AN ABSTRACT OF THE THESIS OF

Kyle E. Ireton for the degree of Honors Baccalaureate of Science in Biochemistry and Biophysics presented on August 28, 2012. Title: Nongenomic Actions of Steroid Hormones in the Ovine Endometrium.

Abstract approved:_____

Dr. Fredrick Stormshak

The steroid hormone estrogen plays a crucial role in regulating certain mammalian tissues, especially female reproductive systems. Estrogen effects physiological changes in the cell by altering transcription activity for certain target genes, as a consequence of interactions between estradiol-17β (E_2) and its nuclear receptor (nER). This mechanism of cellular control is referred to as classical genomic regulation. Changes take hours to manifest, since they depend on gradual changes in protein populations. However, rapid cellular changes – such as activation of MAPK pathways – have been observed within seconds of E_2 exposure *in vivo*. These changes cannot be explained by classical mechanisms of genomic regulation, and thus are defined as nongenomic regulation. Such regulation is understood to be mediated by specific receptors localized to the plasma membrane (PM).

In studies conducted *in vitro* and *in vivo*, in murine models, the specific PM-localized E_2 receptor (mER) responsible for mediating nongenomic regulation has

been shown to be a specially translocated subpopulation of nER. However, these findings have yet to be corroborated *in vivo* in animals larger than mice. The specific purpose of the present study was to ascertain whether a correlation could established between levels of nER expression in the nucleus and expression of mER in the PM in cells of the ovine endometrium, comparing groups of ewes treated with either E_2 or progesterone (P_4). The hypothesis was that significant increases in levels of nER of E_2 treated ewes would be reflected by significant increases in mER, as compared to P_4 treated ewes.

Ovariectomized ewes were treated with E_2 and progesterone (P_4) to condition the endometrium, a rich source of endogenous nER. The ewes were then divided into two treatment groups. Group 1 received additional E_2 injections, known to elevate cellular nER levels, while group 2 received additional P_4 injections, known to suppress nER levels. Results demonstrated significant increases in nER correlated with significant increases in mER in E_2 treated ewes, as compared to P_4 treated ewes, which supports the study's hypothesis. These findings set the stage for further investigations into explicitly characterizing the nature of the mER in an ovine model, as well as continued exploration of nongenomic regulatory responses.

Keywords: Steroid hormone, estrogen, genomic regulation, nongenomic regulation, nuclear estrogen receptor, membrane estrogen receptor, ovine, reproduction

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Nongenomic Actions of Steroid Hormones in the Ovine Endometrium

by

Kyle E. Ireton

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I understand that my project will become part of the permanent collection of Oregon State University, University Honors College. My signature below authorizes release of my project to any reader upon request.

Kyle E. Ireton, Author

Acknowledgments

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Literature Review and Background

Characteristics of steroid hormones and their cellular receptors

Steroid hormones are small, nonpolar molecules that play an important role in regulating the function of certain mammalian tissues, especially by the regulation of cells' nuclear transcription activity. These steroids are derived from cholesterol, which is in turn biosynthesized from acetate. Hormones are produced by specialized organs (e.g. testes, ovaries, adrenal glands) and secreted into the bloodstream for distribution throughout the body, as part of the endocrine system (McCarty and McCarty, 1977). The main classes of hormones are sex hormones and glucocorticoids. The sex hormones testosterone and estrogen are produced in the testes and ovaries, respectively. Glucocorticoids, such as the stress-related hormone cortisol, are produced in other organs such as the adrenal glands. Sex hormones are especially important in regulating the proper development of sex-specific reproductive systems. Glucocorticoids serve a broader role in regulation, affecting a many organ systems (Beato and Klug, 2000).

In the hydrophilic environment of the blood stream, steroid hormones are bound to and transported by serum protein chaperones. For the androgens – estrogen, testosterone and their respective derivatives – the primary transport protein is the testosterone binding globulin. Glucocorticoids are primarily transported by corticoid binding globulin. Serum albumins are also known to weakly bind steroid hormones (McCarty and McCarty, 1977). Since the hormones are small relative to the plasma membrane (PM) and hydrophobic in nature, once

dissociated from their transport globulins they readily slip across the PM to enter cells.

As Jensen et al. (1968) first posited, once inside the cell steroid hormones are understood to move to the nucleus by a two-step mechanism: upon entry to the cell's cytoplasm, a hormone will associate with a specific receptor; the complex is then transported to the nucleus where it can affect transcription activity. In general, the intracellular steroid hormone receptors share four homologous domains: an N-terminal domain of variable length (A/B domain), a DNA-binding domain (DBD, C domain) necessary for genetic regulation, a hinge region to allow flexible movement between the DBD and ligand-binding domain (D domain), and a carboxy-terminal ligand-binding domain (LBD, E/F domain) for its specific steroid hormone partner (Bhasin et al., 2011).

The DBD contains a pair of zinc-coordinated structural modules which are necessary to facilitate DNA interaction. In each module, zinc is coordinated by four cysteine residues. These modules orient toward one another at right angles, forming a globular mass that mediates crucial receptor interactions. One module inserts into the DNA major groove, and contains a sequence of residues necessary for specific recognition of its interaction site. The other module contains a sequence that allows association with the receptor's dimer partner, as well as regions that facilitate less specific DNA interactions to stabilize the whole complex (Helsen et al., 2012)

The LBD is also crucial in mediating regulatory functions of the receptor complex. A region within the domain contains eleven to twelve anti-parallel α-helices arranged like a sandwich. This region can specifically bind a receptor agonist, and contracts to form a more compact structure upon binding. Upon contraction, LXXLL residue motifs are exposed. These motifs are mirrored in various cofactors needed for effective DNA transcriptional regulation, and allow the formation of intermolecular hydrophobic interactions to stabilize the regulatory complex. In addition, the LBD contains regions that contact homo- or heterodimer receptor partners, which are necessary for close dimeric associations and the stabilization of subsequent DNA interactions (Helsen et al., 2012).

Hormone receptors in the cytoplasm are often found in association with heat shock protein (HSP) chaperones. Importantly, HSP70 shelters the premature receptor in the cytoplasm until it assumes its mature conformation. The receptor is then transferred to HSP90, which shelters the receptor and its nuclear localization sequence (NLS) from the hydrophilic environment and transport proteins of the cytoplasm. Upon binding of a steroid to its specific receptor, HSP90 is thought to dissociate from the complex so that the receptor's NLS is exposed. The complex is consequently translocated to the nucleus (Tao and Zheng, 2011).

In the nucleus, the steroid-receptor complexes form homo- and heterodimers that can directly bind DNA. In association with other transcription factor elements, these DNA interactions can either promote or suppress the transcription activity of RNA polymerase II, and thereby control gene expression. The DNA regions

associated with steroid hormone receptor binding are known as hormone response elements (HRE). The HRE are characterized by hexameric inverted repeats of base pairs, separated by three nucleotides. One of the receptor subunits is thought to initially bind just one of the hexamers, which induces a conformational change in the protein or DNA and subsequently facilitates binding of the second dimer subunit to the other hexamer (Helsen et al., 2012).

Thus, steroid hormones are classically understood to modulate and reshape the levels of mRNA production of a wide variety of genes. This regulation therefore changes the expression profile of a cell gradually – over several hours to days. The timescale of regulation depends upon the magnitude of the initial hormone signal, as well as how long it takes for certain changes in transcription to manifest as a change in cellular physiology. Changes in cellular physiology are the result of changes in relative amounts of protein populations. These changes can come about as a result of increased mRNA transcription for certain proteins, or as initially present mRNA transcripts and protein populations are degraded over time as a result of ubiquitination and proteolysis or entropic decay (Pickart, 2001).

Estrogenic cellular regulation

The steroid hormone estrogen plays a crucial role in regulating normal mammalian growth and development, especially in the female reproductive organs and mammary glands. Estrogen is present in three distinct forms in most mammals *in vivo*, including estrone, estriol, and estradiol-17ß (E_2). Of these, E_2

displays the greatest binding affinity – by orders of magnitude – for the specific estrogen receptor (ER) (McCarty and McCarty, 1977). Estradiol is known to specifically bind nucleus-localized estrogen receptors (nER), which exist in the isoforms ERα and ERß. In complex with its receptor, E_2 regulates long-term female sex organ development by binding to specific HRE known as the estrogen response element (ERE) (Klinge, 2001).

Strasbourg mice, a mouse line bred as a double ERα knockout, demonstrate the vital importance of ERα-mediated regulation for normal growth, development, and reproductive function of female mammals. Female Strasbourg mice are wholly infertile, and exhibit phenotypic traits consistent with this condition. Vaginae and uteri are underdeveloped and incapable of mediating fertilization, while mammary glands are also underdeveloped. In addition, the mice display hemorrhagic ovaries with excessively high levels of closed-off follicles (Dupont et al., 2000). These findings indicate that genomic regulation facilitated by ERα is absolutely essential for proper reproductive processes to occur.

Unexpectedly, rapid cellular changes and regulatory activities have also been observed in response to E_2 – on the timescale of seconds to minutes. These changes cannot be satisfactorily explained by the classical mechanism of steroid hormone regulation, due to the time required for changes in transcription regulation to manifest as changes in cellular physiology. Previously observed changes include association with, and activation of, components of mitogen activated protein kinase (MAPK) pathways (Song et al., 2004; Stormshak and Bishop, 2008). Included

among the downstream effects of MAPK pathway activation is the activation of certain transcription factors, meaning E_2 may exert physiological effects independently of classically-described, nER-mediated nuclear transcription regulation (Kitamura et al., 2006).

MAPK pathways are normally triggered by binding of an extracellular chemical ligand to a PM-localized receptor. Thus, it is hypothesized that E_2 may trigger these pathways through an association with a heretofore undescribed pool of PM-localized receptors distinct from nER in the nucleus. This alternative mechanism of cellular regulation is described as being nongenomic or non-classical in nature. Because of E_2's importance in regulating normal growth and development, it is crucial to develop a more in-depth understanding of the all the ways this steroid hormone can regulate mammalian tissues. It is therefore vital to develop a more thorough understanding of the mechanistic pathways that underlie nongenomic cellular regulation and the putative membrane receptors that mediate such mechanisms.

Membrane estrogen receptor characterization

Specific external PM binding sites for estrogen were first observed by Pietras and Szego (1977). Endometrial and hepatic cells were found to tightly bind E_2 which had been covalently arrested to a membrane-impermeable immobile support. Intestinal cells were not able to reproduce this binding affinity. While addition of E_2 was able to displace bound cells, estradiol-17α (an inactive metabolite) was not,

indicating the specificity of the external receptor. This marked the first time the presence of a high-affinity, tissue-specific, external specific receptor for a steroid hormone was reported. Rapid E_2 regulatory mechanisms are believed to be mediated by a pool of these poorly-understood specific membrane receptors.

Ascenzi et al. (2006) demonstrated that rapid cellular responses can be elicited by membrane-impermeable E_2 conjugates, such as E_2 conjugated to bovine serum albumin (E_2-BSA). Rapid cellular responses to E_2 can are therefore mediated by a pool of membrane receptors distinct from nER. This finding shows that high-affinity, specific PM receptors for E_2, like those found by Pietras and Szego (1977), may be responsible for mediating nongenomic regulation. The membrane E_2 receptor (mER) is in fact believed to be a subpopulation of cellular nER – steroid hormone receptors otherwise identical to nER in the nucleus, except for being post-translationally modified and preferentially translocated to the membrane.

In 1999, Razandi et al. demonstrated specialized localization of ER to the PM in transgenic Chinese Hamster Ovary (CHO) cells. The cDNA for ERα was transiently transfected into CHO cells, which are inherently ER-deficient. Northern blot assay revealed the presence of a single ER mRNA transcript, but also specific binding of E_2 in both the PM and nucleus. Up to 3% of CHO cellular ER localized to the PM, offering the first direct evidence that mER could be a specially-distributed subpopulation of nER. The ERα has been reported by Acconcia and Marino (2005) to be specifically targeted for palmitoylation by an as-of-yet undescribed palmitoyl acyl transferase (PAT) enzyme. A nine residue sequence,

centered about cysteine 447 (C447) in the LBD, was necessary to facilitate palmitoylation. Specifically, this residue sequence included a tyrosine or phenylalanine at the -2 position (relative to the acyl-ligated C447 at position 0), an isoleucine or leucine at the +5 position, and a leucine at the +6 location. Mutations in these three residue positions were found to severely reduce measurable quantities of ERα cysteine acylation. Since the addition of acyl-hydrocarbon groups – such as palmitic or myristic acid – in general increases the external hydrophobicity of a protein and therefore its ability to associate with a membrane, these findings help to explain how nER could be targeted to the PM.

The 9 residue sequence required for C447 modification in ERα is in fact an important, highly-conserved sequence found in many other membrane receptors, and in all steroid receptors. The residue sequence takes the general form of $F(X_6)(I/L)L$ (Pedram et al., 2007). A well-described example of such a class of membrane receptors is the heptahelical G-protein coupled receptors (GPCR). Duvernay et al. (2004) reported that this sequence is needed for GPCR to be exported from the endoplasmic reticulum (ER) following translation. Mutations of the -2 F residue, the +5 I/L residue, and the +6 L residue in its key sequence were also found to prevent GPCR from localizing to the PM. Because GPCRs lack a cysteine residue in their key sequence, and thus cannot be acylated like the ER, these findings could indicate that the conserved 9 residue sequence alone – independent of acyl-hydrocarbon modification – may be sufficient for targeting a

receptor such as ER to the PM (where palmitoylation could subsequently occur) (Pedram et al., 2007).

However, with results published in the same study, Pedram et al. (2007) demonstrated otherwise. A mutation in the C447 residue of ERα preventing palmitoyl modification prohibited its PM localization, showing that the nine residue sequence alone was not sufficient for PM association without acylation. Despite this finding, the nine residue sequence could still be necessary and sufficient for directing ERα to the PM, prior to the critical acylation event. To further explore the role the key nine residue sequence plays in PM localization, an N-terminal myristoylation fusion site was cloned into both wild-type ERα (wtERα) and a mutant ERα (mutERα) – which contained key residue F/(I/L)/L mutations. Both wtERα and mutERα were myristoylated at the N-terminus, and thus localized to the PM. Yet, despite PM localization and the presence of an unmodified C447 residue, the mutERα was still not found to be palmitoylated. While these findings do not show if the key nine residue sequence is sufficient to bring ERα into close spatial association with a PM-partitioned PAT, they do seem to indicate that the highly-conserved sequence is required for mediating or stabilizing the interaction of ERα and its PAT.

Pedram et al. (2007) further demonstrated that palmitoyl acylation is needed for mediating the interaction of ERα with caveolin-1, since mutERα displayed substantially less caveolin-1 interaction than wtERα. This is crucial for membrane localization – as siRNA knockdown of caveolin-1 was found to preclude PM ERα

localization. Association with caveolin-1 permits close interactions between ERα and caveolar rafts in the PM. It was further reported in this study that the caveolin-1 mediated caveolar raft association was necessary to facilitate nongenomic, rapid cellular responses to E_2 exposure.

Pedram et al. (2009) then investigated the developmental phenotype of a transgenic mouse line bred to contain only a membrane-localized pool ERα (MOER mouse). Their findings demonstrated the limits of membrane ER-mediated regulation. The females' uteri and vaginas were found to be so severely atrophic that no pregnancies could be produced during breeding of female MOER mice with wild-type (wt) males. The developmental phenotype of these mice can be compared to that of Strasbourg mice, and the study's authors note that their findings demonstrate that the presence of a membrane receptor for E_2 alone is not sufficient to rescue the crippling reproductive deficiencies introduced by the absence of nER. On its own mER, is not able to fully substitute for the same regulatory function of nER *in vivo*. The mER must therefore serve a separate (although possibly related and/or inter-linked) regulatory functions in the cell.

Mechanistic pathways of nongenomic estrogen regulation

Activation of MAPK pathways leads to activation of certain transcription factors, among other downstream effects. Evidence of MAPK activation has been found both *in vitro* and *in vivo*. ERα was found by Song et al. (2002) to associate with Sarc homology collagen (Shc) adaptor protein in a ternary complex with

insulin-like growth factor 1 (IGF1), after stimulation of MCF-7 cells with E_2. Shc normally associates with son-of-sevenless (SOS) and growth factor receptor-bound protein 2 (Grb2) proteins to activate the MAPK cascade via stimulation of the small G-protein ras. In addition, Kitamura et al. (2006) demonstrated phosphorylation of the extracellular-regulated kinases 1 and 2 (ERK1/2) in a rapid response to *in vivo* injections of E_2.

Introduction to Experiment

The purpose of this experiment was to follow up on previously published research by Kitamura et al. (2006) on ovine nongenomic regulation by E_2 by advancing understanding and approaching characterization of the mER in an ovine model. Studies *in vitro* and *in vivo*, in murine models, have demonstrated that a post-translationally modified, subcellular population of nER can function as the mER, but these findings have not yet been corroborated *in vivo* in larger mammals. The present study pushes this field of research forward by expanding studies of the nature of the mER to an ovine model. This was done by determining whether a correlation could be established between levels of mER in the PM and levels of nER in the nuclei of cells in the ovine endometrium (the interior lining of the uterus), in ewes that were treated with E_2 or progesterone (P_4). The hypothesis was that significant increases in levels of nER in E_2 treated ewes would be reflected by significant increases in levels of mER – relative to ewes treated with P_4. Due in part to the recognition of practical limitations involved in obtaining large numbers

of experimental observations with adult ruminant mammals, a significant increase was defined as a variation between the two groups with $p < 0.10$ as determined by an unpaired t-test.

Levels of cellular nER *in vivo* are regulated by the steroid hormones E_2 and progesterone (P_4). Estradiol is known to upregulate its own receptor, making nER more abundant in the cell, while P_4 is inhibitory and decreases nER population levels (Koligian and Stormshak, 1977). In ewes, endogenous steroid hormones are produced by the ovaries in levels that vary over time in a regular, well-described pattern known as the estrous cycle. The estrous cycle develops and enriches the endometrium in preparation for the reception of a fertilized ovum (egg cell). Ovariectomized ewes injected alternately with E_2 and P_4 express physiological effects that essentially mimic the natural estrous cycle (Kitamura et al., 2006). Ewes treated with additional E_2 injections would be predicted to express more nER, while those treated with additional P_4 would be predicted to express less nER. Finding evidence of significant increase of both nER and mER in E_2 treated ewes, relative to P_4 treated ewes, would support the present study's hypothesis.

Because E_2 plays a vital role in regulating endometrium development and function throughout crucial reproductive processes, and because this tissue is naturally rich in nER, endometrial tissue is particularly valuable as an *in vivo* model for studying the importance of E_2 regulatory actions in ewes. The endometrial lining of the uterus is not an entirely uniform surface. It is populated with approximately regularly-spaced, yet asymmetrically distributed, collection of

structures known as caruncles that project upward from the otherwise flat tissue. These structures serve as biological hooks for the purpose of secure adhesion of a developing conceptus to the uterine wall throughout the course of a normal pregnancy. Caruncular composition is less homogeneous, more dense and fibrous, and less abundant in nER than the rest of the tissue lining (Cupps, 1991). Therefore, for the purposes of this study and as previously described (Kitamura et al., 2006), tissue samples were recovered exclusively from the tissue areas between the caruncles, referred to as the intercaruncular space.

Materials and Methods

Ten adult ewes, each weighing approximately between 125 and 150 lbs, were ovariectomized and divided into two treatment groups. These animals were subjected to a 9-day treatment regimen of daily, single subcutaneous of either E_2 or P_4 injections of 25 μg in 2 ml corn oil or 10 mg in 2 ml corn oil, respectively). All ewes received E_2 injections for the first two days, P_4 injections for the next five days, and then two days of either E_2 (group 1) or P_4 injections (group 2). On the following day, no injections were administered, and on day 11 of treatment tissue samples were collected. Ewes were anesthetized by a 3.5 ml intravenous (iv) injection of 100 mg/ml ketamine, followed by a 1.75 ml iv injection of 5 mg/ml diazepam. Anesthesia was maintained with a mixture of isoflurane and oxygen. Ewes were then secured to an elevated surgical table for tissue recovery. Midventral laparotomy (a large incision into the lower abdomen) was used to allow access to the

ewe's uterus. The endometrium was subsequently exposed, and tissue samples of approximately 2.5 g each were recovered from the intercaruncular space of both uterine horns.

Recovered tissues were placed immediately in an ice cold homogenization buffer of 25 mM Tris-HCl, 250 mM sucrose, pH 7.4 and transported from the surgical facilities to the laboratory. In the laboratory, tissue samples were blotted dry with Whatman filter paper, and the dry mass was weighed using glycine paper. Then, the tissue was finely minced with a razor and added to 10 ml fresh homogenization buffer for homogenization with a Tekmar Tissuemizer masticating pulverizer. Tissue samples kept on ice were subjected to 5 s Tissuemizer bursts, with 30 s between bursts to minimize the damaging effects of excessive friction and heating on the samples. The Tissuemizer was rinsed off into the sample solution with ~1 ml buffer, and the tissues were then further homogenized with the use of a glass Duall (no. 21) homogenizer. The Duall was used until any visible pieces of tissue could not be broken down further.

After homogenization, the samples were centrifuged at 1,000 x g for 10 min at 4°C. A nuclear pellet and enriched PM fraction supernatant were recovered. The nuclear fraction was set aside for E_2-binding assays and DNA quantification assays, while the supernatant was centrifuged again at 20,000 x g for 20 min at 4°C. The mitochondrial pellet was discarded, and the PM-containing supernatant was centrifuged once more at 100,000 x g for 1 hr at 4°C. The resulting PM pellet was gently washed three times, for 5 min each in a dilution buffer of 25 mM Tris-HCl,

0.01% sodium azide, pH 7.4. The pellet was then resuspended in ~1.0 ml dilution buffer and centrifuged at 100 x g for 5 min at 4°C to remove membranous aggregates. The PM-containing supernatant was set aside for E_2-binding and protein assays.

Specific binding of E_2 to the nuclear and PM fractions were separately quantified utilizing a [^{3}H]-estradiol-17β radioreceptor assay, as described by Kitamura et al. (2006). One ml aliquots of either fraction were placed into 4 tubes that contained 10 µL of 1 µM [^{3}H]-Estradiol, and 4 tubes that contained 10 µL 100% EtOH (hot tubes), or 10 µL of $1x10^{-6}$ M [^{3}H]-Estradiol and 10 µL 1 mM Diethylstilbestrol (DES) (hot + cold tubes). The tubes were gently vortexed and incubated at 37ºC for 30 min, followed by cooling at 4ºC for 10 min. Two ml 50 mM Tris-HCl buffer was added to all tubes, before centrifugation at 800 x g for 5 min. The supernatant from this centrifugation was discarded, while the pellet was resuspended in 50 mM Tris-HCl buffer and centrifuged for a second time, and then resuspended and centrifuged again. Two ml of 100% EtOH was added to each final pellet, and the tubes were incubated overnight at room temperature. The following day, all tubes were centrifuged once more at 800 x g and the supernatant was decanted into 5 ml liquid scintillation fluid. Each tube's content of [^{3}H]-E_2 was then counted in a Beckman scintillation spectrometer, and specific binding activity was calculated by subtracting the average activity of the hot + cold tubes (measuring nonspecific binding) from the average activity of the hot tubes.

The protein content of the PM fraction was determined by bicinchoninic acid assay (BCA) using BSA to generate a standard curve, and specific binding activity was expressed in units of fmol E_2 bound per mg membrane protein. The DNA content of the nuclear fraction was determined as described by Burton (1956), and specific binding activity was expressed in units of fmol E_2 bound per µg DNA. Means and standard deviations for both treatment groups was calculated, and specific binding activity in the nuclear and PM fractions was compared between the two groups. Statistical significance of the differences in the results from the two groups was determined by an unpaired t-test, with a significant change defined as $p<0.10$.

Results

Group 1 (E_2 treated) ewes (n=5) were found on average to exhibit 0.15 (±0.03) fmol nuclear E_2 specifically bound per µg DNA (Fig. 1). The average value for specific E_2 nuclear binding per µg DNA in group 2 (P_4 treated) ewes (n=5) was 0.06 (±0.02) (Fig. 1). With regard to membrane binding, group 1 ewes were found on average to exhibit 25.03 (±9.19) fmol specifically-bound E_2 per mg protein (Fig. 2). The average value of membrane specifically-bound E_2 per mg protein in group 2 was 1.47 (±0.91) fmol. Variation in average nuclear and membrane binding activity between groups 1 and 2 were found to meet criteria for significant variation ($p<0.10$), with p-values of <0.07 and <0.05 respectively.

Figure 1: Mean (±SE) concentration of endometrial nuclear bound E_2 in ewes treated with E_2 or P_4 on days 8 and 9 of a 9 day treatment regimen.

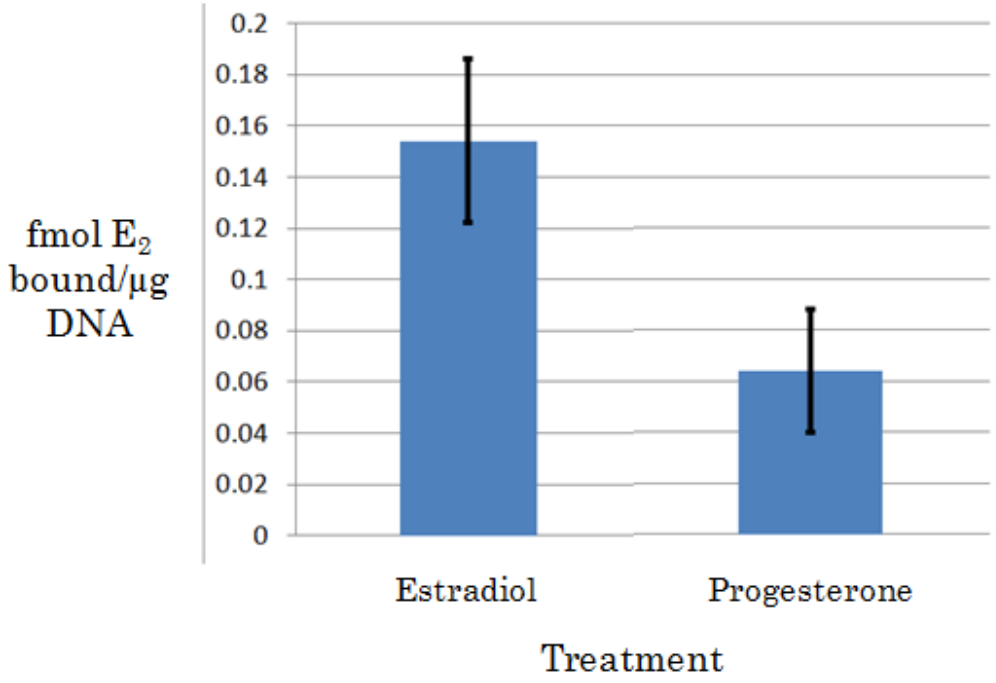
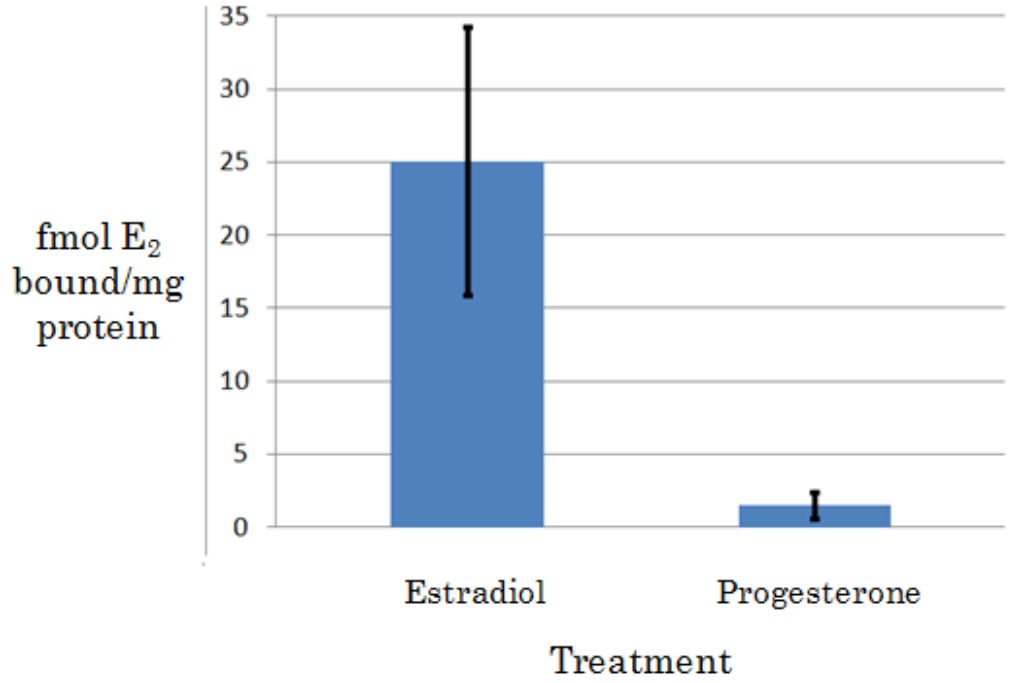


Figure 2: Mean (±SE) concentration of endometrial membrane bound E_2 in ewes treated with E_2 or P_4 on days 8 and 9 of a 9 day treatment regimen.



Discussion

The present study's results indicate, as predicted, that E_2 treated ewes (group 1) displayed significantly increased specific E_2 binding activity in the nucleus, compared to P_4 treated ewes (group 2). Because increased nuclear binding is indicative of higher levels of nER, group 1 ewes can be inferred to express relatively more nER than group 2. The observed difference in specific binding activity and inferred difference in levels of cellular nER between the two groups can be accounted for by the regulatory effects of E_2 and P_4 on nER, since E_2 is known to upregulate its own receptor and P_4 suppresses production of the nER. These data are corroborated by previous findings by Koligian and Stormshak (1977).

In order to support the present study's hypothesis that significantly increased levels of nER are correlated with significantly increased levels of mER in ewes treated with E_2 as compared to those treated with P_4, it would need to be demonstrated that group 1 ewes also display increased levels of mER relative to group 2. Group 1 ewes did in fact display significantly increased levels of specific E_2 binding activity in the PM, relative to group 2 ewes. Increased specific binding to the PM indicates higher levels of mER, thus group 1 ewes can be inferred to have higher cellular levels of mER as compared to group 2. This finding therefore directly supports the study's hypothesis, and demonstrates preliminary *in vivo* experimental evidence to support the model of nER functioning as mER in ewes.

Despite these findings, it of course cannot be explicitly or rigorously stated based on the present study alone whether the mER is in fact a post-translationally

modified population nER in ewes. These findings do, however, open the door to future studies supporting this model. For example, it would be useful to determine whether fully functional mER and nER arise from a single mRNA transcript *in vivo*. This could be assessed by determining whether a knockout or knockdown of the nERα mRNA transcript is sufficient for significantly hampering nongenomic responses in ewes. Finding evidence of this would help to corroborate the findings of Razandi et al. (1999), that a single nERα mRNA transcript in CHO cells *in vitro* gave rise to both mER and nER, with the findings of the present study. Still, the physical observations of the present study do provide partial support for such corroboration, since increased levels of nER – indicating increased levels of the nER mRNA transcript – were correlated with increased levels of mER.

In recent years, Kitamura et al. (2006) and Arreguin-Arevalo and Nett (2006) have both shown evidence of nongenomic, rapid regulatory actions triggered by E_2 treatment in ovariectomized ewes. Kitamura et al. (2006) found evidence of significantly increased phosphorylation of downstream products of the MAPK pathway, using a western blot, in ewes treated with E_2 to upregulate nER as opposed to ewes treated with P_4. Arreguin and Nett (2006) showed that ewes exposed to conjugated E_2 species, e.g. E_2-BSA or E_2 conjugated to a small peptide, were able to rapidly trigger an acute suppression of the secretion of luteinizing hormone (LH) (<20 min, $p<0.01$). However, the same conjugated E_2 species were incapable of inducing the characteristic pre-ovulatory surge of LH which occurs 10 hrs after exposure to E_2 alone. These results were interpreted to mean that the E2

conjugates generated the rapid suppression of LH via nongenomic mechanisms of regulation. These stunning results illustrate the potential physiological and biological significance of such regulation, and corroborate the results of the present study in an ovine model.

Additional observations in an ovine endometrial model would also be useful for establishing a quantifiable correlation between levels of nER and levels of mER, in order to establish more statistically rigorous support for the present study's hypothesis. Studies undertaken to correlate significantly increased quantifiable nongenomic responses along the MAPK pathway, e.g. evidence of Shc association or ERK1/2 phosphorylation as described by Kitamura et al. (2006), with significantly increased levels of nER and mER would also be useful for conclusively establishing that nER/mER are responsible for mediating such rapid cellular changes in large mammals, *in vivo*.

A possible evolutionary explanation for the basis of nongenomic estrogen regulation is that it increases a cell's sensitivity to essential estrogenic regulatory actions. Exposure to E_2 may result in both genomic and nongenomic regulatory downstream effects that make the cell more receptive to future E_2 exposure. Activation of MAPK pathways by E_2 binding to the mER could eventually lead to activation of transcription factors and altered nuclear transcription activity, which may in turn alter the ability of the cell to respond to classical genomic regulation by E_2. This nongenomic regulatory action may or may not be interlinked with classically-understood mechanisms of genomic regulation. Since the two seem to be

happening in the cell simultaneously, it is not beyond question that the nongenomic mechanism does serve to prepare or condition the cell to be receptive to the large-scale changes that are seen in response to classical genomic regulation.

Increases in cellular mER could be triggered by E_2 to further increase sensitivity in a positive feedback mechanism. Changes along these lines could include upregulation of production of nER and its specialized transport to the PM. Downstream effects of MAPK pathway activation may include heightened activity of the PAT responsible for acylating nER. Increasing mER availability would allow the cell to respond more sensitively to subsequent E_2 exposure, ensuring that it would be able to respond adequately to an organism's needs within a reasonable timeframe, thus conferring a reproductive advantage. A better understanding of the mechanisms underlying nongenomic regulation will help to explain the significance behind this evolutionarily-conserved process. Increasing understanding of nongenomic regulatory actions will also assist in understanding the overall importance of the essential role estrogen plays in mediating normal mammalian growth, development, and reproduction.

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