NOTE TO USERS

DISSERTATION

ACUTE EFFECTS OF ESTRADIOL ON GONADOTROPIN-RELEASING HORMONE-INDUCED SIGNAL CASCADE IN GONADOTROPES.

Submitted by

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In partial fulfillment of the requirements

For the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, CO

Spring 2005

COLORADO STATE UNIVERSITY

December 21, 2004

WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY LOUISA RISPOLI ENTITLED "ACUTE EFFECTS OF ESTRADIOL ON GONADOTROPIN-RELEASING HORMONE-INDUCED SIGNAL CASCADE IN CULTURED GONADOTROPES" BE ACCEPTED AS FUFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

ACUTE EFFECTS OF ESTRADIOL ON GONADOTROPIN-RELEASING HORMONE-INDUCED SIGNAL CASCADE IN GONADOTROPES?

The importance of estrogens in numerous physiological systems, including reproduction, cardiovascular, skeletal and immune systems requires a better understanding of the subcellular mechanisms affected by exongenous and endogenous estrogens. Environmental or nutritional estrogenic endocrine disrupting chemicals are thought to mediate developmental and carcinogenic pathologies. Regarding the reproductive system, estrogens affect many target tissues in the male and female, including the mammary glands, uterus, ovaries, testes, prostate, hypothalamus and anterior pituitary gland. It has been demonstrated that estrogens exert both positive and negative effects on the hypothalamus and the anterior pituitary gland to regulate gonadotropin secretion. Classically, effects of estradiol (E_2) are mediated through the nuclear estrogen receptor (ER), which modulates gene expression. In general, this genomic effect occurs over a period of several hours and involves repression or activation of synthesis of new proteins, i.e. gonadotropin-releasing hormone (GnRH), luteinizing hormone (LH) and follicle-stimulating hormone (FSH).

It has been increasingly appreciated in the past decade that steroid hormones, such as E_2, mediate cell functions not only through stimulation or inhibition of transcription, but also via non-genomic mechanisms. The genomic effects of estrogens and other steroid hormones are now relatively well understood, yet we lack understanding of the non-genomic mechanisms. In the present study we replicated previous work on cultured ovine pituitary cells and observed that E_2 decreased GnRH-induced secretion of LH ($P < 0.05$). To evaluate the effects of E_2 on the subcellular mechanisms controlling release of LH, we turned to the homogenous gonadotrope cell line, LβT2. Unlike cultured ovine pituitary cells, treatment of LβT2 cells with E_2 failed to inhibit GnRH-induced release of LH. Interestingly, LβT2 cells express very low levels of estrogen receptor-α (ERα) in contrast to observations on normal anterior pituitary cells. Based on these data, we postulated that increasing expression of ERα in LβT2 cells would restore the negative non-genomic effects of E_2 on GnRH-induced release of LH observed previously in ovine pituitary cells.

LβT2 cells were stably transfected with murine ERα (LβT2-ER). Treatment of these cells with E_2 resulted in decreased release of LH in response to GnRH ($P < 0.01$). Furthermore, membrane impermeable estrogen, E_2 conjugated to bovine serum albumin (E-BSA), inhibited GnRH-induced secretion of LH in this cell line. We concluded that the non-genomic effects of E_2 were likely due to a membrane-associated ERα and were not the result of a non-specific steroid effect. This conclusion is based on the fact that E_2 had no effect on release of LH in the parent LβT2 cells yet decreased GnRH-induced secretion of LH in LβT2-ER cells and the effect could be mimicked using membrane impermeable E-BSA. This was further corroborated by showing that PPT, an ERα

specific agonist, but not DPN, an estrogen receptor-β specific agonist, inhibited GnRH-mediated release of LH.

We investigated the effects of other steroid hormones in an effort to delineate further the non-genomic mechanisms affecting LH secretion. Non-genomic effects for progesterone, cortisol and testosterone have been demonstrated in other tissues. Acute effects of 17α-estradiol depends on the tissue type. In this study using LβT2-ER cells, we demonstrated negative effects of 17α-estradiol, progesterone, testosterone and cortisol on GnRH-induced secretion of LH. Additional studies are required to fully characterize these effects.

Numerous investigators have demonstrated non-genomic effects of E_2 on subcellular mechanisms. Currently, the data on the acute effects of estradiol on GnRH-stimulated signal transduction is limited. In the present study, both LβT2 and LβT2-ER cells responded to GnRH with increased accumulation of IP_3, and E_2 had no effect on the GnRH-stimulated formation of IP_3 in either cell line. We observed a GnRH-evoked spike in intracellular calcium ($Ca^{2+}{}_i$) in both LβT2 and LβT2-ER cells without the characteristic large-amplitude $Ca^{2+}{}_i$ oscillations observed in normal gonadotropes. Interestingly, LβT2 cells seem to have retarded re-uptake of $Ca^{2+}{}_i$ with the inclusion of E_2 in the GnRH treatment. In contrast, in LβT2-ER cells there is a reduction of the increase in intracellular calcium when E_2 is included with the GnRH treatment compared to cells treated only with GnRH. These initial experiments cannot distinguish the Ca^{2+} source, i.e. intracellular or extracellular, responsible for the alteration in $Ca^{2+}{}_i$ levels. Since the effects of E_2 on GnRH-induced increase in $Ca^{2+}{}_i$ are immediate, it is presumed that the effects are mediated non-genomically.

In summary, these results 1) clearly demonstrate the utility of LβT2-ER cells for examination of the non-genomic effects of E_2 on GnRH-evoked secretion of LH; 2) reveal that E_2 has a negative effect on the GnRH-induced increase in $Ca^{2+}{}_i$; and 3) establish that manifestation of the negative effect of E_2 on LH secretion depends upon expression of ERα. Based on the results obtained in the present study, we have concluded that the new cell line, LβT2-ER, exhibits characteristics consistent with those of normal gonadotropes, such as rapid inhibition of GnRH-induced secretion of LH. In the future, it will be possible to use the LβT2-ER cell line not only as a representation of normal gonadotropes to evaluate cellular and molecular events acutely affected by steroid hormones, but also as a model to evaluate both the physiological and pathological non-genomic effects of estrogens.

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ACKNOWLEDGMENTS

Many people donated time and energy to helping me complete my Ph. D. First and foremost, I need to thank my advisor, Dr. Terry Nett for his immeasurable patience, and guidance in assisting me in my pursuit of a Ph.D. I also thank my fellow graduate students, Ryan Ashley, Dr. W Yang, and Dr. Alejandro Arreguin-Arevalo, who were always available to help with experiments and to keep things in perspective. Dr. Tracy Davis assisted me in the end by becoming my friend and being so understanding of how stressful it is at the end of a Ph.D.

Dr. Gordon Niswender and Dr. Colin Clay helped tremendously with answers when I had questions, and the many members of their laboratories (Dr. Jean Escudero, Teresa Slough, Randy Bogan, Dr. Ken Escudero, Dr. Jodi Haller, Jeremy Cantlon, Amy Navratil, Brian Cherrington, Dr. Clay Lents, and Dr. Todd Farmerie) were always available with time, materials and criticism when it was needed. Dr. Heywood Sawyer and Dr. James Bamburg not only kindly agreed to be on my Ph.D. committee but also assisted in the design and the understanding of my research.

I would not have been able to collect the pituitaries that I needed without the liberal assistance of Mike Gallegos and Rick Brandes. Matt Allen's gentle touch with dissociation of pituitary glands helped me have sufficient numbers of cells for my studies. One of the most important pieces of my dissertation would not exist without the generous donation of time, equipment and assistance from Dr. Barbara Sanborn, Dr. Jean Ku and

JP Liao. I owe them a lot for the effort that they put forth to help me get that piece of essential intracellular calcium data. This research would not have been possible without the support of the Colorado Agricultural Experiment Station (COL0020) and NIH training grant (T32HD007031-26).

Last but never least, this is dedicated to my late grandmother and my husband. To the memory of Bonnie West, without your strength of spirit and will as example for me I would not have embarked on this path, nor been able to travel so far along it. I only wish you were here to see that I did reach the end. To Damon Rispoli, I would not have been as happy or as sane as I am today without your love, your support and your faith in my abilities. Sorry for all those long days and nights plus the sheep smell.

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LIST OF ABBREVIATIONS

β-gal:	**β-galactosidase**
[D-Ala^6]GnRH:	**D-Ala^6-desGly^{10}-GnRH-Pro^9-ethylamide**
ANOVA:	**Analysis of Variance**
Ca^{2+}:	**Calcium**
Ca^{2+}_i:	**Intracellular Calcium**
CHOK1:	**Chinese Hamster Ovary K1 cell line**
cs-FBS:	**Charcoal Stripped Fetal Bovine Serum**
DAG:	**Diacyglycerol**
DMEM:	**Dulbecco's Modified Eagle Medium**
DPN:	**Diarylpropionitrile**
DTT:	**Dithiothreitol**
E_2:	**Estradiol**
E-BSA:	**Estradiol-6-(O-carboxymethyl)oxime:Bovine Serum Albumin**
ERα:	**Estrogen Receptor-α**
ERβ:	**Estrogen Receptor-β**
ER:	**Estrogen Receptor**
ERE:	**Estrogen Response Element**
FBS:	**Fetal Bovine Serum**

FSH:	**Follicle Stimulating Hormone**
GAPDH:	**Glyceraldehyde-3-phosphate Dehydrogenase**
GLM:	**General Linear Model**
GnRH:	**Gonadotropin-Releasing Hormone**
GnRH-R:	**Gonadotropin-Releasing Hormone Receptor**
HBS:	**HEPES Buffered Saline**
HEPES:	**n-2-Hydroxyethyl Piperazine Ethane Sulfonic Acid**
HPD:	**Hypothalamic-Pituitary Disconnection**
HRP:	**Horse Radish Peroxidase**
IP_3:	**Inositol-1,4,5-triphosphate**
LH:	**Luteinizing Hormone**
LSD:	**Least Significant Differences**
luc:	**Luciferase**
OVX:	**Ovariectomized**
PBS:	**Phosphate Buffered Saline**
PG:	**Prostaglandin $F_{2\alpha}$**
$PGF_{2\alpha}$:	**Prostaglandin $F_{2\alpha}$**
PIP_2:	**Phosphatidylinositol-4,5-bisphosphate**
PKC:	**Protein Kinase C**
PLCβ:	**Phospholipase Cβ**
PPT:	**Propyl pyrazoletriol**
PRL:	**Prolactin**
RIA:	**Radioimmunoassay**

SDS-PAGE:	**Sodium Dodecylsulfate-PolyAcrylamide Gel Electrophoresis**
SEM:	**Standard Error of the Mean**
TCA:	**Trichloroacetic Acid**

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CHAPTER I: REVIEW OF LITERATURE

A. Introduction

Due to the importance of estrogens in numerous physiological actions, including reproduction, continuance of cardiovascular health, bone maintenance and immune activity, a better understanding of the subcellular mechanisms affected by exongenous and endogenous estrogens is required. Regarding the reproductive system, estrogens affect target tissues in the male and female, including the mammary glands, uterus, ovaries, testes, prostate, hypothalamus and anterior pituitary gland. Estrogens exert both positive and negative effects on the hypothalamus and the anterior pituitary gland to regulate gonadotropin secretion. Classically, effects of estradiol (E_2) are thought to be mediated through the nuclear estrogen receptor (ER), which modulates gene expression. In general, this genomic effect occurs over a period of several hours and involves repression or activation of synthesis of new proteins, i.e. gonadotropin-releasing hormone (GnRH), luteinizing hormone (LH) and follicle-stimulating hormone (FSH). It has been increasingly appreciated in the past decade that steroid hormones, such as E_2, mediate cell functions not only through alteration of a cell's genomic machinery, but also through stimulation of non-genomic mechanisms (Losel and Wehling, 2003).

Pietras and Szego (1977) identified a plasma membrane ER in isolated endometrial cells that was shown to respond rapidly to E_2. Numerous functional studies

of the non-genomic effects of E_2 have increased support for the existence of a membrane ER. In a recent *in vivo* study, administering either E_2 or E_2 conjugated to bovine serum albumin (E-BSA) to ovariectomized (OVX) ewes resulted in a rapid suppression of GnRH-evoked LH release (Arreguin and Nett, 2003). This negative effect of E_2 on secretion of LH appears to be mediated via a non-genomic mechanism, presumably involving membrane ER due to the fact that E-BSA can not cross the plasma membrane. The mechanisms by which E_2 exerts its non-genomic actions are not fully understood, including those resulting in the rapid decrease in secretion of LH. In light of the numerous exogenous sources of estrogenic compounds (i.e. phytoestrogens and environmental estrogens), this understanding may prove more essential than previously thought. The purpose of this chapter is to review the information on gonadotropin secretion in females, with emphasis on the influence of GnRH and estradiol on gonadotropes in the anterior pituitary gland of the ewe.

B. GnRH and its Cognate Receptor

The interaction of GnRH with its cognate receptor plays a fundamental role as the physiologic regulator of reproduction by stimulating the synthesis and secretion of pituitary gonadotropins. GnRH is considered the primary regulator of ovulation and steroidogenesis in females (Desjardins, 1981; Gharib, et al., 1990). Originally, GnRH, a small decapeptide ($pGlu^1$-His-Trp-Ser-Tyr-Gly-leu-Arg-Pro-Gly^{10}-NH_2), was isolated from ovine and porcine brains (Amoss, et al., 1971; Matsuo, et al., 1971). Andrew Schally and Roger Guilleman shared the Nobel Prize for the discovery of the structure and synthesis of GnRH. The structure of GnRH is conserved in all mammals.

Synthesized in the neurosecretory cells in the preoptic area of the hypothalamus, GnRH travels through the hypophyseal portal vasculature to the anterior pituitary gland where it binds to its cognate receptor. GnRH stimulates the synthesis and secretion of LH and, to a lesser extent, FSH from gonadotrope cells of the anterior pituitary gland. Studies of this short-lived hormone (half-life of approximately 2-4 min) have revealed the relationship between structure and biological activity. Simple substitutions or deletions of the histidine in position 2 and the tryptophan in position 3 result in decreased or abolished hormone releasing activity (Boepple, et al., 1986; Karten and Rivier, 1986; Schally, et al., 1976). The amino acids at positions 1, and 4 through 10 are necessary for GnRH binding to receptor (Boepple, et al., 1986; Karten and Rivier, 1986; Schally, et al., 1976). Conservative changes in amino acid composition have resulted in analogs valuable in treatment of a variety of endocrinological disorders and reproductive cancers (Conn and Crowley, 1991; Cook and Sheridan, 2000; Patterson, et al., 2002; Rapkin, 2003; Saltiel and Garabedian-Ruffalo, 1991; Schally, et al., 1990). Analogs synthesized with various D-amino acids in the sixth position results in agonists with 15-200 times the potency of native GnRH (Boepple, et al., 1986; Coy, et al., 1976; Fujino, et al., 1974; Karten and Rivier, 1986). The substitution with a hydrophobic D-amino acid at position 6 also increases the half-life of the molecule (Coy, et al., 1976).

Although a second variant of GnRH has been isolated from the chicken (GnRH-II; His^5, Trp^7, Tyr^8) and found to be present in most species from jawed fish to humans, little information exists to date on the role of GnRH-II in regulating gonadotropin secretion (reviewed by Pawson, et al., 2003; Somoza, et al., 2002). Recent investigations have observed binding of GnRH-II to GnRH-receptor although the biological

significance of these reports is yet to be established (Caunt, et al., 2004; Densmore and Urbanski, 2003). In fact, 16 variants of GnRH have been isolated from various vertebrate species (Somoza, et al., 2002). This review exclusively focuses on function of GnRH-I stimulation of its cognate receptor in mammals.

In most mammals, reproductive function is controlled by GnRH-receptor (GnRH-R) expressed on the membranes of gonadotropes (Childs and Unabia, 1997; Clayton and Catt, 1981). Rodents are an exception with GnRH receptors found in the gonads as well as the gonadotropes (Kaiser, et al., 1992b). However, other tissues bind radioiodinated GnRH agonist suggestive of GnRH-R gene expression. These include specific nuclei in the central nervous system (Jennes, et al., 1997), human placenta (Wolfahrt, et al., 1998), somatotropes (Liebow et al., 1991) and various tumors of the pituitary and pancreas (Emons, et al., 1998). In addition, many sex-steroid hormone dependent tumors (i.e. breast, endometrial, ovarian and prostate) are GnRH-R positive (Limonta, et al., 2001). For the purpose of this review, only GnRH-R expressed on gonadotropes will be discussed.

Identification of the structure of the GnRH-R has furthered our understanding of the physiological requirements for GnRH-induced LH secretion. Based on information concerning second messenger systems activated by GnRH, the GnRH-R was predicted to be a member of the rhodopsin-like G-protein-coupled receptor (GPCR) superfamily containing seven transmembrane domains (TMDs) and an extracellular amino-terminus. Since the TMDs of GPCRs are highly conserved, initial cloning of the mammalian GnRH-R relied on homology of the TMDs of GPCRs. Using this strategy, Tsutsumi and coworkers (1992) were the first to report the cDNA sequence of the murine GnRH-R

present in the gonadotrope-derived αT3-1 tumor cell line. Subsequent cloning has identified the GnRH-R sequence in other mammalian species, including human (Chi, et al., 1993; Kakar, et al., 1992), ovine (Brooks, et al., 1993; Campion, et al., 1996; Illing, et al., 1993), bovine (Kakar, et al., 1993), porcine (Weesner and Matteri, 1994), rat (Eidne, et al., 1992; Kaiser, et al., 1992b; Perrin, et al., 1993), guinea pig (Fujii, et al., 2004) and equine (accession #: AF018072). The amino acid sequence is more than 83% conserved among these eight species. Based on the cDNA sequence, the putative topology of the amino acid sequence of the human GnRH-R is presented in Figure 1. In contrast to most members of the GPCR superfamily, mammalian GnRH-R lacks the characteristic intracellular carboxyl-terminal domain (Eidne, et al., 1992; Tensen, et al., 1997; Troskie, et al., 1998). Further, the GnRH-R has a very short extracellular amino terminus of only 35 residues. Because of these two characteristics, this is one of the smallest GPCRs; rodent GnRH-R consists of 327 amino acids while other mammalian GnRH-R contain an additional amino acid in the second extracellular loop (reviewed by Sealfon and Millar, 1995; reviewed by Sealfon, et al., 1997).

Members of the rhodopsin-like GPCRs have a high degree of conservation suggestive of similar structural framework and mechanism of activation. Ligands and agonists interact with the extracellular face of the receptor, inducing a conformational change of receptor into its active state. This active confirmation propagates signal transduction via associated G-proteins (reviewed by Flanagan, et al., 1997; Gundermann, et al., 1997). Although knowledge of the nature of the GnRH ligand-receptor-G protein relationship remains rudimentary, some specific sites on the GnRH-R for interaction with ligand and G protein have been defined.

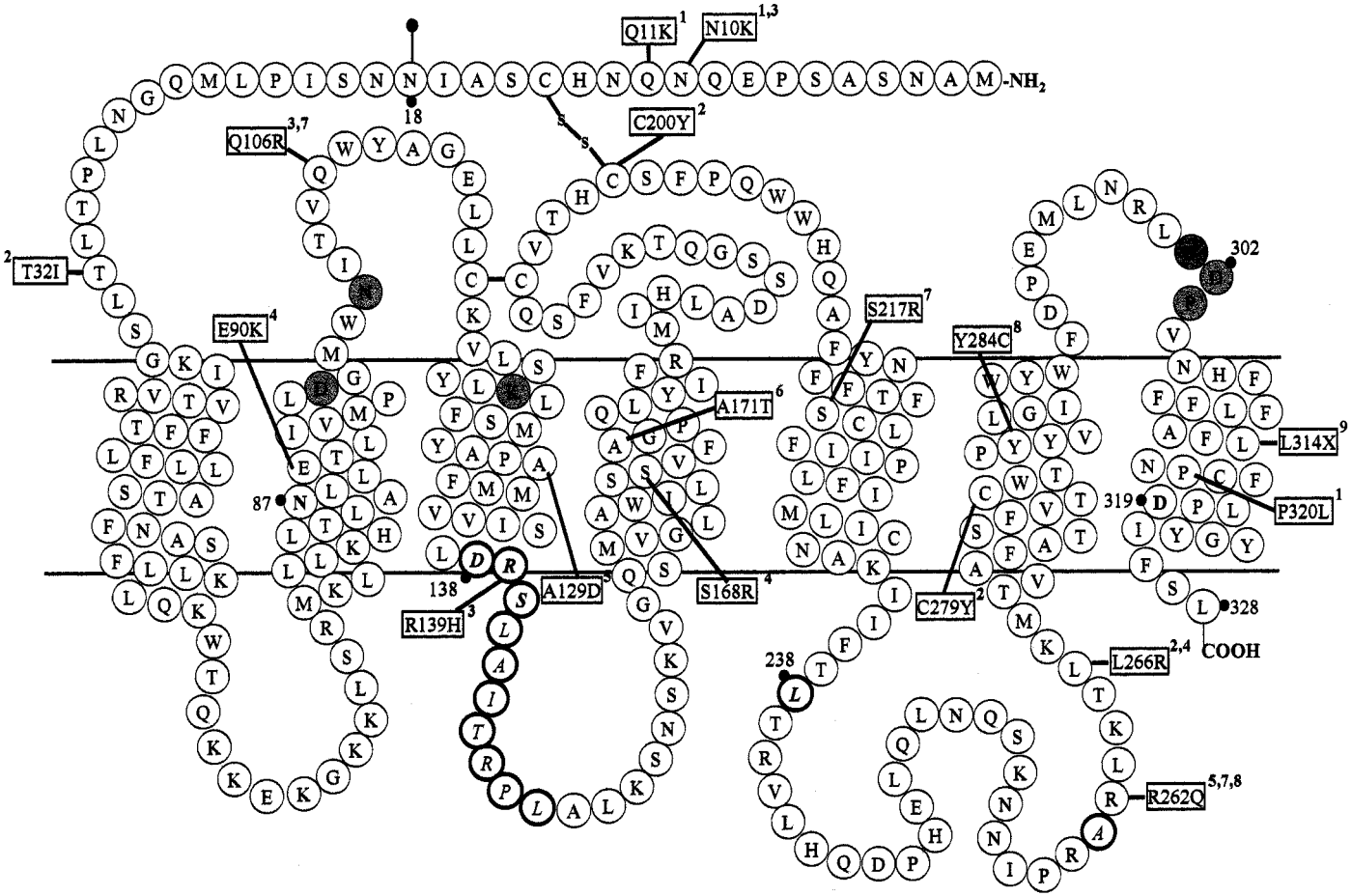

Figure 1: Two-dimensional representation of proposed topology for the human GnRH-R. Numbering is relative to N-terminal amino acid residue. Known N-linked glycosylation sites are marked by symbol —•. Shaded residues have been implicated in forming the proposed GnRH binding pocket. Italized text and bolded circles indicate amino acid residues important for G-protein coupling. The reciprocal mutation of conserved residues to Asp^{87} and Asn^{318} in GnRH-R are designated by bolded text. Common point mutations associated with the human disease hypogonadotropic hypogonadism are denoted in boxes. Superscripts correspond to the following reference(s) for each mutation: [1]Meysing et al., (2004), [2]Bedecarrats et al., (2003), [3]Costa et al., (2001), [4]Brothers et al., (2004), [5]Layman et al., (2002), [6]Karges et al., (2003), [7]de Roux et al., (1999), [8]Layman et al., (2001), [9]Kottler et al., (2000).

For GPCRs that bind small peptides, interaction with ligand appears to occur within the TMDs, which form a hydrophilic pocket. The proposed binding pocket for GnRH involves specific residues of TMD#3 (Lys^{121}; numbering relative to N-terminal amino acid) and at the N-terminal end of TMD#2 (Asp^{98} and Asn^{102})(Davidson, et al., 1996; Flanagan, et al., 2000; Zhou, et al., 1995). In addition, peptide GPCRs require residues in the N-terminal domain for high affinity binding. Deletion of the amino-terminal domain leads to loss of detectable GnRH binding (Sealfon and Millar, 1995). Extracellular loop 3 (ECL#3) has also been implicated in binding of GnRH (Flanagan, et al., 1994; reviewed by Flanagan, et al., 1997; Fromme, et al., 2001; Sealfon, et al., 1997). Specifically, the Glu^{301} in murine and Asp^{302} in human receptor of ECL#3 confers specificity for Arg^{8} in GnRH-I, and potentially induces GnRH-I to assume an active conformation (Flanagan, et al., 1994; Fromme, et al., 2001). Recent cloning of a GnRH-II, which has been demonstrated to be widely expressed in primate brains (Latimer, et al., 2000; White, et al., 1998), indicates a potential need for GnRH-R to distinguish between differing forms of naturally occurring forms of GnRH. In the human GnRH-R adjacent to Asp^{302} is Ser^{301} and Pro^{303}, which have been proposed to function in orientating the side chain of this acidic amino acid into a conformation necessary for interacting preferentially with Arg^{8} in GnRH-I (Fig. 1). Mutation of the GnRH-R amino acid sequence, either Pro^{303}Ala or Ser^{301}Pro/Pro^{303}Gly, results in decreased affinity for GnRH-I but not either Arg^{8}-subsituted GnRH or GnRH-II (Fromme, et al., 2004; Petry, et al., 2002; Wang, et al., 2004). Further research using a peptide specific for residues 293-302 of the murine GnRH-R resulted in folding of GnRH-I into an active conformation (Petry, et al., 2002). Petry et al., (2002) proposed that interaction with ligand stabilized the

ECL#3 leading to conformational changes in TMDs necessary for G-protein activation and subsequent signal transduction.

Sustained agonist binding stimulates desensitization and internalization of GPCRs. Typically, the cytoplasmic carboxyl tail is phosphorylated by GPCR kinases following sustained ligand binding (Hislop, et al., 2001). β-arrestin binds to the phosphorylated domain, uncouples G protein from receptor and targets the densensitized receptor to clathrin-coated vesicles for dynamin-stimulated internalization (Ferguson and Caron, 1998; Goodman, et al., 1996). Mammalian GnRH-R is targeted to clathrin-coated vesicles but utilizes a different mechanism than dynamin for internalization (Hislop, et al., 2001). Once internalized, receptors are either degraded by lysosomes or recycled back to the plasma membrane (Cornea, et al., 1999; Hazum, et al., 1985). Unlike typical GPCRs and non-mammalian GnRH-R, the mammalian GnRH-R lacks a C-terminal tail (Fig. 1). As a result, the mammalian receptor internalizes slower (thus desensitizes slower) than the non-mammalian receptor (Willars, et al., 1999; Willars, et al., 1998). Placing the intracellular C-terminal domain of the rat thyrotropin-releasing hormone receptor (TRH-R) onto the rat GnRH-R accelerated internalization and desensitization (Heding, et al., 1998). Likewise, addition of the catfish GnRH-R C-terminal tail onto the rat GnRH-R results in more rapid down-regulation than seen with wild type receptor (Lin, et al., 1998). In both studies, the chimeric receptors had similar binding affinities for GnRH as their wild type counterparts (Heding, et al., 1998; Lin, et al., 1998). Placing the 26-kDa enhanced green fluorescent protein (eGFP) on the carboxyl terminus of the murine GnRH-R did not alter the internalization rate compared to wild type murine and ovine receptors (Hashizume, et al., 2001). Thus, a specific amino acid sequence at the C-

terminus is required to enhance internalization rates of mammalian GnRH-R. However, this chimera (GnRHR-GFP) was fully functional and has been used to examine movement of the GnRH-R in the plasma membrane prior and subsequent to GnRH-R activation (Horvat, et al., 2001; Nelson, et al., 1999).

Various researchers have shown that GnRH receptor self-association is crucial for signal transduction and subsequent LH release. Hopkins at al. (1981) crosslinked GnRH antagonist bound receptors using an antibody; this resulted in gonadotropin release. Subsequently, Janovick et al. (1996a) treated gonadotropes with GnRH agonists causing receptors to move sufficiently close to one another that a radioiodine molecule could be transferred from a lactoperoxidase molecule covalently linked to a receptor-bound agonist onto an adjacent receptor.

Further research using techniques that are more refined has resulted in evidence for GnRH receptor microaggregation (physical association within 100 – 120 Å). Resonance energy transfer has been used extensively to measure this process, which occurs as quickly as it can be measured (<1 min)(Janovick and Conn, 1996a) and is distinguished from macroaggregation (patching, capping, internalization), an event that occurs later (>20 min)(Hashizume, et al., 2001). Assessing protein-protein interaction using resonance energy transfer requires the fusion of proteins of interest (i.e. GnRH-R) with either fluorescent proteins (i.e. green, red or yellow fluorescent proteins) and/or luciferase. For energy transfer to occur the emission spectra of the donor protein must overlap the absorption spectra of the acceptor and the two light emitting proteins must be within 100 Å of each other. Horvat et al. (2001) demonstrated a GnRH agonist concentration-dependent increase in energy transfer between GnRH receptors fused to

enhanced green and yellow fluorescent proteins. Similarly, energy transfer was observed between GnRH receptors fused to either enhanced green or red fluorescent protein and induced with buserelin (a GnRH agonist)(Cornea and Conn, 2002). This is indicative of hormone driven receptor dimerization or oligomerization. As expected, no significant energy transfer occurs between unoccupied receptors or Antide (GnRH antagonist) treated receptors (Cornea and Conn, 2002; Horvat, et al., 2001). Correspondingly, Kroeger et al. (2001) employed GnRH receptors fused to luciferase (bioluminescence) to donate energy to GnRH receptors fused to humanized green fluorescent protein to determine that GnRH agonist stimulates GnRH receptor self-association in living cells.

It has been suggested that GnRH-R activates several distinct signaling pathways by coupling to multiple G proteins (G_q, G_s, and G_i). The activation of G_s or G_i proteins has not been found in all experimental systems, and their importance in GnRH-R signaling has yet to be determined. Recent research has established that GnRH-R initiates these multiple cascades through exclusive interaction with $G_{q/11}$ proteins (Grosse, et al., 2000). This is consistent with information derived using αT3-1 cells indicating that GnRH-R activates phospholipase Cβ (PLC) and is pertussis-toxin insensitive (Anderson, et al., 1992; Husieh and Martin, 1992).

In activated GnRH-R, the second and third intracellular loops (ICL#2 and ICL#3, respectively) appear necessary to interact with $G_{q/11}$ protein (Ulloa-Aguirre, et al., 1998). The highly conserved Asp^{138}-Arg-Tyr^{140} motif located at the junction of TMD#3 and ICL#2 in many GPCR's appears to participate in G-protein interaction and activation. In GnRH receptors Tyr^{140} is replaced with a Ser, altering the motif from Asp-Arg-Tyr to Asp-Arg-Ser (Fig. 1). Mutations in Arg^{139} of the GnRH-R reduced cell surface

expression, percentage of receptor internalized and its ability to activate $G_{q/11}$ protein (Arora, et al., 1997). Although mutating Asp^{138} decreased the number of GnRH-R expressed on the plasma membrane, it increased percentage of membrane GnRH-R internalized and augmented inositol phosphate production per GnRH-R compared to the wild type receptor (Arora, et al., 1997). In contrast, no alteration in receptor internalization or GnRH-mediated inositol phosphate generation was observed when Ser^{140} was mutated (Arora, et al., 1995). Thus, only Asp^{138} and Arg^{139} of the conserved motif participate in receptor function. Interestingly, in non-mammalian GnRH-R sequences these modifications to Asp^{138}-Arg-Tyr^{140} motif and TMDs do not exist. Unique to GnRH receptors is conservation of the motif from Asp^{138} through Leu^{147} (DRxxxI/VxxPL), which appears to play a role in $G_{q/11}$ protein coupling. Mutation of either Arg^{145} to Pro introduces a Pro-Pro motif know to disrupt secondary structure and leads to defective coupling from $G_{q/11}$ protein (Chi, et al., 1994). Defective coupling with $G_{q/11}$ results from replacement of Leu^{147} with either Asp or Ala (Arora, et al., 1995). Several residues of ICL#3 have also been implicated in GnRH-R activation of G proteins. The size of the residue at position 261 appears to influence coupling of GnRH-R to $G_{q/11}$. If the residue is less than 40 Da, e.g. Ala, then efficient coupling can occur (Myburgh, et al., 1998). In contrast if the residue is greater than 50 Da then uncoupling occurs (Myburgh, et al., 1998). Additionally, efficient coupling of $G_{q/11}$ protein to GnRH-R requires the hydrophobic Leu^{238} (consistently conserved in most GPCRs) at the NH_2-terminal region of ICL#3 (Chung, et al., 1999).

In another departure from sequence conservation found in most GPCRs, the Asp and Asn of TMDs #2 and #7, respectively, are modified in GnRH-R sequence. The

reciprocal mutation to Asn^{87} and Asp^{318} in GnRH-R has assisted in identifying interhelical interactions between TMDS #2 and #7. Recreation of the arrangement found in other GPCRs (Asp^{87} and Asn^{318}) fails to alter GnRH-R function whereas single mutation in either residue disrupts GnRH-R function (Zhou, et al., 1994).

Reports of point mutations leading to inactivation of GnRH-R in hypogonadotropic hypogonadism of humans, a genetically heterogeneous disorder that has been described in both males and females with delayed sexual development and low gonadotropin and sex steroid levels, are becoming more frequent. Mutations are widely distributed across the entire sequence of the GnRH-R resulting in a broad spectrum of phenotypes, ranging from nonfunctional receptors to receptors with a modest degree of function (Fig. 1). These naturally occurring mutations are useful in functional characterization of the structure of the GnRH-R, either for confirmation of previous *in vitro* results (e.g. importance of residues Cys^{200} and Arg^{139}) or as a valuable tool for characterizing the properties of poorly expressed experimental mutants.

The GnRH-activated Gα protein affects the regulatory systems controlling calcium mobilization and protein kinase C (PKC) stimulation (Fig. 2)(Imai, et al., 1993). Specifically, $G\alpha_q$ subunit stimulates PLC to hydrolyze the membrane phospholipid phosphatidylinositol-4,5-bisphosphate (PIP_2) thus releasing diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP_3). IP_3 interacts with the endoplasmic reticulum resulting in mobilization of calcium (Ca^{2+}). The release of sequestered intracellular Ca^{2+} ($Ca^{2+}{}_i$) in the absence of PLC activation is sufficient to initiate secretion of LH, albeit to a lesser degree than that elicited by GnRH stimulation (McArdle and Poch, 1992; Stojilkovic, et al., 1990; Tse, et al., 1993b). Together with DAG, increases in $Ca^{2+}{}_i$ transiently activates

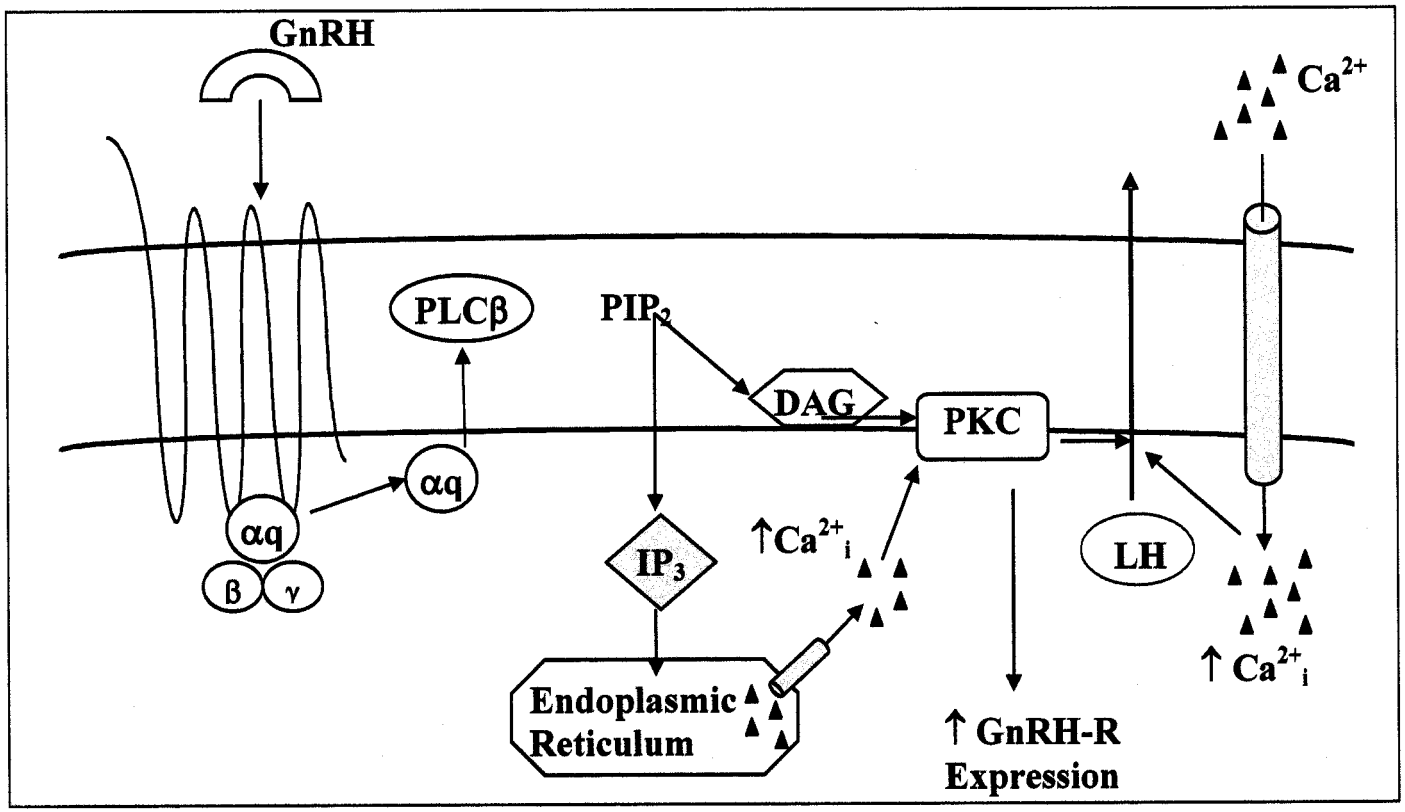

Figure 2: Schematic of signal transduction resulting from ligand binding to GnRH-R. Ligand bound GnRH-R activates the $G\alpha_q$ subunit dissociation from $G\beta\gamma$ subunits and this leads to stimulation of PKC. Abbreviations: Ca^{2+}, calcium; $Ca^{2+}{}_i$, intracellular calcium; DAG, diacylglycerol; GnRH, gonadotropin-releasing hormone; IP_3, inositol-1,4,5-triphosphate; PIP_2, phosphatidylinositol-4,5-bisphosphate; PKC, protein kinase C; PLCβ, phospholipase Cβ.

PKC, which in turn increases transcription of GnRH-R gene (Albarracin, et al., 1994; Duval, et al., 1997a; Ellsworth, et al., 2003a; Ellsworth, et al., 2003b; Fernandez-Vazquez, et al., 1996). Phorbol-12-myristate-13-acetate (an activator of PKC) increased basal secretion of LH 10-fold without elevation of $Ca^{2+}{}_i$, suggesting that PKC has a role in LH exocytosis (Billiard, et al., 1997). Jobin et al. (1995) and Zhu et al. (2002) demonstrated that PKC enhances secretion of LH by increasing the sensitivity of Ca^{2+}-sensing steps of exocytosis, not by increasing the readily releasable pool of secretory granules. Furthermore, synthetic DAGs have been used to stimulate LH release implying that all the components of the PKC activation cascade coordinate GnRH-induced LH release (Catt, et al., 1985; Conn, et al., 1985a; Harris, et al., 1985).

GnRH induces release of LH with rises in $Ca^{2+}{}_i$ not only through calciummobilization from intracellular stores, but also through influx of extracellular Ca^{2+}, predominately via L-type voltage-sensitive Ca^{2+} channels (Heyward, et al., 1995; Stojilkovic, et al., 1988; Tse and Hille, 1993a). The initial increase in $Ca^{2+}{}_i$ results from stimulation of IP_3 receptors on the endoplasmic reticulum, whereas the plateau phase depends on the influx of external Ca^{2+} (Kraus, et al., 2001). Increased exocytosis of LH is seen in pituitary cells exposed to L-type voltage-sensitive Ca^{2+} channel stimulators (Stojilkovic, et al., 1988). Treatment of cultured pituitary cells with either Ca^{2+} channel blockers (Conn, et al., 1983) or chelators of extracellular Ca^{2+} (Marian and Conn, 1979) results in inhibition of GnRH-mediated secretion of LH.

The kinetics of gonadotropin release depends on the source of calcium. In perfused pituitary cells, Hansen et al. (1987) and Tasaka et al. (1988) demonstrated that the immediate LH release seen within minutes of GnRH exposure was a result of

mobilization from sequestered Ca^{2+} stores, whereas sustained GnRH-stimulated LH release requires Ca^{2+} influx (Naor, et al., 1988; Tasaka, et al., 1988).

C. GnRH and Estradiol Regulate Reproduction.

Reproduction in mammals is controlled by interactions among the hypothalamus, anterior pituitary gland and gonads. The hypothalamus secretes pulses of GnRH; E_2 influences the duration, magnitude and timing of these pulses. GnRH binds with high affinity to its cognate receptor on pituitary gonadotropes to stimulate release of the gonadotropic hormones, LH and FSH that in turn regulate production of gametes and gonadal hormones. LH and FSH are glycoprotein hormones comprised of a common glycoprotein hormone α subunit and unique β subunit; LHβ and FSHβ, respectively. In the cycling female, FSH acts upon the ovary to stimulate follicular development. Consequently, E_2 is secreted from the developing follicle and feeds back upon the hypothalamus and the anterior pituitary gland. E_2 alters hypothalamic secretion of GnRH from an episodic to a continuous pattern of secretion (Caraty, et al., 1995). This massive, prolonged release of GnRH from the hypothalamus not only induces secretion of LH but also increases pituitary sensitivity to GnRH (discussed later in the review), which contributes to pre-ovulatory surge of LH. This substantial release of LH from the anterior pituitary gland results in ovulation, the release of the ovum (egg) from the follicle.

Estradiol exerts both positive and negative effects on secretion of LH at the level of the hypothalamus. During estrous or menstrual cycles, basal levels of E_2 are insufficient to alter pulsatile secretion of GnRH. When E_2 levels are increased from a

basal level to a late follicular phase or proestrous level, there is an initial negative effect on GnRH secretion. GnRH pulse amplitude and sometimes frequency is suppressed in what is termed negative feedback. Seasonal breeders, such as sheep, enter a period of infertility when females are more sensitive to E_2 negative feedback. Changes in photoperiod are responsible for this alteration in reproductive activity. Ovariectomized ewes bearing subcutaneous E_2 implants exhibit decreased secretion of LH during the normal time of anestrus in intact animals (Goodman, et al., 1982; Martin, et al., 1983), and this same effect can be induced when the ewes are exposed to artificial long-days (Karsch, et al., 1986). It appears that the neurons of the retrochiasmatic (RCh) area respond to E_2 to inhibit pulsatile LH release (Gallegos-Sanchez, et al., 1997). Although it has been established that estrogen receptor-α mediates seasonal changes in the RCh (Hardy, et al., 2003), no classical ERs have been found in neurons of this area (Lehman, et al., 1997).

Chronic administration (greater then 7 days) of E_2 to OVX ewes results in decreases in levels of mRNA for LHβ and serum concentrations of LH (Herring, et al., 1991; Nett, et al., 1990). Further, pulsatile administration of GnRH was unable to override the decline in release of LH observed in OVX ewes chronically treated with E_2; this is suggestive of a direct effect of E_2 at the level of the anterior pituitary gland (Nett, et al., 1990). Additional studies have used OVX ewes with hypothalamic-pituitary-disconnection (HPD) to remove the confounding effects of not only endogenous steroids but also hypothalamic input on the anterior pituitary gland. In OVX-HPD ewes pretreated with GnRH, chronic administration of E_2 resulted in reduction of levels of mRNA for LHβ and serum concentrations of LH to a lesser extent than seen in OVX

ewes (Girmus and Wise, 1991). Girmus and Wise (1991) observed decreased levels of mRNA for LHβ and no effect on secretion of LH in OVX-HPD ewes pulsed with exogenous GnRH prior to and during implantation with E_2 for 7 days.

The rapid effects (< 24 hr) of E_2 on release of LH differ greatly from the chronic results. Herring et al. (1991) implanted silastic capsules of E_2 into OVX sheep and observed an initial decline in serum concentrations of LH (hours 1-10) followed by a pre-ovulatory-like surge of LH (hours 11-22). Similar effects have been demonstrated in other species; steers (Kesner, et al., 1981), rhesus monkeys (Spies and Norman, 1975), rats (Strobl and Levine, 1988), and humans (Nagahara, et al., 1984). If E_2 treatment is continued beyond 24 hr, the results are always a reduction in secretion of LH.

Specific events are stimulated by E_2 during the first 24 hr of exposure to produce the pre-ovulatory surge of LH. At the outset, the genomic machinery of gonadotropes is activated by E_2 to increase expression of the GnRH-R gene (Quyyumi, et al., 1993). The increase in numbers of GnRH-R in the anterior pituitary gland increases sensitivity of the anterior pituitary gland to GnRH (Goubillon, et al., 1999; Gregg and Nett, 1989, 1990; Hamernik, et al., 1995; Harris, et al., 1998; Laws, et al., 1990b; Turgeon, et al., 1996; Turzillo, et al., 1994; Turzillo, et al., 1998; Wise, et al., 1984). The entire process takes 4-8 hours as determined from direct measurement in an ovine study (Nett, et al., 1984).

The density of GnRH receptors on gonadotropes determines their ability to respond to GnRH (Wise, et al., 1984). This density is highest just prior to ovulation and likely is important for complete expression of the pre-ovulatory surge of LH (Fig. 3). Therefore, knowledge regarding what regulates the density of GnRH-R is essential to understanding changes in pituitary sensitivity to GnRH and ultimately, to expression of

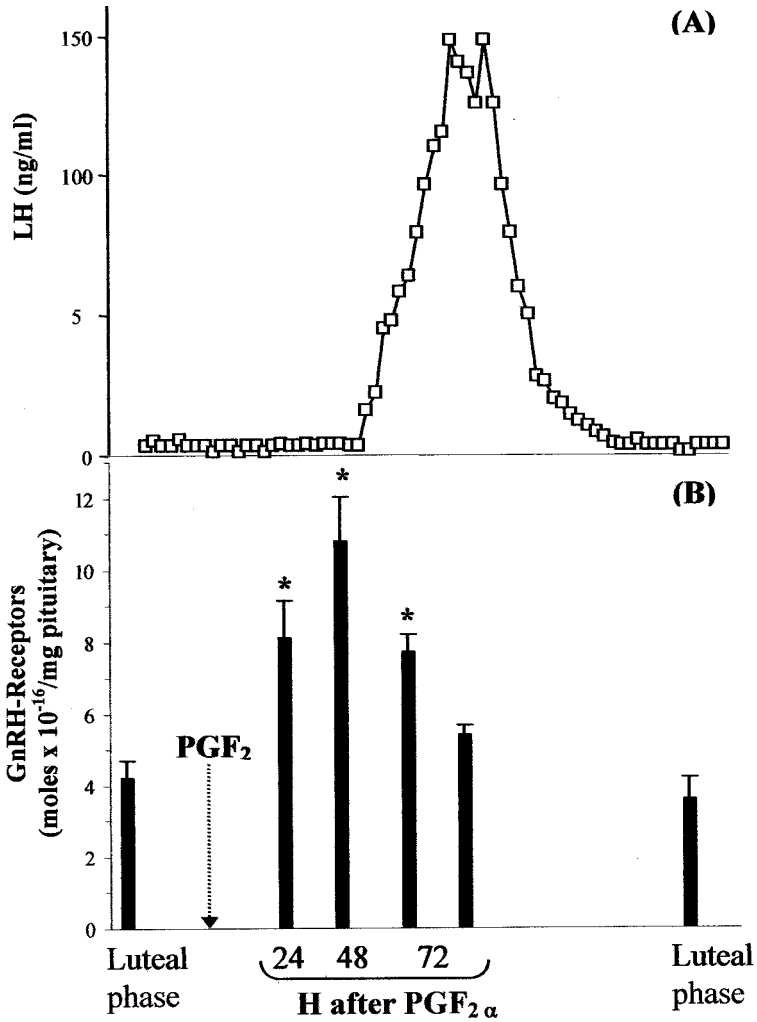

Figure 3: Serum concentrations of LH (A) and numbers of receptors for GnRH (B) during the periovulatory period in sheep (adapted from Nett, et al., 2002). Values marked with an asterisk (*) are different from luteal phase values ($P < 0.05$).

the LH surge. Regulation of GnRH-R gene expression is influenced by a multitude of factors including gonadal steroid hormones, inhibin, activin and perhaps most importantly GnRH itself.

The hormone that has been examined in the greatest detail with respect to regulation of pituitary sensitivity to GnRH is estradiol. It has long been known to increase pituitary responsiveness to GnRH (Reeves, et al., 1971). Moreover, it has been shown to increase both mRNA (Turzillo, et al., 1998) for and numbers of GnRH receptors in many species including sheep (Clarke, et al., 1987; Gregg and Nett, 1989), cattle (Schoenemann, et al., 1985), and several species of laboratory animals (Bauer-Dantoin, et al., 1995; Yasin, et al., 1995). This action occurs directly on gonadotropes, but the mechanism is yet to be defined. Factors other than E_2 may also participate in stimulating the pre-ovulatory increase in GnRH-R noted prior to the pre-ovulatory surge. In anestrous ewes, when ovulation was stimulated with small doses of GnRH and luteal regression was induced using prostaglandin-$F_{2\alpha}$ ($PGF_{2\alpha}$), both mRNA for and numbers of GnRH-R rose to levels similar to those observed during normal luteal phase (Nett, et al., 2002). In control ewes treated with $PGF_{2\alpha}$ but not administered pulses of GnRH, there was no increase in mRNA for or numbers of GnRH receptors indicating that basal concentrations of estradiol, even in the absence of progesterone were not sufficient to increase synthesis of GnRH-R. Furthermore, hourly pulses of GnRH were capable of increasing concentrations of GnRH-R in the pituitary gland even when the corpus luteum was fully functional (Fig. 4). Two important conclusions may be drawn from these observations. First, it appears that the increased frequency of GnRH pulses following regression of the corpus luteum may be the most important factor regulating expression

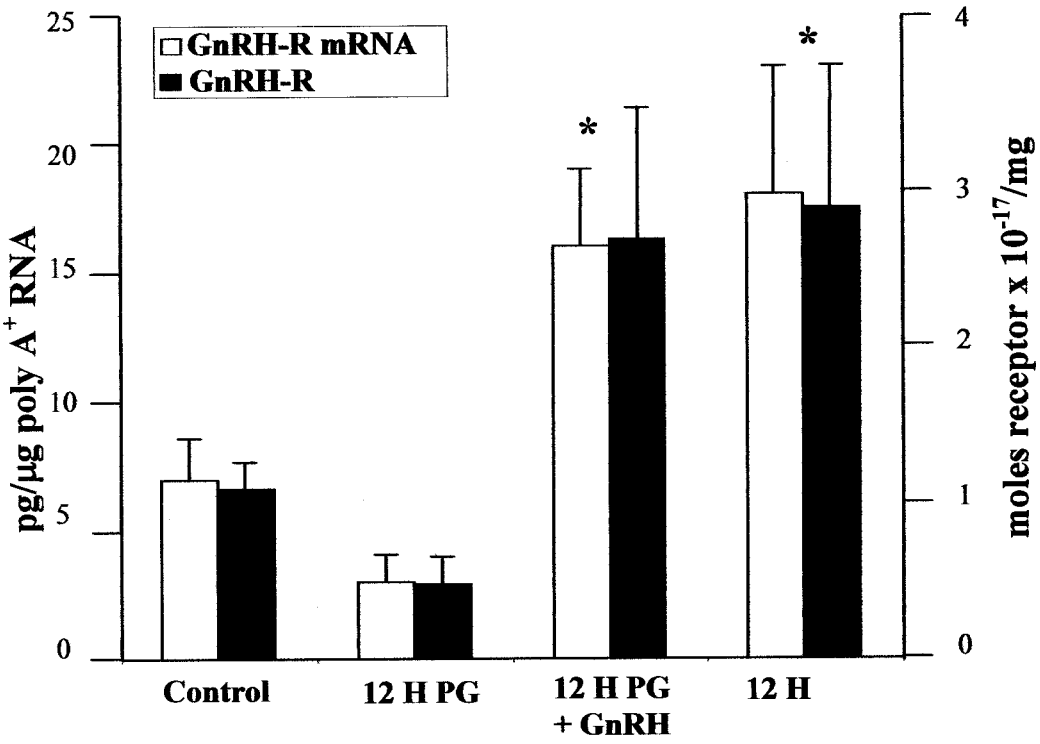

Figure 4: Depiction of amounts of mRNA for GnRH-R and numbers of receptors for GnRH after treatment with GnRH and/or $PGF_{2\alpha}$ (PG) in ewes pharmacologically induced to ovulate during anestrous. Measurements were made 12 h after administration of $PGF_{2\alpha}$; GnRH was administered hourly for 12 h either alone or beginning immediately after $PGF_{2\alpha}$. Data are mean ± SEM. Values with an asterisk (*) are different from controls, $P < 0.05$ (Nett, et al., 2002).

of the GnRH-R gene during the pre-ovulatory period. Second, the mechanism(s) by which GnRH and estradiol regulate expression of the GnRH-R gene appear to be completely separate. This conclusion stems from the fact that progesterone can inhibit the estradiol-induced expression of the GnRH-R but has no affect whatsoever on regulation of GnRH-R gene expression induced by GnRH itself. While the mechanisms described above have been most closely evaluated in sheep, it appears that hormonal regulation of the GnRH-R gene expression is probably very similar in other species of domestic livestock (Schoenemann, et al., 1985; Silvia, et al., 1987).

Surgical disconnection of the hypothalamus from the anterior pituitary gland results in decreased mRNA for and numbers of GnRH-R in OVX ewes (Clarke, et al., 1987; Gregg and Nett, 1989; Turzillo, et al., 1995), presumably due to removal of GnRH input to the anterior pituitary gland. Likewise, treatment of ewes with an antiserum to GnRH (Sakurai, et al., 1997; Turzillo and Nett, 1997) or with a GnRH antagonist (Brooks and McNeilly, 1994) to prevent GnRH from interacting with its receptor also drastically reduces the number of GnRH-R in the anterior pituitary gland. In contrast, administration of GnRH pulses to HPD ewes restores GnRH-R numbers to levels seen prior to HPD (Clarke, et al., 1987; Hamernik and Nett, 1988). Thus, normal secretory patterns of GnRH appear to be essential for maintaining a full complement of GnRH-R in the anterior pituitary gland. In contrast to the normal pulsatile pattern of GnRH secretion, continuous delivery of GnRH dramatically decreases both amounts of mRNA for and number of GnRH-R in sheep (Nett, et al., 1981; Turzillo, et al., 1995) and cattle (Vizcarra, et al., 1997). This appears to occur as a result of GnRH-R being internalized and degraded more rapidly than it can be replaced. Interestingly, the pituitary becomes

unresponsive to further challenges with GnRH after continuous exposure to GnRH, but an increase in GnRH-R can be stimulated by administration of E_2, even in the face of continued administration of GnRH (Nett, et al., 1981; Turzillo, et al., 1995). The pathway by which GnRH regulates transcription of GnRH-R has been clearly established (Duval, et al., 1997b; Ellsworth, et al., 2003a; Ellsworth, et al., 2003b; Kraus, et al., 2001; Liu, et al., 2002; Mulvaney and Roberson, 2000; White, et al., 1999). However, the lack of a consensus estrogen response element in the promoter region of GnRH-R suggests that E_2 regulates transcription of the GnRH-R gene via a yet to be identified mechanism. This further substantiates that GnRH and E_2 appear to regulate the number of GnRH-R by independent mechanisms.

D. Non-genomic Effects of Estradiol

The biological effects of E_2 are mediated by estrogen receptors, which belong to the nuclear receptor superfamily, a family of ligand-regulated transcription factors. Primarily, two isoforms of ER have been characterized, α and β, although isoform is an incorrect term to use because ERα and ERβ are distinct proteins encoded by separate genes located on different chromosomes. A third more distantly related ER was recently cloned, ERγ, which to date has only been found in teleosts (Hawkins, et al., 2000). While α and β estrogen receptors both bind estrogen as well as other agonists and antagonists, the two receptors have distinctly different localizations and concentrations within the human body. Structural differences also exist between ERα and ERβ allowing for a wide range of diverse and complex processes to take place. The ligand-binding domain is conserved in both estrogen receptors (Fig. 5) and each exhibits a similar affinity for E_2.

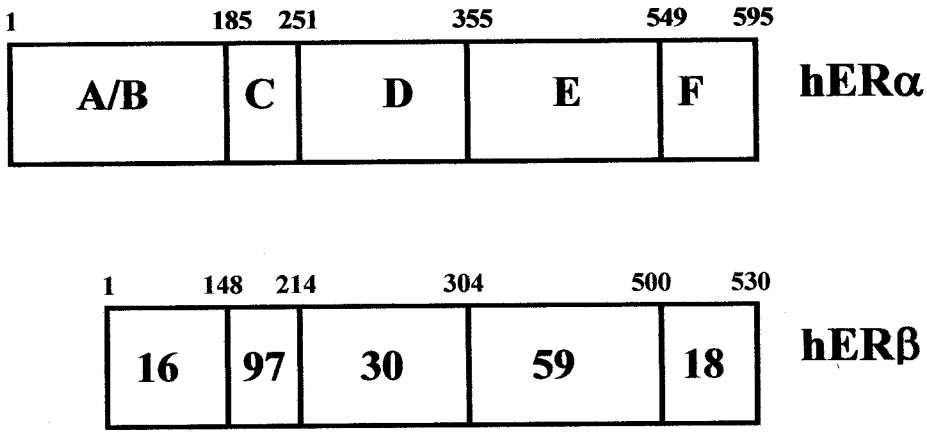

Figure 5: Structure of the two forms of human estrogen receptors, adapted from review by Gustafsson (1999). The figures above represented receptors indicate number of amino acids, with number 1 the most N-terminal. The separate domains are identified in the ERα diagram; the numbers in the ERβ diagram show the amino acid sequence identity as percentage. Nomenclature: A/B, AF-1 binding and transcriptional activation domain; C, DNA binding domain; D, hinge region; E, AF-2 binding and ligand binding domain; and F, agonist/antagonist distinction region.

The α and β receptors are products of different genes and exhibit tissue-specific expression; both are co-expressed in numerous tissues, including cardiovascular system, central nervous system, urogenital tract, bone, and breast tissue (Gustafsson, 1999). Although both isoforms are expressed in pituitary, mammary and uterine tissue, it appears that ERα plays a more important role in the physiology of these tissues and is mainly involved in reproductive events (Couse and Korach, 1999; Hess, 2003; Hewitt and Korach, 2003).

Estradiol is produced primarily in ovaries and testes and is thought to diffuse out of cells in which it is produced. In the classical model of action in target tissues, E_2 crosses the plasma membrane (presumably by diffusion or facilitated transport), binds to ER within the cytoplasm (Jensen and DeSombre, 1973) and then the steroid-receptor complex translocates to the nucleus. Using immunocytochemical detection, unoccupied ER's have also been found within the nucleus (King and Greene, 1984; Welshons, et al., 1984). Once bound by E_2, the ER undergoes a conformational change and binds as either homodimers (Kumar and Chambon, 1988) or heterodimers (Cowley, et al., 1997) to estrogen response elements in the promoters of many estrogen-responsive genes. The DNA-binding domains of ERα and ERβ share a high degree of sequence homology (Fig. 5) and bind with similar specificity and affinity to estrogen response elements. Next, the ER recruits an array of transcriptional cofactors (coactivators and corepressors) that bind to the receptor and interact with other transcription factors, thereby modulating transcription of target genes (McKenna and O'Malley, 2001).

Increasing evidence suggests that distinct pools of estrogen receptors that localize to the plasma membrane play important roles in estrogen-dependent responses. Razandi

et al. (1999) demonstrated by transient transfection that the same gene product responsible for ERα and ERβ nuclear transcripts results in a small pool of estrogen receptors localized to the plasma membrane. Although the precise mechanism and structural features of ERs that result in membrane localization have not been determined, recent research suggests caveolin-1 (an integral protein of caveolae) may assist in translocating ERα to the membrane (Razandi, et al., 2002).

Numerous functional studies of the acute (< 1 hr), non-genomic effects of E_2, has increased support for the existence of a membrane ER. Following are a few examples of non-genomic effects of E_2 in various cell types. Picotto et al. (1999) showed that E_2 could rapidly stimulate entry of Ca^{2+} into isolated duodenal enterocytes through a PLC-dependent mechanism. Moreover, Le Mellay et al. (1997) demonstrated that within a few seconds to minutes, E_2 could activate PLC. With the aid of confocal reflective microscopy and immunocytochemistry using antibodies to ERα, Nadal et al. (1998) were able to show specific localization of ER at the plasma membrane of mouse pancreatic β-cells and further demonstrated a rapid (<20 min) release of insulin when these receptors bound E_2. Estradiol can also stimulate production of cyclic nucleotides in pancreatic β-cells (Ropero, et al., 1999). In human endothelial cells, Russell et al. (2000) provided evidence that E_2, or the cell-impermeable compound, E-BSA, rapidly stimulates cGMP formation and nitric oxide production, and activates extracellular-regulated kinase (ERK). Also in this study, with the use of labeled E-BSA, cell surface binding occurred, presumably to a membrane ER (Russell, et al., 2000). Studies employing MCF-7 breast cancer cells also provided evidence that E_2 can stimulate the signaling cascade that activates ERK (Migliaccio, et al., 1996). Furthermore, E_2 can activate c-fos transcription

in a human neuroblastoma cell line (SK-N-SH), which probably occurs through extracellular signal-regulated kinase (Watters, et al., 1997). Administration of estradiol stimulates rapid release of prolactin in cultured rat pituitary cells (Christian and Morris, 2002; Morris, et al., 2002). Norfleet et al. (1999) demonstrated the presence of membrane ERs on GH3/B6/F10 rat pituitary tumor cells. Later Norfleet et al. (2000)used antibodies against the hinge region of ERα to mimic the stimulatory effects of E_2 on PRL secretion further substantiating the theory that E_2 induces acute effects via membrane estrogen receptors.

It has been known for at least three decades that E_2 has a negative effect on secretion of LH (Brown, et al., 1969). It has been assumed that the negative effects were mediated by inhibition of GnRH secretion, thereby leading to a decrease in secretion of LH (Karsch, et al., 1987). However, Nett et al. (1984) using OVX ewes infused with GnRH at a constant rate, demonstrated a negative effect of E_2 on secretion of LH at the level of the anterior pituitary gland (Fig. 6). Each ewe was ovariectomized as this allows expression of robust pulses of GnRH and LH secretion, thereby facilitating detection of suppressed pulsatility following E_2 treatment. Further, this model ensures that changes in GnRH and LH secretion are not caused by altered ovarian steroid secretion. Using this model, a negative effect on secretion of LH was seen within 15-30 minutes of administration of E_2 (presumably an immediate effect on the gonadotrope since the half-life of LH in sheep is ~25 min (Akbar, et al., 1974)). This was followed several hours later by a positive effect on the release of LH (apparently due to synthesis of new GnRH-receptors). Similar rapid negative effects of estrogen on LH release were observed in steers (Kesner, et al., 1981), rhesus monkeys (Spies and Norman, 1975), rats

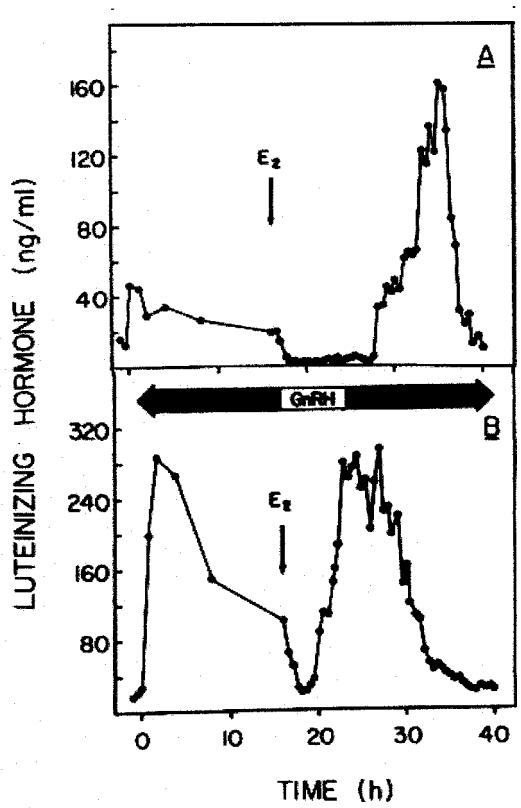

Figure 6: OVX ewes were treated with estradiol (E_2) only (panel A) or given a continuous infusion of GnRH and then treated with E_2 (panel B) adapted from Nett et al. (1984). Note that in either case, there was an immediate decrease in secretion of LH after administration of estradiol. The rapid decline in LH secretion during the GnRH infusion indicates that at least some of the negative effects of estradiol were mediated at the level of the anterior pituitary gland.

(Strobl and Levine, 1988), and humans (Nagahara, et al., 1984). Additionally, injection of Raloxifene, a selective estrogen receptor modulator, into OVX rats caused a comparable rapid effect, an immediate decrease in secretion of LH (Pinilla, et al., 2001). From these and other studies, it appears that E_2 has a non-genomic effect on gonadotropes.

In some of these studies, it might be argued that the rapid reduction in secretion of LH could have resulted from a negative effect on secretion of GnRH from the hypothalamus. However, this is certainly not the case in the GnRH-clamp model employed by Nett et al. (1984) which eliminates effects of E_2 on the hypothalamus. In a recent *in vivo* study, administration of either E_2 or E-BSA to OVX ewes resulted in a rapid suppression of basal LH release (Arreguin and Nett, 2003). Similar results were observed in cultured pituitary cells treated with either E_2 or E-BSA (Arreguin and Nett, 2002). Therefore, it appears that the inhibition of LH secretion by E_2 results not only from a non-genomic effect mediated at the level of the anterior pituitary gland, but also via a membrane associated ER.

E. Modeling Gonadotropes for Subcellular Studies

Primary pituitary cells are comprised of a heterogeneous population of well-differentiated, secretory cell-types and non-endocrine cells. These include corticotropes, somatotropes, lactotropes, thyrotropes, and gonadotropes. Based on observations of rat anterior pituitary glands, somatotropes and lactotropes comprise approximately 70% of the secretory cells of the anterior pituitary gland (Dada, et al., 1984). Less than five percent of the anterior pituitary cells are corticotropes (Dada, et al., 1984). Gonadotropes

comprise approximately 6-15% of the endocrine cells of the anterior pituitary gland (Dada, et al., 1984; Gharib, et al., 1990; Ibrahim, et al., 1986). In the postpubertal animal, the anterior pituitary gland of the female has more lactotropes and gonadotropes, while that of the male has a higher percentage of somatotropes (Chen, 1988; Gonzalez-Parra, et al., 1998; Ho, et al., 1986; Ho, et al., 1988; Hoeffler and Frawley, 1986; Sasaki and Sano, 1982; Takahashi and Kawashima, 1982; Tan, et al., 1998). Each cell type responds to a hypothalamic-releasing hormone with secretion of a specific anterior pituitary gland hormone. Corticotropes synthesize and secrete adrenocorticotropin as well as other hormones including lipotropins, endorphins and enkephalin in response to corticotrophin-releasing hormone. Thryotropin-releasing hormone stimulates thyrotropes to release thyroid-stimulating hormone and lactotropes to release PRL. Somatotropes release growth hormone when stimulated with growth hormone-releasing hormone and gonadotropes secrete LH and FSH in response to GnRH. Folliculostellate cells, star-shaped and nongranular, comprise an extensive communication network between endocrine cells of the anterior pituitary gland and are a source of paracrine factors that act locally to modulate pituitary responses to hypothalamic and peripheral signals (Allaerts, et al., 1990; Fauquier, et al., 2002; Soji and Herbert, 1989). The heterogeneity of the cell population and of the cellular responses in anterior pituitary gland causes difficulties for researchers aspiring to examine the subcellular mechanisms mediating hormone secretion.

Gonadotropes are the only cell type of the anterior pituitary gland that secrete LH. Studies examining the effects of either GnRH and/or E_2 on secretion of LH generally use cultured pituitary cells (Campen and Vale, 1988; Fowler, et al., 1992; Hopkins, et al.,

1981; Jinnah and Conn, 1985; Krsmanovic, et al., 2000; Laws, et al., 1990b; Marian and Conn, 1979; Moss and Nett, 1980). Reverse hemolytic plaque assays have been used successfully to assess the release of LH from individual gonadotropes (Cassina and Neill, 1996). Some researchers enrich the cultures of pituitary cells for gonadotropes using either Percoll step gradient centrifugation (Heyward, et al., 1995) or differential centrifugation (Childs and Unabia, 2001; Drouva, et al., 1990). These procedures rely on the difference in size of the cells of the anterior pituitary gland for separation. Unfortunately, the resulting "purified" gonadotropes are frequently contaminated with somatotropes (Childs, et al., 1994; Childs, et al., 2000). Given that somatotropes respond to GnRH and E_2 (Klausen, et al., 2002; Silverman, et al., 1988), contamination of the "purified" population of gonadotropes complicates determination of the effects of either GnRH or E_2 on subcellular events. Masumoto et al. (1991) developed a protocol using fluorescence-activated cell sorting and argon laser treatment of the cells labeled with anti-luteinizing hormone antibody to purify gonadotropes from whole anterior pituitary glands. This protocol requires extensive "handling" and decreases the biological potential of the gonadotropes. None of the discussed procedures are convenient or timely to perform.

The development of immortalized gonadotrope cell lines has greatly facilitated the study of molecular and cellular biology of this cell type. Using directed expression of simian virus-40 T-antigen oncogene fused to the promoter/enhancer region from the human glycoprotein hormone α-subunit, Windle et al. (1990) was able to create anterior pituitary gland tumors in transgenic mice specifically of gonadotrope origin. The cell lines generated from the αT3-1 tumor are stably diploid, express glycoprotein α-subunit

and respond to GnRH with elevated α-subunit mRNA comparable to what has been observed in normal gonadotropes (Windle, et al., 1990). This immortalized cell line has proven invaluable to research for GnRH-R gene cloning (Tsutsumi, et al., 1992) and transcription regulation (Cheng and Leung, 2001), GnRH-dependent signaling (Grosse, et al., 2000), plus GnRH-mediated receptor internalization (Hashizume, et al., 2001; Nelson, et al., 1999) and desensitization (McArdle, et al., 1996; Willars, et al., 1998; Willars, et al., 2001). Unfortunately, the αT3-1 cells lack expression of the glycoprotein β-subunits for LH and FSH and cannot be used to study the endocrinology of gonadotropes. αT3-1 cells appear to represent precursor gonadotropes that were arrested in an early stage of development (Windle, et al., 1990). Mellon and co-workers (1991) generated another immortalized gonadotrope cell line, LβT2, utilizing the rat LHβ promoter linked to T-antigen for targeted oncogenesis. The cells of this line express mRNA for GnRH-R and for both the α- and β-subunits of LH (Turgeon, et al., 1996). As seen in normal gonadotropes, LβT2 cells secrete LH in response to pulsatile GnRH in a concentration-dependent manner and the presence of E_2 and dexamethasone modulates GnRH-mediated LH release (Turgeon, et al., 1996). The LβT2 cell line has been employed to study the regulation of transcription of the α-subunit (Fowkes, et al., 2002), GnRH-induced LHβ and FSHβ protein expression (Liu, et al., 2002; Nguyen, et al., 2004; Pernasetti, et al., 2001), and the roles of PKC, cyclic AMP, and Ca^{2+} in GnRH-induced signaling (Liu, et al., 2003; Thomas, et al., 1996; Vasilyev, et al., 2002). Our understanding of the endocrinology and molecular biology of gonadotropes has been substantially advanced with the development of the LβT2 cell line.

F. Summary and Goals of this Research

The importance of estrogens in numerous physiological systems, including the reproductive, cardiovascular, skeletal and immune systems, requires a better understanding of the subcellular mechanisms affected by exongenous and endogenous estrogens. Environmental or nutritional estrogenic endocrine disrupting chemicals are thought to mediate developmental and carcinogenic pathologies (Colborn, et al., 1993; McLachlan, 1993). The genomic effects of estrogens and other steroid hormones are now relatively well understood, but we lack comprehension of the non-genomic mechanisms. As indicated previously, evidence supports the fact that E_2 acutely affects LH secretion at the level of anterior pituitary gland. The review of literature created two questions to be addressed in this thesis. Question one, which signaling events associated with GnRH-induced release of LH are negatively influenced by E_2? Although, primary pituitary cultures provide sufficient gonadotropes to examine the endocrinology of gonadotropes, the heterogeneity of the cell population complicates the determination of the effects of either GnRH or E_2 on subcellular events of gonadotropes. Thus question two, can LβT2 cells be employed as a model for cultured gonadotropes to evaluate how GnRH-induced secretion LH is acutely affected by E_2?

CHAPTER II: *IN VITRO* MODELING OF ACUTE EFFECTS OF ESTRADIOL ON GONADOTROPES

A. Introduction

Review of the literature revealed a biphasic effect of E_2 on pre-ovulatory release of LH. In ovariectomized (OVX) ewes administered a single dose of E_2 (Nett, et al., 1984) or implanted with silastic capsules of E_2 (Herring, et al., 1991), an initial decline in serum concentrations of LH (hours 1-10) was observed. This decline was followed by a pre-ovulatory-like surge of LH approximately 11 to 22 hours later. Similar effects have been demonstrated in other species; steers (Kesner, et al., 1981), rhesus monkeys (Spies and Norman, 1975), rats (Strobl and Levine, 1988), and humans (Nagahara, et al., 1984). To date, studies investigating mechanism by which E_2 induces the initial decline in LH are not definitive. In some of these studies, it might be argued that the rapid reduction in secretion of LH could have resulted from a negative effect on secretion of GnRH from the hypothalamus. However, this is certainly not the case in the GnRH-clamp model employed by Nett et al. (1984). In a recent *in vivo* study, administration of either E_2 or E-BSA to OVX ewes resulted in a rapid suppression of basal LH release (Arreguin and Nett, 2003). Arreguin and Nett (2002) performed studies on cultured ovine pituitary cells and were able to produce comparable inhibition of GnRH-induced LH secretion by treatment with either E_2 or E-BSA. It appears that the inhibition of LH secretion by E_2

results not only from a non-genomic effect mediated at the level of the anterior pituitary gland, but also from a membrane associated ER.

Unfortunately, progress in identifying signaling intermediates involved in this process has been impeded by the heterogeneity of cultured ovine pituitary cells. Previous research has demonstrated that LβT2 cells exhibit functional characteristics consistent with those of normal pituitary gonadotropes, such as secretion of LH in response to GnRH, an effect potentated by pretreatment with estradiol (Turgeon, et al., 1996). Therefore, LβT2 cells should be a useful tool for delineating the cellular and molecular events involved in the non-genomic effect of E_2 on GnRH-induced release of LH. Unfortunately, data regarding the acute effects of E_2 on LH secretion does not exist. Hence, we need to determine whether LβT2 cells respond to E_2 with decreased GnRH-induced release of LH as observed in cultured ovine pituitary cells (Arreguin and Nett, 2002). This chapter presents characterization of acute secretion of LH in response to simultaneous treatment with E_2 and GnRH in LβT2 cells and in cultured ovine pituitary cells handled similarly.

B. Materials and Methods

Reagents

Collagenase, hyalurondisae, deoxyribonuclease, gonadotropin-releasing hormone, 17β-estradiol, and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Cell culture reagents, including Dulbecco's Modified Eagle Medium (DMEM), penicillin, streptomyocin, non-essential amino acids, cell culture dishes and flasks were purchased from Gibco BRL (Carlsbad, CA). Fetal bovine serum (FBS) was purchased from Gemini

Bio-Products (Woodland, CA). For experimental cultures, FBS was treated with charcoal-dextran to remove lipophilic material thus reducing the serum concentration of hormones such as estradiol, progesterone, testosterone and cortisol. Once treated, the charcoal-stripped FBS (cs-FBS) was sterilized by passage through a 0.22 μm filter and stored in aliquots at -20°C. LβT2 and αT3-1 cells were generous gifts from Dr. Pamela Mellon (UC San Diego, La Jolla, CA). ChemiBlot molecular weight markers were procured from Chemicon International, Inc (Temecula, CA). The primary antibodies used for immunoblotting, rabbit anti-mouse ERα (MC-20) and rabbit anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH; ab9485) were obtained from Santa Cruz Biotechnology, Inc (Santa Cruz, CA) and AbCam Ltd (Cambridge,MA), respectively. The secondary antibody, goat anti-rabbit IgG conjugated to horseradish peroxidase (HRP) and SuperSignal West Dura Extended Duration Substrate were purchased from Pierce (Rockford, IL).

Anterior Pituitary Gland Dissociation

Anterior pituitary glands were collected from adult OVX ewes that had been exsanguinated prior to collection. Ovariectomies were performed a minimum of 30 days prior to anterior pituitary gland collection. Anterior pituitary glands were dispersed into single cells by collagenase-hyaluronidase-deoxyribonuclease treatment as described by Gregg et al. (1990). Following collection, anterior pituitary glands were sectioned with a Stadie-Riggs tissue slicer and washed twice with dissociation medium (137 mM NaCl, 5mM KCl, 10 mM glucose, 25 mM n-2-hydroxyethyl piperazine ethane sulfonic acid (HEPES), pH 7.3 freshly prepared for each dissociation). The sectioned tissue was transferred to 35 ml culture flasks containing dissociation medium, to which collagenase

(Type II, 1 mg/ml), hyaluronidase (Type V, 1 mg/ml), and deoxyribonuclease I (0.02 mg/ml) were added. Flasks were incubated at 37° C in a Dubnoff metabolic shaker for 45 min. Following the initial incubation, undigested tissue was gently passed through a sterile Pasteur pipette to mechanically dissociate cells. Incubation was continued for an additional 45 min at 37° C during which the remaining tissue was mechanically manipulated at 15 min intervals. After 90 min, the supernatant containing dispersed cells was transferred to sterile conical tubes and the cells were washed 5 times with dissociation media lacking the digestive enzymes. After the final wash, cells were resuspended into pituitary culture media; DMEM without phenol red supplemented with 4.5 mg/ml glucose, 30% cs-FBS, 1% non-essential amino acids, 100 IU/ml Penicillin, and 50 μg/ml Streptomycin.

Cell Culture Conditions

All cell cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air. LβT2 cells were cultured in 150 mm dishes and passaged weekly by trypsin dispersion. The LβT2 cells were maintained in DMEM with 4.5 mg/ml glucose, 10% FBS, 100 IU/ml Penicillin, and 50 μg/ml Streptomycin. For experiments, LβT2 cells from a cell passage number of less than 30 were used as Campbell et al. (2002) demonstrated the potential for large declines in both membrane and intracellular ER numbers with increased passage number of cultured tumor-derived cells.

Measurement of LH Secretion from Cultured Cells

For evaluation of LH secretion, anterior pituitary cells were seeded into 24-well dishes at a density of 0.5 x 10^6 cells/well and LβT2 cells were seeded into 6-well dishes

at a density of 2 x 10^6 cells/well. Three days prior to the experiment, cells were primed with pulses of GnRH as described by Turgeon et al. (1996) and depicted in Figure 7. Day of plating was designated day 0. After approximately 24 hours, cells were placed into incubation medium for 30 min. Phenol-red free DMEM was used in experiments because phenol-red has been shown to have weak estrogenic properties (Berthois, et al., 1986). Incubation media contained a similar percentage of serum and antibiotics as the culture media for anterior pituitary cells and LβT2 cells except that the phenol-red free DMEM was supplemented with cs-FBS and 0.2 nM E_2. After 30 min, medium was replaced with GnRH-containing incubation medium (2 nM for pituitary cells, 10 nM for LβT2 cells). The 15 min GnRH incubation was followed by removal and replacement with fresh incubation medium and incubation was continued for 75 min. The treatment was repeated three more times for a total of four pulses of GnRH. Following the last exposure to GnRH, cells were incubated 18 hours in incubation medium. The regimen was replicated on days 2 and 3 with exception that on day 3 subsequent to the four GnRH pulses, cells were cultured in incubation medium lacking E_2. Immediately prior to an experiment, cells were washed twice with prewarmed phosphate buffered saline (PBS; 150 mM NaCl, 10 mM $NaPO_4$, pH 7.4). Anterior pituitary and LβT2 cells were challenged with GnRH in the presence or absence of E_2 for 30 min and medium collected. We chose to use a 10 nM concentration of E_2 and an equivalent dose for other estrogens for the remaining experiments; this was the minimal dose required for maximal inhibition of GnRH-stimulated secretion of LH in the cultured pituitary cells (Arreguin and Nett, 2002). Estradiol was dissolved in 100% ethanol at a 10 μM concentration and diluted 1000-fold in experimental medium. The ethanol concentration used as control

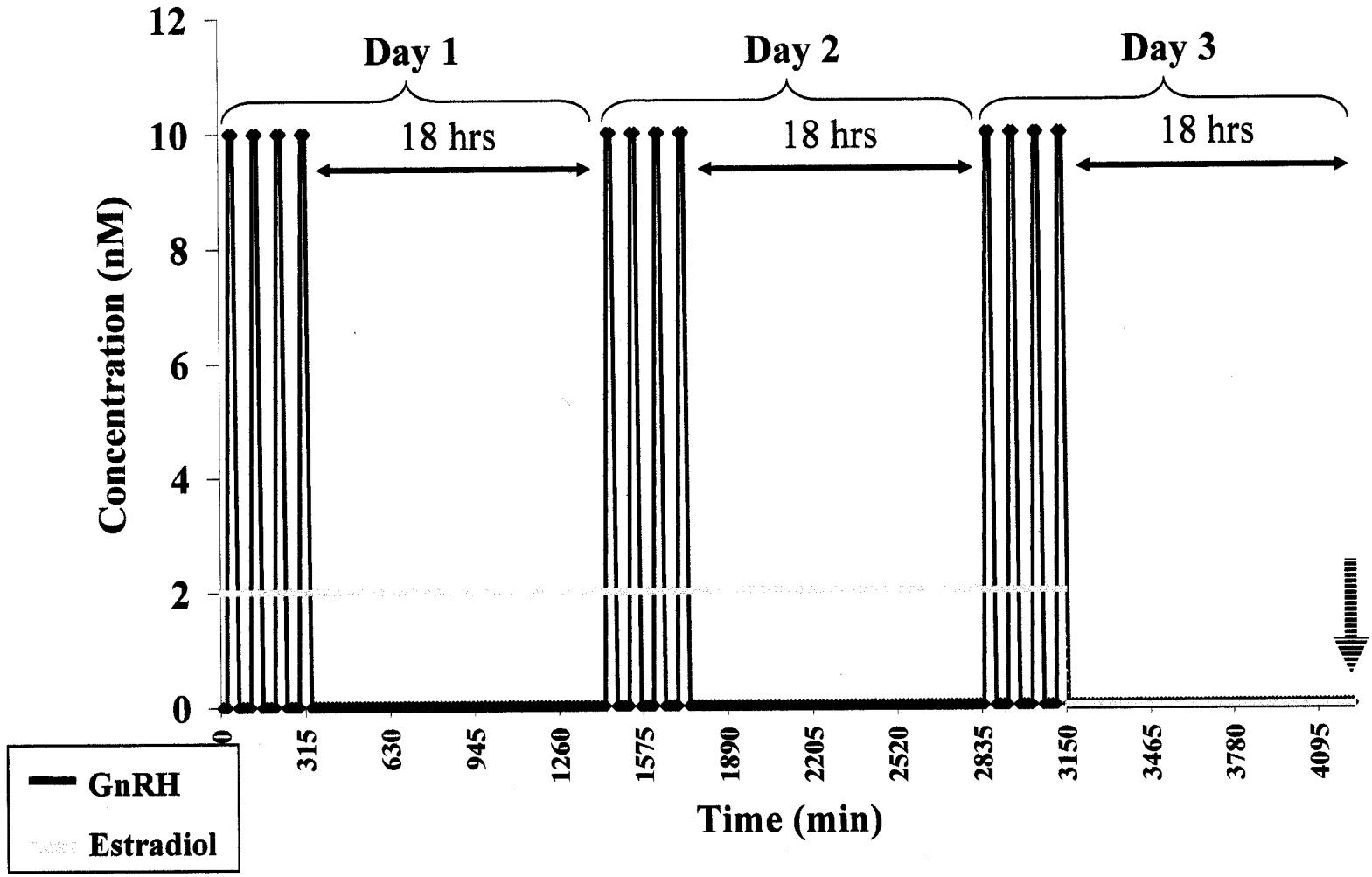

Figure 7: Graphic representation of experimental protocol for pulsing cultured cells with GnRH. Presence or absence of either 2 nM GnRH or 0.2 nM E_2 are designated by black line and grey line, respectively. Pulse procedure was initiated at the same time each day, approximately 18 hours following last 15 min pulse of GnRH of the previous day. Large dashed arrow indicates time of experiment following final pulse on Day 3.

was 0.1%. Media samples were stored at -20° C until the LH content was quantified by radioimmunoassay (RIA) as described for ovine (Niswender, et al., 1969) and rodent LH (Niswender, et al., 1968).

Evaluation of GnRH Receptor Numbers

Anterior pituitary cells were plated onto 6 well dishes at a density of 5 x 10^6 cells/well and were cultured for 48 hours in pituitary culture medium. Twelve hours prior to GnRH receptor assay, medium was replaced with fresh culture medium containing either 0.01% ethanol or 4 nM E_2. Cells were washed four times with room temperature PBS prior to removal from wells using a rubber policeman. Wells were bathed with PBS twice and washes pooled with removed cells. Cell were centrifuged at 3,000 x g for 10 min and the resulting pellets resuspended in ice-cold GnRH-R assay buffer (10 μM Tris, 100 nM $CaCl_2$, 0.1% BSA, pH 7.4). Approximately 1 x 10^6 cells from individual wells were aliquoted into plastic 12 x 75mm tubes to which ice-cold GnRH-R assay buffer and 5 x 10^4 cpm of radiolabeled GnRH agonist (15.3 fmol) were added for a final volume of 250 μl. D-Ala^6-desGly^{10}-GnRH-Pro^9-ethylamide ([D-Ala^6]GnRH) was radioiodinated using a glucose-oxidase procedure and reaction products were separated on QAE Sephadex (Sigma-Aldrich) column as described by Wagner et al. (1979). Non-specific binding was determined in the presence of 21.1 pmol unlabeled [D-Ala^6]GnRH. Tubes were vortexed for 10 sec preceding 4 hr incubation at 4°C. Following incubation ice-cold GnRH-R assay buffer (3 ml) was added and tubes centrifuged at 16, 000 x g for 15 min at 4°C. Unbound ^{125}I-[D-Ala^6]GnRH was decanted with the supernatant and bound radioiodinated GnRH was quantified in the remaining pellet. Femtomoles of GnRH-R per 1 x 10^6 cells were calculated for each experiment.

Sample Preparation and Western Blot Analysis

Proteins were extracted from adherent cells using an SDS-extraction protocol as previously described (Meberg and Bamburg, 2000). LβT2 and αT3-1 cells were seeded on 150 mm dishes to achieve 80% confluency. LβT2 cells were subjected to pulses of GnRH as previously described for secretion studies. On the day of extraction, cells were washed quickly 4 times with ice-cold PBS prior to addition of SDS lysis buffer (2% SDS, 10 mM Tris, pH 7.5, 10 mM sodium floride, 5 mM dithiothreitol (DTT), 2mM EGTA). Cells were then harvested from plates, boiled for 3-5 min and sonicated briefly. The protein concentration was determined using Coomassie Plus (Bradford) protein assay (Pierce) and SDS-extracts were stored at -70° C until western analysis.

Proteins from SDS-extracts were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE; 12% acrylamide) and electrophoretically transferred to nitrocellulose using a Bio-Rad Mini Trans-Blot apparatus (Bio-Rad Laboratories, Inc; Hercules, CA). ChemiBlot molecular weight markers were used to estimate size of immunostained protein bands. Membranes were blocked with 5% non-fat dried milk (NFM) in PBS with 0.1% Tween-20 (PBST) for 1 h at room temperature. Then, membranes were first probed overnight at 4° C with rabbit anti-mouse ERα diluted to 40ng/ml in 5% NFM in PBST. Following overnight incubation, membranes were rinsed with PBST 3 times and probed with the secondary antibody, goat anti-rabbit IgG-HRP diluted 1:2,500 (final concentration 4 ng/ml) in 5% NFM/PBST for 2 h at room temperature. Antibody stained bands were visualized with chemiluminescent substrate (SuperSignal West Dura Extended Duration Substrate) and images of the autoradiograms were scanned into a computer file. Scion Image (NIH Image equivalent for Windows;

Scion Corp.; Frederick, MD) was used to analyze the area of protein bands. Membranes were re-probed with rabbit anti-GAPDH antibody diluted 1:5,000 in 5% NFM/PBST overnight at 4° C after stripping with western blot stripping buffer (62.5 mM Tris, pH 6.8, 2 % SDS, 0.7 % 2-mercaptoethanol). GAPDH protein was used to assess consistency of protein loading onto the polyacrylamide gel.

Statistical Analysis

Anterior pituitaries were kept separate and each used for a single experiment. Treatment means and standard error of the mean (SEM) were calculated from a minimum of three experiments consisting of triplicate samples for each treatment. Control group (cells treated with 0.1% ethanol only) was used to normalize data and to obtain fold differences (control = 1.0). Data from studies on anterior pituitary cells was subjected to examination by Tukey's multiple comparison test. Data from studies on LβT2 cells was subjected to examination by analysis of variance (ANOVA) using General Linear Model (GLM) procedure of SAS (SAS Institute, Inc; Cary, NC). When significant F-values were found, differences among treatments were separated using Least Significant Differences (LSD) tests. In all cases, $P \leq 0.05$ was considered significant.

C. Results

GnRH-Stimulated LH Secretion During Non-Breeding Season

Estradiol exerts both positive and negative effects on LH secretion at the level of the hypothalamus. Seasonal breeders, such as sheep, enter a period of infertility when females are more sensitive to E_2 negative feedback (February through August). Changes in photoperiod are responsible for this alteration in reproductive activity. Cells from

pituitary glands collected during this period responded to GnRH with an increase in LH secretion above basal levels (Fig. 8, upper panel). Removing pituitary cells from the suppressive influence of the hypothalamus for three days and subjecting the cells to GnRH pulses in the presence of E_2, eliminated some of the variability seen previously in responsiveness to GnRH. Interestingly as seen in Figure 8, upper panel, it appears that ovine pituitary cells collected from ewes during the non-breeding season lack the non-genomic response to E_2 observed previously in cultured pituitary cells collected from ewes during the breeding season (Arreguin and Nett, 2002). To assess whether anestrous pituitary cells are also deficient in genomic responsiveness to E_2, we examined changes in numbers of GnRH receptors in response to 12 hr treatment with E_2. Estradiol stimulated an increase in expression of GnRH receptors (Fig. 8, lower panel).

Estradiol's Acute Effects on LH Secretion in Cultured Pituitary Cells

Preliminary research on OVX ewes administered E_2 demonstrated a rapid suppression of GnRH-evoked LH secretion (Arreguin and Nett, 2003). The first aim was to determine whether cultured ovine pituitary cells pulsed with GnRH were capable of responding to E_2 as previously observed *in vivo* and *in vitro*. We expected the GnRH pulse regimen to be optimal for secretion studies by culturing the cells using a procedure to model more closely physiological GnRH and E_2 conditions. The concentration of LH in medium from anterior pituitary cells was elevated above control with treatment of 2 nM GnRH (Fig. 9). As seen in Figure 9, E_2 inhibits GnRH-induced secretion of LH yet not to control levels as observed previously (Arreguin and Nett, 2002).

Estradiol Fails to Inhibit GnRH-induced LH Secretion in LβT2 Cells

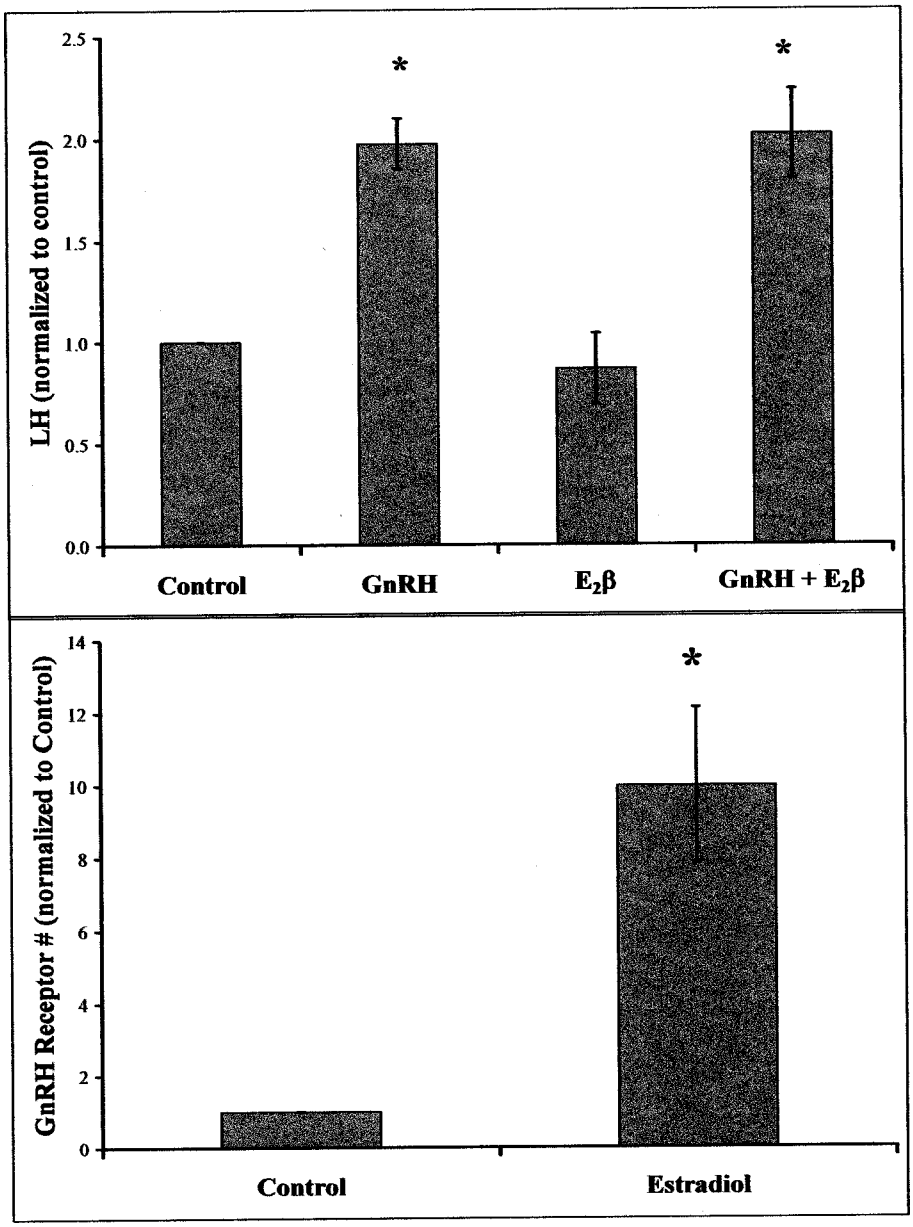

Figure 8: Upper panel –LH content of medium from cultured ovine pituitary cells from anestrous ewes during a 30 min incubation. Lower panel – Using radioiodinated [D-Ala^6]GnRH, we determined GnRH receptor numbers in cultured pituitary cells treated for 12 hr with either 0.1% ethanol or 4 nM E_2. Data are presented as mean ± SEM of averaged fold difference (control = 1.0) for three experiments. Data were analyzed by Tukey's multiple comparison test and means identified with * are different from control ($P< 0.05$).

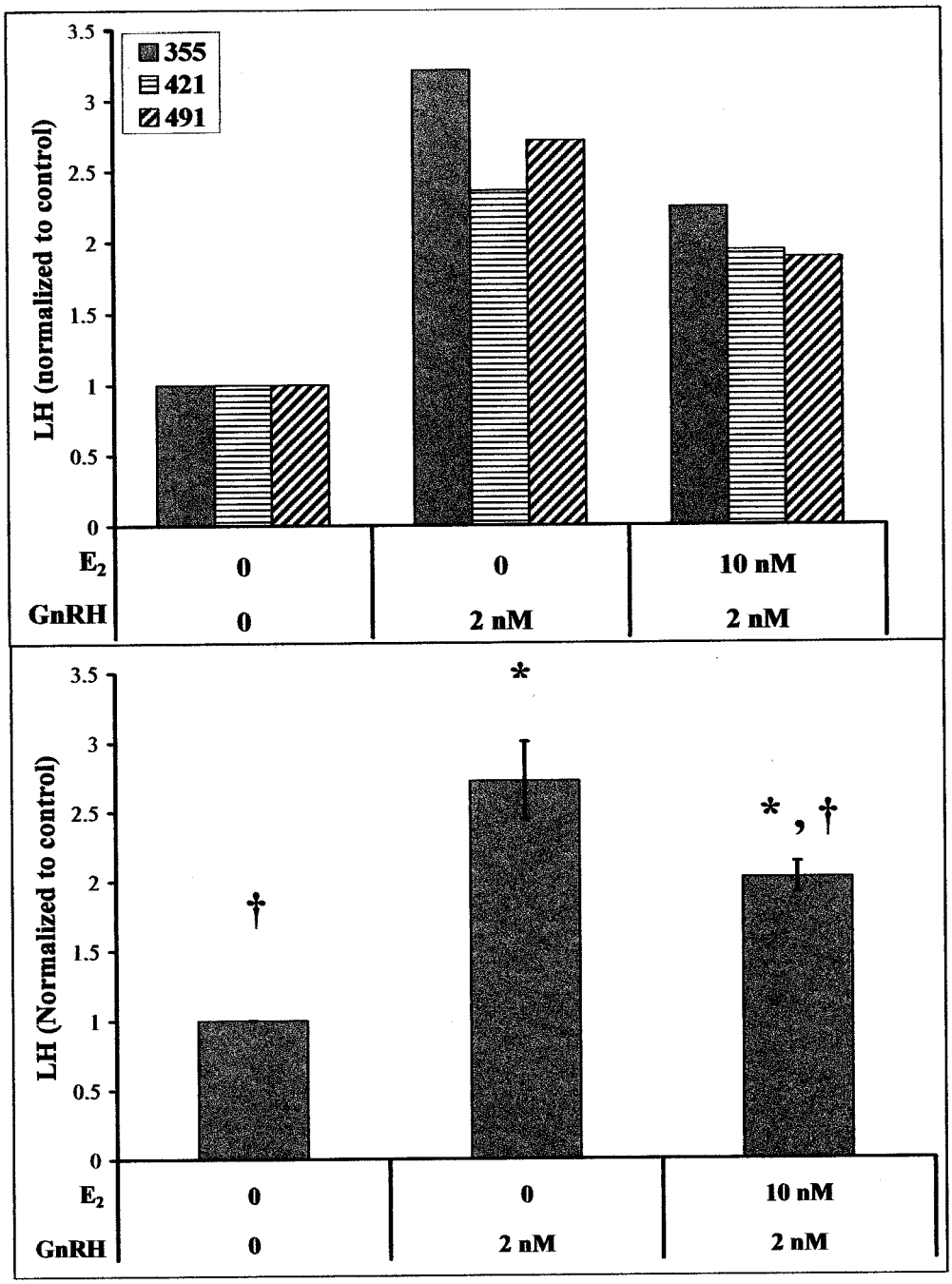

Figure 9: Medium concentrations of LH from cultured ovine pituitary cells treated with 2 nM GnRH in the presence or absence of 10 nM E_2. Means of fold changes were calculated within experiment (upper panel) and averaged (lower panel). Legend in upper panel depicts animal number from which the pituitary was collected. Averaged fold-differences that are different from 0.1% ethanol control and GnRH alone are denoted with * and †, respectively ($P< 0.01$).

Ideally, all our experiments would have used dissociated anterior pituitary cells to determine effects of E2 on gonadotropes. Unfortunately, only ~10% of anterior pituitary cells are gonadotropes and the other cells of the pituitary potentially contain membrane estrogen receptors in addition to similar signal transduction machinery. Methods for isolating the gonadotropes from the anterior pituitary cell population are difficult and unreliable. Therefore, we employed the mouse differentiated gonadotrope cell line LβT2 to investigate the acute effects of E2 on GnRH-induced release of LH. LβT2 cells express the glycoprotein hormone α subunit and the LH β subunit as well as GnRH receptors. Additionally, LβT2 cells not only secrete LH in response to the GnRH stimulus, but also respond to challenge with GnRH with a 2-fold increase in intracellular calcium (Thomas, et al., 1996). We wanted to determine if LH secretion from LβT2 cells would be altered similarly to that seen in anterior pituitary cells. Similar to cultured pituitary cells, we observed an approximate 2.5-fold difference between control and GnRH treatment ($P < 0.001$) (Fig. 10). Inclusion of E2 with the GnRH treatment failed to inhibit GnRH-stimulated LH secretion from LβT2 cells as seen previously with cultured ovine pituitary cells.

Western Analysis for ERα

While examining the potential reasons for the lack of the negative response of E_2 we found that LβT2 cells express very low levels of ERα (Fig. 11). We included protein extracts from αT3-1 cells in our protein blots as a positive control. It has been reported that the anterior pituitary gland contains greater amounts of mRNA for and numbers of

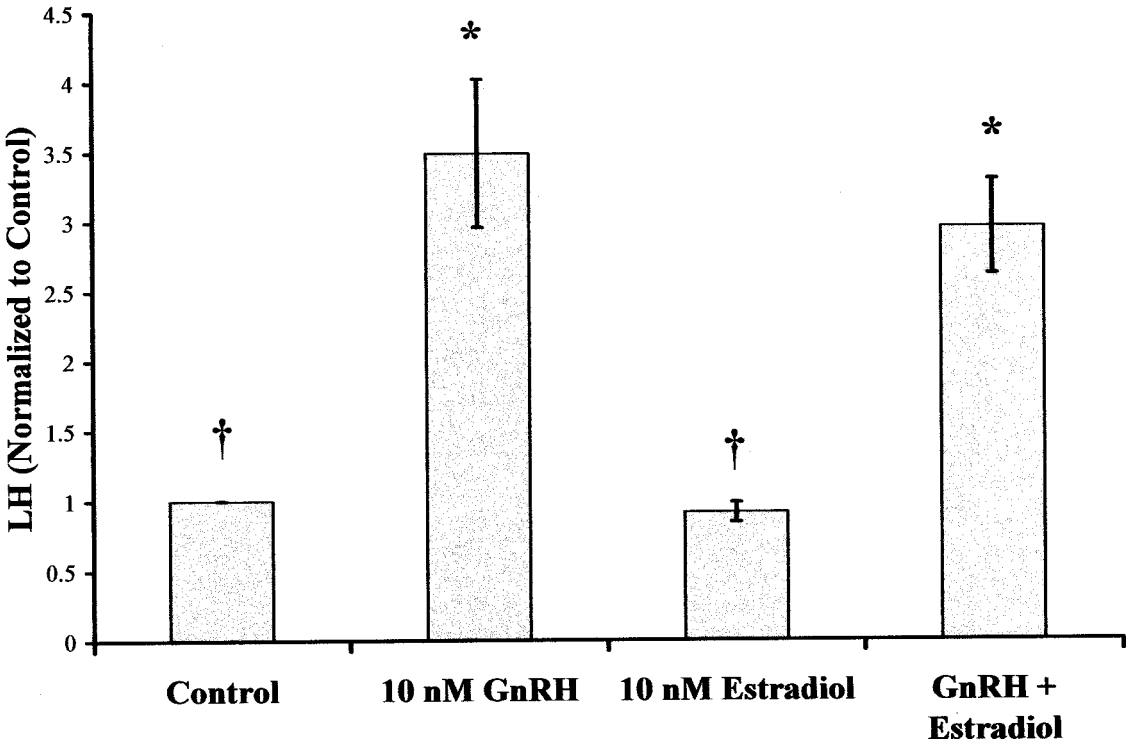

Figure 10: Acute effects of E_2 on LH release from LβT2 cells. Means that are different from solvent control and GnRH alone are denoted with * and †, respectively (n = 5 and $P < 0.05$).

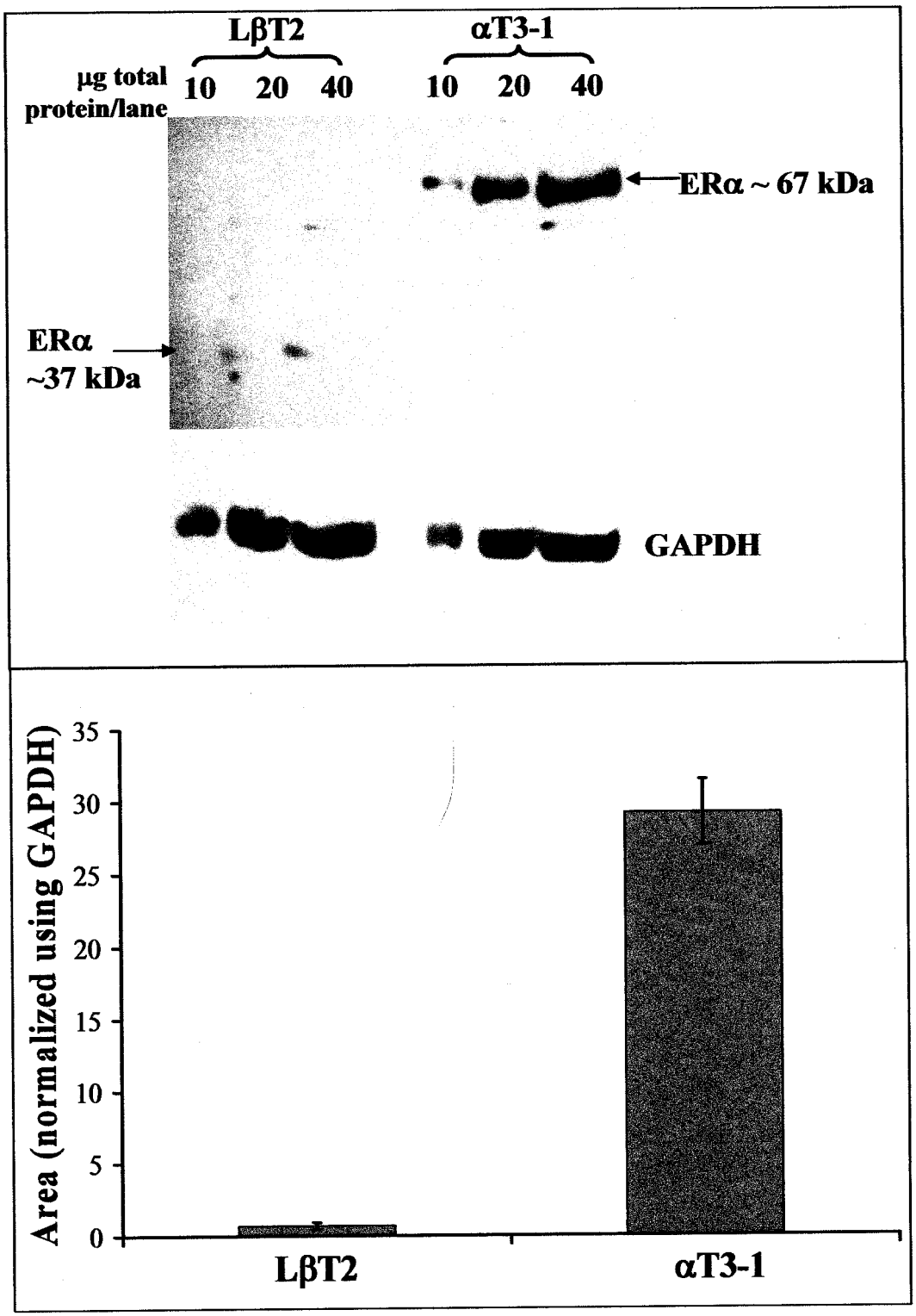

Figure 11: Upper panel – Western blots for ERα and GAPDH from extracts of αT3-1 and LβT2 cells. Lower panel – relative amounts of full-length ERα found in each cell type. Statistical analysis was not performed on this data due to small sample size.

ERα than ERβ (Kuiper, et al., 1997; Mitchner, et al., 1998; Shughrue, et al., 1998; Wilson, et al., 1998).

D. Discussion

We began our research trying to use dispersed anterior pituitary cells to model the non-genomic effects of E_2 on release of LH. Several studies have demonstrated effects of E_2 on cultured pituitary cells (Emons, et al., 1988; Henderson, et al., 1989; Misiti, et al., 1998; Nett, et al., 2002). In this study we established that cultured ovine pituitary cells collected from OVX ewes during the non-breeding season lack responsiveness to acute inhibition of E_2 on GnRH induced release of LH. The lack of an inhibitory effect of E_2 during the non-breeding season in cultured pituitary cells is not unique to the acute affects of E_2. It has been demonstrated in various studies that gonadotropin surge-attenuating factor (GnSAF, a hormone produced by the developing follicle after stimulation by FSH) suppresses LH secretion induced by GnRH *in vitro*. Fowler et al. (1992) determined that human GnSAF has effect on GnRH-stimulated secretion of LH from cultured ovine pituitary cells collected during the breeding season but not during anestrus . In addition, Glass et al. (1984) found that in both rams and ewes, there were more ER in the anterior pituitary gland during the breeding season than during anestrus. In light of these data, we decided to conduct our studies using cultured pituitary cells during the breeding season. Pituitary cells collected during the breeding season respond to GnRH with an increase in release of LH. As seen in Figure 9, E_2 inhibited GnRH-induced secretion of LH, although not to the same extent as observed previously (Arreguin and Nett, 2002).

Our ultimate goal is to characterize the specific events involved in the rapid suppression of LH secretion by E_2. Gonadotropes comprise only a small percentage of cells in the anterior pituitary gland and a convenient, efficacious method to purify gonadotropes has yet to be developed. Thus, it is difficult to use cultured pituitary cells to evaluate subcellular mechanisms mediating LH release. Therefore, we turned to LβT2 cells to evaluate the subcellular mechanism responsible for this non-genomic effect of E_2. Although these gonadotrope derived cells respond to GnRH with increased LH secretion, we failed to observe the negative effects of E_2 on LH secretion as demonstrated in the cultured pituitary cells (Fig. 10).

We demonstrated that LβT2 cells express very low levels of ERα in contrast to αT3-1 cells, another immortalized gonadotrope cell line (Fig. 11). This is inconsistent with studies on pituitaries collected from adult animals, which report levels of mRNA for and numbers of ERβ are significantly lower than ERα (Mitchner, et al., 1998; Shughrue, et al., 1998). Studies using ERα null mice revealed that females require ERα for normal regulation of the hypothalamic-pituitary gonadal axis and release of LH (review by Couse and Korach, 1999; review byHewitt and Korach, 2003; review by Rissman, et al., 1997). We postulated that if expression of ERα in LβT2 cells was increased we could recover the acute effects of E_2 on LH secretion observed in cultured ovine pituitary cells. To examine this hypothesis, we stably transfected LβT2 cells with cDNA for murine ERα and evaluated the effects of E_2 on GnRH-induced secretion of LH (Chapter III).

CHAPTER III: DEVELOPMENT OF A MODEL TO STUDY ESTRADIOL'S ACUTE EFFECTS ON GONADOTROPES

A. Introduction

In the past decade, it has been demonstrated that E_2 and estrogenic compounds have non-genomic effects on various physiological systems. We have recently begun novel studies on the non-genomic effects of E_2 on GnRH-controlled gonadotropin secretion. Our current in *vivo* and *in vitro* research suggests that E_2 negatively affects LH secretion via a non-genomic effect expressed at the level of the anterior pituitary gland (Arreguin and Nett, 2002, 2003). We have effectively used cultured pituitary cells to evaluate rapid effects of estradiol on exocytosis of LH. The heterogeneity of the pituitary cell population and the limited timeframe which pituitary cells can be used during the year complicates studies on intracellular mechanisms affected by acute treatment with E_2. Until a convenient method to purify gonadotropes from anterior pituitary glands is developed, unraveling the multiple regulatory mechanisms involved in suppressing LH secretion requires development of a reliable cell culture system.

The established gonadotrope-derived cell lines, αT3-1 and LβT2, cannot be used to assess the immediate signaling pathways affected by E_2 to inhibit LH secretion induced by GnRH. Created by targeted tumorgenesis using the human glycoprotein α-subunit, αT3-1 cells lack expression of the glycoprotein β-subunits. Using similar

techniques substituting the rat LHβ promoter to target oncogene expression, Mellon et al. (1991) created the LβT2 cell line, which expresses both LHβ and FSHβ subunits. Unfortunately, we have demonstrated that E_2 fails to attenuate GnRH-induced LH secretion in LβT2 cells. This appears to be due to the fact that LβT2 cells express very low levels of ERα, certainly much less than αT3-1 cells. Based on these findings, we theorized that if we increased the expression of ERα in LβT2 cells, we would be able to regain the negative effects of E_2 on LH release observed in cultured pituitary cells. The intention of these studies was to evaluate the responsiveness of LβT2 cells stably transfected with murine ERα cDNA to simultaneous treatment with E_2 and GnRH.

B. Materials and Methods

Reagents

Luciferase reporter vector (PGL_2), and pBK-RSV expression vector were purchased from Promega (Madison, WI) and Stratagene (La Jolla, CA), respectively. Murine estrogen receptor-α (ERα) cDNA, estrogen response element-luciferase (ERE-luc) expression vector, and β-galactosidase expression vector (CMV-βgal) were generous gifts from Dr. Malcolm G. Parker (Institute of Reproductive and Developmental Biology, Imperial College London, London, United Kingdom), Dr. Colin M. Clay (Colorado State University, Fort Collins, CO), and Dr. Gordon W. Niswender (Colorado State University), respectively. Reagents for β-galactosidase and luciferase assays were purchased from Applied Biosystems (formerly Tropix; Foster City, CA) and Promega. Cortisol, testosterone, progesterone, 17α-estradiol and other chemicals were purchased

from Sigma-Aldrich (St. Louis, MO). Estradiol-6-(O-carboxymethyl)oxime:BSA (E-BSA) was procured from Steraloids, Inc (Newport, Rhode Island). Following receipt, E-BSA was dissolved in PBS and extracted 4 times with diethyl ether and dialyzed to remove the 3-5% unconjugated E_2 contamination (Stevis, et al., 1999; Taguchi, et al., 2004). Propyl pyrazoletriol (PPT) and diarylpropionitrile (DPN) were purchased from Tocris Cookson, Inc (Ellisville, MO).

Construction of Murine ERα Clone for Transfection

The cloned mouse ERα cDNA (White, et al., 1987) was supplied in a SV40-based expression vector without selectable antibiotic resistance (pSG5; Stratagene) and was altered for stable transfection. The eukaryotic selectable expression plasmid containing the cDNA for ERα was created by digesting the SG5-ER plasmid with EcoRI and subcloning into pBK-RSV vector, which had been altered such that it no longer contained the *lacZ* promoter or the endogenous start codon (RSV^{-lac}). Plasmid DNA was prepared from overnight bacterial cultures using DNA plasmid columns according to the suppliers's protocol (Qiagen; Valencia, CA). Clones were screened for correct orientation using BglII and BamHI restriction sites.

Transfections and Cell Culture

Chinese hamster ovary tumors cells (CHOK1) were co-transfected with RSV-ERα clones and ERE-luc plasmid to assay relative clone functionality. CHOK1 cells normally do not express either ERα or ERβ proteins. Using Polyfect Reagent (Qiagen) according to manufacturer instructions, CHOK1 cells were co-transfected with 0.25 μg CMV-βgal and either 1 μg of RSV-ERα or RSV^{-lac} plasmids plus 0.5 μg of either ERE-

luc or PGL_2. Following an overnight incubation, medium was replaced with culture medium (DMEM supplemented with 4.5 mg/ml glucose, 10% FBS, 100 IU/ml Penicillin, and 50 μg/ml Streptomycin) lacking phenol red and serum. Cells were incubated an additional 24 h prior to treatment with either 10 nM E_2 or 0.1% ethanol in fresh medium. Cells were harvested 18 h later for β-galactosidase and luciferase assays. The luciferase activity was normalized to β-galactosidase control.

Calcium phosphate precipitation transfection was performed as described previously by Vasilyev et al. (2002). LβT2 cells were plated into 6-well dishes at a density of 0.5 x 10^6 cells/well. On the following day, cells were transfected for 16 h with 1 μg/well RSV-ERα DNA that was precipitated in sterile H_2O with 0.25 M $CaCl_2$ and buffered with HBS buffer (135 mM NaCl, 4 mM KCl. 1 mM Na_2HPO_4, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM Glucose, 20 mM HEPES, pH 7.0). After incubation, cells were subjected to glycerol shock (10% glycerol in PBS for 90 sec) and washed twice with prewarmed phosphate buffered saline (PBS; 150 mM NaCl, 10 mM $NaPO_4$, pH 7.4). Cells were incubated overnight in fresh culture medium (DMEM with 4.5 mg/ml glucose, 10% FBS, 100 IU/ml Penicillin, and 50 μg/ml Streptomycin). Cells were detached by trypsin dispersion and transferred to 100 mm dishes containing selection medium (culture medium plus 500 μg/ml G418 sulfate). G418 sulfate is an aminoglycoside used as a selection agent for transfected mammalian cells. Resistance is conferred by the bacterial gene for aminoglycoside-3'- phosphotransferase in the pBK-RSV vector that can be expressed in eukaryotic cells. Cells expressing ERα (LβT2 -ER) were expanded and maintained in selection medium at 37°C in a humidified atmosphere of 5% CO_2 in air. LβT2-ER cells were cultured in 150 mm dishes and passaged weekly by trypsin

dispersion. For reasons discussed in chapter II, we used only LβT2-ER cells from a passage number of less than 30 for experiments.

Sample Preparation and Western Blot Analysis

Proteins were extracted from LβT2 and LβT2-ER cells using SDS-extraction protocol and subjected to Western analysis as previously described in Chapter II. Similar to experiments in Chapter II, membranes were probed with anti-ERα and anti-GAPDH primary antibodies to evaluate relative expression.

Measurement of LH Secretion

For evaluation of LH secretion, LβT2-ER cells were seeded into 6-well dishes at a density of 2 x 10^6 cells/well. Cells were challenged with GnRH at four pulses/day on days 1-3 using the same protocol as described in Chapter II. Immediately prior to an experiment, cells were washed twice with prewarmed PBS. LβT2-ER cells were exposed to 10 nM GnRH in the presence or absence of either steroid, E-BSA or an ER isoform specific agonist for 30 min and media were collected. Steroids were dissolved in 100% ethanol at a 10 μM concentration and diluted 1000-fold in experimental medium. The ethanol concentration used as solvent control was 0.1%. Media samples were stored at -20° C until assayed for LH content by RIA.

Statistical Procedures

Luciferase to β-galactosidase ratios were averaged from three experiments with triplicate wells used within an experiment to calculate individual group ratio. For multiple comparisons across transfected wells, ratios were evaluated by ANOVA using

GLM procedure of SAS and significant differences among ratios were determined using Tukey's multiple comparison test. In addition, Student's *t* test was employed to determine the specific *P* value for the single comparison of treated versus untreated of the RSV-ERα and ERE-luc transfection. For secretion studies, mean of fold difference and standard error of the mean (SEM) were calculated from a minimum of three experiments consisting of triplicate samples for each treatment. Solvent control was used to normalize secretion data and to obtain fold changes (control = 1.0). A Fisher's protected LSD test was used to identify significant differences among treatments. In all cases, $P \leq 0.05$ was considered significant.

C. Results

Relative Activity of the RSV-ER Clone

CHOK1 cells originate from a Chinese hamster ovarian tumor and normally are devoid of ER. Razandi et al. (1999) used this cell line to demonstrate that functional membrane and nuclear estrogen receptors may arise from a single transcript. The ERE-luc construct consists of two copies of the Xenopus vitellogenin estrogen response element (5' AGGTCACAGTGACCT 3') inserted upstream of the thymidine kinase minimal promoter and luciferase gene (Duval, et al., 2000). In the presence of a biologically active ER and E_2, ERE-luc plasmid is stimulated resulting an expression of luciferase (Duval, et al., 2000). Co-transfection with any of the following combinations does not result in stimulation of luciferase expression (Fig. 12): 1) RSV^{-lacZ} and ERE-luc constructs; 2) RSV-ERα and PGL_2 plasmids or 3) RSV-ERα and ERE-luc cDNA. However, inclusion of E_2 in culture medium increased luciferase expression 3.3-

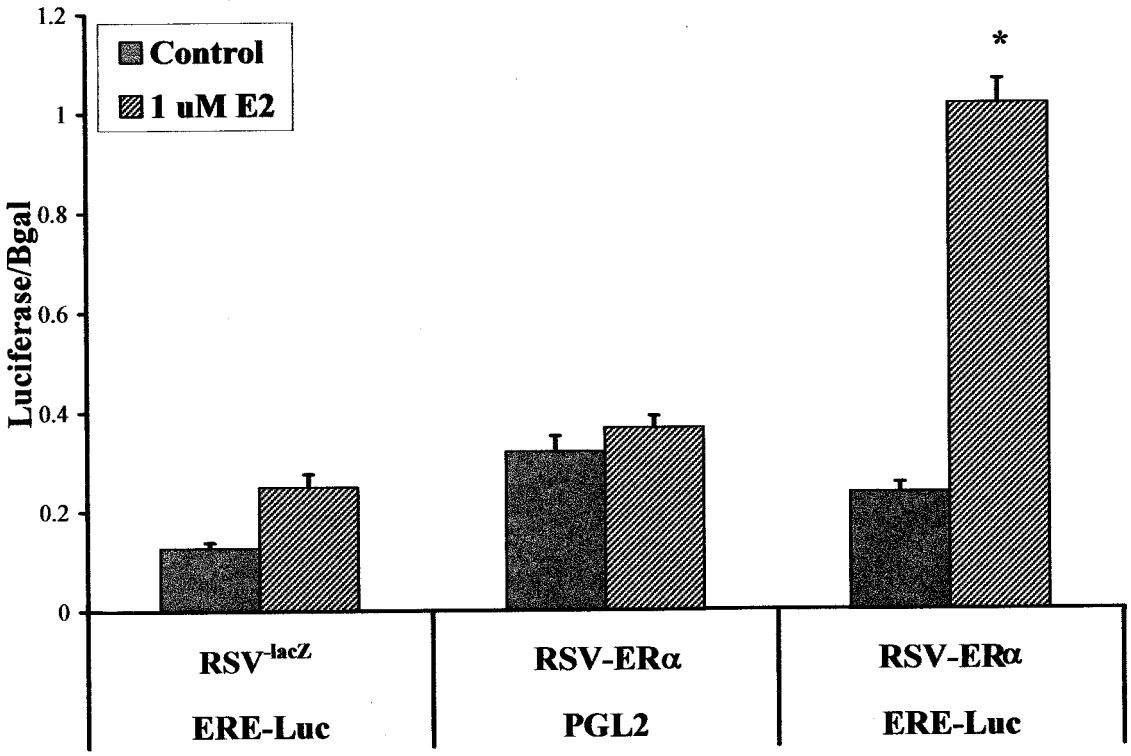

Figure 12: Stimulation of ERE-luciferase plasmid in CHOK1 cells transfected with RSV-ERα construct. Means designated with * differ from untreated; comparisons only done within transfection group (n = 3; $P < 0.0001$).

fold above untreated cells for the RSV-ERα and ERE-luc co-transfection only (Fig 12; P < 0.0001).

ERα Protein Expression in LβT2 Cells Transfected with ERα cDNA

The concentration of G418 sulfate used for selecting and maintaining LβT2-ER cells was sufficient to kill 99.9% of cells not expressing the RSV-ERα construct. To determine if LβT2-ER cells express more ERα than the parent cell line, we evaluated relative expression of protein using western blot analysis. We found expression levels of ERα to be greater in LβT2-ER than found for LβT2 parent cell line (Fig. 13). Multiple specific ERα bands were detected using the antibody against a peptide mapping to the carboxyl terminus of the mouse ERα. Specificity of detection was checked by pre-absorbing primary antibody with an equivalent weight of peptide prior to incubating with western blots. The full length ERα protein is estimated to be 67 kDa; while the smaller form has been estimated to be 37 kDa. It appears that LβT2 cells express both full length and truncated forms of the ERα and that LβT2-ER express more of the both 67 kDa and 37 kDa versions of ERα protein (Fig. 13, panel B). The smaller form ERα must arise from truncation of receptor at N-terminus given that the primary antibody binds to the C-terminus of the protein.

GnRH-Induced LH Secretion in LβT2-ER Cells

Having increased expression of ERα in LβT2 cells, we examined the ability of E_2 to inhibit GnRH-induced release of LH release in LβT2-ER cells. As expected, GnRH stimulated LH secretion in LβT2-ER cells ($P < 0.001$) (Fig. 14). Unlike the parent LβT2

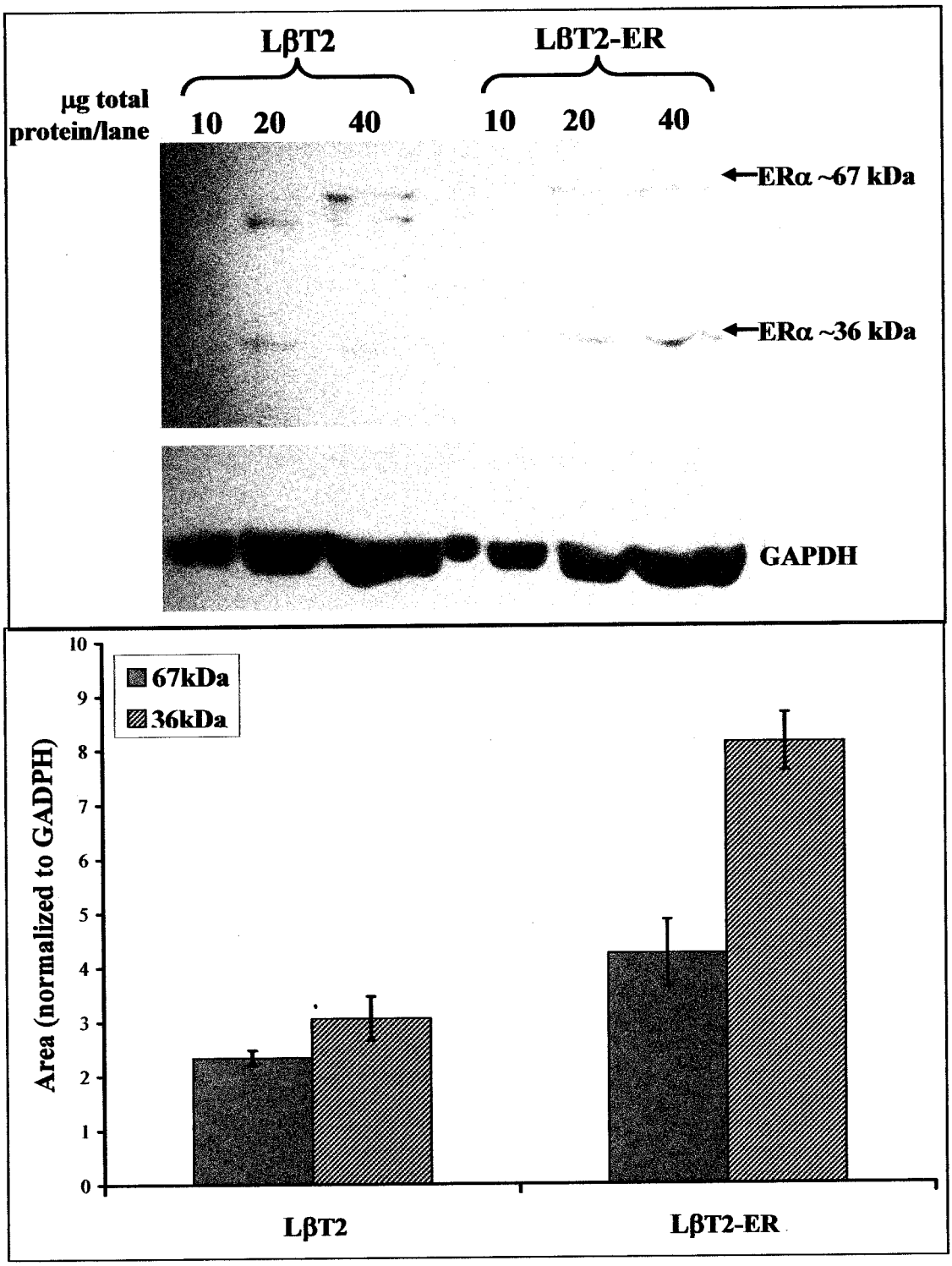

Figure 13: Upper panel – western analysis for ERα and GAPDH from LβT2-ER cells and LβT2 cells. Lower panel – relative amounts of 67 kDa and 36 kDa ERα found in each cell type. Statistical analysis was not performed on this data due to small sample size.

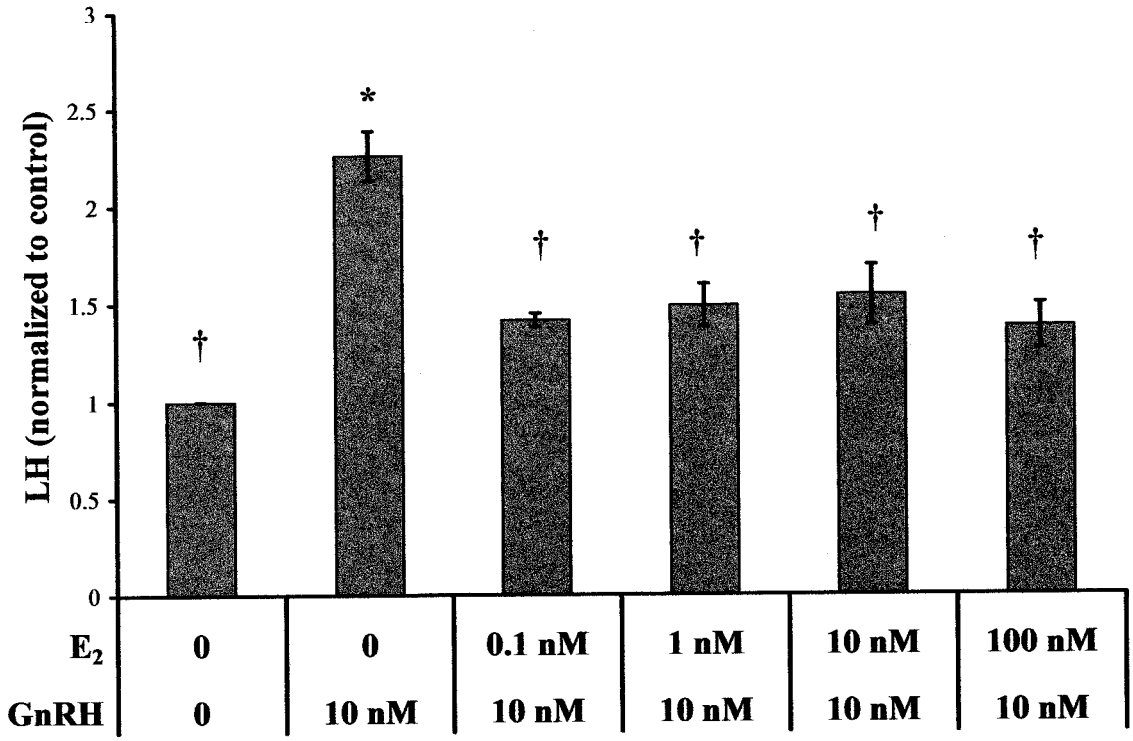

Figure 14: Effect of increasing dose of E2 on LH release in LβT2-ER cells. Fold differences were calculated from three experiments. Statistical analysis was performed comparing control (0.1% ethanol) to 10 nM GnRH to evaluate biological activity of GnRH (difference indicated by *; $P < 0.001$). Means that are different from 10 nM GnRH alone are denoted with † ($P < 0.01$).

cells, inclusion of E_2 with the GnRH treatment suppressed GnRH-stimulated LH secretion in cells stably transfected with the RSV-ERα (Fig. 14; $P < 0.0001$).

Verification of Specificity of Steroid Response

Studies on cultured pituitary cells have been used to evaluate the differential effects of steroids on gonadotropin release. Androgens suppress GnRH-stimulated secretion of FSH and LH in primary pituitary cultures from adult male rats (Campen and Vale, 1988). Progesterone interferes with ability of inhibin to increase GnRH-R numbers and to promote GnRH-stimulated LH secretion, yet progesterone alone has no effect on release of LH (Laws, et al., 1990a). 17α-estradiol is a stereoisomer of E_2 with weak activity at nuclear ER compared to that observed for E_2. In most cases 17α-estradiol is considered to be inactive given that genomic effect of 17α-estradiol upon tissue is negligible compared to E_2. In human subjects treated with 17α-estradiol for three to six weeks, no discernable effects on blood pressure, breast development, menstrual periods, serum liver enzymes, serum proteins, plasma growth hormone, insulin, serum clotting factors, serum triglycerides, serum copper or serum ceruloplasma were observed (Meyer, et al., 1976). In contrast, 17α-estradiol increased skin prolyl hydroxylase activity, increased soluble collagen content in the skin and increased urinary hydroxyproline excretion (Meyer, et al., 1976). In these examples, treatment with steroid lasted longer than 24 hr. Given that acute effects of these steroids on LH release in response to GnRH have yet to be determined, we were unsure what affect they may have on GnRH-evoked

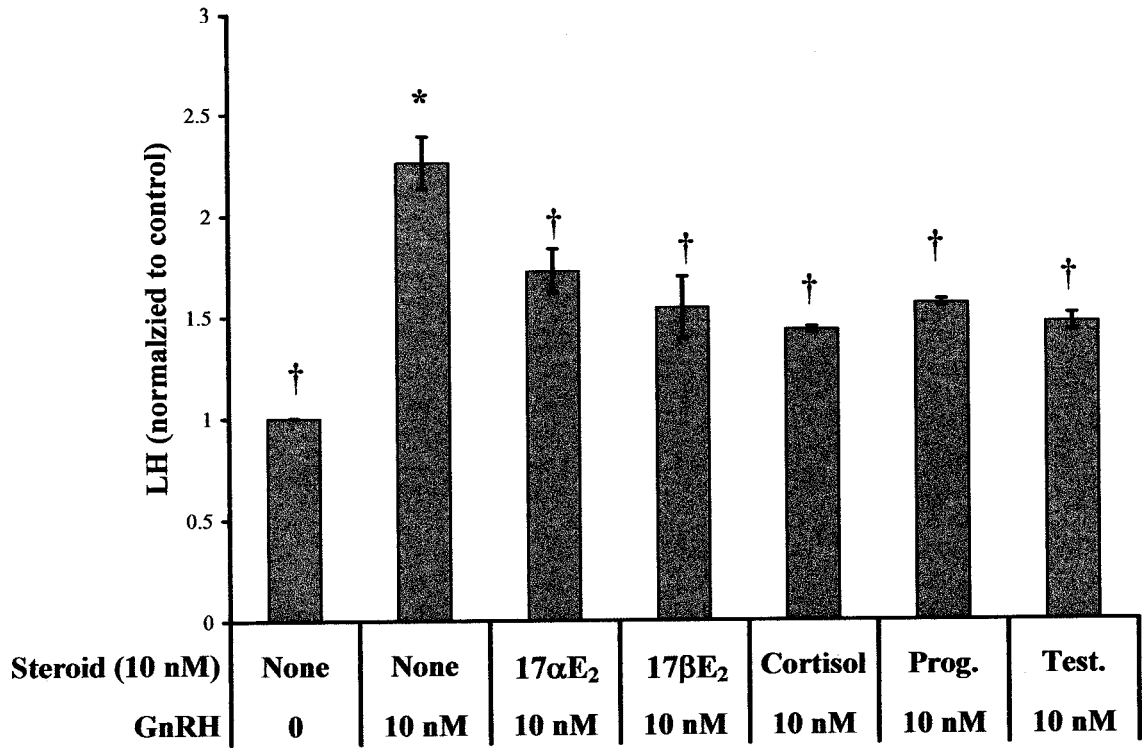

Figure 15: Changes in GnRH-mediated LH release by various steroid compounds in LβT2-ER cells. Fold differences were calculated from three experiments. Statistical analysis was performed comparing control (0.1% ethanol) to 10 nM GnRH to evaluate the effect of various steroid hormones on GnRH induced release of LH (difference indicated by *; $P < 0.001$). Means that are different from 10 nM GnRH alone are denoted with † ($P < 0.01$). Abbreviations: 17α-estradiol as 17αE2, 17β-estradiol as 17βE2, progesterone as Prog. and testosterone as Test.

release of LH. As seen in Figure 15, treatment of GnRH with 17α-estradiol, progesterone, cortisol, and testosterone negatively affected GnRH-mediated secretion of LH.

LH Release in Response to E-BSA and ER Specific Agonists

Given that LβT2-ER cells seem to model gonadotropes due to the fact that they mimic the non-genomic effects of E_2 previously observed *in vivo* and *in vitro*, we deemed it necessary to determine if a membrane impermeable estrogen would also inhibit GnRH-induced secretion of LH. Simultaneous treatment with GnRH and E-BSA resulted in inhibition of LH secretion similar to that observed with E_2 (Fig. 16; $P = 0.0004$). Levels of mRNA for ERα and ERβ are reported to be very low in LβT2 cells (Schreihofer, et al., 2000). Increasing expression of ERα in LβT2 cells resulted in recovery of negative effect of E_2 on GnRH-induced secretion of LH. Hence, ERα appears to be necessary for this non-genomic effect of E_2. Our studies have demonstrated detectable levels of ERβ by western analysis in both LβT2 and LβT2-ER cells; therefore it appears that ERβ does not participate in inhibition of LH secretion by E_2. Using ER specific agonists, we have attempted to evaluate the contributions of ERα and ERβ to exocytosis of LH. We examined the effects of PPT, DPN and a combination of PPT and DPN on GnRH-stimulated LH release using doses equivalent to 10 nM E_2 as determined by their relative binding affinities for ERα and ERβ (Meyers, et al., 2001; Stauffer, et al., 2000). As shown in Figure 17, PPT mimics the effects of E_2 to reduce LH secretion, while DPN has little to no effect on GnRH-induced LH secretion. Combining PPT and DPN with the GnRH treatment results in a loss of PPT's negative effect on LH release (Fig. 17).

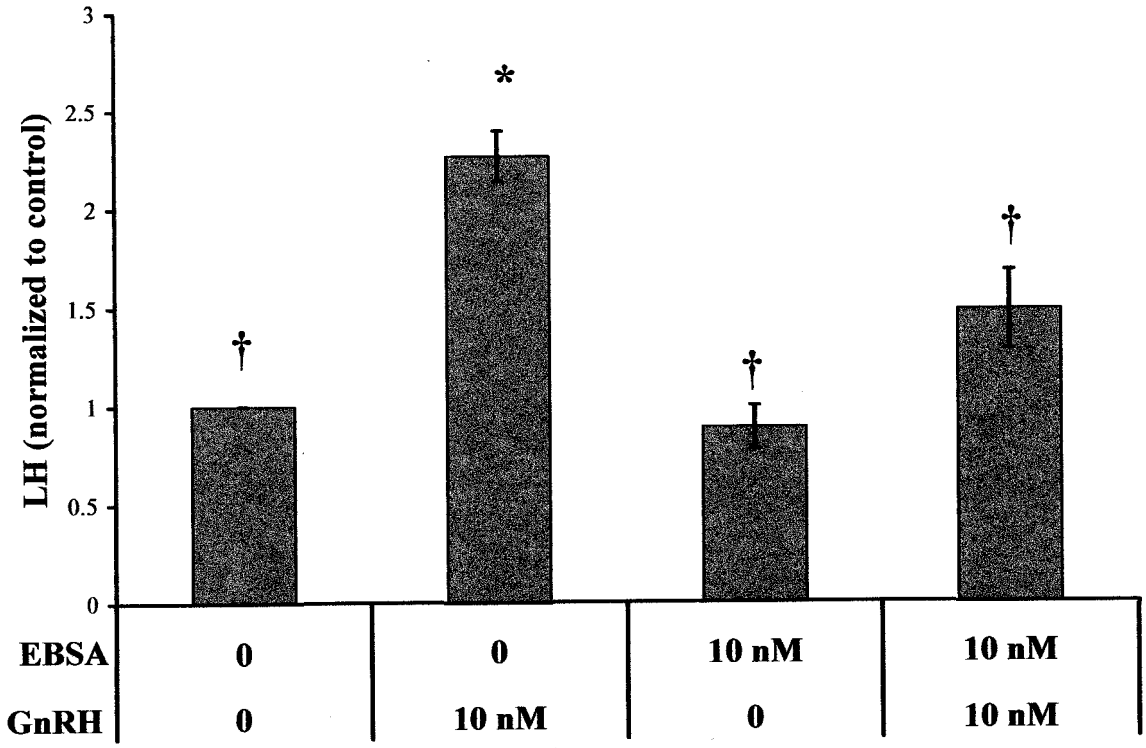

Figure 16: A membrane impermeable form of estradiol, E-BSA, inhibits GnRH-mediated secretion of LH from LβT2-ER cells. Fold differences were calculated from four experiments. Statistical analysis was performed comparing control (0.1% ethanol) to 10 nM GnRH to evaluate the effect of E-BSA on GnRH-induced release of LH (difference indicated by *; $P < 0.001$). Means that are different from 10 nM GnRH alone are denoted with † ($P = 0.0004$).

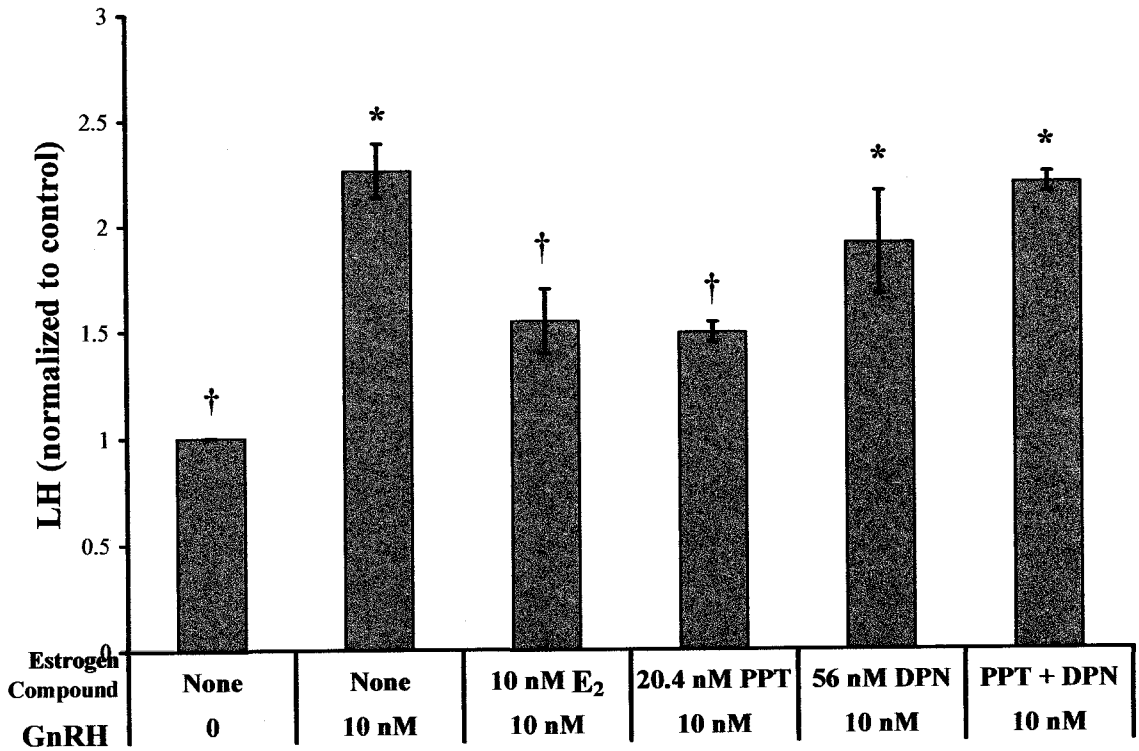

Figure 17: ER specific agonists affect GnRH-stimulated LH secretion in LβT2-ER cells. Fold differences were calculated from three experiments. Statistical analysis was performed comparing control (0.1% ethanol) to 10 nM GnRH to evaluate biological activity of GnRH (difference indicated by *; $P < 0.001$). Means that are different from 10 nM GnRH alone are denoted with † ($P< 0.01$). PPT is an ERα specific agonist and DPN is an ERβ specific agonist.

D. Discussion

Transfecting the full-length cDNA for the murine ERα increased expression of all variations of ERα typically found in LβT2 cells (Fig. 14). We detected at least three forms of ER using the antibody against a peptide mapping to the carboxyl terminus of the mouse ERα. Two variants, one sized 67 kDa and presumed to be the full-length version of ERα and a second sized approximately 36 kDa, possibly a N-terminus truncated adaptation of ER,α were predominate. This was not unexpected as previous immunoblotting for ERα on total protein from rat pituitary detected proteins running at the following approximate molecular weights; 59.2 kDa, 36.8 kDa, 20.5 kDa, and 19.0 kDa (Friend, et al., 1995). ER variants, the result of base pair insertions, transitions and deletions, as well as alternative splicing, were once considered abnormal due to their prevalence in breast cancer. This concept has been altered dramatically as recent research has identified functions for these ERs in the brain as well as in extraneural tissues (Toran-Allerand, 2004).

Additionally, truncated products of ERα have been implicated in membrane-initiated estrogen actions. An amino-terminal truncated product of full-length ERα, ER46, has been identified in the plasma membrane, cytosol, and nucleus of human endothelial cells (Li, et al., 2003). Li et al. (2003) reported that ER46 triggers nitric oxide release via a phosphatidylinositol 3-kinase/Akt/endothelial nitric-oxide synthase pathway more efficiently then full-length ERα. Although a putative membrane ER has yet to be identified in the rodent anterior pituitary gland, variants of ERα have been described. Friend et al. (1995) identified two transcripts of mRNA for ERα in the rat

anterior pituitary gland. Similar to ER46, truncated estrogen receptor product (TERP)-1 and -2 have shortened amino termini and lack A/B, DNA-binding and hinge regions normally found in full-length ERα. It appears that TERP-1 is generated by a promoter located between exon 4 and 5 which stimulates expression of a unique exon 1 and exons 5-8 common to the full-length ERα (Schausi, et al., 2003). The truncated product we detected by immunoblotting appears to be larger then the expected translation products of TERP-1 and -2; this could be a result of the differences between protein electrophoresis systems and further investigation into the 37 kDa protein is required prior to any inference about how the product was generated. The 36 kDa protein that we detected may act similarly to ER46 and mediate membrane-initiated estrogen events. We have inferred the existence of a membrane ER based on the evidence that E-BSA inhibits LH release comparable to E_2. LβT2-ER cells express relatively more 36 kDa protein than full-length, which may contribute to the recovery of the negative effect of E_2 on GnRH-stimulated release of LH in LβT2-ER cells. Whether ERα variants exist in the ovine anterior pituitary remains to be demonstrated.

Gonadotrope-derived tumor cells provide models to study specific intracellular functions of gonadotropes. Previously, LβT2 cells have been utilized to examine the effects of GnRH, E_2 and dexamethonsone on LH release (Turgeon, et al., 1996). As seen in cultured pituitary cells and the parent LβT2 cell line, we can stimulate LH secretion with GnRH in cells stably transfected with ERα (Fig. 14). By increasing ERα expression we can significantly reduce the GnRH-stimulated LH secretion with E_2 similar to what we have observed in cultured pituitary cells (Fig. 9 and 14).

Non-genomic effects for progesterone, cortisol and testosterone have been demonstrated in multiple tissues (Borski, et al., 2001; Farrar and Rodnick, 2004; Frye, 2001; Kang, et al., 2003; Peluso, 2003; Sutter-Dub, 2002). Acute effects of 17α-estradiol depends on the tissue type. To date, studies exist demonstrating a non-genomic effect similar to E_2 in epithelial cells (Singh, et al., 2000), on human neuronal nicotinic receptors (Nakazawa and Ohno, 2001), and in superior cervical ganglionic neurons (Uki, et al., 1999). In this study, we demonstrated negative effects of 17α-estradiol, progesterone, testosterone and cortisol on GnRH-induced secretion of LH (Fig. 15). Recent research on cultured ovine pituitary cells established that neither progesterone, testosterone or cortisol had any effect on GnRH-evoked secretion of LH (Arreguin and Nett, unpublished). One explanation for this contradiction is that although LβT2 cells are arrested in a later developmental stage of gonadotropes than αT3-1 cells, hence the ability to secrete LH and FSH, LβT2 cells may not be derived from fully differentiated gonadotropes, therefore LβT2 cells respond to progesterone, testosterone and cortisol. Another explanation is that tumorigenesis resulted in partial dedifferentiation plus loss and gain of functions (Windle, et al., 1990). We had to increase expression of ERα in LβT2 cells in order to recover the non-genomic response to E_2. It is possible that LβT2 cells have gained responsiveness to other steroids during their generation. This is consistent with research on human gonadotrope adenomas. Asa et al. (1988) studied tumors formed from well-differentiated gonadotropes. The tumors originated from men of a similar age yet differed in their response to gonadal steroids (Asa, et al., 1988). Estradiol, progesterone and testosterone stimulated release of LH, FSH and α-subunit from one tumor and the other tumor had no significant response to E_2, progesterone or

testosterone (Asa, et al., 1988). It appears that the ability of the tumors to respond to gonadal steroids was a result of tumorigenesis and may explain why LβT2-ER cells respond to progesterone, testosterone and cortisol unlike cultured ovine pituitary cells.

The estrogen receptor has been observed to bind non-steroidal ligands with remarkably high affinity. A number of important physiological processes can be affected by these estrogenic agents. In many instances, these agents have mixed agonist-antagonist effects depending on the tissue stimulated. In many cases these compound positively influence the development and function of female reproductive system and the maintenance of bone density and cardiovascular health, while undesirably stimulating the growth of breast and uterine cancers. The desire for safer and more effective pharmaceuticals has lead to the development of ER ligands that not only display agonist versus antagonist behavior, but also selectivity for specific ER isoform.

Harris et al. (2002) have used PPT effectively to increase uterine weight and markedly reduce plasma cholesterol levels in mature animals. It was demonstrated that PPT has a 400-fold higher affinity for ERα than for ERβ and is a potent agonist of ERα devoid of any activity on ERβ (Stauffer, et al., 2000). Meyers and co-workers (2001) have demonstrated that DPN has a 70-fold relative binding affinity for ERβ compared to ERα and acts as a full ERβ agonist. Hence, we selected PPT and DPN in our studies to determine the respective biological roles of ERα and ERβ in the negative effect of E_2 on gonadotropin release.

Estrogen receptor and other steroid receptors are thought to act primarily as homodimers, although ERα-ERβ dimers can be observed on DNA and in solution (Cowley, et al., 1997; Pettersson, et al., 1997; Schreihofer, et al., 1999). Because the

isoforms are expressed in a tissue- and cell-specific manner and have differential affinities for synthetic and environmental ligands (Kuiper, et al., 1998), dimer formation can have important biological consequences. The ratio of the two forms could determine cellular responses to estradiol, explaining why with increased expression of ERα in LβT2 cells we can inhibit with E_2 GnRH-induced secretion of LH (Fig. 17). Interestingly, using a combination of PPT and DPN had no effect on stimulation of LH release by GnRH. It appears that using the combination of PPT and DPN to stimulate ERα and ERβ does not recapulate GnRH-evoked secretion of LH as observed with treatment with E_2. In a recent study, Razandi et al. (2004) demonstrated that ERα and ERβ form and function as homodimers at the plasma membrane in the presence of E_2. Heterodimerization of ERα and ERβ occurs primarily when both receptors are expressed at high levels, such as in endothelial cells (Razandi, et al., 2004). This information combined with the fact that little is known about how ERs are translocated to the membrane, makes it difficult to interpret the PPT/DPN results without further investigation.

It was expected that derived-pituitary cell lines may not mimic normal gonadotropes perfectly but valuable insight into the subcellular mechanisms regulating LH secretion may be gained. LβT2 cells stably transfected with ERα respond to E_2 similar to what we have observed in cultured pituitary cells. These data combined with the evidence that PPT impedes GnRH-mediated release of LH, indicate ERα is required for the non-genomic effect of E_2. Given that E-BSA imitates non-genomic effects of E_2 we have inferred that a membrane-associated or –bound variant of ERα influences exocytosis of LH. The increased expression of ERα in LβT2-ER cells provides a suitable model to further investigate specifics of ERs necessary for non-genomic effects of E_2

(Chapter IV).

CHAPTER IV: EVAULATION OF ACUTE EFFECTS OF ESTRADIOL ON GNRH-INDUCED SIGNAL CASCADE.

A. Introduction

Dissection of the signaling pathways activated by GnRH in pituitary gonadotropes has resulted in considerable insight into the molecular events necessary for LH secretion. Upon GnRH binding, GnRH receptors decrease their lateral diffusion and aggregate to stimulate $G_{q/11}$ protein (Conn and Venter, 1985; Hazum, et al., 1980; Hopkins, et al., 1981; Horvat, et al., 2001; Janovick and Conn, 1996a; Nelson, et al., 1999). Immediately following ligand binding to the GnRH-R, $G\alpha_{q/11}$ subunit is activated leading to stimulation of PLC that converts phosphatidylinositol-4,5-bisphosphate (PIP_2) to diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP_3). Subsequently, IP_3 rapidly acts on the endoplasmic reticulum to mobilize Ca^{2+} which, in turn, leads to activation of PKC. Simultaneoulsy, GnRH binding to receptor activates influx of calcium through voltage-gated Ca^{2+} channels leading to a sustained increase in intracellular Ca^{2+} (Ca^{2+}_i) secondary to IP_3-induced increase. Various components of this signaling cascade play important roles in stimulating exocytosis of LH, and thus providing potential targets for E_2 to affect. From studies in other cell types, E_2 has been observed to affect IP_3 formation and PKC activation. Within minutes of E_2 treatment, Razandi et al. (1999) observed stimulation of $G\alpha_s$ and $G\alpha_q$ proteins and adenylate cyclase, in addition to

increased IP_3 formation, in CHOK1 cells overexpressing ERα. Sylvia and co-workers (2000) found that the rapid stimulation of PKC activation by E_2 in female rat chondrocytes is dependent on G protein-coupled PLC. These data only suggest possible avenues of influence by estradiol. Having developed a reliable cell line to study the acute effects of estradiol on gonadotropes, we have begun to examine the effects of estradiol on the GnRH-induced receptor activation and subsequent signaling events. We chose to investigate estradiol's effect on GnRH-stimulated formation of IP_3 as an indicator of GnRH receptor activation and subsequent induction of $G_{q/11}$ protein-mediated activation of PLC. Given the necessity for an increase in $Ca^{2+}{}_i$ to stimulate exocytosis of LH, we also evaluated the effect of E_2 on the ability of GnRH to increase $Ca^{2+}{}_i$.

B. Materials and Methods

Reagents

D-myo-inositol (1,4,5) triphosphate and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Fresh bovine adrenal glands were procured from HyClone Labs (Greeley, CO). Tritiated inositol (1,4,5) triphosphate (^3H-IP_3) was purchased from Perkin Elmer Life Sciences, Inc (Boston, MA). Matrigel for coating glass coverslips was obtained from Becton Dickinson (San Jose, CA). Fura-2/acetoxymethylester (Fura-2 AM) and pluronic acid F127 were purchased from Molecular Probes (Eugene, OR)

Cell Culture Conditions

All cell cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air. LβT2 and LβT2-ER cells were cultured in 150 mm dishes and passaged weekly by trypsin dispersion. The LβT2 cells were maintained in DMEM with 4.5 mg/ml glucose, 10% FBS, 100 IU/ml Penicillin, and 50 μg/ml Streptomycin. Medium for LβT2-ER cells contained 500 μg/ml G418 sulfate in addition to the reagents in LβT2 culture medium. G418 sulfate is related to gentamycin, a selective agent used to maintain stably transfected mammalian cells expressing the bacterial gene for aminoglycoside-3'-phophotransferase. Continuation of LβT2 cells expressing the RSV-ERα construct required inclusion of G418 at the above concentration. Passage and maintenance of LβT2 and LβT2-ER cells were performed as described in Chapters II and III. Cells were subjected to the GnRH pulse procedure diagramed in Figure 7 prior to experimentation.

Radioreceptor Assay for Inositol (1,4,5)Triphosphate

Cells were plated into 12-well culture dishes at a density of 0.5 x 10^6 cells/well for both LβT2 and LβT2-ER cells. On day 4, approximately 18 h post GnRH pulses, cells were washed and incubated at 37° C for 30 min with Krebs/HEPES buffer (10 mM HEPES, 4.2 mM $NaHCO_3$, 11.7 mM glucose, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 4.7 mM KCl, 118 mM NaCl, 1.3 mM $CaCl_2$, and 10 mM LiCl, pH 7.4). Buffer was aspirated from cells and replaced with fresh buffer containing 0.1% ethanol and/or 10 nM GnRH in the absence or presence of 10 nM E_2. Following 30 min incubation at 37° C, reactions were stopped by addition of an equivalent volume of ice-cold 15% TCA/2 mM EDTA solution. Cells were incubated an additional 30 min on ice and then harvested from plates into low-binding eppendorf tubes. TCA homogenate was centrifuged at 2,000 x g

for 15 min at 4°C to sediment denatured proteins. The supernatant was transferred to a glass 12 x 75 mm tube for diethyl ether extraction as described by Sun et al. (1995) with minor modifications. TCA was removed from samples by washing with five volumes of water-saturated diethyl ether three times. Samples were dried under nitrogen for 30 min removing any residual ether. Samples were reconstituted with 400 µl Krebs/HEPES buffer and stored at -70° C until assayed for IP_3 content.

Using the protocol established by Challiss et al. (1990), we assessed the concentration of IP_3 in our samples using a radioreceptor assay. Adrenal-cortical protein was prepared from bovine adrenals. Fresh adrenals were decapsulated and demedullated to obtain adrenal cortex. Using a pre-cooled polytron, adrenal cortex was homogenized in eight volumes (on per gram basis) of homogenization buffer (20 mM $NaHCO_3$, 1 mM DTT, pH 8.0). Homogenate was centrifuged at 5,000 x g for 15 min at 4° C and the supernatant was collected. The pellet was resuspended in four volumes of homogenization buffer and re-centrifuged. Pooled supernatants were centrifuged at 38,000 x g for 20 min at 4° C and the pellets were recovered. Adrenal cortical protein was resuspended at a concentration of ~10 mg/ml into homogenization buffer and stored in aliquots at -70° C until future use. On the day of assay, adrenal cortical protein was thawed on ice and homogenized with a cooled glass dounce prior to addition to the assay. In low-binding eppendorf tubes, equal volume of sample or standard were combined with assay buffer (100 mM Tris, 4 mM EDTA, 4 mg/ml BSA, pH 8.5; diluted 4-fold in final volume and made fresh the day of assay). Approximately 7,000 cpm of $[^3H]IP_3$ in a 100 µl volume was added to all tubes. Total count tubes held only $[^3H]IP_3$ and tubes for B_0 contained Krebs/HEPES buffer instead of sample or standard. For non-specific binding,

630 μg/ml D-myoIP$_3$ was substituted for sample volume. Finally, adrenal-cortical protein was added to all tubes except total count tubes. The assay was incubated on a rotating platform for 30 min at 4° C. Bound [^{3}H]IP$_3$ was separated from free by centrifuging tubes in an Eppendorf centrifuge (model 5215; Brinkmann; Westbury, NY) at maximum speed for 15 min at 4° C. The supernatant was carefully aspirated using a pulled glass pipette and the remaining pellet was dissolved in 200 μl H_2O and transferred to a scintillation vial containing 3 ml ScintSafe solution (Fisher Scientific; Hampton, NH). Radioactivity was quantified in a liquid scintillation spectrometer (LS5000CE, Beckman Coulter; Fullerton, CA) for 10 min/vial to obtain a ~2-3 % counting error (see Appendix).

Measurement of Intracellular Ca^{2+}

Cells were plated onto Matrigel-coated glass inserts in 35 mm dishes (Mat Tek; Ashland, MA) at 0.5 x 10^5 cells/ml in 1 ml culture medium for both LβT2 and LβT2-ER cells. Before Ca^{2+} measurements were made, cells were primed with pulses of GnRH for 3 days as described in Chapter II. Cells were prepared for $Ca^{2+}{}_i$ measurements as described by Shlykov and Sanborn (2004) with minor modifications. Cells were loaded with 5 μM Fura-2 AM and 0.08% pluronic acid F127 in Fura-2 buffer (145 mM NaCl, 5 mM KCl, 1 mM Na_2HPO_4, 0.5 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM HEPES, 5 mM glucose, pH 7.4) at 37° C for 45 min. Unincorporated dye was removed by washing cells twice with Fura-2 buffer. Cells were incubated an additional 30 min at room temperature in Fura-2 buffer. Prior to measuring changes in $Ca^{2+}{}_i$ concentrations, cells were rinsed twice with Fura-2 buffer. Individual cell measurements were made within 30-45 min following room temperature incubation. Cells were imaged in a fixed volume of 1 ml

Fura-2 buffer and treatment solutions (50 µl volume; 20X final concentration) were added directly into coverslip dishes during imaging. Final concentration of ethanol was 0.005% in these experiments. Using an InCyt2 imaging system (Intracellular Imaging Inc; Cincinnati, OH), cells were measured at 340 and 380 nm excitation and 510 nm emission wavelengths. Changes in $Ca^{2+}{}_i$ concentrations over time were expressed using 340/380 nm fluorescence ratio with interpolation of intracellular concentrations from a Ca^{2+} calibration curve.

Data Collection and Analysis

Cells not subjected to steroid or GnRH treatment were used to normalize IP_3 data and to obtain fold differences (control = 1.0). For IP_3 studies, mean of fold difference and standard error of the mean (SEM) were calculated from a minimum of three experiments consisting of triplicate samples for each treatment. IP_3 assays were performed on duplicate aliquots of each sample. Concentrations of IP_3 contained in samples were generated by interpolation from a 12 point standard curve (1.5 nM to 3 µM). Data from IP_3 assays were subjected to analysis of variance using GLM procedure of SAS. When significant F-values were found, differences among treatments were separated using Tukey multiple comparison tests. In all cases, $P \leq 0.05$ was considered significant.

For $Ca^{2+}{}_i$ measurements, 4-6 dishes of cells per treatment group were examined. Approximately 15-30 cells were measured per dish but only cells that responded to treatment were used for data analysis. A cell was classified as a responder when following addition of treatment the 340 nm reading exceeded the 95% Confidence Interval (CI) of baseline and the 380 nm reading was less than the 95% CI of baseline.

Baseline was defined by 60 sec of $Ca^{2+}{}_i$ measurement prior to addition of treatment. Changes in concentrations of $Ca^{2+}{}_i$ overtime are presented with baselines set at 0 to normalize data. The areas under the peaks were calculated and responses per dish were averaged. Data for area under the peak were analyzed by Student's *t*-test and significance was taken as a value of $P < 0.05$.

C. Results

Effect of Estradiol on GnRH-Induced IP_3 Formation

The present study characterized the IP_3 response to GnRH in the presence of E_2 in gonadotrope-derived cells. Treatment of either LβT2 or LβT2-ER cells with GnRH for 30 minutes significantly increased the formation of IP_3 compared to solvent control (Fig. 18). No changes in amount of IP_3 accumulation were observed with the inclusion of E_2 with GnRH treatment compared to GnRH alone in either cell line.

Changes in $Ca^{2+}{}_i$ Mediated by GnRH and Estradiol

The effect of E_2 on GnRH-induced Ca^{2+} mobilization and influx from extracellular stores was investigated in LβT2 and LβT2-ER cells. Measurement of submicromolar concentrations of $Ca^{2+}{}_i$ is possible using fluorescent dyes such as Fura-2, whose fluorescent properties change when they bind calcium. Fluorescence excitation shifts occur when Fura-2 binds Ca^{2+}, i.e. the excitation efficiency increases at 340 nm and decreases at 380 nm. Ratios of values obtained at 340/380 nm therefore represent changes in $Ca^{2+}{}_i$. Calibration was performed by scanning the emission of Fura-2 in the presence of eight different Ca^{2+} concentrations.

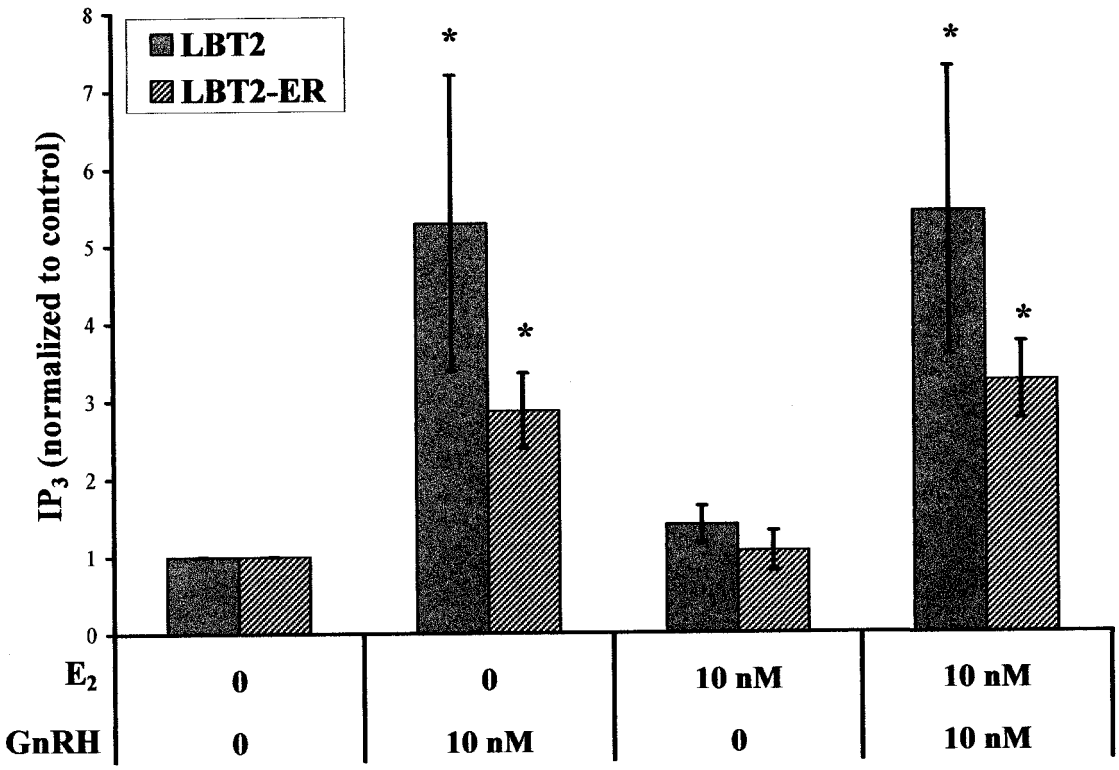

Figure 18: GnRH-induced IP3 formation was unaffected by inclusion of E2 in the medium. Fold differences were calculated from four experiments. Means that are significantly different from control are denoted with *; comparisons done within cell type only ($P < 0.01$).

The effect of GnRH on Ca^{2+}_i in LβT2 cells is shown in figure 19, upper panel (trace colored black). A maximal response was observed immediately following addition of GnRH, rising 400 ± 75 nM from baseline (averaged from 4 dishes of cells). Within 25 sec, Ca^{2+}_i had returned to baseline levels. Inclusion of E_2 had no effect on maximal response of Ca^{2+}_i to GnRH as seen in figure 19, upper panel (trace colored grey). LβT2 cells treated simultaneously with E_2 and GnRH returned to baseline levels of Ca^{2+}_i after approximately 37 sec. These results suggest that re-uptake of Ca^{2+} following GnRH-induction is delayed by the presence of E_2.

Similar to the parent cell line, LβT2-ER cells respond to GnRH with a rapid spike in Ca^{2+}_i with some difference as seen in figure 20, upper panel. GnRH stimulates a transient increase in Ca^{2+}_i of 49 ± 5 nM, which returns to baseline in approximately 60 sec. Unlike LβT2 cells, the simultaneous treatment with GnRH and E_2 resulted in a significant decrease in maximal response of Ca^{2+}_i (Fig. 20, grey colored trace) and the duration of the increase was shorter (Table 1). We concluded that this negative effect of E_2 on Ca^{2+}_i likely results in the blunted release of LH discerned previously (Chapter III). Of the number of LβT2 cells examined for these studies, only 56 percent responded to GnRH. Similar results are seen for the stably transfected, LβT2-ER cells. These data are summarized in Table 1. Cells that respond to GnRH challenge can be classified into 2 categories; low responders (AUC < 1000) and high responders (AUC > 1000). Of the cells that responded, most LβT2 cells are high responders. LβT2-ER cells are evenly split between high and low responders. Estradiol appears to increase number of cells that respond to GnRH, but only in LβT2 cells (Table 1).

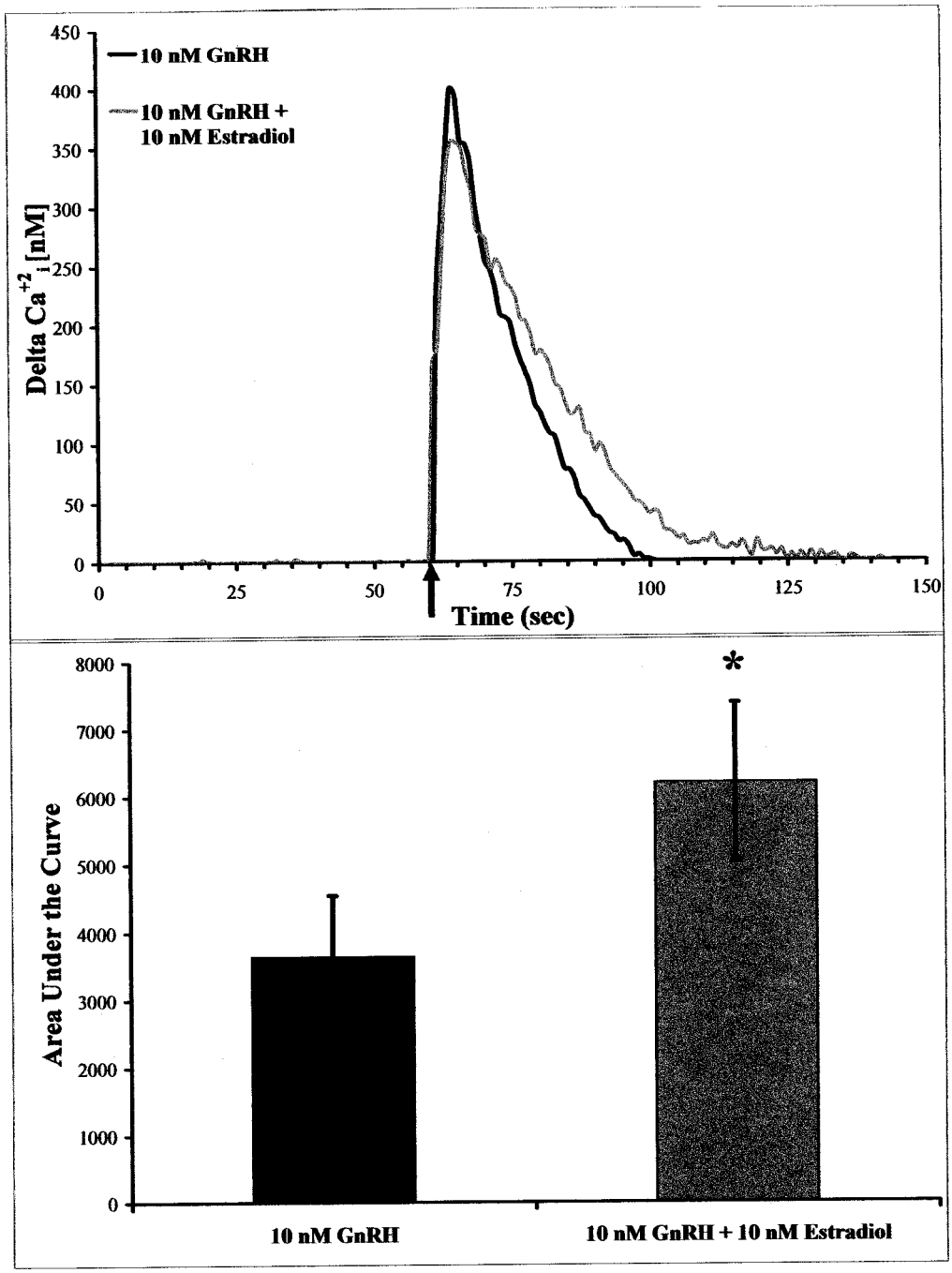

Figure 19: Upper panel – Tracings of changes in intracellular Ca^{2+} concentrations over time averaged from 4 dishes of LβT2 cells either treated with 10 nM GnRH (black) or 10 nM GnRH + 10 nM E_2 (grey). Arrow indicates time of addition of the treatment. Baselines were normalized to zero for presentation. Lower panel – Area under the curve calculated from tracings of LβT2 cells (mean ± SEM) with baselines normalized to zero. Mean marked with * is different versus GnRH alone (n = 4 dishes; $P < 0.05$).

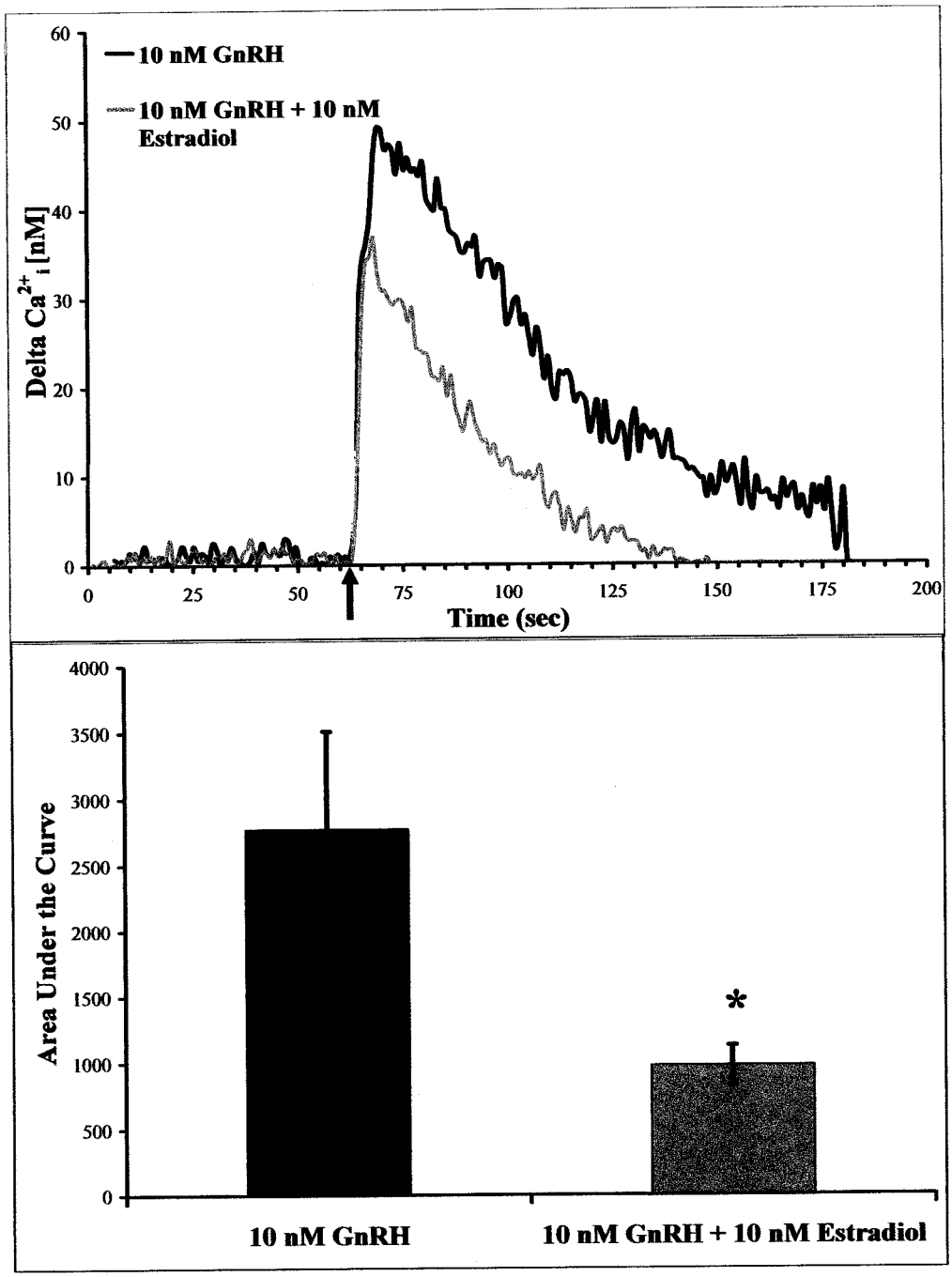

Figure 20: Upper panel – Tracings of changes in intracellular Ca^{2+} concentrations over time averaged from 6 dishes of LβT2-ER cells either treated with 10 nM GnRH (black) or 10 nM GnRH + 10 nM E_2 (grey). Arrow indicates time of addition of the treatment. Baselines were normalized to zero for presentation. Lower panel – Area under the curve calculated from tracings of LβT2-ER cells (mean ± SEM) with baselines normalized to zero. Mean marked with * is different versus GnRH alone (P = 0.02).

	Number cells measured	Percent of cells responding	Number of Cells with AUC < 1000	Number of Cells with AUC > 1000	Averaged Baseline Values (nM)	Maximal Response Above Baseline (nM)	Average duration of response (sec)
LβT2							
GnRH	119	56	4	63	300 ± 2	400 ± 75	24 ± 1
GnRH + E_2	169	70	8	111	313 ± 6	356 ± 86	37 ± 5*
LβT2-ER							
GnRH	174	53	36	56	206 ± 3	49 ± 5	63 ± 16
GnRH + E_2	192	54	59	45	194 ± 1	37 ± 4*	39 ± 5

Table 1: Tabulation of results for cells measured for changes in intracellular calcium following treatment with 10 nM GnRH in the presence or absence of 10 nM E2. Average time of response and maximal response are presented as mean ± sem. Statistical analysis was performed on maximal response and average duration of response. Comparisons were done within cell type only, denoted by brackets. *, Value is different from GnRH only value ($P < 0.05$). Abbreviation: AUC, area under curve.

D. Discussion

Investigation of molecular mechanisms involved in the cellular responses to simultaneous treatment with GnRH and E_2 has been complicated by the lack of a homogenous source of pituitary gonadotropes which maintain their differentiated function. The immortalized gonadotrope-derived cell lines, αT3-1 and LβT2, have been used extensively in the past to delineate the subcellular responses activated by GnRH. Unlike primary pituitary cells, LβT2 cells do not exhibit the non-genomic effects of E_2 observed in cultured pituitary cells. However, LβT2 cells stably transfected with ERα cDNA simulate the non-genomic effects of E_2 on LH secretion observed in primary pituitary cells.

GnRH-bound receptor activates signal transduction coupled to $G_{q/11}$ proteins leading to a series of events necessary for LH exocytosis. Hydrolysis of PIP_2 by a PLC dependent mechanism is an early event in the action of GnRH. Currently, there are no data available on E_2's participation in the signaling cascade following GnRH-R activation. As expected, both LβT2 and LβT2-ER cells respond to GnRH with increased accumulation of IP_3 (Fig. 18). As demonstrated in figure 18, E_2 had no effect on the formation of IP_3, whether in the presence or absence of GnRH. This lack of effect leads to several conclusions. Given that phosphodiesterase cleavage of PIP_2 is the only known mechanism for IP_3 formation in mammalian cells (Berridge, et al., 1984; Nishizuka, 1984) and that GnRH-induced PIP_2 breakdown occurs through the action of PLC, we infer that E_2 does not alter GnRH-mediated secretion of LH by affecting PLC hydrolysis of PIP_2. Additionally, events prior to PLC dependent mechanisms, such as ligand initiated GnRH-R microaggregation (Conn, et al., 1985b; Cornea and Conn, 2002;

Janovick and Conn, 1996a) or receptor induction of $G_{q/11}$ protein-coupled PLC activation, are inferred to be unaffected by inclusion of E_2 with GnRH treatment. Events that occur after IP_3 formation, e.g. mobilization of $Ca^{2+}{}_i$ and DAG activation of PKC, could be potential targets for the negative influence of E_2.

Calcium mobilization and influx stimulated by GnRH involves a variety of signal transduction pathways, including activation of PKC and exocytosis of LH. Intracellular levels of Ca^{2+} have been shown to increase rapidly (5 sec) following treatment with GnRH. Previous work in pituitary and αT3-1 cells has established that ligand binding to GnRH-R produces an initial rapid but transient spike in $Ca^{2+}{}_i$ concentrations followed by a reduced but sustained secondary plateau phase. In conjunction with GnRH-induced Ca^{2+} oscillations, this stimulus-activated $Ca^{2+}{}_i$ spike/plateau is believed to operate as an exocytosis signaling code, which induces secretion of LH.

We observed GnRH-evoked changes in $Ca^{2+}{}_i$ in both LβT2 and LβT2-ER cells. As with previous studies on LβT2 and αT3-1 cells, neither cell line exhibit the characteristic large-amplitude $Ca^{2+}{}_i$ oscillations observed in normal gonadotropes (Stojilkovic, et al., 1990). The $Ca^{2+}{}_i$ responses observed are qualitatively similar between LβT2 and LβT2-ER cells, but not quantitatively similar. Changes in intracellular calcium were greater in LβT2 cells compared to LβT2-ER cells (400 ± 75 nM versus 49 ± 5 nM). This divergence in calcium response may explain the differences in secretion of LH in response to GnRH observed between the two cell lines, a 2.5-fold increase above control in LβT2 cells compared to a 1.3-fold increase above control in LβT2-ER cells (Chapters II and III). Interestingly, LβT2 cells seem to have the initial spike in $Ca^{2+}{}_i$ normally found in pituitary cells (Fig. 19, upper panel) without the secondary plateau phase. In

contrast, LβT2-ER cells exhibit both the primary spike and secondary plateau (Fig. 20, upper panel). Based on these results, we have concluded that the new cell line, LβT2-ER, model gonadotrope responses to GnRH more closely than the parent cell line.

Although we have created an *in vitro* model for gonadotropes we must be cautious about any conclusions derived from these studies concerning the biology of the primary gonadotropes. These initial experiments cannot distinguish the source of calcium, either sequestered intracellular or extracellular, responsible for the fluctuation in $Ca^{2+}{}_i$ levels. Thapsigargin, a sesquiterpane lactone which selectively inhibits Ca^{2+}-ATPase in the endoplasmic reticulum thereby decreasing its ability to store calcium, has been used to demonstrate that the initial $Ca^{2+}{}_i$ spike predominately involves the mobilization of intracellularly sequestered Ca^{2+} (Anderson, et al., 1992). Anderson et al. (1992) used nifedipine, an L-type Ca^{2+} channel blocker, to markedly reduce the secondary phase of the GnRH calcium response. Further experiments using pretreatment of LβT2-ER cells with thapsigargin and nifedipine are required to evaluate the change in concentrations of $Ca^{2+}{}_i$ in response to GnRH and E_2 to establish which, if either, mechanism E_2 negatively affects. Additionally, we need to determine whether a membrane ER is required for this effect, possibly by using a conjugated estradiol.

CHAPTER V: SUMMARY AND DISCUSSION

> The most exciting phrase to hear in science, the one that heralds new discoveries, is not 'Eureka' (I found it) but rather, 'hmm ... that's funny...'
>
> -Isaac Asimov

Humans today are regularly consuming xeno-estrogens, either nutritionally or pharmacologically. Women and men are beginning to take soy supplements (containing phytoestrogens) as "alternative" remedies for menopause and prostate cancer prevention (Messina, 2003; Sarkar and Li, 2003). Tamoxifen, a nonsteroidal anti-estrogen, is widely used for the treatment of breast cancer (Hanstein, et al., 2004). Frequently these treatments are chosen without either sufficient evidence for the benefits or efficacy of treatment, or without proper acknowledgment of adverse side effects. Only further research into the mechanisms by which estrogens affect a specific tissue will result in nutraceuticals and pharmaceuticals with beneficial consequences and without negative side effects. Our research on non-genomic effects of E_2 on GnRH-evoked release of LH contains the potential to provide insight that could lead to new pharmaceutics.

Oral contraceptives are one of the most commonly used products that contain estrogenic compounds. Formulations are constantly being revised to improve safety, yet retain acceptable cycle control. Although relative amounts of estrogens, types of progestin and mode of administration are frequently altered to produce a new

contraceptive product, no new concept for birth control has been developed in decades. Virtually all chemical contraceptive products rely on the progestin/estrogen combination or progestin alone to suppress the hypothalamic-pituitary-gonadal axis and to prevent ovulation. The acute action of estradiol on gonadotropes may provide insight that could lead to future development of a new generation of birth control products. Once we understand the basic nature of the rapid action of E_2 upon LH secretion, it may be possible to take advantage of the information for contraceptive research and offer novel treatment regimens never before considered.

Our recent review of the literature revealed that there is little information available concerning the acute effects of E_2 on subcellular mechanisms controlling release of LH. The genomic effects of E_2 resulting in pre-ovulatory surge of LH are relatively well understood, but we lack comprehension of the non-genomic mechanisms activated by E_2 to initially prevent release of LH. Although we have focused our literature review on sheep, similar rapid negative effects of E_2 on LH release have been observed in numerous other species, including humans (Nagahara, et al., 1984).

In the present study we replicated previous work on cultured ovine pituitary cells (Arreguin and Nett, 2002) using an alternate culture procedure and demonstrated that E_2 reduced GnRH-induced secretion of LH (Fig. 9). To evaluate the effects of E_2 on the subcellular mechanisms controlling release of LH, we turned to the homogenous gonadotrope cell line, LβT2. Given that the non-genomic effect of E_2 was not confined to a single species (Chapter I), we reasoned that information obtained using a mouse-derived gonadotrope cell line would be applicable to other species. While our studies on ovine pituitary cells confirmed our expectations, treatment of LβT2 cells with E_2 did not

inhibit GnRH-induced release of LH (Fig. 10). Thus, we began to examine the literature for an explanation for the inability of LβT2 cells to acutely respond to E_2. Studies using RT-PCR and/or ribonuclease protection assay (RPA) indicated that ERα mRNA is the predominant ER isoform in the uterus, mammary gland, testis, pituitary, liver, kidney, heart, and skeletal muscle, whereas ERβ transcripts predominate in the ovary and prostate (Fig. 21)(review by Couse and Korach, 1999). Interestingly, based on our observations, LβT2 cells express very low levels of ERα compared to ERβ. Although ER knockout studies indicate that ERα and ERβ may have redundant functions, their differences in tissue distribution and response to certain ligands indicate the presence of distinct roles fulfilled by each. Knockout of ERα in mice (αERKO) results in effects on the hypothalamic-pituitary-gonadal axis similar to what is seen in OVX animals. Despite being chronically exposed to elevated levels of E_2, αERKO mice exhibit increased levels of α-subunit, LHβ and FSHβ transcripts (review by Couse and Korach, 1999). Additionally, there are significant elevations in concentrations of serum LH in αERKO mice (review byCouse and Korach, 1999). Similar effects are observed in mice in which both ERα and ERβ have been knocked out (review by Hewitt and Korach, 2003). Interestingly, levels of LH and FSH appear to be normal in ERβ null mice (βERKO), indicating that ERα is the primary ER isoform regulating LH secretion (Hewitt and Korach, 2003). Based on these data, we hypothesized that increasing levels of ERα expression in LβT2 cells would restore the negative non-genomic effects of E_2 on release of LH observed previously in ovine pituitary cells (Arreguin and Nett, 2002).

LβT2 cells overexpressing murine ERα substantiated our theory and treatment

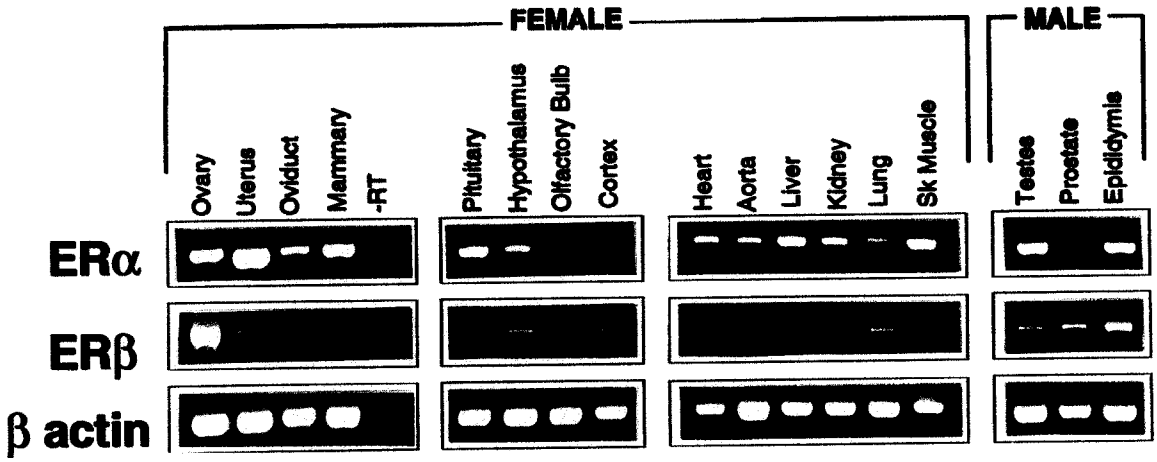

Figure 21: RT-PCR for ERα and ERβ mRNA in various tissues of the wild-type mouse. RT-PCR was carried out on 0.5 μg of total RNA pooled from adult wild-type mice using primers specific for the mouse ERα and ERβ transcripts (adapted from Couse and Korach, 1999). Equal amounts of the individual RT-PCR reactions were then fractionated on an agarose gel. Note the broad tissue distribution of ERα mRNA, whereas ERβ transcripts are primarily expressed in the ovary, hypothalamus, lung, and male reproductive tract. RT-PCR for β-actin was carried out as a positive control. (-RT) indicates a negative control, *i.e.*, PCR on total ovarian RNA minus reverse transcriptase, indicating the specificity of the ER primers for cDNAs generated by the reverse transcriptase enzyme.

with E_2 resulted in decreased release of LH in response to GnRH (Fig. 14). Furthermore, E-BSA inhibited GnRH-induced secretion of LH (Fig. 16) similar to observations in cultured ovine pituitary cells (Fig. 9). Given the structural analogy between cholesterol and steroid hormones, it is possible that such hormones could alter the dynamic properties of membrane bilayers and thereby influence exocytosis. In fact, previous research has demonstrated an effect of E_2 on membrane fluidity (Whiting, et al., 1995).

We presumed that the non-genomic effect of E_2 was due to a membrane-associated ERα not via a non-specific steroid effect because E_2 had no affect on release of LH in LβT2 cells yet had an effect in LβT2-ER cells and the effect could be mimicked using membrane impermeable E-BSA. This was further corroborated by showing that PPT, an ERα specific agonist, and not DPN, an ERβ specific agonist, inhibited GnRH-mediated release of LH (Fig. 17).

We investigated the effects of other steroid hormones in effort to delineate further the non-genomic mechanisms affecting LH secretion. Studies on cultured pituitary cells were used to evaluate the differential effects of steroids on gonadotropin release. Androgens, progesterone and cortisol all have been shown to influence gonadotropin secretion to a certain extent (Campen and Vale, 1988; Debus, et al., 2002; Laws, et al., 1990a). 17α-estradiol, an stereoisomer of E_2 inactive at nuclear ER, has been suggested for therapeutic use in certain human diseases due to its low-density lipoprotein-lowering effects (Lundeen, et al., 1997), its ability to prevent neuronal degeneration (Nakazawa and Ohno, 2001) and its inhibition of ER activated contractions of bladder smooth muscle (Ratz, et al., 1999). In above studies, treatment with steroid lasted longer than 24 hr. We were unsure what to expect for non-genomic effects following treatment with

GnRH and the following steroids: testosterone, progesterone, and 17α-estradiol. Recently, Debus et al. (2002) demonstrated that stress-like levels of cortisol on OVX ewe negatively affect LH secretion within 1-2 hr of treatment.

As seen in Figure 15, treatment of GnRH with 17α-estradiol, progesterone, cortisol, and testosterone negatively affected GnRH-mediated secretion of LH. Non-genomic effects for progesterone, cortisol and testosterone have been demonstrated in other tissues (Borski, et al., 2001; Farrar and Rodnick, 2004; Frye, 2001; Kang, et al., 2003; Peluso, 2003; Sutter-Dub, 2002). Acute effects of 17α-estradiol depends on the tissue type; studies exist demonstrating a non-genomic effect similar to E_2 in epithelial cells (Singh, et al., 2000), in human neuronal nicotinic receptors (Nakazawa and Ohno, 2001), and in superior cervical ganglionic neurons (Uki, et al., 1999). In hypothalamic neurons (Morales, et al., 2003), astrocyte (Sato, et al., 2003), hippocampal pyramidal neurons (Carrer, et al., 2003), and chondrocyte (Sylvia, et al., 2000), 17α-estradiol does not mimic acute of effects of E_2. In this study, we demonstrated negative effects of 17α-estradiol, progesterone, testosterone and cortisol on GnRH-induced secretion of LH. As discussed in Chapter III, these results contradict recent research on cultured ovine pituitary cells. This discrepancy may arise because LβT2 cells are either not derived from fully differentiated gonadotropes or have dedifferentiated as result of tumorigenesis. Furthermore, the disparity in response to gonadal steroids may be due to species differences; LβT2 cells are derived from mouse gonadotropes. In cultured ovine pituitary cells, treatment with progesterone for 48 hr results in a decrease in GnRH binding but no effect on GnRH-stimulated release of LH compared to control (Laws, et al., 1990a). Similar treatment of cultured rat pituitary cells with progesterone results in reduced

responsiveness to GnRH and decreased LH secretion compared to control (Emons, et al., 1992; Janovick and Conn, 1996b). Additional studies are required to fully characterize these various steroid effects, e.g. dose curve studies, specificity of binding to LβT2-ER cells, and effect of various steroids on GnRH-mediated release of LH in the parent cell line, LβT2.

Numerous investigators have demonstrated non-genomic effects of E_2 on subcellular mechanisms. Transcription can be stimulated from E_2 signaling initiated at the membrane through either a membrane-associated or -bound receptor (Pedram, et al., 2002). Other investigators using membrane impermeable E_2 have been able to activate membrane-bound receptor to stimulate prolactin release (Christian and Morris, 2002), to induce a rapid and dose-dependent increase in $Ca^{2+}{}_i$ due to Ca^{2+} influx and release of Ca^{2+} from intracellular stores (Benten, et al., 1998; Benten, et al., 2001) and to mediate nitric oxide release (Kim, et al., 1999). Increases in intracellular concentrations of IP_3 and Ca^{2+} are indicators of GnRH receptor activation. Currently, there are no data on the acute effects of estradiol on GnRH-stimulated signal transduction.

In the present study, both LβT2 and LβT2-ER cells respond to GnRH with increased accumulation of IP_3 and E_2 has no effect on the GnRH-stimulated formation of IP_3 (Fig. 19). This lack of effect lead us to conclude that signal intermediaries prior to PLC hydrolysis of PIP_2 to form IP_3, e.g. ligand-initiated GnRH-R self-association (Conn, et al., 1985b; Cornea and Conn, 2002; Janovick and Conn, 1996a), or receptor activation of $G_{q/11}$ protein-coupled PLC, appear to be unaffected by inclusion of E_2 with GnRH treatment. Events that occur either after or simultaneous to IP_3 formation, such as

mobilization of Ca^{2+}_i, DAG activation of PKC or stimulation of adenylate cyclase, could be potential targets for the negative influence of E_2.

Calcium mobilization and influx stimulated by GnRH involve a variety of signal transduction pathways, including activation of PKC and exocytosis of LH. GnRH stimulates an initial spike in Ca^{2+}_i followed by a secondary sustained increase in Ca^{2+}_i. Together with GnRH-induced Ca^{2+} oscillations, this stimulus-activated Ca^{2+}_i spike/plateau is believed to control exocytosis of LH. In the present study we observed GnRH-evoked spike in Ca^{2+}_i without the characteristic large-amplitude Ca^{2+}_i oscillations observed in normal gonadotropes (Stojilkovic, et al., 1990) in both LβT2 and LβT2-ER cells (Fig. 19 and 20). Interestingly, LβT2 cells seem to have retarded re-uptake of Ca^{2+}_i when E_2 is included with GnRH treatment (Fig. 19, upper panel). In contrast, LβT2-ER cells exhibit both blunting of the primary spike and secondary plateau in response to addition of E_2 to GnRH treatment (Fig. 20, upper panel). These initial experiments cannot distinguish the source of Ca^{2+}_i, intracellular or extracellular, responsible for the increase in Ca^{2+}_i levels. Further experiments using pretreatment with thapsigargin, a selective inhibitor of Ca^{2+}-ATPase, and nifedipine, a L-type Ca^{2+} channel blocker, are required to establish the source of Ca^{2+} that is negatively impacted by E_2. Additionally, we need to determine if a membrane ER is required for this effect, possibly by using a membrane impermeable form of estradiol.

Based on the results obtained in the present study, we have concluded that the new cell line, LβT2-ER, mimic normal gonadotrope responses to GnRH and E_2 more closely than LβT2 cells. Although we have created a biochemical model for gonadotropes we must be cautious with respect to any conclusions derived from these

studies concerning the biology of the primary gonadotropes. A variety of caveats must be considered to acknowledge the potential limitations of LβT2-ER cells. One of the most important limitations of using *in vitro* models is the lack of input from other cell types. For example, activins, isolated from ovarian follicular fluid (along with their functional antagonists, the inhibins and follistatins), modulate the synthesis and secretion of FSH (Ying, 1988). The lack of extrapituitary input results in imperfect information on regulation of gonadotropin secretion. We recognize that potential endocrine factors important to GnRH-evoked release of LH have been eliminated by using an *in vitro* cell system. Additionally, using LβT2 cells, a homogenous cell population, instead of the heterogeneous primary pituitary cultures, eliminates any paracrine effects that could influence gonadotropes *in vivo*. Folliculostellate cells, nonendocrine cells of the anterior pituitary gland, also produce activin and follistatin (Kaiser, et al., 1992a; Nett, et al., 2002; Ying, 1988). Estradiol suppresses the expression of the activin gene in the pituitary, in turn inhibiting the formation and release of FSH (Nett, et al., 2002). The loss of paracrine effects may account for the different results obtained in LβT2 and LβT2-ER cells compared to cultured pituitary cells, e.g. neither cell line exhibit the characteristic large-amplitude $Ca^{2+}{}_i$ oscillations observed in normal gonadotropes (Stojilkovic, et al., 1990). Despite these limitations, LβT2-ER cells can be used as a source of information for subcellular and molecular mechanisms specific to gonadotropes.

We have examined a portion of the GnRH-activated signaling cascade and more research is required to define which signaling intermediates are responsible for the non-genomic response to E_2. In the future, it may be possible to use this system not only as a representation of normal gonadotropes to evaluate subcellular mechanisms acutely

affected by steroid hormones, but also as a model to evaluate both the physiological and pathological non-genomic effects of estrogens.

APPENDIX: INFORMATIVE DATA

A. Radioreceptor- And Radioimmuno-assay Precision

Co-efficients of variation (CV) were calculated for inter- and intra-assay as an estimate of accuracy of assays. Assays with CV values of 10 % or less were considered to have good method performance and CV values greater than 10 % were considered to be of suspect performance. The intra-assay CV for IP_3 was 10.4 % (n = 8). The CV between assays was 9.7 % (n = 11) using repeated measurement of the same samples in successive assays. The sensitivity of radioreceptorassay, defined as the amount of IP_3 needed to reduce the B_0 binding by two standard deviations was 238 pg.

For LH radioimmunoassay, CV for intra-assay was 9.3 % (n = 12) and 7.6 % (n = 10) for murine and ovine assays, respectively. The CV for inter-assay for murine and ovine LH radioimmunoassays was 6.2 % (n = 13) and 5.5 % (n = 14), correspondingly.

B. Counting Precision For [^{3}H]Inositol (1,4,5)Trisphosphate

The average counting error for the inositol (1,4,5) triphosphate assays was 2.46 ± 0.02 %. Counting error establishes the 95 % confidence interval for the count. Thus a value of 2.46 % indicates that 95 out of 100 cases, the counts per minute obtained will be within 2.46 % of the mean and in the remaining 5 cases out of 100 may be outside that

2.46 %. This results from the randomness of the radioactive decay process and not from any variation within the instrument.

C. Membrane Localization of ERα

Preliminary research repeating the 1984 experiment of Nett et al. on ovarectomized ewes substituting E-BSA for E_2 indicates that the rapid decline in LH secretion is due to activation of membrane estrogen receptors (Arreguin and Nett, 2003). Immediately following injection of E-BSA a rapid decline in LH resulted, but unlike E_2 treatment, E-BSA failed to stimulate the pre-ovulatory-like surge of LH several hours later (Arreguin and Nett, 2003). From this *in vivo* study we concluded that the rapid decline in basal LH secretion is likely due to activation of membrane ERs. Given that ERα appears to be necessary for E_2 to inhibit secretion of LH (Chapter III), we have inferred that the non-genomic effects of E_2 we have observed occur via a membrane ERα. The purpose of the following preliminary study was to evaluate the presence of ERαs on the membranes of ovine gonadotropes

Immunocytochemistry

The primary antibodies used for immunocytochemistry, guinea pig anti-mouse LHβ and rabbit anti-mouse ERα (MC-20) were obtained from National Hormone & Peptide Program (Dr. A.F. Parlow; Torrance, CA) and Santa Cruz Biotechnology, Inc, respectively. The secondary antibodies, goat anti-guinea pig IgG conjugated to FITC, and goat anti-rabbit IgG conjugated to Cy5, were purchased from Rockland (Gilbertsville, PA) and Molecular Probes (Eugene, OR), respectively. In addition,

concanavalin A-rhodamine conjugate was also purchased from Molecular Probes. Gelmount, Permount, glass coverslips, and microscope slides were purchased from Fisher Scientific (Hampton, NH).

Anterior pituitary cells were plated at a density of 0.5 x 10^6 cells/well into 6-well dishes containing a single sterile coverslip per well and incubated in pituitary culture medium for 24 hours prior to being washed with twice with PBS and stained with Concanavalin A-rhodamine conjugate (20 ug/ml) in PBS for 10 min at room temperature. Following staining, cells were fixed in 4% paraformaldehyde for 10 min on ice and then permeabilized using 0.2% Triton-X 100 in PBS for 2 min at room temperature. After blocking non-specific binding sites with PBS containing 2.5% normal goat serum (PBS/NGS), cells were incubated overnight at 4° C with primary antibodies, anti-mouse LHβ and anti-mouse ERα diluted 1:200 in PBS/NGS. Cells were washed twice with PBS prior to incubating for 2 hours at room temperature with secondary antibodies. Secondary antibodies were diluted 1:200 in PBS/NGS. Evaluation of antibody specificity was carried out by omitting primary antibodies. Unbound antibodies were washed from cells using PBS and coverslips were mounted onto glass microscope slides using Gelmount and Permount. Images were collected using an inverted scanning confocal microscope. The confocal imaging was performed on an Olympus Fluoview inverted microscope (Melville, NY), equipped with a Nikon 60x objective and argon, krypton and helium-neon ion lasers (488, 565, and 633 nm, respectively) to excite FITC (green), rhodamine (red) and Cy5 (far red) fluorescence. Image processing and the quantitative evaluation of the co-localized flurochromes were performed using Adobe Photoshop (Adobe; San Jose, CA) and Scion Image, respectively. Using a minimum of 20

gonadotropes identified by LHβ staining, we calculated the value for Cy5 fluorescence of the entire cell and Cy5 fluorescence specific to the membrane using the computer program, Scion Image. These values were then converted to percent of total estrogen receptors for each cell.

Results

Norfleet et al. (2000) demonstrated the presence of ER in the membrane of rat pituitary GH(3)/B6/F10 cells, a somatolactotrope cell line, by immunocytochemistry. Concanavalin A labels lectins on the plasma membrane of living cells. We used this compound to delineate the location of plasma membrane. Currently there is no published evidence that membrane ER exist on gonadotropes. A representative gonadotrope cell imaged in green, red and far red channels is shown in Figure 22. Panels A, B, and C show Concanavalin A, ERα, and LHβ signal localization, respectively. The merging of the three images in Figure panel D shows an overlapping of red (Concanavalin A) and green (ERα) colors as yellow. Gonadotrope cells, identified by blue (LHβ), were analyzed individually to determine relative co-localization of the two signals. Our data indicate that approximately 20 ± 2% of the total gonadotrope ERα co-localized with Concanavalin A-rhodamine

Discussion

Researchers have demonstrated that a single gene transcript for estrogen receptors produced both the membrane and nuclear receptors (Razandi, et al., 1999). Razandi et al. (1999) successfully produced functional estrogen receptors in CHOK1 cells by introducing (transfecting) the cDNA for estrogen receptors into the cells. Approximately

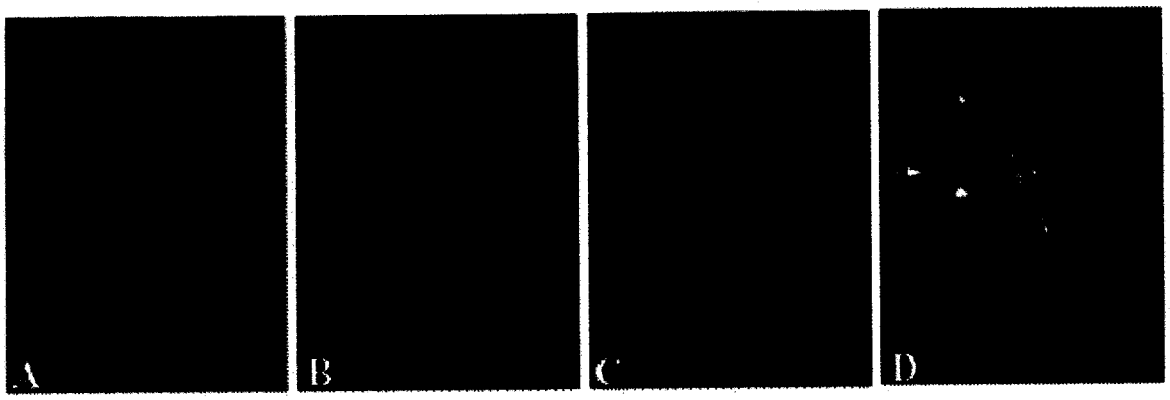

Figure 22: Immunocytochemistry and confocal microscopy on dissociated ovine pituitary cells using rabbit antibodies against ERα and guinea pig antibodies against LH. Cells were fixed in 4% paraformaldehyde and permeabilized using 0.2% Triton-X 100. Panel A – Concanavalin A-rhodamine marker that delineates membranes of living cells; Panel B – Antibody against rabbit IgG conjugated to Cy5 denotes estrogen receptor-α location; Panel C – Antibody against guinea pig IgG conjugated to FITC indicates cells containing LH; and Panel D – composite of previous three panels. Arrow points to representative cell used for calculating percent of ERα located at the membrane.

3% of the estrogen receptors were localized to the membrane (Razandi, et al., 1999). Our preliminary data suggest that one-fifth of the total estrogen receptor-α population in ovine gonadotropes is localized to the plasma membrane. The discrepancy between our data and the published results may exist for several reasons. First Razandi et al. (1999) used a tumor cell line that does not normally express ERs (Chen, et al., 1999). Second Razandi et al. (1999) used a system of gene expression that tends to lead to overexpression (greater than physiological levels) of the gene of interest. This may result in a lesser percentage of total ER becoming associated with the membrane. Their data came from an artificial system while our study was conducted on normal anterior pituitary cells known to express ERs (Glass, et al., 1984; Schreihofer, et al., 2000).

Studies using ERα null mice reveal that females require ERα for normal regulation of the hypothalamic-pituitary gonadal axis (Couse and Korach, 1999; Hewitt and Korach, 2003; Rissman, et al., 1997). Previous studies have shown that ERα is expressed primarily in lactotropes and gonadotropes (Mitchner, et al., 1998). We hypothesized that the negative effects of estradiol on LH secretion result from activation of ERα. In addition, we have inferred that this effect is due to a membrane-associated ERα since previous studies can mimic estradiol's negative effects on LH secretion using a conjugated estradiol (E-BSA) that cannot cross the plasma membrane. Detecting the presence of membrane ERαs using immunocytochemistry provides substantiation for this hypothesis to be correct. Unfortunately, we can only infer the presence of membrane ERα and not definitively say that ERαs are localized to the membrane. Using a protocol either without permeabilization of cells (Norfleet, et al., 1999) or incubating cells with primary anti-ERα prior to fixation (Benovic, 2000) may ameliorate some of the problems

associated with concluding that membrane-associated estrogen receptors are present in gonadotropes. Using either procedure would eliminate the possibility of detecting intracellular receptors located near or associated with the plasma membrane.

D. Alternative to E_2 Conjugated to BSA

Recently investigators have begun using steroids conjugated to horseradish peroxidase (HRP) instead of BSA for research on non-genomic effects of steroids (Marin, et al., 2003a, 2003b; Peluso, et al., 2001, 2002; Ropero, et al., 1999). The preference for an HRP conjugate versus a BSA conjugate arises for several reasons. Unlike the E-BSA conjugate that contains 30-40 moles of E_2 per mole of BSA, HRP conjugates have 2-3 moles of E_2 per mole of HRP. In addition, HRP exhibits less nonspecific binding than BSA and could be used for histocytochemistry. We conjugated HRP onto carbon six of E_2 (illustrated in Figure 23) instead of purchasing pre-conjugated E-HRP. Commercially available E-HRP has HRP positioned at carbon 17 (Sigma-Aldrich) of E_2. Conjugations at this position prevents binding to estrogen receptor and results in a biologically inactive compound (Arunachalam, et al., 1979).

Steroids conjugated to HRP has been used not only for studies on non-genomic effects of steroids (Marin, et al., 2003a, 2003b; Peluso, et al., 2001, 2002; Ropero, et al., 1999), but also for histocytochemistry for detection of steroid receptors (Sakabe, et al., 1986). Following are protocols for conjugating E_2 to HRP, determining relative activity of the resulting conjugate and employing E-HRP for histocytochemistry to detect membrane associated ERs.

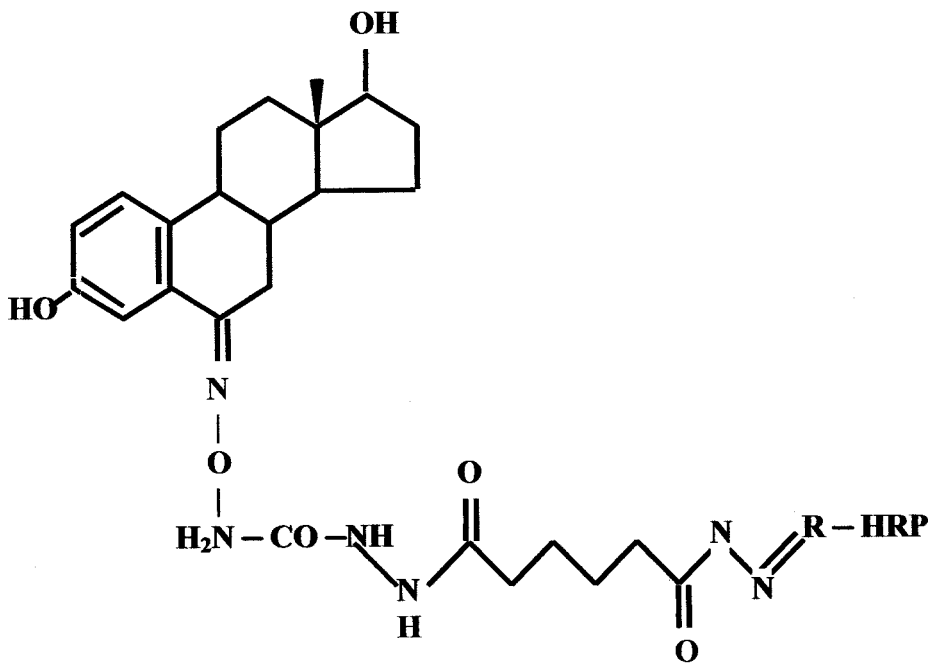

Figure 23: Structure of 17β-Estradiol-6-(O-Carboxymethyl)oxime-adipic acid dihydrazide-horse radish peroxidase

Conjugation of Estradiol to Horseradish Peroxidase

The conjugation of HRP to E_2-CMO through adipic acid dihydrazide (ADH) as the link is based on a previously described protocol for conjugating cortisol to HRP (Basu, et al., 2003). Activation of HRP requires the addition of 10 µl of 0.1 M freshly prepared aqueous solution of sodium meta-periodate per ml of 10 mg/ml HRP in H_2O. Following 30 min incubation at room temperature in the dark, the reaction mixture was passed through a Sephadex G-25 column previously equilibrated with 10 mM ammonium carbonate, pH 9.3. In a vial containing 100 mg ADH, the brown colored fraction of activated HRP was collected. After vortexing to mix, the reaction was incubated overnight at 4° C to form a hydrazone bond. Following overnight incubation, 10 µl of 5 M sodium cyanoborohydride in 1 mol/L NaOH was added to the reaction mixture. After vortexing, the reaction mixture was incubated 3 h at 4° C and passed through a Sephadex G-25 column previously equilibrated with PBS. The brown-colored fraction containing the HRP-ADH conjugate was collected and stored at less than -20° C for future use.

The steroid was activated by adding 200 µl of dimethyl formamide, 200 µl of dioxin and 100 ul nanopure H_2O containing 10 mg *N*-hydroxysuccinimide and 20 mg of 1-ethyl-3-(3-dimethyl-amino-propyl) carbodiimide-HCl to 5 mg E_2-CMO. Following vortexing to mix, the steroid mixture was incubated overnight at 4° C. Approximately 1 mg (1 ml) of the HRP-ADH conjugate was added to the activated steroid mixture and allowed to form a diimide bond over 24 h at 4° C. The resulting E-HRP conjugate (Fig.23) was passed through a Sephadex G-25 column previously equilibrated with PBS containing 0.1% thimerosal. Fractions containing enzyme activity were pooled and extracted with diethyl ether twice. Prior to conjugation ether extraction was shown not to

affect peroxidase enzymatic activity. Ether extracted and non-extracted HRP was combined with DAB peroxidase solution (3,3' diaminobenzidine FastTab, Sigma-Aldrich) and read in an microplate reader at 450 nM. The average 450 nm reading for extracted HRP (1.36; n = 5) was not significantly different compared to average 450 nm reading for non-extracted HRP (1.33; n = 5). To the extracted conjugate solution, 1 g/L of sucrose, ammonium sulfate and BSA were added. The E-HRP conjugate was aliquoted and stored at -20° C for future use. E-HRP was subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE;12% acrylamide) and electrophoretically transferred to nitrocellulose. The membrane probed with rabbit anti-E-BSA antibody was used to immunolocalize a band of approximately 44 kDa implying successful conjugation of E_2 to HRP (data not shown).

Relative Activity of E-HRP

The relative estrogenic activity of the E-HRP was assessed by comparing E-HRP and unlabeled E_2's ability to displace [^{3}H]E_2. Preparation of ovine uterine cytosol for generation of E_2 inhibition curve was adapted from Amann et al. (1986). A precooled Polytron homogenizer was used to homogenize fresh ovine uterine tissue in TEDG buffer (10 mM Tris, 1.5 mM EDTA, 1 mM DTT, 10% glycerol, pH 7.4). The homogenate was centrifuged at 30,000 x g for 1 h at 4° C; the supernatant was collected and stored in aliquots at -70°C for future use. The competitive binding curves were produced using a single concentration of [^{3}H]E_2 in the presence of increasing concentrations of E_2 or E-HRP. To 12 x 75 mm glass tubes, TEDG buffer, 1 nM [^{3}H]E_2, either unlabeled E_2 or E-HRP and 100 µl uterine cytosol preparation was added to a final volume of 500 µl. The tubes were vortexed for 10 sec and incubated for 60 min in a 30° C waterbath. Following

incubation, tubes were transferred to an ice bath and 100 µl TEDG buffer containing 4% charcoal and 0.4% dextran was added to each tube except the total counts tube. The tubes were incubated for 10 min on ice and centrifuged at 2,000 x g for 15 minutes at 4°C. To ascertain amount of [^{3}H]E_2 bound, the supernatant was transferred to scintillation vials containing 3 ml ScintSafe solution (Fisher Scientific; Hampton, NH) and radioactivity quantified in liquid scintillation system (LS5000CE, Beckman Coulter; Fullerton, CA) for 10 min/vial to obtain a ~2-5 % counting error. Biopotency of the E-HRP was determined by comparing the inhibition of [^{3}H]E_2 from uterine cytosol obtained using serial dilutions of E-HRP and E_2 (Fig. 24).

Counting Precision For [^{3}H]Estradiol

The average counting error for the estradiol binding assays was 4.44 ± 0.20 %. Counting error establishes the 95 % confidence interval for the count. Thus a value of 4.44 % indicates that 95 out of 100 cases, the counts per minute obtained will be within 4.44 % of the mean and in the remaining 5 cases out of 100 may be outside that 4.44 %. This results from the randomness of the radioactive decay process and not from any variation within the instrument.

Histocytochemistry Using E-HRP

The following protocol, based on a technique devised by Sakabe et al. (1986) to histochemically localize progestin receptors, was developed to visualize membrane estrogen binding sites on primary gonadotropes. Anterior pituitary cells were plated at a density of 0.5 x 10^6 cells/well into 6-well dishes containing a single sterile coverslip per well and subjected to GnRH challenges as described in Chapter II. On day four, medium

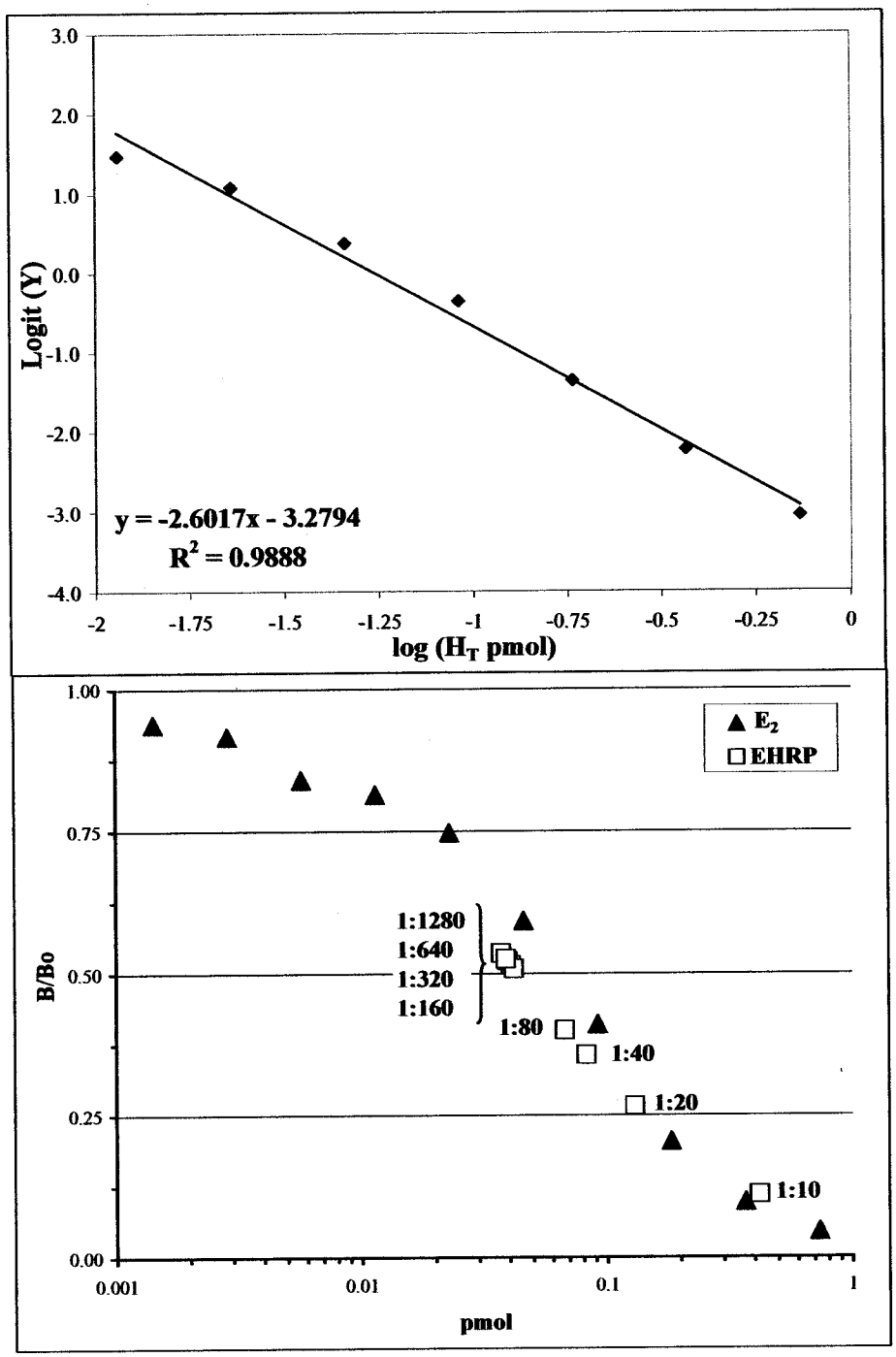

Figure 24: Upper panel – standard curve generated from inhibiting binding of [3H]E_2 to uterine cytosol preparations using estradiol. To constant amounts of [3H]E_2 (27.24 pg/ml) and uterine cytosol preparation (300 µg/tube), increasing amounts of unlabeled E_2 were added to generate an equation to extrapolate relative affinity of E-HRP. Lower panel – inhibition curve depicting biopotency of serial dilutions (indicated) of E-HRP compared to E_2.

on cells was replaced with fresh culture medium containing 10 nM E-HRP and incubated for 30 min at 37° C. Control wells either lacked E-HRP or were pre-incubated with unconjugated E_2 for 30 min. Cells were rinsed twice with ice-cold PBS prior to fixing cells with 4% paraformaldehyde in PBS for 15 min on ice. Cells were washed twice with ice-cold PBS and incubated in diaminobenzidine peroxidase solution until the desired stain intensity develops. Development was stopped by washing cells with PBS. Cells were incubated with biotinylated concanavalin A (5 μg/ml 10 mM HEPES, 0.15 M NaCl, pH 7.5) for 30 min at room temperature. Cells were washed three times with PBS prior to incubation in Vectastain Elite ABC reagent for 30 min at room temperature. Following additional washing with PBS, cells were incubated with NovaRed substrate solution until desired stain intensity developed. Washing cells with PBS halted development of color. To identify gonadotropes, cells were subjected to immunofluorescent staining for LHβ as described in immunocytocehmistry section. Unfortunately, the current procedure failed to produce specific staining on cultured pituitary cells using 10 nm E-HRP for staining. We believe that further refinement of the method may be required or increased dosage with E-HRP may be needed.

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