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Studies of the mammalian ovarian endothelin system

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STUDIES OF THE MAMMALIAN OVARIAN ENDOTHELIN SYSTEM

Marietta Felicidad Wright

Thesis

Submitted to the Eberly College of Arts and Sciences
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ABSTRACT

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Marietta Felicidad Wright

There is recent evidence in porcine ovaries that endothelin-1 (ET-1) may have an autocrine/paracrine role in follicular development. Endothelin-converting enzyme-1 (ECE-1) is the key enzyme in converting the biologically inactive big endothelin-1 to the active ET-1 peptide. Previously, ET-1 and ET type A receptors (ETA) were detected in the granulosa cells of large antral follicles in porcine ovaries. However, the porcine ovarian source of ECE-1 had not been investigated. Porcine ovaries were collected from pre-pubertal pigs (65-95 kg body weight), frozen and sectioned. *In situ* hybridization was used to determine the ovarian compartment that synthesizes ECE-1. Expression of ECE-1 mRNA was detected in the granulosa cells of large (about 10 mm) and medium (about 3 mm) porcine antral follicles. This study, in conjunction with previous studies suggests a novel autocrine role of ET-1 as a possible local ovarian factor involved in follicular development.

Recent evidence in the cow suggests that ET-1 plays a role during prostaglandin (PG) F₂ alpha-induced luteal regression. We have examined the effects of treatment with PGF₂ alpha during the early and mid-luteal phases on three components of the endothelin system: ECE-1, ETA and ET-1 in the bovine corpus luteum. Cyclic beef cows were injected (time 0) on Day 4 or Day 10 with either saline or the PGF₂ alpha analog, Lutalyse (15 mg). The corpora lutea (CL) were collected 2 (n = 11), 10 (n = 23), 24 (n = 15), or 48 h (n = 12) after treatment. The cows in which CL were removed after 10 h were comprised of two experimental groups. The first group (n = 11) received one injection; the second group (n = 12) received two injections, one at 0 h and another at 8 h. The cows in which the CL were collected after 24 and 48 h received one injection every 8 h. Semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) was used to evaluate the mRNA encoding ECE-1, ETA and ET-1. The ECE-1 and ETA proteins were evaluated by semi-quantitative Western Blotting. ET-1 was the most likely target of the endothelin system for PGF₂ alpha regulation during the mid-luteal phase. The ETA and ECE-1 genes were constitutively expressed in the Day 4 and Day 10 CL. A practical application of this observation is that it may be possible to target the ET-1 gene as a way to manipulate the luteolytic actions of PGF₂ alpha.

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CHAPTER I

INTRODUCTION

The development of the ovarian follicle and the corpus luteum (CL) is regulated by cyclical changes. Some of the mechanisms underlying these changes are uncertain. Although it is known that endocrine components are involved, there is evidence that autocrine and/or paracrine factors may take part in some of these mechanisms [1,2]. It has been suggested that endothelin-1 (ET-1) may be one of these autocrine/paracrine factors [3,4,5], however the specific role of ET-1 is unknown.

Ovarian Follicles

Ovarian follicles are the functional units of the ovary. Histologically, the follicle consists of at least four cell types: the oocyte, granulosa cells, thecal cells and endothelial cells. It is the interplay of these cells, responding to various factors, which regulate follicular development.

Most female mammals are born with their lifetime supply of oocytes enclosed in follicles, known as primordial follicles. Primordial follicles consist of a single layer of flattened granulosa cells and a primary oocyte, arrested in prophase of the first meiotic division [2,6]. The cohort of primordial follicles is referred to as a resting pool. Those factors that determine when a primordial follicle leaves the resting pool and enters the growth phase, a process called recruitment, remains to be determined [6]. The gonadotropins, follicle stimulating hormone (FSH) and luteinizing hormone (LH), do not appear to be critical factors for early follicular growth [1,6]. However, it has been suggested that autocrine/paracrine factors may be involved in this process [1,2].

Follicles that have been recruited and possess one layer of a mixture of flattened and cuboidal granulosa cells are considered transitory follicles [2]. As the growth phase

continues, the follicle and oocyte increase in size, forming the primary follicle. Continued development involves the formation of a glycoprotein coat and zona pellucida around the oocyte [7, 8]; the granulosa cells undergo mitosis to form two layers [2]. These follicles that continue to grow, develop the theca, which is separated from the granulosa by a basement membrane, and are termed secondary, or preantral, follicles [7,8]. From among these preantral follicles, one or more follicles continue to develop to form tertiary, or antral, follicles. They are denoted by the formation of the theca interna and theca externa cell layers, more than five layers of granulosa cells, and the formation of the antrum, a cavity filled with follicular fluids [2,8]. Antral follicles consist of a range of sizes, from small (about 400 micrometers in diameter, in pigs) to large (about 10 mm in diameter, in pigs) [3, 9]. Continued growth of preantral and antral follicles is accompanied by the expression of FSH receptor mRNA localized on granulosa cells and the expression of LH receptor mRNA localized on thecal cells [1]. It appears that both gonadotropins are necessary for the continued survival of growing antral follicles, however, the cellular mechanisms underlying the insurance for the survival of early antral follicles remains to be determined [7].

Further follicular development occurs through the selection of the antral follicles. As the selected follicle(s) continues to grow, the antrum enlarges and the oocyte completes the first meiotic division, now known as the secondary oocyte [6,8]. The selected, antral, follicle(s) is associated with increased expressions of gonadotropin receptor mRNA [1]. As the follicle becomes dominant, expressions of the gonadotropin receptors are even higher [1]. Thecal cells are stimulated by LH to convert cholesterol into androstenedione (AD) and testosterone (T) by cytochrome P450 side chain cleavage

(scc) oxidases and 3-beta hydroxy-steroid dehydrogenase (3 beta-HSD) [6]. The granulosa cells are stimulated by FSH to convert AD and T into estrone and estradiol (E2), respectively, by aromatase [6]. The number of follicles that become dominant is species-specific and some of these will continue on to ovulate. Follicular dominance is characterized by rapid development in an environment where growth and development of other follicles is inhibited [1,10]. Those follicles that do not ovulate undergo a process known as atresia, which may occur at any stage of follicular development [5,7,11]. Atresia occurs via apoptosis, or programmed cell death. Of all the ovarian follicles that a female possesses, 99.9% will undergo atresia [10,11].

The Corpus Luteum

Following ovulation, the ruptured follicle collapses and fills with a blood clot while it begins to form the corpus luteum (CL) [8]. The mature CL consists of at least three cell types: large luteal, small luteal and endothelial cells. The steroidogenic cells are the large luteal cells, which appear to be of granulosa cell origin and the small luteal cells, which appear to be of thecal cell origin [12]. The CL acts as an endocrine gland and secretes progesterone (P4). Secretion of P4 by the CL is essential for the maintenance of pregnancy. When normal pregnancy occurs, the CL continues to secrete P4 throughout pregnancy. If pregnancy does not occur, the CL undergoes luteolysis, P4 secretion declines, and consequently, the next ovarian cycle occurs [13].

Luteolysis is brought about by the direct actions of prostaglandin F2 alpha (PGF2 alpha) on the CL. A combination of PGF2 alpha-induced changes results in cessation of luteal P4 production and involution of luteal tissues with luteal cell death [14,15,16]. The

luteolytic actions of PGF2 alpha are mediated by specific plasma membrane receptors, which belong to the seven transmembrane G-protein-coupled receptor superfamily [17]. Interestingly, in those species in which PGF2 alpha is luteolytic, the early CL has been shown to be resistant to the luteolytic actions of PGF2 alpha. Insensitivity to PGF2 alpha has been observed in cows, in marmoset monkeys, in pigs and in rats [18]. The mechanisms responsible for this insensitivity of the early CL to the luteolytic actions of PGF2 alpha are poorly understood, however, it is not due to the lack of high affinity receptors for PGF2 alpha [18]. Indeed, recently, it has become clear that PGF2 alpha can regulate cellular and molecular processes, such as ascorbic acid depletion and inhibition of mRNA for steroidogenic enzymes and prostaglandin receptors in the early and mid-luteal phase CL [18]. Luteal regression might involve endocrine as well as paracrine/autocrine actions of PGF2 alpha [14,16]. It has been suggested that ET-1 may play a role as a mediator of PGF2 alpha-induced luteal regression [4,13,20].

The Endothelin System

Endothelin-1 is found in various mammalian tissues and serves many roles. Endothelin-1 is a member of the endothelin system, which is composed of the endothelin peptide family, the endothelin-converting enzymes and the endothelin receptors. Each component of the endothelin system is essential in the diverse and important physiological roles of ET-1.

The endothelin peptide family includes the endothelins and the sarafotoxins. Endothelin-1 was discovered by Yanagisawa et al. [20] in porcine endothelial cells as a 21 amino acid potent vasoconstrictor peptide. Since this discovery, two additional

Figure 1. Amino acid sequences of peptides from the endothelin family. Amino acids shaded in yellow are different from those in the endothelin-1 sequence.

isopeptides of endothelin have been sequenced and cloned. These three distinct isoforms, ET-1, ET-2 and ET-3 are encoded by three separate genes, and have an overall 70% sequence homology [21]. The endothelins and the sarafotoxins contain two disulfide bonds, between positions 1 and 15, and between 3 and 11 (Figure 1). The sarafotoxins are venoms found in the burrowing asp snake, *Attractaspis engaddensis*, which are structurally very similar to the endothelins. The four sarafotoxins that have been identified possess high sequence homology to the endothelins' carboxyl termini, allowing them to compete for the endothelin receptor [22].

Endothelins are synthesized initially as a precursor peptide, preproendothelin (ppET), consisting of about 200 amino acids. The ppET is first cleaved by signal peptide peptidases and then, cleaved proteolytically by an endopeptidase specific for a pair of dibasic amino acids [23]. This results in the intermediate, biologically-inactive form, big endothelin [23]. Big endothelin is then cleaved at the Trp21-Val22 bond (in ET-3, it is the Trp21-Ile22 bond) by an endothelin-converting enzyme (ECE) to produce the biologically-active endothelin [20,23,24]. ECE is the key enzyme in converting the biologically-inactive big endothelin to the active endothelin peptide.

Two ECE genes have been cloned: ECE-1 and ECE-2, and both belong to a family of zinc metalloendopeptidases. Their sequences are approximately 59% identical [25]. Endothelin-converting enzyme-1 is an integral membrane protein with a short N-terminal cytoplasmic tail, a transmembrane hydrophobic domain that represents the uncleaved signal peptide, and a large putative extracellular domain containing the catalytic site and the motif HEXXH, typical of many zinc peptidases [24]. The molecular mass of ECE-1 is estimated to be 130 kD under reducing conditions and 300 kD under nonreducing

conditions, suggesting that ECE-1 is composed of two disulfide-linked subunits [26]. Endothelin-converting enzyme-1 is localized on cell surfaces and intracellular vesicles [22].

The localization of ECE-1 within the cell and on the cell surface led investigators to explore the existence of several ECE-1 isoforms. Indeed, the ECE-1 N-terminal domain showed variability and was responsible for the existence of different isoforms in humans, ECE-1a, ECE-1b, ECE-1c, and ECE-1d. These isoforms are derived from distinct promoters of the ECE-1 gene [27,28,29]. ECE-1a is strongly expressed at the plasma membrane, ECE-1b is almost exclusively intracellular. ECE-1c is moderately expressed at the cell surface. ECE-1d is more strongly expressed at the cell surface than ECE-1c, but less than ECE-1a, and it is present also in endosomal structures [29].

ECE-2 is an integral membrane protein, however, its C-terminal catalytic domain faces the lumen of secretory vesicles and functions at a low pH [25]. Because of this characteristic, the conversion of big endothelin to the active endothelin by ECE-2 may occur within cellular compartments in which the optimal pH is 5.5-5.7. It is suggested that this conversion may occur in the trans-Golgi network [25]. ECE-2 may function in these acidified compartments during the secretory pathway of endothelin [25].

The endothelins act on at least two distinct endothelin receptor subtypes of the seven transmembrane G-protein-coupled receptors, endothelin type A (ETA) and endothelin type B (ETB). A separate gene encodes each receptor type. Although both receptor types possess high homology in their seven transmembrane domains, they differ in the extracellular amino termini and in the intracytoplasmic carboxy termini [30]. ET-1

and ET-2 bind to ETA with higher affinity than ET-3 [31]. All three isopeptides bind to ETB with equal affinity [31].

The Endothelin System of Ovarian Follicles

Expressions of the endothelin system components have been identified in the ovarian follicles of several mammalian species, including human being, rat, and pig. In human ovaries, ET-1 mRNA and protein were detected in the granulosa cells of tertiary follicles [5]. In this same study, follicles of women who were undergoing gonadotropin treatment had high concentrations of ET-1-LI (like immunoreactivity) in the follicular fluids [5]. From these two observations, it was suggested that gonadotropins might modulate the production of ET-1 of the granulosa cells in the later stages of follicular development [5]. In another study involving women undergoing follicular aspiration for in vitro fertilization, ET-1-LI was found in the follicular fluid [32]. Small antral follicles (<15 mm) contained higher concentrations of ET-1-LI in the follicular fluids than in medium (15-25 mm) or large (≥ 25 mm) follicles [32]. Interestingly, a positive correlation was observed between ET-1-LI, FSH and insulin-like growth factor-II (IGF-II) [32]. The role of IGF-II in follicular development is unknown, however, it is suggested that it may be a general stimulator in the proliferation and differentiation of ovarian granulosa cells [32]. These observations led investigators to suggest that ET-1 may play a role in follicular development [32]. Yoshioka et al. [33] reported that in an immunohistochemical study of human ovaries, the preovulatory follicles expressed ECE-1 with moderate intensity on the internal thecal cells and weakly on the granulosa cells. However, Korth et al. [34] found that in the human ovary, ECE-1 was highly distributed

in the follicular granulosa and theca, while the surrounding ovarian stromal cells contained low to moderate ECE-1. ETA and ETB receptor mRNAs were detected only in the vasculature of human ovaries by *in situ* hybridization experiments [35].

In the rat ovary, ET-1 expression was found in cultured granulosa cells of small and medium follicles [30]. Follicular fluids in superovulated rats contained ET-1-LI, suggesting that ET-1 may play a role in follicular development [30]. Also, ETA and ETB gene expression was detected in rat ovaries. However, *in situ* hybridization assays revealed that ETB was the dominant receptor in the granulosa cells of developing follicles [30].

Immunocytochemistry and *in situ* hybridization assays detected a greater likelihood of ET-1 mRNA in the granulosa cell compartment of large antral follicles than in small antral follicles of porcine ovaries [3]. In this same study [3], ET-1 mRNA was expressed intensely in the vasculature. Evidence based on studies of cultured porcine granulosa cells suggests that ET-1 may be modulating steroidogenesis [9, 36]. ET-1 binding studies indicated that ETA receptors were detected in the granulosa cells of large antral follicles [36]. All of these observations in various mammalian ovaries have led authors to suggest that ET-1 has a potential autocrine/paracrine role in follicular development.

The Endothelin System of the Corpus Luteum

In the mammalian corpora lutea, authors who have identified components of the endothelin system have suggested that ET-1 may play a role in luteolysis. In the human CL, ET-1 mRNA and protein were detected [37]. The expression of ECE-1 was detected

on large and small luteal cells of the menstrual and pregnant CL of human ovaries [33]. However, in another study, ECE-1 mRNA signal was observed in the luteinized granulosa cells and not in the luteinized thecal cells of the human CL [35]. Western blotting revealed that ECE-1 protein was expressed strongly in the early and mid-luteal phase than in the late luteal phase of the human CL [33]. ETA and ETB receptor genes were both detected in human luteal cells, however, ETA appeared to be the prominent receptor type [34].

Studies in the bovine CL have indicated the presence of the endothelin system. ET-1 mRNA and protein were detected in bovine CL in the early, mid-luteal and late phases of the estrous cycle [19]. ECE-1 expression in endothelial and steroidogenic cells of the bovine CL was demonstrated by Meidan et al. [38]. Also, ETA receptor mRNA expression was found in the large and small luteal cells of the bovine CL [39].

There is recent evidence in the cow that ET-1 plays an essential role during PGF2 alpha-induced luteal regression [4]. ET-1 mRNA and peptide were elevated within 2 hours after in vivo or in vitro treatment with PGF2 alpha [4,19]. High concentrations of ET-1 inhibit P4 production by luteal cells [4]. The inhibitory effects of ET-1 have been shown to be exerted via the selective ETA receptors [19]. Indeed, administration of PGF2 alpha during the mid-luteal phase was able to induce both ET-1 and the ETA receptors [13]. In contrast, during the early luteal phase, administration of PGF2 alpha did not alter the expression of ET-1 and its type A receptor [13]. These observations have led Levy et al. [13] to suggest that the lack of ET-1 synthesis and response at the early luteal phase may render the early CL insensitive to PGF2 alpha.

Thesis Objective

One purpose of understanding the processes of follicular development and luteolysis is to further advance the technologies of biological reproduction in humans and in domestic animals. Our laboratory is interested in understanding the physiological role of ET-1 in these two ovarian processes. Thus, each aspect of the endothelin system requires examination. In the porcine ovary, granulosa cells appear to be the site of ET-1 synthesis and ET receptors [3,36]. However, the ovarian source of ECE-1 has not been investigated. One objective of these studies was to determine the follicular compartment associated with ECE-1 expression. The localization of ECE-1 mRNA was investigated by *in situ* hybridization of the porcine ovary. The second objective was to examine the effects of administration of PGF2 alpha during the early and mid-luteal phases on the mRNA expression encoding ECE-1, ETA receptors, ppET-1, and the amounts of ETA and ECE-1 protein in the bovine CL.

CHAPTER II
MATERIALS AND METHODS

Animals and Tissue Collection

(I) Follicular Development Study

Porcine ovaries were collected as previously described [3]. Briefly, prepubertal pigs (65-95 kg body weight) were housed in confinement with ad libitum access to food and water. Sequential administration of eCG and hCG were used to stimulate follicular growth and to induce ovulation. Gilts received an intramuscular (i.m.) injection of 1000 IU eCG (Sigma chemical Company, St. Louis, MO) followed by an i.m. injection of 750 IU hCG (Sigma). Ovulation occurred 36 h after hCG administration, and ovaries were then collected to obtain follicles at maximum development or after 48 h to observe follicles after ovulation.

Gilts were ovariectomized under Tilemine and Zolazepan anesthesia (Telazol, A.H. Robins, Richmond, VA). After tracheal intubation, the gilts were ventilated with Halothane during ovariectomy. Both ovaries were removed and cut into 1.5 cm cubed pieces. The ovarian fragments were frozen on dry ice and stored at -80°C until sectioning. For sectioning, the frozen tissue was embedded in cryoform (International Equipment Co., Needham, MA), equilibrated at -20°C , and 10-micrometer sections were prepared using a Zeiss microtome cryostat (HM 505 E, Fisher Scientific, Pittsburgh, PA). Frozen sections were thaw-mounted onto gelatin and chrome alum-coated slides. Slides were stored at -80°C until *in situ* hybridization assay.

(II) Luteolysis Study

Cyclic beef cows were treated on Day 4 ($n = 31$) or Day 10 ($n = 30$) with either saline or PGF2 alpha (15 mg Lutalyse; Pharmacia and Upjohn, Inc., Kalamazoo, MI). Ovaries were collected by blunt dissection after ovariectomy via supravaginal incision

under epidural anesthesia (6-9 ml, 2% lidocaine for cows, 450-700 kg) [40]. The ovaries were removed at 2 (n = 11), 10 (n = 23), 24 (n = 15), or 48 h (n = 12) after treatment. The experimental group in which the ovaries were removed at 2 h received a single injection. The experimental group in which the ovaries were removed at 10 h included two sets, one that received a single injection at time zero (n = 11) and another one that received two injections: one at time zero and the second at 8 h. The experimental groups in which the ovaries were collected at 24 h and 48 h received an injection every 8 h. The corpora lutea were dissected immediately out from the collected ovaries, frozen in liquid nitrogen, and stored at -70°C .

Total RNA Isolation and Primer Design

Total RNA was isolated from porcine ovaries with Trizol Reagent according to the manufacturer's instructions (Gibco BRL, Gaithersburg, MD) and DNase I treated. RNA was quantified spectroscopically at 260 nm. The primers for ECE-1 (Table 1) were designed based on known DNA sequences of human (*Homo sapiens*) and bovine (*Bos taurus*) ECE-1. Reverse transcriptase-polymerase chain reaction (RT-PCR, Gibco BRL, One step PCR, Gaithersburg, MD) generated a 500 base pair (bp) product. ET_A primers (Table 1) were designed based on known ETA DNA sequences. The PCR product generated for ETA was 500 bp. It was sequenced (Davis Sequencing, CA) and verified to represent genuine ETA receptor via NCBI BLAST [41]. The primers for ET-1 were those designed by Levy et al [13]. These primers (Table 1) have been verified to amplify genuine ET-1 [13]. We have used their sequence to search the nucleic acid database at NCBI with the software BLAST [41] and corroborated their specificity. Primers for two

constitutively expressed genes, beta-actin (Promega; Madison, WI) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as internal standards (Table 1).

Cloning ECE-1 cDNA Fragment from Porcine Ovary

Total RNA was isolated from porcine ovary as described above. RT-PCR (Gibco BRL One step PCR; Gaithersburg, MD) was utilized to generate the cDNA. The PCR cycling was as follows: initiation step: 42C for 30 min, 94C for 2 min; elongation: 94C for 30 sec, 51C for 30 sec, 72C for 1 min for 40 cycles; termination: 72C 10 min and 4C. The RT-PCR products were run on a 1.5 % agarose gel and viewed. A 500 bp band was cut and extracted from the gel using the protocol from QIAEX II Agarose Gel Extraction kit (Qiagen; Valencia, CA). The extracted band was determined to be pure by running it on a 1.5% agarose gel and observing a single band at 500 bp.

The cDNA fragment was cloned into a bacterial vector using the TOPO Cloning Reaction and One Shot Chemical Transformation Reaction kit (Invitrogen; Carlsbad, CA), according to the manufacturer's instructions. Briefly, three plates were used in this experiment: (1) control, pUC18 without TOPO vector, (2) GAPDH positive control with the TOPO vector, and (3) ECE-1 fragment with TOPO vector. The plates were incubated overnight at 37C. The plates were checked on the following day and all the plates contained colonies. The plate with the ECE-1 fragment grew 46 colonies. PCR was performed with several of the colonies to ensure that the fragment was incorporated into the vector. Briefly, 0.5 microliters of the forward ECE-1 primer and 0.5 microliters of the reverse ECE-1 primer, 25 microliters of the PCR Master mix, a bacterial colony chosen from the plate and 24 microliters of sterile water were added into the mixture. PCR

cycling parameters were as follows: initiation step: 94C for 2 min for denaturing, elongation: 35 cycles for annealing was performed at 94C for 20 sec, 51C for 30 sec, 72C for 1 min, termination: 72C for a 10 min extension and then held at 4C. The expected 500 bp band was viewed in a 2% agarose gel. A band at 500 bp was observed. Plasmids containing the ECE-1 fragment were isolated using the Qiagen plasmid MaxiPrep kit (Valencia, CA) according to the manufacturer's instructions. The isolated plasmids were subjected to spectrophotometry at 260 nm to give the concentration.

Sequencing of ECE-1 cDNA Fragment in the Porcine Ovary

The cDNA fragment was first sequenced in the laboratory [42] and subsequently, sent to Davis Sequencing (Davis, CA) for verification. Briefly, in the lab, the double stranded DNA was denatured using approximately 4 micrograms of plasmid DNA, 1 microliter of 2M NaOH, 2 mM EDTA and incubated at 37C for 10 min. One microliter of 3M NaOAc (pH 4.5-5.5) and 25 microliters of 100% EtOH were added and stored at -80C for 15 min. The mixture was centrifuged at 4C and the supernatant pipetted off. A small pellet was observed and washed with 70% EtOH. The pellet was redissolved in 7 microliters of double distilled water and 2 microliters of Sequenase reaction buffer. The mixture was heated for 2 min at 65C in a water bath and cooled slowly to less than 35C over 30 min. The mixture was centrifuged briefly and chilled on ice. The termination mix was prepared and labeled using alpha 35S. The DNA samples and termination mixtures were loaded onto the sequencing gel. The sequencing gel was dried for 4 h. The dried gel was exposed to film for 30 hours and developed. The sequence of the fragment was determined and verified with Davis Sequencing (Davis, CA).

Preparation of RNA Probes (for in situ hybridization of porcine ovaries)

Restriction sites in the plasmids containing the ECE-1 fragment were mapped so that the plasmid DNA could be linearized by restriction enzymes and utilized as a template. The restriction enzymes, Bam H1 and T7 were utilized. Bam H1 was used to include the T7 promoter, and Not 1 was used to include the Sp6 promoter. These DNA templates were then utilized to make 2 digoxigenin (DIG)-labeled probes, sense (Sp6) and antisense (T7) using the Ambion Maxiscript (Austin, TX) reaction assay. Briefly, 2 microliters of 10X buffer, 1 microliter of ATP, 1 microliter of CTP, 1 microliter of GTP, 0.8 microliters of UTP-DIG, 0.2 microliters of UTP, 2 or 5 microliters of the DNA template, 2 .0 microliters of RNA polymerase, and T7 or Sp6 and DEPC-H₂O were added into the tubes. The mixture was incubated at 37C for 2 h, then at 95C for 5 min, and put on ice. One microliter of DNase I was added and incubated at 37C for 15 min. The products were purified using G-150 chromatography microcolumns (Amersham Pharmacia Biotech, Piscataway, NJ). The transcripts were electrophoresed and visualized on a 1% agarose gel to ensure purity.

In situ Hybridization (porcine ovaries)

In situ hybridization was performed as previously described [3]. Prehybridization step: Frozen sections of porcine ovary were dried and fixed with 4% paraformaldehyde in DEPC-PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, and 0.05% DEPC) for 15 min at 4C. Sections were washed in a series of buffers: twice in DEPC-PBS for 5 min, twice in DEPC-PBS containing 100 mM glycine for 5 min, once in DEPC-PBS containing 0.3% Triton X-100 for 15 min, twice for 5 min in DEPC-PBS and

twice with 0.1 M TEA (Triethanolamine) buffer (pH 8.0) containing 0.25% acetic anhydride. Sections were incubated for 10 min at 37C with prehybridization buffer (40% formamide, 1x denhardt's solution, 4x SSC (3.0 M NaCl, 300 mM sodium citrate, pH 7.2), 1 mg/ml yeast t-RNA, 1 mg/ml denatured and sheared salmon sperm DNA and DEPC-H₂O). The prehybridization buffer was drained and sections were overlaid with hybridization buffer (300 microliters of hybridization buffer was prepared for each probe: 40% formamide, 1x denhardt's solution, 4x SSC, 1 mg/ml yeast t-RNA, 1 mg/ml denatured and sheared salmon sperm DNA and DEPC-H₂O, 10% dextran sulfate, with 22.5 microliters of the appropriate DIG-labeled probe). The control sections did not contain a probe in the hybridization buffer. DIG-labeled sense probes were used to assess background signals when comparing signals generated from DIG-labeled antisense probes. Sections were incubated overnight at 55C in a humid chamber.

Posthybridization: Sections were washed in a series of buffers: 2x SSC (3.0 M NaCl, 300 mM Sodium citrate, pH 7.2) at 37C for 15 min twice, 1x SSC at 37C for 15 min twice, NTE containing 20 micrograms/ml of RNase A at 37C for 30 min, 0.1x SCC at 37C for 30 min twice.

Detection: A detection protocol using sheep anti-DIG-alkaline phosphatase (AP) (Roche) complex was utilized. Sections were washed twice with Buffer 1 (100 mM Tris Base, 150 mM NaCl, pH to 7.5) for 10 min. Sections were incubated for 30 min with 150 microliters of a blocking solution (buffer 1, containing 0.1% Triton X-100 and 2% Normal sheep serum (NSS)). The blocking solution was drained and the sections were incubated with 100 microliters of Buffer 1, containing 0.1% triton X-100, 1% NSS and sheep anti-DIG-AP antibody (1:400)) for 2 h. Sections were washed twice in Buffer 1 for

10 min and then in Buffer 2 (100 mM Tris base, 125 mM NaCl, 50 mM MgCl₂, pH 9.5) for 10 min. Sections were incubated with a substrate solution (1 Fast Red tablet (Roche), 2 ml of 10 mM Tris-HCl, pH 8.2) until the desired signal was achieved, usually overnight at 37°C. A positive signal was characterized by the appearance of a red product at the site of the reaction. Sections were washed twice in double distilled water for 15 min, counterstained with haematoxylin for 2-3 min, washed with double distilled water, mounted with Crystal mount and protected with a coverslip. Once sections were dried, they were viewed under an Olympus AX70 microscope equipped with Nomarski differential interference contrast (DIC) objectives. Images were photographed on Kodacolor 200 ASA film (Kodak, Rochester, NY).

Semi-quantitative Reverse Transcriptase-Polymerase Chain Reaction (bovine CL)

Total RNA was isolated from bovine CL with Trizol Reagent according to the manufacturer's instructions (Gibco BRL, Gaithersburg, MD) and DNase I treated. RNA was quantified spectroscopically at 260 nm.

Semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) assays were performed using the QIAGEN One-Step RT-PCR Kit (Valencia, CA). The parameters for the RT-PCR assays were optimized for semi-quantitative analysis of expression of ECE-1, ETA, ET-1, beta-actin and GAPDH transcripts in the bovine CL. The number of cycles for ECE-1 was tested in the range of 20 to 30 cycles with a total RNA concentration of 600 ng/microliters (Appendix, Figure 1, Panel A).

Total RNA concentration for ECE-1 and beta-actin was tested in the range of 5 to 50 ng/microliters (Appendix, Figure 1, Panels B & C). When optimal parameters were

determined for ECE-1 and beta-actin, the RT-PCR assays were performed using 50C for 30 min for reverse transcription reaction and 95C for 15 min for activation of RT-PCR enzyme. Thirty cycles were carried out as follows: 94C for 30 sec for denaturing, 52C for 1 min for annealing, and 72C for 1 min for extension followed by a 10 min final extension at 72C. The beta-actin housekeeping gene was determined to be beyond the linear range for the optimal total RNA concentration for ETA (data not shown). Therefore, GAPDH was used as the internal standard for ETA. The number of cycles for ETA was tested in the range of 20 to 28 cycles at 600 ng/microliters (Appendix, Figure 2, Panel A). Total RNA concentration for ET_A and GAPDH was tested in the range of 30 to 600 ng/microliters (Appendix, Figure 2, Panels B & C). When optimal parameters were determined, the RT-PCR assays were performed using 50C for 30 min for reverse transcription reaction and 95C for 15 min for activation of RT-PCR enzyme. Twenty-five cycles for GAPDH and 28 cycles for ETA were carried out as follows: 94C for 50 sec for denaturing, 52C for 1 min for annealing and 72C for 1 min for extension followed by a 10 min final extension at 72C. The number of cycles for ET-1 was tested in the range of 20 to 30 cycles at 100 ng/microliters (Appendix, Figure 3). After optimal parameters were determined, the RT-PCR assays were performed using 50C for 30 min for reverse transcription reaction and 95C for 15 min for activation of RT-PCR enzyme. Twenty-six cycles for GAPDH and 30 cycles for ET-1 were carried out as follows: 94C for 50 sec for denaturing, 52C for 1 min for annealing and 72C for 1 min for extension followed by a 10 min final extension at 72C.

All PCR products were electrophoresed on 2% agarose gel, stained with ethidium bromide and viewed using the Fluor-S MultiImager by Bio-Rad Laboratories (Hercules,

CA). Data were collected using the densitometric analysis of the Quantity One Quantitation software package (version 4.0; Bio-Rad Laboratories, Hercules, CA).

Western blotting analyses of ECE-1 and ETA (bovine CL)

Protein samples from saline- and PGF2 alpha-treated Day 4 and Day 10 Bovine CL collected after 24 and 48 h were obtained from the tissues collected as described under Treatments and Tissue Collection. Proteins were isolated from individual bovine corpus luteum using the Trizol reagent (Gibco BRL, Gaithersburg, MD) as specified by the manufacturer. Protein concentration in the samples was determined with the BioRad Protein Assay (BIORAD, Hercules, CA). The optimal protein concentration needed to detect ECE-1, ETA receptor and actin was determined by testing 10, 20, 50 and 100 micrograms of isolated proteins from the CL (Appendix, Figure 4).

Sodium Dodecyl Sulfate-PAGE was carried out as previously described [43]. Briefly, protein samples were loaded onto an 8% polyacrylamide gel. After electrophoresis at 150 V for 1 h, the resolved proteins were transferred to PVDF membranes (Biotechnology Systems, Boston, MA) using a Mini-V 8.10 Blot Module (Gibco BRL, Gaithersburg, MD). The membranes were blocked in 1% BSA, 0.05% Tween-20 in Tris-buffered saline (TBS-T), pH 7.5 for 2 h at room temperature. The membranes were incubated with the primary antibody for 2 h at room temperature and washed three times for 10 min in TBS-T. Subsequently, the membranes were incubated with anti-rabbit (1:5000, Amersham Pharmacia Biotech, Piscataway, NJ) or -mouse (1:30,000, Gibco, Grand Island, N.Y.) horseradish peroxidase-conjugated antibodies for 1 h. After three TBS-T washes, visualization was achieved using an enhanced

chemiluminescence (ECL) detection system (Amersham Pharmacia Biotech, Piscataway, NJ) and Kodak Biomax Light Film (Eastman Kodak Company, Rochester, N.Y.). This technology was selected because of its versatility in allowing the stripping of primary and secondary antibodies from membranes for sequential re-probing of membranes with a variety of antibodies. Stripping of membranes was performed with Restore Western Blot Stripping Buffer (Pierce, Rockport, IL.). Stripping conditions were tested to demonstrate complete removal of antibodies; 1 h at room temperature was found effective for all antibodies used in this study. Sequential re-probing with the same antibody demonstrated that the signal intensity did not decrease up to five strippings of the membranes (data not shown).

Images of the detected proteins were captured using the Fluor-S Multimager by Bio-Rad Laboratories (Hercules, CA). Densitometry of the bands of interest were performed using the quantitation software program, Quantity One (Version 4.0; Bio-Rad Laboratories; Hercules CA). The intensity of the signal corresponding to the ECE-1 and ET_A proteins was standardized by the corresponding intensity of the Actin protein control. Normalization of the data allowed us to estimate, in a semi-quantitative manner, the amount of protein in the sample of interest. Semi-quantitative Western blotting methodology has been described earlier [44]. Three primary antibodies were used in these studies. A mouse anti-Actin monoclonal antibody (Chemichon International Inc, Temecula, CA), used at a dilution of 1:3000. The rabbit anti-endothelin receptor A polyclonal antibody (Chemichon International Inc, Temecula, CA) was used at a dilution of 1:200. And the rabbit polyclonal anti-bovine ECE-1 antibody (a generous gift of Dr. Masashi Yanagisawa from the Howard Hughes Medical Institute, University of Texas

Southwestern Medical Institute) was used at a dilution of 1:2000. The specificity of each primary antibody was confirmed using antibody-specific epitope peptides pre-incubated with the primary antibody before addition to membranes.

Statistical Analysis

Statistical analyses were performed using JMP, a statistical software program for the Apple Macintosh [45]. For the RT-PCR, semi-quantification was based on the ratios of the densitometric analyses of ECE-1 to beta-actin, ETA to GAPDH and ET-1 to GAPDH. Thirty-one samples were used for Day 4 and 30 samples used for Day 10. The average values for each time point were calculated and saline control versus PGF2 alpha treatments were compared. For the Western blot, semi-quantification was based on the ratios of the densitometric analyses of ECE-1 and ETA to Actin. Fifteen samples were used for Day 4 and 12 samples used for Day 10. The average values for each time point were calculated and saline controls versus PGF2 alpha were compared. Data are presented as the mean $\pm$ SEM. Statistical significance was determined using the Student's t-test [45]. Table 2 of the Appendix lists the results of the t-tests, degrees of freedom (df) and p values.

Table 1. Primers list.

Gene		Primers
beta-actin	sense	5'-TCATGAAGTGTGACGTTGACATCCGT-3'
	antisense	5'-CCTAGAAGCATTTGCGGTGCACGATG-3'
ECE-1	sense	5'-GATGGTGATGTTGGCCAGCG-3'
	antisense	5'-GGGAACACAACCAGGCCATC-3'
GAPDH	sense	5'-TGTTCCAGTATGATTCCACCC-3'
	antisense	5'-TCCACCACCCTGTTGCTGTA-3'
ETA	sense	5'-TTCTACTTCTGCATGCCCCTGGT-3'
	antisense	5'-GTCCTTGTGGCTGCTCCTCTCAG-3'
ET-1	sense	5'-TGTCTTCATCAGCAGCTCG-3'
	antisense	5'-GTTTCTCCCTGAAATGTGCC-3'

CHAPTER III

RESULTS

cDNA Fragment Sequence

Figure 2 reveals the sequence of the 500 base pair (bp) porcine cDNA fragment that was isolated in this study. Using NCBI Blast (www.ncbi.nlm.nih.gov/blast) [43], this sequence was found to be 93% identical with the *Bos taurus* mRNA for ECE-1a (Figure 2), 91% identical with the *Homo sapiens* mRNA for ECE-1c (data not shown) and 89% identical with the rat mRNA for ECE-1 (data not shown).

Figure 2.

porcine	1	tggaacacaaccaggccatcatcaagcacctcctcgaaaactccacag-cagtgtgagc	59
bovine	538	tggaacacaaccaagccatcatcaagcacctccttgaaaactccacggccagcgtgagc	597
	60	ga-gcaga-aagaaggcccaggtatacttaccgtgcgtgtatgaacgaaaccaggattga	117
	598	gaggcagagaggaaggcccaggtgtac-taccgagcctgcatgaacgaaaccaggattga	656
	118	ggagctcaaagccaaaccctgatggagctgattgagaagctcgggggctggaacatcac	177
	657	ggagctcaaggccaaaccctgatggagctcattgagaagctcgggggctggaacatcac	716
	178	agggccctgggacaaggacaacttcaggacaccctacaggtggtcacgtcccactaccg	237
	717	ggggccctgggacaaggacaacttcaggacaccctgcaggtggtcacatcccactacca	776
	238	cacctgcccttcttctccgtctacgtcagcgccgactccaagaactccaacagcaacgt	297
	777	cacctcccccttcttctccgtctacgtcagtcgagcactccaagaattccaacagcaacgt	836
	298	gatccaagtggaccagctctggcctgggcttaccctcgagggactattacctgaacaagac	357
	837	gatccaagtggaccagctctggcctgggcttaccctcaagagattattacctgaacaaaac	896

358 cgaaaatgagaaggtgctgacgggatacctgaactacatgggccagctggggaagctact 417
897 cgagaatgagaaggtgctgacgggatacctgaactacatgggccagctggggaagctgct 956
418 gggcggcggggacgaggacaccatccgacccagatgcagcagatcctggactttgagac 477
957 gggaggaggggccgaggacaccatccgacccagatgcagcagatcctggactttgagac 1016

Localization of ECE-1 in the Porcine ovary

Four pigs were used for the *in situ* hybridization assays. Of these 4 pigs, 2 pigs produced positive (i.e., generated a red signal) results. From these two pigs, a total of 24 porcine ovarian sections were used in the assays. Expression of ECE-1 mRNA was detected in the granulosa cells of large (about 10 mm) antral follicles when the antisense probe was used (Figure 3, Panel A). Atretic follicles did not produce a red signal when the antisense probe was used (Figure 3, Panel B). Intense hybridization signal in the granulosa cell layer of a medium (about 3 mm) antral follicle was observed with the antisense probe (Figure 3, Panel C). The sense probe did not generate a red signal, and thus, demonstrated the specificity of the assay (Figure 3, Panel D). ECE-1 mRNA expression was not detected in the theca cells, ovarian stroma, primary and atretic follicles.

Effect of PGF2 alpha on Luteal ECE-1 mRNA

The amounts of luteal mRNA encoding ECE-1, ETA receptor and ET-1 were evaluated in the early and mid-luteal phase of the cycle at 2 and 10 h after a dosage of 15

mg PGF2 alpha. The sensitivity of the early and mid-luteal CL was evaluated further by the response to repeated injections of 15 mg PGF2 alpha every 8 h.

As reported elsewhere [40], the concentration of PGF2 alpha used in this study was effective in reducing plasma concentration of progesterone in the Day 4 and the Day 10 CL. Figure 4, panel A shows a representative photograph of the RT-PCR products obtained. This example was performed with total RNA from the Day 4 CL collected after 48 h from the group receiving multiple injections. Lanes 1-4 represent samples from cows treated with saline. Lanes 5-8 represent samples from cows treated with PGF2 alpha. Figure 4, panel B shows a representative photograph of the RT-PCR products obtained. This example was performed with total RNA from the Day 10 samples. The tissues were collected after 48 hours. Lanes 1-6 represent samples from cows treated with saline. Lanes 7-10 represent animals treated with PGF2 alpha. Figure 4, panel C shows that in the early CL, PGF2 alpha treatment had no effect on the amount of mRNA encoding ECE-1 at any of the time points examined ($P > 0.1$). After 48 h there was a slight decrease in the amount of ECE-1 mRNA, but this difference was not statistically different from its saline control. This finding was seen also at the protein level.

In the mid-luteal phase of the cycle, one or multiple injections of PGF2 alpha had no effect on the amount of mRNA encoding the ECE-1 up to 10 h (Figure 4, Panel C, $P > 0.05$). In contrast, after 24 h, PGF2 alpha had induced a significant ($P = 0.05$) 20% reduction in the amount of ECE-1 mRNA. By 48 h, this PGF2 alpha-induced inhibition was more pronounced, about 60% ($P < 0.01$). This effect of PGF2 alpha also was observed in the amount of ECE-1 protein.

Effect of PGF2 alpha on Luteal ETA mRNA

Figure 5, panel A shows that the amount of mRNA encoding ETA was significantly higher in the Day-10 than in the Day-4 CL ($P < 0.05$). However, administration of PGF2 alpha did not affect the mRNA for ETA at any of the time periods examined at the Day-10 (Figure 5, Panel B) or Day-4 CL (data not shown).

Effect of PGF2 alpha on Luteal ECE-1 protein concentration

In the early CL, PGF2 alpha treatment had no effect on the amount of ECE-1 protein at any of the time points examined ($P > 0.1$, Figure 6, Panel A). In the mid-luteal phase of the cycle, only the 48 h treatment with PGF2 alpha induced a statistically significant 12% reduction in the amount of ECE-1 protein ($P < .01$, Figure 6, Panel A).

Effect of PGF2 alpha on Luteal ETA protein concentration

Administration of PGF2 alpha did not affect the amount of ETA protein at any of the time periods examined in the Day-10 (Figure 6, Panel B) or Day 4 CL. Surprisingly, no statistical differences were observed in the amount of ETA protein present in the Day-4 CL from the amount detected in the Day-10 CL (Figure 6, panel B).

Panels A and B in Figure 7 show representative Western blots corresponding to the 48 h treatment for the Day-10 (panel A) and Day-4 (panel B) CL. Protein bands of 130, 57 and 43 kDa were immunodetected with the antibodies for ECE-1, the ETA receptor and Actin respectively.

Effect of PGF2 alpha on Luteal ET-1 mRNA

Figure 8, panel A shows a representative photograph of the RT-PCR products obtained with ET-1 primers. This example was from total RNA of the Day-4 CL treated with two injections of saline or PGF2 alpha and collected after 10 hours. Lanes 1-6 represent samples from animals treated with saline. Lanes 7-12 represent animals treated with PGF2 alpha. Figure 8, panel B shows a representative photograph of the RT-PCR products obtained with the ET-1 primers in the Day-10 samples. The CL was treated with two injections of saline or PGF2 alpha and collected after 10 hours. Lanes 1-6 represent samples from animals treated with saline. Lanes 7-12 represent animals treated with PGF2 alpha. Figure 8, panel C shows that in the early CL, PGF2 alpha treatment had no effect on the amount of mRNA encoding ET-1 at any of the time points examined ($P > 0.1$). In the mid-luteal phase of the cycle, a single injection of PGF2 alpha had no effect on the amount of mRNA encoding the ET-1 up to 10 h. In the mid-luteal CL collected after 10 h, the two injections of PGF2 alpha at 0 and 8h induced a statistically significant 55% increase ($P < 0.05$) in the amount of mRNA encoding ET-1. By 24 h, the amount of ET-1 mRNA was increased 66%, though this was not statistically significant. After 48 h, the amount of ET-1 mRNA was increased by approximately 54% ($P = 0.059$) from the saline control.

Figure 3. Detection of ECE-1 mRNA by in situ hybridization in sections of porcine follicles. Red color denotes hybridization of the probe to the target mRNA in the tissue. A) Intense hybridization signal in the granulosa cell layer of a large antral follicle when antisense probe was used. B) A red signal was not observed in an atretic follicle using the antisense probe. C) Intense hybridization signal in the granulosa cell layer of a medium antral follicle. D) Sense RNA probe did not generate any hybridization signal in the tissue. The bar in panel A indicates 100 micrometers. (g = granulosa cell layer; t = theca cell layer; se = surface epithelium; os = ovarian stroma; o = oocyte)

Figure 4. Effects of PGF2 alpha on the amount of mRNA encoding ECE-1 during the early and mid-luteal phases of the bovine CL. Ten ng of total RNA, extracted from bovine CL, were subject to RT-PCR. The RT-PCR products were electrophoresed on 2% agarose gel, stained with ethidium bromide and photographed. The marker was a 100 bp marker with the brightest band representing 500 bp. (A) A representative photograph of the RT-PCR products obtained from total RNA from the Day 4 CL collected after 48 h. Lanes 1-4 represent samples from animals treated with saline. Lanes 5-8 represent samples from animals treated with PGF2 alpha. (B) A photograph of the RT-PCR products of the Day 10 samples. The CL was collected at 48 hours. Lanes 1-6 represent samples from animals treated with saline. Lanes 7-10 represent animals treated with PGF2 alpha. (C) The amount of mRNA encoding ECE-1 during Day 4 and Day 10. Data are the mean $\pm$ SEM of the densitometric analyses of ECE-1 relative to beta-actin mRNA. The treatments labeled 10a received a single injection of saline or PGF2 alpha and the treatments labeled 10b received two injections of saline or PGF2 alpha. **P < 0.01 versus saline control.

A

B

Figure 5. The amount of mRNA encoding ETA during the early and mid-luteal phases of bovine CL and the effects of PGF2 alpha administration during the mid-luteal phase. Data are the mean $\pm$ SEM of the densitometric analyses of ETA relative to GAPDH mRNA. (A) The amount of mRNA encoding ETA during the early phase (Day 4) and mid-luteal phase (Day 10) of the bovine CL. * $P < 0.05$ (B) Effects of PGF2 alpha administration on the amount of mRNA encoding ETA during Day 10. The treatments labeled 10 hrs a received a single injection of saline or PGF2 alpha and the treatments labeled 10 hrs b received two injections of saline or PGF2 alpha.

A

B

Figure 6. Summary of the Western blot analyses quantified by scanning densitometry. The effects of saline and PGF2 alpha on the amount of ECE-1 and ETA protein during the early ($n = 15$) and mid-luteal ($n = 12$) phases of the bovine CL at 24 and 48 h are presented. Data are the mean $\pm$ SEM of the densitometric analyses of ECE-1 or ETA relative to Actin protein. (A) ECE-1 protein. ** $P < 0.01$. (B) ETA protein.

Figure 7. Representative Western blots demonstrating the effects of PGF2 alpha on ECE-1 and ETA in bovine CL. Thirty micrograms of protein samples were used in SDS-PAGE. Visualization of bands was achieved using ECL detection system. The intensity of the signal corresponding to the ECE-1 and ETA proteins were standardized by the corresponding intensity of the Actin protein control. The sizes of the proteins are: ECE-1, 130 kDa, ETA, 57 kDa, and Actin 43 kDa. (A) A photograph of the Western blot analysis of ECE-1 and ETA protein samples obtained from the Day 10 CL collected after 48 h. Lanes 1-3 represent samples from animals treated with saline. Lanes 5-8 represent samples treated with PGF2 alpha. (B) A photograph of the Western blot analysis of ECE-1 and ETA protein samples obtained from the Day 4 CL collected after 48 h. Lanes 1-2 represent samples treated with saline. Lanes 4-8 represent samples treated with PGF2 alpha.

Figure 8. Effects of PGF2 alpha on the amount of mRNA encoding ET-1 during the early and mid-luteal phases of the bovine CL. 100 ng/microliter of total RNA, extracted from bovine CL, were subject to RT-PCR. The RT-PCR products were electrophoresed on 2% agarose gel, stained with ethidium bromide and photographed. The marker was a 100 bp marker with the brightest band representing 500 bp. (A) A photograph of the RT-PCR products obtained from total RNA from the Day 4 CL treated with two injections of saline or PGF2 alpha and collected after 10 hours. Lanes 1-6 represent samples from animals treated with saline. Lanes 7-12 represent animals treated with PGF2 alpha. (B) A photograph of the RT-PCR products of the Day 10 samples. The CL was treated with two injections of saline or PGF2 alpha and collected after 10 hours. Lanes 1-6 represent samples from animals treated with saline. Lanes 7-12 represent animals treated with PGF2 alpha. (C) The amount of mRNA encoding ET-1 during Day 4 and Day 10. Data are the mean $\pm$ SEM of the densitometric analyses of ET-1 relative to GAPDH mRNA. The treatments labeled 10a received a single injection of saline or PGF2 alpha and the treatments labeled 10b received two injections of saline or PGF2 alpha. *P < 0.05 versus saline control.

CHAPTER IV
DISCUSSION

Localization of ECE-1 in Porcine Ovarian Follicles

The evidence presented in this study has demonstrated that the granulosa cell is the compartment that expresses ECE-1 mRNA in medium and large porcine antral follicles. ET-1 gene expression was demonstrated in the granulosa cells of large antral follicles [3] and autoradiographic studies indicated that ET receptors resided in the granulosa cell layer of antral follicles [36]. The direct actions of ET-1 on granulosa cells appears to be regulation of steroidogenesis [9,36]. In cultured porcine granulosa cells, ET-1 has been shown to modulate production of P4. Although there is controversy whether ET-1, mediated by FSH, inhibits P4 [36] or increases P4 production [9], it appears that ET-1 may be involved in the regulation of steroidogenesis. Taking these observations altogether, we strongly suggest that ET-1 acts in an autocrine manner.

The *in situ* hybridization clearly demonstrates that ECE-1a isoform mRNA is expressed in the granulosa cells of the medium to large antral follicles of the porcine ovary. This is in contrast with the evidence in the monkey and in the human in which ECE-1 was expressed intensely in the internal thecal layers and weakly in the granulosa cells [33]. Korth, et al. [34] determined that ECE-1 mRNA expression was in the granulosa and thecal layers of the human ovary. However, previous studies [3,9,36] and the evidence presented in this study suggest an autocrine role for ET-1 in developing porcine follicles: big ET-1 synthesized by the granulosa cells of the developing antral follicle [3] would be secreted out of the cell, cleaved at the plasma membrane by ECE-1 of the granulosa cell and then bind to its receptor on the granulosa cells [9]. If ET-1 regulates steroidogenesis [9,36] during the later stages of follicular development, it may be possible that ET-1 may be one of many local ovarian factors that influence a particular

follicle to be selected for further development. It is also possible that ET-1 may be one of several factors that determine whether a follicle becomes atretic. The detection of ET-1 [3], ETA [36] and ECE-1 in the large antral follicles suggests a novel autocrine role of ET-1 as a possible local ovarian factor involved in follicular development. However, the mechanisms for the role of ET-1 in follicular development remain uncertain.

Effects of PGF2 alpha on the Endothelin System of the Bovine CL

This study has demonstrated a novel action of PGF2 alpha in the mid-luteal phase of the bovine CL; it regulates the amount of mRNA encoding ECE-1. This novel luteal action for PGF2 alpha was inhibitory in nature. It was first observed after 24 h in corpora lutea of cows that received multiple injections of PGF2 alpha. Importantly, this novel action of PGF2 alpha was phase of the cycle specific, because it was not observed in the early CL. This finding supports and expands the work by Meidan [38] who reported the expression of ECE in the bovine CL.

In the CL, it was observed that PGF2 alpha up-regulated ET-1 during the mid-luteal phase [13]. However, results of the present study are interpreted to imply that a PGF2 alpha-mediated up-regulation of the ECE-1 gene is not required for PGF2 alpha to up-regulate ET-1 during the mid-luteal phase. In fact, PGF2 alpha, in a phase-specific fashion, down-regulated the expression of the ECE-1 gene in the Day 10 CL. It is unlikely that this phase-specific action of PGF2 alpha on the ECE-1 gene contributes to the complex mechanism responsible for the insensitivity of the early CL to the luteolytic actions of PGF2 alpha. A more likely candidate gene contributing to the insensitivity of the early CL would be the phase-specific action of PGF2 alpha on ppET-1 mRNA

observed in this study and reported by Levy et al. [13]. This later observation reported by Levy et al. [13] and corroborated in the current study, supports the proposed participation of endothelin in mediating the luteolytic actions of PGF2 alpha in the CL.

The mechanism responsible for the resistance observed in the early CL to the luteolytic actions of PGF2 alpha is not fully understood, but several potential components have been implicated. Silva et al. [46] recently demonstrated that luteal prostaglandin dehydrogenase (PGDH) activity in the CL of the ewe is greater on Day-4 than on Day-13 of the estrous cycle. PGDH is responsible for converting PGF2 alpha to its inactive metabolite, and therefore, its higher activity in the early CL could account for lower intraluteal concentrations of PGF2 alpha in the early CL, leading to its resistance. Tsai and Wiltbank [47, 48] have reported that a single injection of PGF2 alpha upregulated mRNA encoding prostaglandin G/H synthase 2 (PGHS-2, COX 2) in the mid- and late-cycle ovine and bovine CL, but was not effective in animals on Day-4 of the estrous cycle. Therefore, these investigators proposed that secretion of PGF2 alpha by the late-cycle CL amplified the luteolytic signal from the uterus in an autocrine/paracrine manner [47]. Because this amplification was not observed in the early CL, this could account for its resistance to the luteolytic actions on PGF2 alpha. In contrast, Sayre et al. [40] found that repeated treatment with PGF2 alpha upregulated PGHS-2 and PGF2 alpha synthase in the Day-4 CL.

Levy et al. [13] have reported another possible cause for the resistance of the early CL. These investigators have reported that administration of PGF2 alpha during the early bovine luteal phase does not alter expression of ET-1 and its type A receptor. In contrast,

ET-1 and its type A receptor mRNA were markedly induced in the mid-luteal phase of the CL, when exposed to a similar dose of PGF2 alpha.

Although we were able to detect a higher amount of mRNA encoding ETA receptors in the Day-10 than in the Day-4 CL, this was not the case at the protein level. Furthermore, we failed to detect any regulatory effect by PGF2 alpha on this gene, at the mRNA and protein level. This observation is in contrast with the report by Levy et al. [13], who found that PGF2 alpha administered during the mid-luteal phase was able to induce both ET-1 and ETA receptors. These investigators reported that during the early luteal phase, administration of PGF2 alpha did not alter the expression of the ETA receptor [13]. In spite of the fact that we examined CL at 2, 10, 24, and 48 h and after multiple challenges with PGF2 alpha we never observed a stimulatory effect on this gene by PGF2 alpha in the Day 4 or Day-10 bovine CL. The reason for this discrepancy is not clear. However, in the study by Levy et al. [13], the dairy cows used for the mid-luteal stage varied from Days 10-17. It is possible that if their mid-luteal samples were closer to Day-17, the luteal tissue could have developed greater PGF2 alpha sensitivity. Our observation would imply that the mRNA encoding ETA receptor is up-regulated from levels observed on Day-4 to those seen on Day-10 by PGF2 alpha-independent mechanisms. However, as mentioned earlier, this was not translated into a greater amount of ETA receptor protein.

It is of interest to stress that a stimulatory effect on the ETA gene by PGF2 alpha was not observed and we did not see any inhibitory effect, even after 48 h with multiple injections. Clearly this is a gene that is regulated very differently than the ECE-1 gene. The results of our studies indicate that if the endothelin system mediates the luteolytic

action of PGF2 alpha, an up-regulation of the endothelin system from the Day-4 to the Day-10 CL does not require an increased expression of the ETA and ECE-1 genes by PGF2 alpha. However, as argued above, it is possible that an increased sensitivity of the CL for PGF2 alpha to up-regulate these components of the endothelin system is reached later than the luteal stages examined here.

If ET-1 participates in mediating the luteolytic actions of PGF2 alpha, the gene expression of the ETA receptor and ECE-1, do not appear to be the limiting factors of the CL endothelin system. Our observation that the amount of mRNA encoding ECE-1 is down-regulated by PGF2 alpha in the Day 10 but not in the Day-4 CL, may be a result of the increased ET-1 by PGF2 alpha at these developmental stages of the CL. A similar finding has been reported in the vascular system of the rat pulmonary endothelial cells, where ET-1 inhibits ECE-1 expression [49].

Clearly, these actions of PGF2 alpha in the mid-luteal phase of the CL can be dissociated from its actions on P4. First, a single injection of PGF2 alpha during the mid-luteal phase of the CL, which effectively reduced plasma concentration of P4, had no effect on the amount of mRNA encoding ECE-1 and ET-1 by 10 hours. Second, multiple injections of PGF2 alpha in the early CL were effective in reducing plasma levels of P4 without any effect on mRNA encoding ECE-1 or ET-1. It is possible that unchanged mRNA concentrations for ECE-1 were enough to maintain the necessary amount of bioactive ET-1. This is an issue that needs to be explored further in future studies. In fact, a reduction in mRNA encoding ECE-1 was detected after 24 h, but a reduction in the amount of ECE-1 protein was not detected until 48 h. It will be necessary to determine the effect of PGF2 alpha on the actual ECE-1 activity. Certainly the amount of ppET-1

mRNA was increased by PGF2 alpha at a time when both ECE-1 mRNA and protein were reduced by the PGF2 alpha treatment. It is likely that this increase in ppET-1 mRNA induced by PGF2 alpha results in an increase in ET-1 peptide [50]. If this is the case, three scenarios can be envisioned. First, it is possible that the observed reduction in ECE-1 mRNA is not to a level at which the ECE-1 protein and activity would be reduced and would become rate limiting in the production of bioactive ET-1. Another possible scenario is that ECE-1 is not the enzyme responsible for generating bioactive ET-1 in the bovine CL. A third alternative scenario is that an ECE-1 isoform different from the one we have examined (ECE-1a) is compensating for the reduced ECE-1 mRNA and protein. In the human vascular system, there is evidence indicating that, by alternative splicing, the ECE-1 gene can generate several ECE-1 isoforms [30]. At this time, these possible interpretations can't be distinguished.

If ET-1 participates in mediating the luteolytic actions of PGF2 alpha, then the results of this study indicates that the ET-1 gene is the component of the endothelin system most likely for regulation by PGF2 alpha. ECE-1 and the ETA receptor appear to be constitutively expressed in the Day-4 and Day-10 CL.

Summary

In summary, ECE-1 mRNA expression is localized within the granulosa cell compartment of porcine antral follicles. This observation supports the idea that ET-1 may be acting in an autocrine manner in ovarian regulation. Further studies in this cellular compartment would need to be conducted in order to examine the precise role of ET-1 as a novel ovarian regulator. In this study, a novel action of PGF2 alpha in the mid-luteal

phase of the bovine CL was found; it regulates the amount mRNA encoding ECE-1. This novel luteal action for PGF2 alpha appeared to be inhibitory in nature. Importantly, this novel action of PGF2 alpha was phase of the cycle specific, because it was not observed in the early CL. It is suggested that if ET-1 participates in mediating the luteolytic actions of PGF2 alpha, then the most likely component of the endothelin system in the bovine CL as a target for PGF2 alpha regulation is the ET-1 gene. Finally, an important practical implication of this observation is that it may be possible to target the ET-1 gene as an effective manner to manipulate the luteolytic actions of PGF2 alpha.

APPENDIX

Detection of ECE-1, beta-Actin, ETA Receptor, ET-1 and GAPDH Transcripts in Bovine CL

The validity of the RT-PCR assays for semi-quantitative evaluation was supported by at least three criteria: first, by selecting, for each target, an RNA concentration yielding amplification in the exponential phase of the RT-PCR (Appendix figures 2, 3 & 4); second, by repetitive observations of results within experimental groups (at least two assays per data point); and third, by using an appropriate standard control.

The RT-PCR assays were optimized for semi-quantitative analysis of expression of ECE-1, beta-actin, ETA, ET-1 and GAPDH transcripts in bovine CL. In the case of ECE-1, (Appendix figure 2, Panel A) plotting the intensity of the RT-PCR signal (as expressed by absolute optical density values) against the number of amplification cycles revealed a linear relationship between cycles 24 and 30 ($r^2 = 0.61$). Appendix figure 2, Panel B, shows the 285 bp and 500 bp RT-PCR products generated with primer pairs 1 and 2 (Table 1). The 500-bp band was sequenced (Figure 6) and shown to be genuine ECE-1. The intensity of the band produced with the primer pair corresponding to beta-actin was stronger than the one produced with the primer pair corresponding to ECE-1. However, the intensity of the bands produced was well correlated with the amount of total RNA tested (in the range of 5 - 50 ng) (Appendix figure 2, Panel C). Based on this finding, in subsequent RT-PCR experiments the total RNA concentration used to evaluate the expression of these two genes was selected at 10 ng. The number of cycles for the RT-PCR was fixed at 30 cycles and under these conditions, the level of expression of both genes was in the linear range.

The amount of gene expression for beta-actin was much greater than that for the ETA receptors (data not shown). The GAPDH gene was found to be a better internal standard than beta-actin when evaluating the amount of mRNA encoding the ETA receptor. As expected, the primer pairs designed for the GAPDH and ETA receptor (primer pairs 3 and 4, Table 1) generated RT-PCR products of 900-bp and 500-bp. These RT-PCR products were sequenced and shown to represent genuine ETA receptor and GAPDH (data not shown). The optimal number of cycles for the ETA receptor was fixed at 28, because a good correlation was found between the signals generated when the cycles were tested from 22 to 28 cycles ($r^2 = 0.60$, Appendix figure 3, Panel A). Because the GAPDH gene was expressed at a higher number of copies than the ETA receptor gene (Appendix figure 3, Panels B & C), the number of cycles for GAPDH was selected at 25 cycles. When optimizing the RT-PCR conditions for the ETA receptor and GAPDH, there was a good correlation (ETA receptor $r^2 = 0.64$, GAPDH $r^2 = 0.61$) between the intensity of the bands produced and the amount of total RNA used in the range of 30 to 600 ng (Appendix figure 3, Panels B and C). The primer pair for ET-1 generated a RT-PCR product of 580 bp. The optimal number of cycles determined for ET-1 was 30 cycles ($r^2 = 0.98$) and GAPDH was 26 cycles ($r^2 = 0.96$, Figure 4). Total RNA concentration was fixed at 100 ng.

Detection of ECE-1, Actin, ETA Receptor Proteins in Bovine CL

Single bands of approximately 130 (ECE-1), 57 (ETA) and 43 (Actin) kDa were detected when the protein samples were loaded at concentrations of 10 micrograms or greater; at lower protein concentration, no bands were detected (data not shown). The

molecular weights of the detected proteins corresponded closely to the published size bands detected for ECE-1, ETA and Actin [25,51] by their respective antibodies. Based on this, we chose 30 micrograms protein samples to evaluate the effect of PGF2 alpha on the amount of these proteins in the samples (Appendix figure 4). The specificity of ETA antibody was confirmed by pre-absorption of the antibody with excess peptide (data not shown). The specificity of the bands detected with the ECE-1 and Actin antibodies have been confirmed similarly elsewhere [25,51].

Table 2. Statistical results.

Treatments		Tissue Collection after Treatment
A	Saline/PGF2 alpha given at 0 h	2 h
B	Saline/PGF2 alpha given at 0 h	10 h
C	Saline/PGF2 alpha given at 0 & 8 h	10 h
D	Saline/PGF2 alpha given every 8 h	24 h
E	Saline/PGF2 alpha given every 8 h	48 h

Day-4 ECE-1 mRNA, Saline vs. PGF2 alpha

Treatment	t-test	df	p value
A	0.3893	4	0.7169
B	0.9242	3	0.4235
C	0.5556	4	0.6081
D	0.1949	6	0.8519
E	2.0972	4	0.1040

Day-10 ECE-1 mRNA, Saline vs. PGF2 alpha

Treatment	t-test	df	p value
A	0.7285	3	0.5190
B	0.6263	4	0.5651
C	0.4105	4	0.7025
D	2.4560	5	0.0575
E	5.3012	4	0.0061**

Day-4 ET-1 mRNA, Saline vs. PGF2 alpha

Treatment	t-test	df	p value
A	0.4191	4	0.6967
B	0.9334	3	0.4195
C	0.1508	4	0.8875
D	0.7991	6	0.4547
E	1.9066	5	0.1149

Table 2. (continued)

Day-10 ET-1 mRNA, Saline vs. PGF2 alpha

Treatment	t-test	df	p value
A	0.8272	3	0.4688
B	0.2595	4	0.8081
C	4.1519	4	0.0142*
D	1.5195	5	0.1890
E	2.4327	5	0.0592

Day-10 ETA mRNA, Saline vs. PGF2 alpha

Treatment	t-test	df	p value
B	0.2378	4	0.8237
C	0.3721	3	0.7345
D	1.3106	4	0.2602

Day-4 ECE-1 protein, Saline vs. PGF2 alpha

Treatment	t-test	df	p value
D	1.8728	6	0.1102
E	0.0789	5	0.9402

Day-10 ECE-1 protein, Saline vs. PGF2 alpha

Treatment	t-test	df	p value
D	0.1044	3	0.9234
E	5.0559	5	0.0039**

Day-4 ETA protein, Saline vs. PGF2 alpha

Treatment	t-test	df	p value
D	1.2406	6	0.2610
E	1.5384	5	0.1846

Table 2. (continued)

Day-10 ETA protein, Saline vs. PGF2 alpha

Treatment	t-test	df	p value
D	0.4178	3	0.7042
E	0.6278	5	0.5577

*, ** indicates statistical significance

Appendix figure 1. Determination of optimal parameters for RT-PCR assays for ECE-1 and beta-actin. A) The number of cycles for ECE-1 was tested in the range of 20 to 30 cycles at 600 ng. There was a linear relationship between cycles 24 and 30 ($r^2 = 0.61$). B) Total RNA concentration (ng/microliter) for ECE-1 and beta-actin was varied to determine optimal concentration. A photograph of the RT-PCR products: lanes 1-2 contain 5 ng/microliter, lanes 3-4 contain 10 ng/microliter, and lanes 5-6 contain 50 ng/microliter (amplification of 30 cycles and 52°C annealing temperature). The RT-PCR products were electrophoresed on 2% agarose gel, stained with ethidium bromide and photographed. ECE-1 is 500 bp and beta-actin is 285 bp. The marker was a 100 bp marker with the brightest band representing 600 bp. C) A graphical representation of the photograph shown in panel B. Optimal concentration was determined to be 10 ng for ECE-1 and beta-actin.

A

B

C

Appendix figure 2. Determination of optimal parameters for RT-PCR assays for ET_A and GAPDH. A) The number of cycles for ETA was tested in the range of 20 to 28 cycles at 600 ng. There was a linear relationship between cycles 22 and 28 ($r^2 = 0.60$). Total RNA concentration (ng/microliter) for ETA (B) and GAPDH (C) was varied (30 ng to 600 ng) to determine optimal concentration. The RT-PCR products were electrophoresed on 2% agarose gel, stained with ethidium bromide and photographed. Total RNA concentrations for ETA and GAPDH were plotted against optical density. Optimal concentrations for ETA and GAPDH was determined to be 600 ng.

Appendix figure 3. Determination of optimal parameters for RT-PCR assays for ET-1 and GAPDH. The number of cycles for ET-1 and GAPDH was tested in the range of 20 to 30 cycles at 100 ng/ml. The optimal number of cycles for ET-1 was 30 cycles ($r^2 = 0.98$) and GAPDH was 26 cycles ($r^2 = 0.96$). Total RNA concentration was fixed at 100 ng.

Appendix figure 4. Determination of optimal total protein concentration in the bovine CL to detect ECE-1 and ET_A. Protein concentration in the samples was determined with the BioRad Protein Assay (BIORAD, Hercules, CA).

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