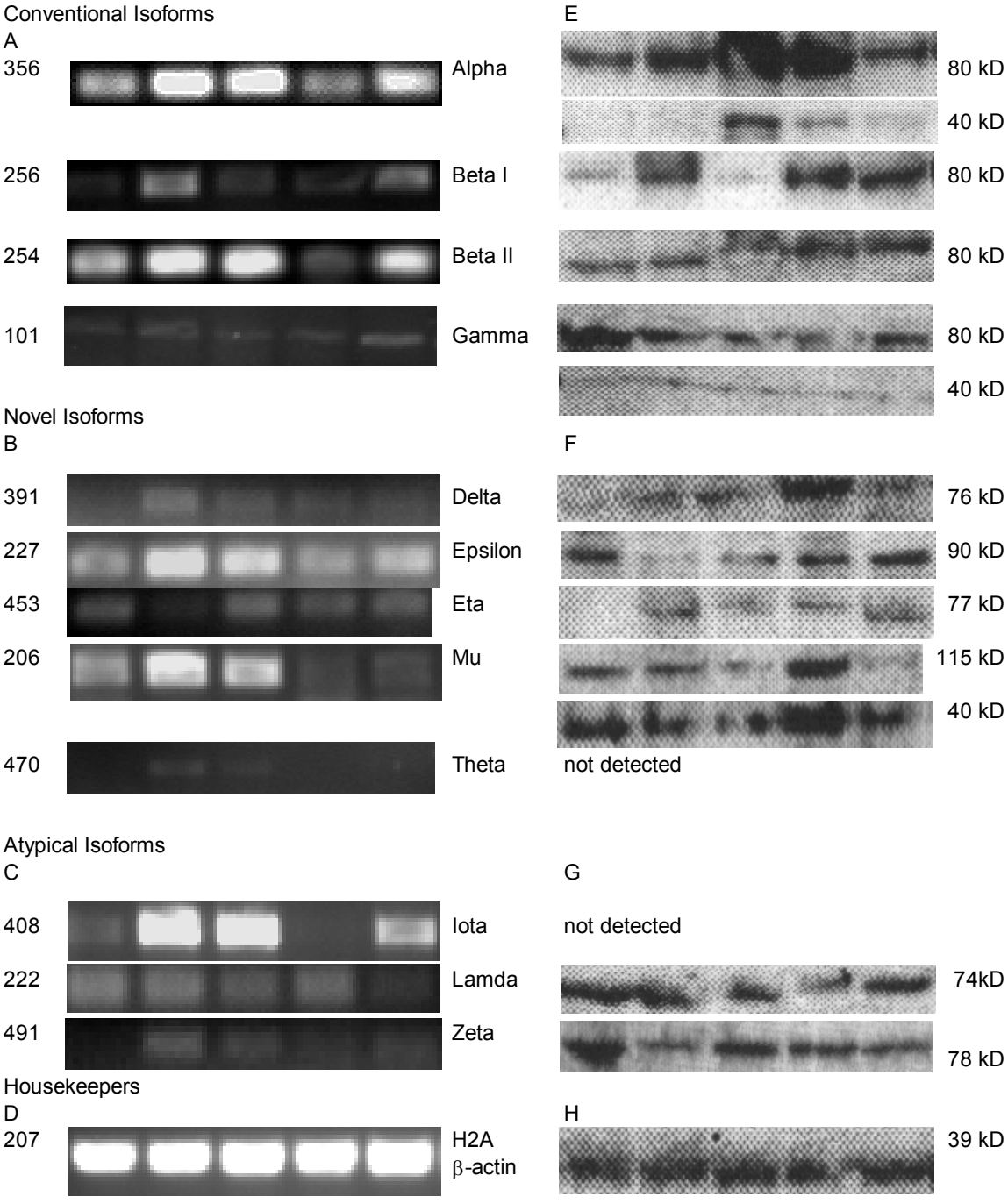


Fig. 10. Proposed Luteal Cell Model: PKC Epsilon Mediating PGF-2α Response. Endothelial cells express ppET-1, which is cleaved at the target cell (LLC) by ECE-1. Active ET-1 then binds to ET_A receptor, which upregulates PKC ε by translocating to membrane of LLC. PKC ε then facilitates PGF-2α signaling to decrease progesterone to cause luteolysis. PKC ε may also act in a positive feedback mechanism to increase ECE-1 expression in an autocrine or paracrine manner.

Fig. 11. Representative mRNA and protein analysis by RT-PCR and Western blotting of porcine luteal cells throughout the estrous cycle. Panels A-C show mRNA bands and bp sizes for each family of PKCs. Panel D shows housekeeping gene H2A as control. Panels E-G show protein bands and molecular weight for each family of PKCs. Panel H shows housekeeping protein β-actin as control.


Conventional Isoforms
A
356
Alpha
256
Beta I
254
Beta II
101
Gamma
E
80 kD
40 kD
80 kD
80 kD
80 kD
40 kD
Novel Isoforms
B
391
Delta
227
Epsilon
453
Eta
206
Mu
470
Theta
F
76 kD
90 kD
77 kD
115 kD
40 kD
not detected
Atypical Isoforms
C
408
Iota
222
Lamda
491
Zeta
G
not detected
74kD
78 kD
Housekeepers
D
207
H2A
β-actin
H
39 kD

Spatial and Temporal Expression Patterns of Apoptotic Genes in the Porcine Corpus Luteum[1]

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[1] This project was supported by National Research Initiative Competitive Grant no. 2004-35203-14777 from the USDA Cooperative State Research, Education and Extension Service.

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ABSTRACT

Prostaglandin-F-2α (PGF-2α) has long been recognized as the physiological luteolysin in the porcine corpus luteum (CL); however the mechanism by which the CL acquires sensitivity to PGF-2α (LS) is not understood. Tumor necrosis factor (TNF)-α is known to be involved in luteolysis during the late stages of the cycle. However recently macrophages, which produce TNF-α, were shown to infiltrate the CL much earlier in the cycle (days 4-8), suggesting that TNF-α may play a significant role earlier than previously demonstrated. Additionally, many other apoptotic-associated genes have been implicated in the late luteal stages (Fas, Nitric Oxide, Bcl-2 family members and Caspases); however how these genes may play a role earlier in the cycle has not been studied. Therefore, we investigated the expression (mRNA by RT-PCR) of genes associated with apoptosis (TNF-α, TNFR, iNOS, eNOS, Fas, Bax, Bcl-x, p53, NFκB, Caspase-8 and -3) in porcine CLs collected throughout the estrous cycle (days 4, 7, 10, 13 and 15). Additionally, we determined the cellular localization of select components using immunofluorescent labeling. Our results indicate that TNFR, p53 and eNOS mRNA expression are all significantly increased on day 7 of the cycle, coinciding with the onset of LS in the porcine CL. Additionally iNOS mRNA expression was elevated on days 7 and 10 of the cycle. Ironically Bax (pro-apoptotic) mRNA expression was highest on days 4 and 7 of the cycle. The expression of the above mentioned apoptosis-associated genes shown in this study reveal a complex pattern of regulation in the porcine CL during acquisition of LS. The increased expression of TNFR on day 7 suggests a significant role for TNF-α much earlier in the cycle than previously suggested, and the roles of the other genes examined also provide interesting evidence and implicate a role in acquisition of LS in the porcine CL.

INTRODUCTION

The porcine corpus luteum (CL) is unusual in that it does not show a luteolytic response to an exogenous dose of PGF-2α until after day 12 of an 18-21 day cycle [1-3]. This is in marked contrast to other farm animal species in which luteolysis can be induced after about 6 days of the estrous cycle [4, 5]. It has been suggested that acquisition of luteolytic sensitivity (LS, ability to respond to PGF-2α) in the pig is not due to a lack of PGF-2α receptors, as they are present and functional as early as day 5 [6, 7], but due to a lack of post-receptor signaling (i.e. Protein Kinase C) [8, 9]. Therefore understanding the lack of sensitivity to PGF-2α earlier in the cycle is critical for the discovery of new approaches for estrous cycle regulation in this species. Thus in the present study we examined the expression patterns and localization of several genes associated with apoptosis in the porcine CL throughout the estrous cycle, to investigate whether changes in gene expression my underlie control of luteolytic sensitivity in the pig.

TNF-α, produced by macrophages, is a 17 kDa non-glycosylated protein [10], which when physiologically active circulates as a homotrimer [11]. Depending upon the cell type, TNF-α has a variety of actions such as proliferation, differentiation, eliciting inflammatory responses, mediating steroidogenesis and apoptotic or necrotic cell death [12]. It has been suggested that TNF-α is involved in the later stages of luteolysis by inhibiting steroidogenesis [13, 14], stimulating luteal PGF-2α synthesis [13, 15, 16], and activating apoptosis [17]. However, recently macrophages have been identified in the early and mid-cycle CL [18, 19], which suggests that TNF-α may play a role before the onset of luteolysis. Additionally, TNF-α is found in the CL from the early to regressing CL (RT-PCR) [20], confirmed by Hehnke-Vagoni and collegues (1995) in the mid and late CL by IHC and Western blot analysis [21]. Macrophages and their capacity to produce TNF-α, have been shown to dramatically increase (four-fold) between the early (day 4-6) and mid (day 8-12) stages of the porcine luteal cycle, further increasing an additional 1-2 fold in the late luteal stage [18]. Moreover, TNF-α receptors have been characterized in the porcine CL to be present and unchanged from days 4-12 of the cycle, but increased on day 15 [22, 23]. TNF-α receptors have been demonstrated on small

luteal cells (SLC), large luteal cells (LLC) and Endothelial cells (EC) in the porcine [22] and bovine CL [17].

NO, generated by immune cells, is a functional part of the hypothalamus-pituitary-gonadal axis, and plays a role in luteal development and function [24, 25]. Endogenous NO is synthesized and regulated from L-arginine by a family of NO synthase (NOS) isozymes [endothelial NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS)] [26]. nNOS and eNOS are constitutive and are responsible for basal release of NO, whereas iNOS is expressed in response to cytokines (i.e. TNF-α) and lipopolysaccarides [27]. eNOS and nNOS are activated by increases in Ca^{2+}, and therefore release of NO is within several minutes, whereas iNOS is calcium-independent, responds to immune or inflammatory signals and produces large amounts of iNOS for up to several days [28, 29]. NO can induce apoptosis in several cell types, including the ovary, but also NO has been shown to have anti-apoptotic effects by activating NFκB and modifying capases so that they do not induce cell death [30].

Apoptosis in the CL is characterized by the loss of steroidogenic potential and luteal cell death [31, 32], and is believed to be stimulated by PGF-2α [33, 34], FasL [35, 36] and TNF-α [17]. Luteal cell apoptosis thought to be a key process in CL regression [33], and FasL binding to its receptor Fas, is one way, via the extrinsic pathway, apoptosis in the CL could be initiated. However in the CL, the intrinsic cascade is thought to play an important role in controlling the rate of apoptosis [37]. Apoptotic family members such as Bcl-2 (anti-apoptotic), Bcl-x (anti-apoptotic) and Bax (pro-apoptotic) have all been found to be expressed in the human CL [38-40]. Although apoptosis has been extensively studied in the porcine follicle, there has been no data thus far that examined apoptosis-related genes in the CL, specifically during LS.

Although control of luteolytic sensitivity has been studied in other species, it is not well understood in the porcine CL. Thus in the present study we examined the expression patterns and localization of several genes associated with apoptosis (TNF-α, TNFR, iNOS, eNOS, Fas, Bax, Bcl-x, NFκB, p53, Caspase-3, Caspase-8) in the porcine CL throughout the estrous cycle, to investigate whether changes in these genes may underlie control of acquisition of luteolytic sensitivity in the pig.

MATERIALS AND METHODS

Chemicals and Reagents

Ketamine, xylazine and halothane were purchased from Webster Veterinary Supply (Sterling, MA). Tri-reagent® and DNAse kit were purchased from Ambion (Austin, TX). Omniscript® and MasterMix® kits were purchased from Qiagen (Valencia, CA). Primers were synthesized by Sigma-Genosys (Woodlands, TX). 4-12% Bis-Tris gels, molecular weight markers and secondary antibodies for immunofluorescence (Alexafluor) were purchased from Invitrogen (Carlsbad, CA). PVDF membrane was purchased from Pall Company (Pensacola, FL). Antibodies were purchased from Chemicon (NFκB, Bcl-x, Caspase-3, Fas Ligand), Upstate Laboratories (Bax, p53) or ABCAM (β-actin, von Willibrand Factor). ECL kits were purchased from GE Healthcare (Piscataway, NJ). Unless noted all other chemicals and reagents were purchased from Sigma (St. Louis, MO).

Animals

30 crossbred (females- Landrace and Yorkshire, males- Durco and Hamshir) cycling gilts were obtained from 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. On the day of surgery (days 4, 7, 10, 13 and 15 of the cycle; n = 6 per day), animals were anesthetized with i.v. injection of Ketamine (2.2 mg/kg) and Xylazine (0.4 mg/kg). Gilts were then maintained on halothane and oxygen/nitrous oxide for the remainder of the procedure. Ovaries were collected via midventral laparotomy and CL were dissected from the ovary. CL were snap frozen in liquid nitrogen and stored at -80°C until further use. All animals were housed at NCSU Swine Unit and all protocols were approved by NCSU Institutional Animal Care and Use Committee (IACUC).

RNA Isolation

Total RNA was isolated from CL using Tri-Reagent® according to the manufacturer's protocol. Briefly, CL were homogenized in 1ml Tri-Reagent®/mg tissue. Chloroform was added, mixed and incubated for 15 minutes. Samples were centrifuged at 12,000g for 15 minutes and the aqueous phase containing RNA was removed. Ethanol was subsequently added and mixed. Samples were then transferred into an RNAqueous filter cartridge (Ambion) and centrifuged at 12,000g. RNA in collection tube was washed and eluted. RNA quality and concentration were determined by agarose gel electrophoresis and by NanoDrop spectrophotometry at 280 nm (Wilmington, DE), respectively. Samples were then DNAase treated using the DNAse treatment kit (Ambion). RNA was subsequently quantified using the NanoDrop Spectrophotometer.

Reverse Transcription- Polymerase Chain Reaction (RT-PCR)

As a measure of mRNA expression, steady state levels of mRNA for all genes were determined by semi-quantitative (sq) RT-PCR, as described below. DNAse-treated RNA was used to synthesis cDNA using the Omniscript® kit (Qiagen). Negative controls (no reverse transcriptase) were also run on each sample to ensure that DNAse treatment was successful in removing any genomic DNA contamination. Primers were designed porcine sequences when available, and other primers were designed based on the sequence availability and expected homology to porcine sequences (All porcine sequences except H2A- bovine, NFκB-sheep). Primers were synthesized based upon the published GenBank sequences to produce the expected product sizes (see Table 1 for primer sequences and bp sizes). All primers were validated for semi-quantitative (sq) RT-PCR by running each primer at 20, 25, 30, 35 and 40 cycles and choosing a cycle number that corresponded to the linear range of amplification (all 33 cycles except H2A, 28 cycles). Once primers were validated, sq RT-PCR was run using 1 ug of cDNA and the Taq PCR Mastermix® Kit (Qiagen). Thermocycler conditions were as follows: 3 minute initial denaturation 94°C, followed by 28 to 33 cycles of denaturation, 30 seconds 94°C; annealing, 1 minute 50°C; extension, 1 minute 72°C; followed by a 5 minute 72°C final extension. All gene products were separated on 2% agarose gels and stained with ethidium bromide. Products were quantified using Lab-Works imaging system (UVP

Imagining Company, Upland, CA). Values were calculated as the ratio of gene product band intensity/H2A band intensity; H2A was used as a housekeeping gene since its levels of expression did not vary with stage of estrous cycle.

Cell Dissociation

Day 13 CL were collected from cycling gilts and transported in M199 with antibiotics and antimycotics on ice to the laboratory. CL were dissociated as previously described [1]. Briefly, CL were dissociated in a collagenase containing enzyme preparation for 30 minutes at 37°C. Dissociated cells were removed and remaining tissue was incubated for another 30 minutes. This was repeated until all cells were completely dissociated. Cells were washed and placed in Percoll to remove red blood cells. Large luteal cells were then counted by Trypan blue exclusion. Cell viability was determined to be over 90%.

Immunofluorescence

Chamber slides (Fisher Scientific, Waltham, MA) were coated with 0.1% Gelatin. 25,000 cells (based on LLC count) were plated Medium 199 and incubated at room temperature for 1 hour. Medium was then removed and the plated cells were fixed in ice cold acetone for 10 minutes. Cells were rinsed with phosphate buffered saline (PBS) and blocked with 1% Normal Goat Serum in PBS for 30 minutes at room temperature. Primary antibodies (anti-rabbit antibodies plus anti-mouse von Willibrand factor, or anti-mouse antibodies plus anti-rabbit LH receptor to double stain cells) in 1% BSA in PBS (1:40) were added and incubated overnight at 4°C. Slides were then washed with PBS, and Alexa Fluor secondary antibodies (1:800, anti-rabbit and anti-mouse) were added and incubated for 2 hours at room temperature. DAPI (Vector Laboratories, Burlingame, CA) was added to visualize nuclei and slides were viewed at 40X magnification using a Nikon Eclipse E400 Microscope.

Statistical Analysis

Values were calculated by dividing densitometric values from gene of interest divided by the housekeeping gene (H2A). Values from day of cycle (n=6) were the

averaged. All mRNA statistical analysis was carried out using Repeated Measures followed by Duncan's test for significance using Statistical Analysis Software (SAS, Cary, NC). Repeated measures for mRNA analysis was performed because each RT-PCR reaction was performed twice for each sample and gene. Housekeeping gene (H2A) was analyzed across the days of the cycle. All means are reported as least-squares means +/- SEM. Differences were considered significant at $p < 0.05$, unless otherwise noted.

RESULTS

Expression of Apoptotic-associated gene levels throughout the estrous cycle

The expression of apoptotic-associated mRNAs throughout the estrous cycle was determined (Figure 1). H2A values were analyzed across all days of the cycle and no differences in days were found (data not shown). TNFR mRNA was significantly increased on day 7 versus the other days of the cycle, ~3 fold higher on day 7 compared to days 4, 13 and 15, and ~2 fold higher than on day 10 ($p< 0.01$, Fig. 1B). eNOS expression showed a similar expression pattern to TNFR, and mRNA was expressed most abundantly in day 7 CL versus the remaining days of the cycle, ~6 fold higher than on days 4, 13 and 15 and ~4-fold higher than on day 10 ($p<0.01$, Fig. 1D). iNOS mRNA expression was also highest on day 7, and ~10-fold higher than on day 4 and day 13, and ~6-fold higher than day 15 ($p<0.01$, Fig. 1C). mRNA expression on day 10 was not significantly different than day 7 or day 15. p53 mRNA expression also demonstrated this pattern, significantly higher on day 7 versus all other days of the cycle ($p<0.01$, Fig. 1I), approximately 3-fold higher than all other days. Bax mRNA expression was significantly higher on days 4 and 7 versus the other days of the cycle ($p<0.01$, Fig. 1G). In contrast, TNF-α, FP, Fas, Bcl-x, NFκB, Caspase-8 and Caspase-3 showed no significant differences across the stages of the cycle.

Immunofluorescence in Day 13 CL

The cellular localization of some of the apoptotic-associated genes was examined in day 13 CL. The pro-apoptotic protein Bax was found to be expressed in all luteal cell types (LLCs, SLCs and ECs), however expression was found in many more luteal cells than ECs (Fig. 2B). Caspase-3 expression was localized to SLCs (Fig. 2F), and does not

appear to be expressed in either LLCs or ECs. Bcl-x was also localized to all cell types, however not many cells were immuno-positive for this anti-apoptotic protein (Fig. 2K). FasL was localized only to ECs, however again not many cells were immuno-positive (Fig. 2O). NFκB protein was expressed in both SLCs and ECs (Fig. 2S). p53 protein was expressed in ECs only (Fig. 2W).

DISCUSSION

The present study characterized and quantified the expression and cellular localization of some apoptotic-associated genes in porcine CL throughout the estrous cycle to determine if any of these genes may be involved in LS. This data provided some evidence that elevated TNFR, iNOS, eNOS and p53 mRNA may be involved in acquisition of LS, which is hypothesized [41] to occur between days 7 and 13.

Evidence in the CL has long suggested that TNF-α is involved in luteolysis by inhibiting steroidogenesis [13, 14], stimulating luteal PGF-2α synthesis [13, 15, 16], and activating apoptosis [17, 42, 43]. Macrophages and their capacity to produce TNF-α, have been shown to increase dramatically (four-fold) at a much earlier period of the estrous cycle (between day 4-6 and day 8-12) in the pig [18]. Such observations have prompted the suggestion that TNF-α may have critical functions within the CL several days before luteolysis [18], and we have hypothesized that TNF-α may play a role in the acquisition of LS in the pig [41]. In other studies, TNF-α was also found to be expressed in the CL throughout the estrous cycle from early development right up to the time of luteal regression (mRNA, by RT-PCR, [20]), and in the mid and late CL (protein, by IHC and Western blot analysis) [24]). Moreover, TNF-α receptors have been characterized in the porcine CL to be present and unchanged from days 4-12 of the cycle, but increased on day 15 [22, 23]. These data support the contention of macrophages and TNF-α playing an important role in luteolysis in the pigs.

Our current data confirms the observations published previously in the bovine CL [44, 45], that TNF-α mRNA is produced as early as day 4 and that mRNA levels do not change over the course of the cycle (Fig 1A). However, our data also shows that TNFR mRNA is present as early as day 4 (similar to above studies), but that TNFR mRNA levels increase 3-fold on day 7, compared to days 4, 13 and 15, and 2-fold over day 10

(Fig. 1B). These data are not in agreement with the observation of Miyamoto and collegues (2002) who showed that TNF-R determined by receptor binding studies were unchanged throughout the estrous cycle [46]. This discrepancy could be explained by the fact that our study examined mRNA expression and the prior study examined TNFR expression at the protein level. Nevertheless, if confirmed in future studies, our observation of an increase in TNFR on day 7 of the cycle could provide additional support for our hypothesized role of TNF-α in the acquisition of LS which occurs between days 7 and 13 [41].

TNF-α effects are mediated by two distinct receptor types, which trimerize with itself upon TNF-α binding, with molecular sizes 55 (TNFRI) and 75 (TNFRII) kDa [47]. Activation of the 55kDa TNF-α receptor (TNFRI) contains an intracellular death domain, which is required for apoptotic signaling pathways [48]. TNF-α binds to TNFRI which contains an intracellular death domain (DD), the intracellular DD of the receptor interacts with the DD of the adaptor protein (TNFR-associated DD protein; TRADD) [49-51]. The DD of TRADD then binds with the DD of another adaptor protein (Fas-associated DD protein; FADD), which then activates initiator caspase (procaspase-8); which initiates apoptosis (named the TNFR1-TRADD-FADD-caspase-8 signaling axis) [49-51]. In the CL, TNFRI been associated with luteolytic actions [17, 45, 52] and most likely acts through this pathway.

Ligand binding to TNFRI can also lead to cell survival and differentiation, and may be doing so in the porcine CL around day 7 of the cycle (based on our mRNA data, Fig. 1B). When TNF-α acts as a survival/anti-apoptotic factor, TNF-α binds to the extracellular domain of TNFRI and the intracellular DD of the receptor interacts with the DD of TRADD. The DD of TRADD binds with the DD of the receptor interacting protein (RIP). RIP then interacts with TNFR-associated factor-2 (TRAF-2). TRAF2 mediates the physical interaction of the TNFRI signaling complex with the nuclear factor (NF)-κB-inducing inhibitor of κB kinase (IKK) and the inhibitor of apoptosis proteins (cIAP) 1 (named the TNFRI-TRADD-RIP-TRAF2 signaling axis), and consequently, expression of survival/anti-apoptotic genes is up-regulated [49-51]. At this point in the cycle, TNF-α actions do not cause luteolysis (via increasing PGF-2α and decreasing

progesterone), however TNF-α may be playing a luteotrophic role, acting through this pathway by promoting steriodogenesis and playing a protective role against apoptosis.

We also examined the expression of NFκB mRNA throughout the cycle to determine whether there was an increase in mRNA expression that correlated to an increase in the increase in TNFR. Although there is no statistical significance in mRNA expression throughout the cycle, there is a slight trend showing an increase on day 7 versus day 10 (Fig. 1J), possibly suggesting that TNF-α binding to TNFR at this point in the cycle does induce the expression of survival and anti-apoptotic genes. We detected NFκB in both SLCs and ECs (Fig. 2S), and TNFR have been shown on both of these cell types [22], which could suggest that TNF-α does act to induce NFκB expression in SLCs and/or ECs early in the cycle. Further examination of this pathway is required to determine the significance of the presence of NFkB this early in the cycle.

Another apoptotic-associated component is Nitric Oxide (NO), which can induce apoptosis in several cell types, including the ovary, via the mitochondrial pathway [30]. In general long-lasting production of NO acts as a pro-apoptotic modulator by activating caspases via cytochrome c release, upregulation of p53, activation of JNK/SAPK and altering the Bcl-2 family of proteins [36]. The four ways in which NO can act as a pro-apoptotic inducer are: 1) directly inducing cytochrome c release through mitochondrial membrane potential loss [36]; 2) activation of caspase through NO-induced p53 expression due to DNA damage [52]; 3) activation of JNK/SAPK and p38MAPK which activates Caspase 3 via NO donors or NOS [29, 53, 54]; and 4) NO increasing ceramide levels through nSMase and caspase-3 activity [55, 56]. In cultured bovine luteal cells, NO caused an increase in Bax as well as an increase in expression and activation of Caspase-3 [57].

NO promotes apoptosis in some cells (as described above) but can inhibit it in others [30]. Low concentrations of NO prevents cells from undergoing apoptosis which occurs when there are low levels of certain apoptotic factors such as Fas, TNF-α and lipopolysaccaride [30]. NO can also act in an anti-apoptotic manner by modifying a cysteine (by nitrosylation) at the enzyme catalytic site on all caspases [58], and therefore the caspases can not be activated. NO can also induce the expression of several cytoprotective genes inducing HSP70 and HSP32 which protect cells from apoptosis

[36]. NO can also increase Bcl-2 expression (mRNA and protein) to prevent apoptosis in cultured B cells [59].

In the CL, there are conflicting reports of NO regulation depending on stage of development and species. In a study by Jo and collegues (1995), IFN-γ and TNF-α synergistically increased NO production in mouse luteal cells, and they suggested that NO produced in response to multiple cytokines plays a role in decreasing progesterone in luteal cells [62]. It has also been suggested that NO plays a role in luteolysis, as NO stimulates PGF-2α synthesis in the human [60] and bovine [61] CL. It has also been demonstrated that PGF-2α increases NOS activity to further NO production, causing a positive feedback mechanism to be activated in the rabbit [62] and rat [24, 25], leading to further decreases in progesterone production in the rat [63], rabbit [64], and bovine [61] CL. Inhibition of NO synthesis (using a NOS inhibitor) during the late luteal stage in the bovine CL counteracts spontaneous and PGF-2α-induced luteolysis by increasing progesterone production and prolonging the luteal cycle [65], which strongly suggests that NO plays a significant role in luteolysis. However, in mid-stage rat CL, NO stimulates progesterone production via iNOS mediated-NO [66-68], therefore demonstrating that NO can have dual functions, depending on the stages of the cycle.

Our mRNA data shows a significant increase in iNOS expression on day 7 versus days 4, 13 and 15 (Fig. 1C). Expression on days 4 and 13 were the lowest, and day 10 was no different than day 7 or 15. Additionally, eNOS expression was significantly increased on day 7 (~5-6 fold) versus all other days of the cycle (Fig. 1D). Our data in the porcine CL could suggest NOS act in multiple ways. Firstly, elevated NO (via increased iNOS and eNOS) early in the cycle, could be the result of the increased numbers of endothelial cells as the vascular supply develops during the early formation/development of the CL, similar to what was seen in other species [63,64,67,71] and may function to increase progesterone production. The relative amounts of mRNA detected in our samples show that even on day 7 when expression of both iNOS and eNOS are highest, eNOS (produced from endothelial cells) is ~6 times higher than iNOS and, therefore, suggests in our luteal cells the majority of NOS produced at this time in the cycle is from endothelial cells. The timing of this increase is certainly in line with the idea that NOS/NO may also play a role in control of LS,

although further studies will be required to investigate this hypothesis. Secondly, in some species the presence of NOS in the later stages of the cycle implicates its role in luteolysis, and our data showing a significant increase on day 7 of the estrous cycle, is difficult to reconcile, in view of the clear role of NOS/NO in functional luteolysis in other species [63,65,66]. Furthermore, increased NO has been demonstrated to increase the pro-apoptotic genes p53 and caspases to activate Bcl-2 pro-apoptotic members [69], and thus is believed to play a role in inducing apoptosis during structural luteolysis (described below). However, it should be emphasized that our data reflect only steady state mRNA values and that the expression at the protein level may be very different, as should be revealed once additional studies to examine protein expression are carried out. Thus, although iNOS and eNOS mRNA levels are elevated early in the cycle, their translation to mature, functional (enzymatically active) protein may not occur until later in the cycle, as suggested from data in other species [63,65,66].

Apoptosis in the CL is characterized by the loss of steroidogenic potential and luteal cell death [31, 32], and is believed to be stimulated by PGF-2α [32-34, 69, 70] Fas ligand (FasL) [35, 36] and TNF-α [17]. Luteal cell apoptosis thought to be a key component in CL regression [32-34, 69, 70]. FasL, produced by T lymphocytes, is a member of the TNF super family and is recognized to have important apoptotic actions in the CL [10]. Fas antigen (Fas) is a cell surface receptor that when bound to FasL, induces apoptosis via the extrinsic apoptotic pathway [71, 72] and structural luteolysis [10]. The Fas signaling pathway involves protein-protein interactions that result in a cascade of protease activities, cleavage of cellular substrates and cell death [73]. The apoptotic signaling mechanism involves FasL binding to Fas, and the recruitment of FADD, an adaptor protein which then recruits the initiator caspase-8 [72]. The caspase cascade continues, leading to DNA fragmentation and cell death [74].

Fas and FasL are expressed during the luteal phase in rat and mouse CL [75, 76], and activation of Fas occurs around the time that luteolysis [75, 77] and atresia [78-82], occur. Additionally, Fas is expressed in human [83] and bovine CL [35] and at higher levels in the regressed CL (mouse- [76]; human- [83]). In our study, we determined the levels of Fas mRNA expression, the temporal expression patterns of Fas during the estrous cycle. However, our data did not show any significant differences throughout the

cycle (Fig. 1F). We also examined the expression of FasL protein in day 13 luteal cells by immunofluorescence. Although we saw limited expression, the only positive cells were small cells presumed to be ECs, as the positive cells for LH receptor (assumed to be SLC, Fig. 2N) were not the same cells that expressed FasL protein (Fig. 2O). It is possible that some of these positive cells are actually other small cell types present in the CL such as T-lymphocytes, as ECs normally represent 50% of the luteal cells in the CL [84, 85], however this would need to be determined by double labeling the cells with both FasL and von Willibrand Factor (EC marker). Examination with alternate cell markers (such as an immune cell marker) may clarify the actual localization of FasL in the CL. As discussed above, further examination of protein levels of Fas/FasL during the cycle is needed before we can draw any conclusions regarding the role of these two components in regulating LS and/or in mediating luteolysis in the porcine CL.

We also examined mRNA expression of genes normally associated with the apoptotic cascades to determine if these genes were suppressed during the onset of LS in the pig. We examined the mRNA expression of p53, as it targets Bax in p53-mediated apoptosis [86]. p53 also is known to play a role in apoptosis in granulosa cells [87] and has been localized to granulosa cells in atretic follicles [88]. Additionally, p53 has also been shown to be associated with lutenization and steriodogenesis by regulating steriodogenic enzymes in the primate CL [89]. Our data shows a significant (~6 fold) increase in p53 mRNA on day 7 of the cycle versus all other days (Fig. 1I), and protein localization exclusively to small cells that are not LH receptor positive, perhaps EC (Fig. 2W). As suggested above, this increase could be coupled with the increases seen with iNOS and eNOS to promote apoptosis, leading to transcription of Bax. However, this significant increase we observed occurred in developing CL (day 7) CL, during the period when we hypothesize that LS is acquired (days 7-13) and thus may also be associated with steriodogenesis, similar to those effects seen in the primate CL. Therefore, exploring p53 protein expression patterns as well as determining which pathways it is regulating this early in the cycle is critical to understanding how p53 is involved in the porcine cycle.

In the CL, apoptosis via the intrinsic cascade is also thought to play an important role in controlling the rate of cell death [37]. Apoptotic family members such as Bcl-2

and Bcl-x (both anti-apoptotic) and Bax (pro-apoptotic) have all been found to be expressed in the human CL [38-40]. An apoptosis-inducing member of the Bcl-2 family is Bax, which acts on the mitochondrial membrane to form pores which allows mitochondrial proteins (i.e. cytochrome C) to escape [90]. Increased expression of Bax over Bcl-2 (which counteracts Bcl-2 death repressor activity; [90]) has been correlated with the occurrence of apoptosis in granulosa cells during atresia, and in bovine and human luteal cells during regression [91, 92]. Furthermore, higher Bax mRNA levels have been observed in the bovine regressing CL [93].

Examination of the intrinsic (or mitochondrial) pathway revealed the pro-apoptotic gene, Bax, was actually ~3-fold higher on days 4 and 7 than on days 10, 13 or 15 (Fig. 1G), and was localized to all luteal cell types (LLCs, SLCs and ECs, Fig. 2B). This was somewhat of a surprise, as this gene is normally upregulated during apoptosis, and the cellular changes that occur in the early CL are not normally associated with apoptosis, as this is the major time for cellular growth. Nevertheless, its expression during this early period may suggest a role in control of LS. However, the presence of Bax expression in all of the cell types in day 13 of the CL suggest that Bax expression during the late stages of the cycle may certainly regulate luteal cell apoptosis in the porcine CL. Examination of the anti-apoptotic gene, Bcl-x, showed no significant differences across the cycle (Fig. 1H), and expression appears to be localized to all three luteal cell types (SLCs, LLCs and ECs, Fig. 2K). However, Bcl-x mRNA may not be indicative of protein levels with in the CL, and therefore protein expression may actually represent somewhat of a different pattern. This particular protein may be involved in luteal apoptosis, as there was expression in all of the day 13 cell types, examined by immunofluorescent examination of Bcl-x protein is necessary to fully understand its involvement in luteal apoptosis. Additionally, since we do not see a significant increase in mRNA expression in the early stages of the cycle, we suggest that Bcl-x is not playing a significant role in acquisition of LS in the pig.

Lastly, we examined the mRNA expression patterns of two of the caspases to determine the role in LS. First we determined the expression of caspase-8, which is associated with the DD of the TNF superfamily of receptors (i.e. Fas), and thus the extrinsic pathway. Mice treated with PGF-2α 48 hours post ovulation displayed a 22-fold

increase in caspase-8 activity, even though the PGF-2α receptor is not known to be coupled with caspase-8 [36]. We showed no significant differences in mRNA expression across the estrous cycle, however there was a tendency (p=0.1) for an increase on day 13 versus day 7 of the cycle (Fig. 1K). This may suggest that caspase-8 is involved in apoptosis later in the cycle, and that the lack of caspase-8 mRNA earlier (i.e. day 7) is due to the lack of Fas activation. However further studies are warranted to determine protein levels of caspase-8 to determine if this is true. We also determined levels of caspase-3 mRNA expression, as the presence of caspase-3 in the cell is indicative of apoptosis, as it is the final executioner caspase, leading to DNA degradation. Carambula and colleagues (2002) showed that activation of caspase-3 is required for structural involution of the mouse CL [94]. More recently, the same authors demonstrated that caspase-3 deficient mice were resistant to apoptosis induced by PGF-2α *in vivo*, and that caspase-3 was a required mediator of PGF-2α induced luteolysis. They additionally suggested that PGF-2α initiated luteolysis involved in part, increasing bioactivity and bioavailability of cytokines such as FasL to activate caspase-3 driven apoptosis [36]. Furthermore, expression of caspase-3 was stimulated by inhibiting intra-luteal progesterone action in bovine luteal cells, which suggests that intra-luteal progesterone may suppress apoptosis in by activating a survival pathway via the inhibition of Fas and caspase-3 mRNA expression [95]. Our mRNA analysis shows that the levels of caspase-3 mRNA are constant throughout the cycle (Fig. 1L). Because caspases are regulated at the protein level, mRNA profiles of caspases might not be indicative of protein levels in the cells, and therefore caspase-3 protein levels should be examined throughout the cycle to determine whether or not caspase-3 plays a role in apoptosis. However, caspase-3 mRNA expression does not seem to indicate that it is involved in acquisition of LS in the pig.

Understanding the acquisition of LS in the porcine CL is of major economical interest for estrus cycle regulation in the pig. Therefore, further studies are warranted to determine the causes of the refractoriness of the early CL to PGF-2α, and how apoptotic-related genes may play a physiological role.

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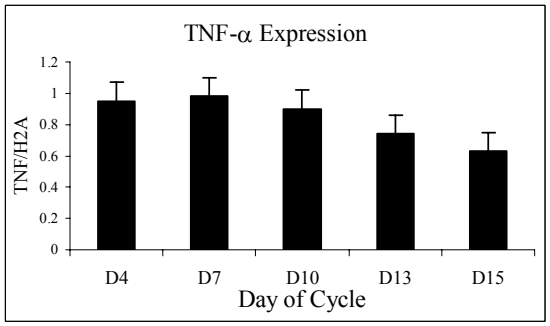
Table 1. Primer sequences used for PCR[a]

Gene	Accession Number	Product Size		Primer Sequences	Cycles	Annealing Temp.
H2A	X52318	209	F	AGGACGACTAGCCATGGACGTGTG	28	50
			R	CCACCACCAGCAATTGTAGCCTTG		
TNF-α	NM_214022	223	F	TTTCCTCACTCACACCATCAGCCG	33	50
			R	CCCAGATTCAGCAAAGTCCAGATAG		
TNFR	NM_213969	183	F	AGAGAGAGTTTGTGTCCCCAAGG	33	50
			R	CAGGCATTGTGTGAGGTGGTTC		
iNOS	U59390	333	F	TGCGTTATGCCACCAACAATG	33	50
			R	GCTCCTGGAACCACTCGTATTTG		
eNOS	AY266137	384	F	ACCCTCAGGTTCTGTGTGTTCG	33	50
			R	GCTTTGTAGGTTTTCCACCGAGAG		
FP	AY043485	429	F	GGCATCGTGACTACAAAATTCAAGCA	33	50
			R	AGAGATTCTTAAGGACTGCCTTCCG		
Fas	AY781398	131	F	CCAACCAGCAACACCAAATGC	33	50
			R	CACCGTCTTTTCACCTCCCTG		
Bax	AM233489	296	F	CGATTGGAGATGAACTGGACAG	33	50
			R	CAGGCTGAAATCAAGAGGGCAC		
Bcl-x	AJ001203	210	F	GAGCAGGTATTGAACGAACTCTTCC	33	50
			R	TTCCACAAAAGTGTCCCAGCC		
p53	NM_214145	455	F	TACTCCCCTGCCCTCAATAAGC	33	50
			R	CACAAACACGCACCTCAAAGC		
NFκB	AF283892	139	F	GGTTCGGTTCTACGAGGATGATG	33	50
			R	CACGGTTACAGGACGCTCAATC		
Caspase-8	NM_0010317	112	F	TCCCAGGATTTGCCTC	33	50
			R	AAGCCAGGTCATCACTGTC		
Caspase-3	NM_214131	202	F	CAAGTTTCTTCAGAGGGGACTGC	33	50
			R	TCGCCAGGAATAGTAACCAGGTGC		

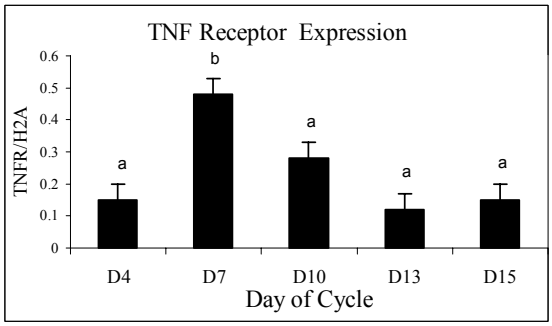
[a] All primers are listed 5'-3'

Fig. 1. mRNA expression in porcine CL throughout the estrous cycle. mRNA expression on days 4, 7, 10, 13 and 15 of the cycle was determined by semi-quantitative RT-PCR using H2A as a housekeeping gene. All values represent gene of interest/H2A. A) TNF-α, B) TNFR, C) iNOS, D) eNOS, E) FP, F) Fas, G) Bax, H) Bcl-x, I) Caspase-8 and J) Caspase-3. Bars represent the least-squared means +/- SEM of the densitometric analysis (n=6). Different letters denote significant differences between groups ($p<0.05$).

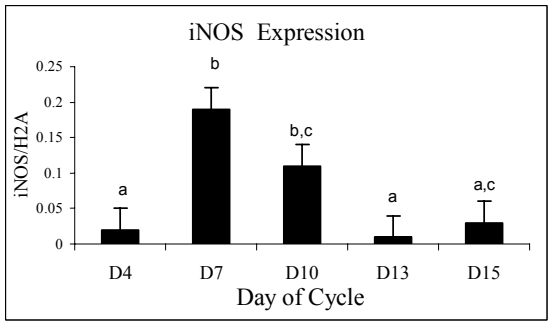
A


TNF-α Expression
TNF/H2A
1.2
1
0.8
0.6
0.4
0.2
0
D4
D7
D10
D13
D15
Day of Cycle

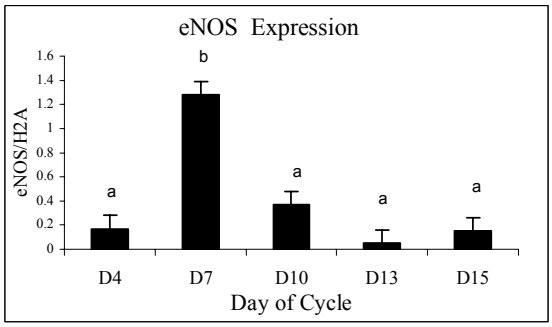
B


TNF Receptor Expression
TNFR/H2A
0.6
0.5
0.4
0.3
0.2
0.1
0
a
b
a
a
a
D4
D7
D10
D13
D15
Day of Cycle

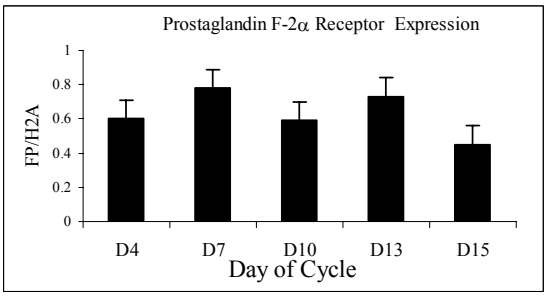
C


iNOS Expression
iNOS/H2A
0.25
0.2
0.15
0.1
0.05
0
a
b
b,c
a
a,c
D4
D7
D10
D13
D15
Day of Cycle

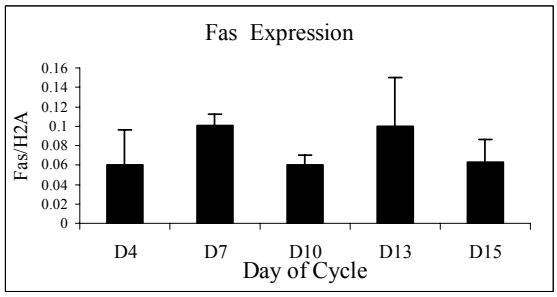
D


eNOS Expression
eNOS/H2A
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
a
b
a
a
a
D4
D7
D10
D13
D15
Day of Cycle

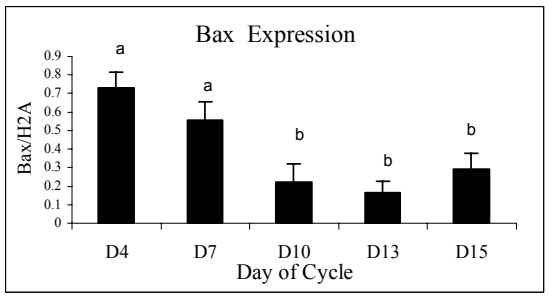
E


Prostaglandin F-2α Receptor Expression
FP/H2A
1
0.8
0.6
0.4
0.2
0
D4
D7
D10
D13
D15
Day of Cycle

F


Fas Expression
Fas/H2A
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
D4
D7
D10
D13
D15
Day of Cycle

G


Bax Expression
Bax/H2A
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
a
a
b
b
b
D4
D7
D10
D13
D15
Day of Cycle

H

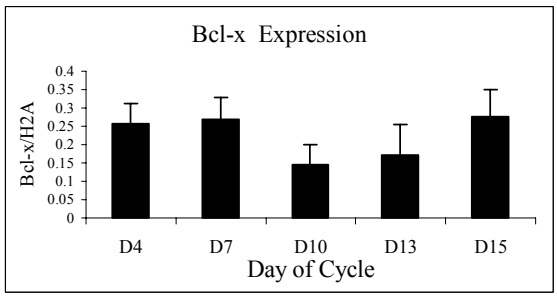
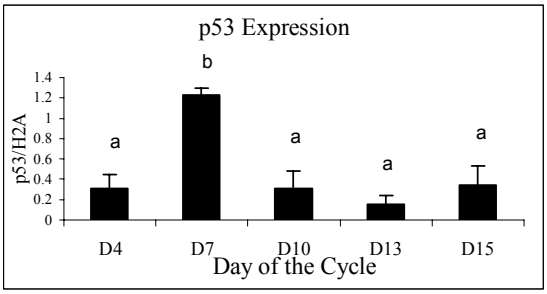
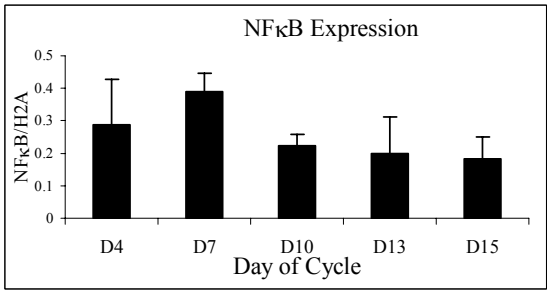

Bcl-x Expression
Bcl-x/H2A
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
D4
D7
D10
D13
D15
Day of Cycle

Figure 1 continued

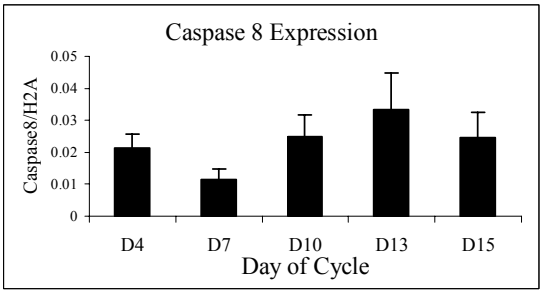
I



J



K



L

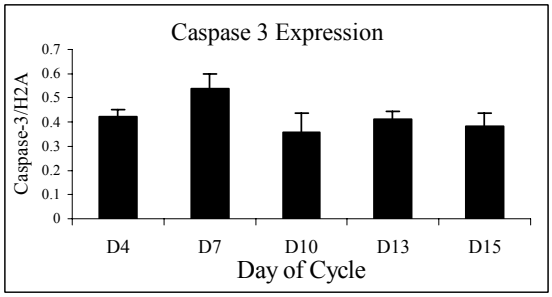
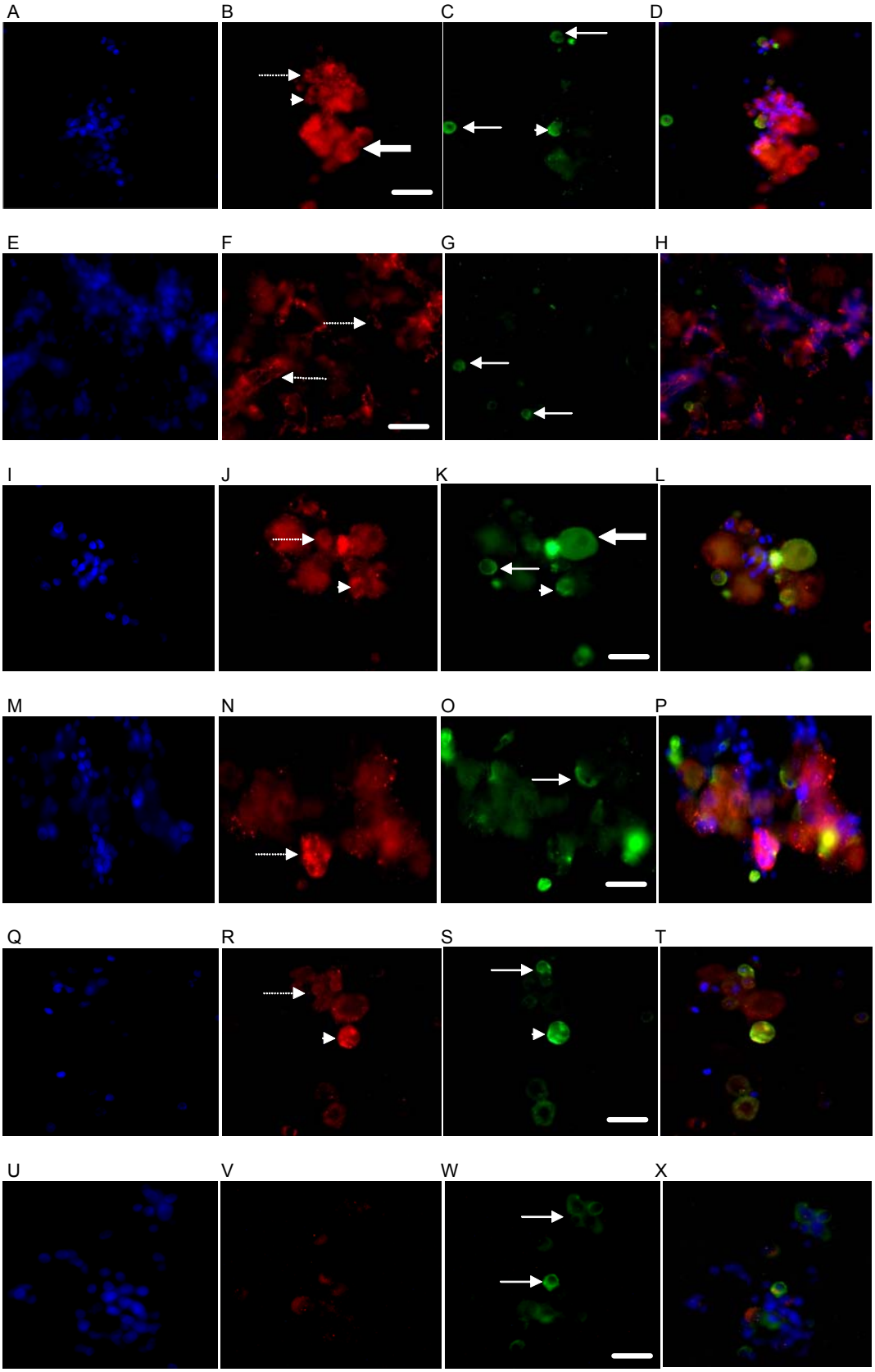


Fig 2.: Detection of protein in porcine luteal cells by Immunofluorescence. Protein localization was determined in day 13 porcine luteal cells. Panels A, E, I, M, Q and U represent DAPI staining of nuclei. Panels B and F represent immuno-positive staining of Bax and Caspase-3. Panels C and G represent von Willibrand positive cells (EC marker). Panels J, N R and V represent positive immunostaining of LH receptor (SLC marker). K, O, S and W represent immuno-positive staining of Bcl-x, FasL, NFκB and p53 respectively. Panels D, H, L, P, T and X show DAPI/Immunostaining overlays. Large thick arrows represent LLCs, small dotted arrows represent SLCs, thin arrows represent ECs, and arrowheads represent double staining in either ECs (Bax and Caspase-3) or in SLCs (Bcl x, FasL, NFκB and p53). All panels are at 40x magnification. Bar represents 60 μm.


A
B
C
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I
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K
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X

Overall Conclusions

Since the pig is unique in that is does not respond to an exogenous dose of PGF-2α before day 13 of an 18-21 day cycle, unlike other domestic species, understanding acquisition of luteolytic sensitivity in the porcine corpus luteum is critical for estrous cycle regulation in this agriculturally important species. The aim of these studies was to investigate the roles of several factors (TNF-α, ET-1, PKC) that have been suggested to be involved in luteolysis in other species (i.e. cow and sheep), however none of which have been examined in the porcine estrous cycle, nor have then been examined to determine how they might play a role in acquisition of LS, which occurs earlier in the cycle. Therefore these studies provide new evidence as to control of LS in the pig.

The purpose of the first study was to develop and establish an in vitro cell culture system where we could enzyme dissociate and culture luteal cells for a period of days. Additionally we wanted to test our hypothesis that TNF-α (a product of luteal macrophages present in the early to mid luteal cycle) plays a role in LS by sensitizing cells to PGF-2α. This study showed that TNF-α cultured at doses as low as 0.1 ng/ml for 48 hours (TNF-α "priming") followed by exposure to PGF-2α with as low of a dose as 0.1 ng/ml showed a sensitizing effect to PGF-2α, (i.e. no TNF-α or PGF-2α effect on progesterone production alone, but with TNF-α pretreatment, followed by PGF-2α treatment, a significant decrease in progesterone occurred). Additionally this study provided preliminary evidence that TNF-α dose-dependently increases ET-1 (peptide), ET_A (protein), PKC βII and PKC ε (protein) on after 48 hours of treatment, as well as ET_A (protein) after 96 hours of treatment, suggesting a role for both the endothelin "system" and various PKC isoforms.

Our next study was to determine the spatial and temporal expression patterns of the ET-1 "system" throughout the stages of the estrous cycle, by examining mRNA and protein expression, as well as localization studies (by immunofluorescent labeling). This study showed that although ppET-1, ET_A and ET_B protein expression did not change throughout the estrous cycle, ECE-1 protein expression patterns did. ECE-1 showed a significant increase in expression on day 10 versus 4, 7 and 13, suggesting an increase in ET-1 peptide at this time. Additionally, our IF data showed ppET-1 localized to endothelial cells, ECE-1 localized to both ECs and LLCs (suggesting ET-1 action on

these cell types), ET_A localization to LLCs (suggesting ET-1 acts on LLCs around the time of LS) and ET_B localization to ECs. Overall this study provides evidence that the ET-1 system is involved in LS in the pig.

The purpose of the third study was to evaluate the expression and localization of the PKC isoforms, to determine if one or more of the isoforms was involved in LS as suggested in the cow. This was the first known comprehensive study done in the pig to evaluate all of the PKC isoforms in the CL. Our data showed all 12 isoforms were expressed in the cycle (mRNA), however protein was detected for 10 isoforms (no PKC ι or θ), and the only PKC isoform whose protein expression pattern changed over the cycle was PKC ε, which showed a significantly higher expression (30%) on day 13 versus day 10 of the cycle. Additionally, when PKC ε was evaluated in the cytosol and membrane fractions, expression in the membrane was significantly higher on day 13 versus day 10, suggesting that PKC ε is activated on day 13 of the cycle (around the time that LS occurs in the CL). Additionally, all PKC isoforms were evaluated with immunoflurescent labeling in day 13 luteal cells. PKC ε was localized on all luteal cell types (EC, SLC and LLC); however immuno-positive staining was most prominent on LLCs. Therefore this study provides important evidence that PKC, specifically PKC ε, is increased (protein expression) and expressed on LLCs, strongly suggesting a role for PKC ε in LS in the pig.

Finally our fourth study evaluated various genes associated with apoptosis to understand how these genes are regulated early in the cycle (as we know much about them in the later stages of the cycle). This study provides preliminary evidence that TNF-R and nitric oxide (iNOS/eNOS) may play a role around the time of acquisition of LS, as suggested by the mRNA expression patterns (significantly higher on day 7 versus the other days of the cycle). Additionally, p53 mRNA was also found to be significantly higher on day 7 versus the other days of the cycle, and Bax (normally associated with pro-apoptotic signals) mRNA expression was significantly higher on both days 4 and 7 of the cycle. Therefore further evaluation, particularly at the protein level, is critical to determine the roles of these genes early in the cycle.

Overall, these studies provide important evidence which suggests that TNF-α plays a role in sensitizing luteal cells to the actions of PGF-2α, earlier in the cycle than

previously suggested. Additionally, we provide evidence for the first time in the pig that the ET-1 system is involved through increasing ECE-1 (and thus ET-1 peptide) around the time of LS, and probably acts through the ET_A receptor present on LLCs (Figure 1). Furthermore, we suggest that PKC ε may also be involved in LS, as in the cycling animal protein expression is significantly higher on day 13 than day 10 of the cycle. We also suggest that genes normally associated with apoptosis may also play a role earlier in the cycle, however further investigation is necessary to determine the exact functions. Generally these studies help in understanding acquisition of luteolytic sensitivity in the porcine corpus luteum, and will assist in designing further studies to elucidate the precise mechanism in which luteolytic sensitivity occurs.

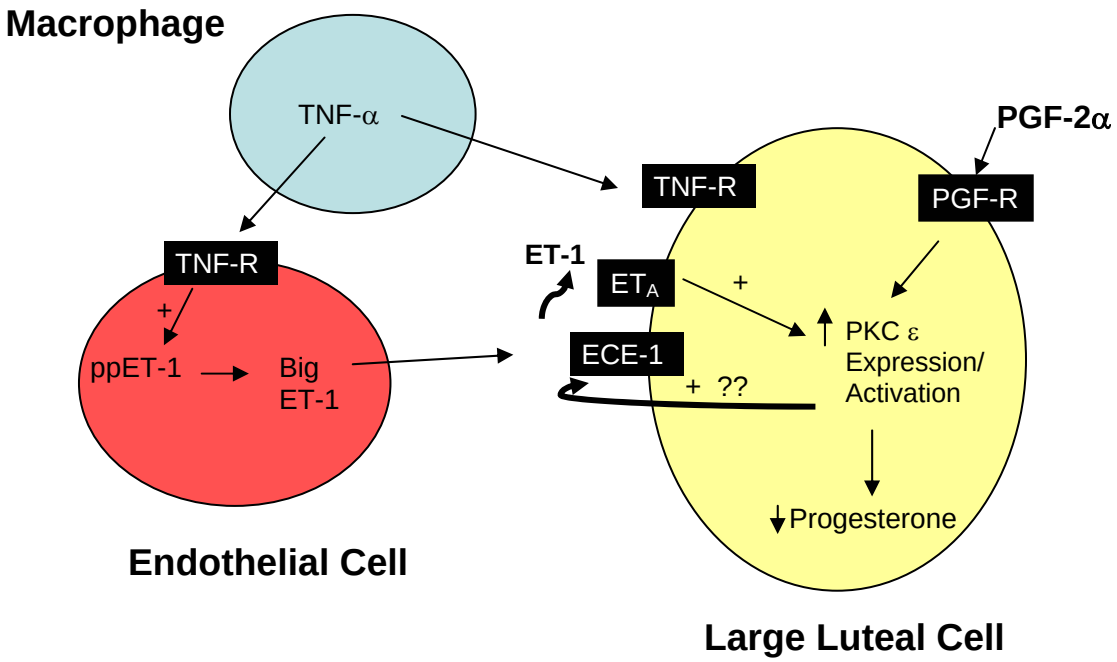

Figure 1: A Model of Acquisition of LS in the Porcine Corpus Luteum

In the early-mid CL, macrophages infiltrate the CL, secreting TNF-α. TNF-α acts on its receptor (presumably on ECs and LLCs) to increase ppET-1 expression. ppET-1 protein is produced and ECE-1 cleaves Big ET-1 at the LLC membrane, producing bioactive ET-1. ET-1 then acts on its ET_A receptor (on LLCs) to increase PKC ε expression and activation. PKC ε may then act to increase ECE-1 expression on LLCs to further increase ET-1 production. PKC ε then mediates PGF-2α response causing a decrease in Progesterone secretion and initiation of luteolysis. How the expression of apoptosis-related genes mediates acquisition of LS requires further analysis.