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Changes in Estrogen Receptor Alpha (ERα) Phosphorylation in Human T Cells

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CHANGES IN ESTROGEN RECEPTOR ALPHA (ERα) PHOSPHORYLATION IN HUMAN T CELLS

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For my family,

especially my parents and my sister,

who taught me I could do anything I put my mind to.

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An Abstract of the Thesis by
Samantha Meneely

Estrogen has two receptor proteins, estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ). ERα can undergo multiple post-transcriptional modifications (PTMs); however, relatively little is known about the function and regulation of any of the PTMs that ERα can potentially undergo, especially *in vivo*. In total, 19 phosphorylation sites have been identified in ERα thus far, and most sites contain a Ser-Pro motif. Different pathways are responsible for the phosphorylation of different sites. These pathways include mitogen-activated protein kinase (MAPK) signaling, IkappaB kinase complex alpha (IKKα), cyclin-dependent kinase 7 (CDK7), a subunit of transcription factor II H, protein kinase B (PKB), glycogen synthesis kinase-3 beta (GSK3β), mammalian target of rapamycin (mTOR/p70S6K), ribosomal s6 kinase (Rsk), and casein kinase II. Here, phosphorylation of three sites between resting and activated human T cells are compared. T cells were purified and total proteins were extracted from both resting and activated T cells. Changes in ERα were investigated via immunoprecipitation and Western blot. The amount of phosphorylation at each site was compared between resting and activated T cells, and the amount of phosphorylated receptor was adjusted to the total ERα in each sample. The results for a sample size of ten indicated that when ERα is at 100%, Ser 104/106 resting T cells are 89.30% and active are 92.00%, Ser 118 resting T cells are 80.08% and activated are 87.54%, and Ser

167 resting T cells are 86.44% and activated are 78.35%. Statistical analysis revealed the results were significant for both resting and activated T cell for ERα Ser 118 and Ser 167, but not for Ser 104/106 in those same conditions. These results provide a baseline for studying the phosphorylation changes in SLE patients. It is known that the MAPK-ERK1/2 pathway is abnormal in SLE; therefore, it is hypothesized there will be a decrease in phosphorylation in activated T cells compared with control.

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CHAPTER I

INTRODUCTION

Estrogen and its Receptors

Estrogen, a class of female steroid hormones, induces cellular changes to different mechanisms throughout the body (1). The systems include the bone, brain, uterus, breast tissue, ovaries, heart, and fat (1). The hormone serves as a ligand for two specific receptor proteins termed estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ) (2). These nuclear receptor complexes are dynamic transcription factors that can shuttle between the cytoplasm and nucleus in order to bind to specific DNA sequences of target genes and alter transcription rates (3, 4, 5). ERα and ERβ bind estradiol, a type of estrogen, with equal affinity, and both receptor subtypes interact with the estrogen-response element (ERE), an inverted-repeat DNA sequence found in the promoter regions of many estrogen-responsive genes and regulates the expression of ER-dependent genes (4, 5). Dimerization of the ER is required for transcriptional activity and in cells that express both receptor subtypes ERα and ERβ, can form heterodimers (5). ERα is unusual among nuclear hormone receptors, in that its turn-over rate is more rapid than other nuclear receptors with a half-life of approximately 4 hours

in breast cancer cells and in normal target tissues such as the uterus, indicating dynamic regulation by modulating factors (6, 7, 8, 9). In contrast, ERβ has a half-life of 8 hours and is predominately expressed in glanulosa cell-derived tissues, and to the lesser extent in mucinous tissue of epithelial origin (1, 10, 11).

Steroid receptors (SR) share a common structure of a carboxyl-terminal ligand binding domain (LBD) and an amino-terminal domain (NTD) (3) (Appendix A). The LBD is linked by a hinge region (H) to a highly conserved DNA-binding domain (DBD), comprising the hormone response element, and contains a hormone-dependent coactivator interface called activation function 2 (AF2) (3). Coactivators have a common signature motif, LXXLL, with which they can interact with ERα in a hormone dependent manner (4, 12). Crystallography shows that, when an antagonist, such as tamoxifen, binds to the LBD, helix 12 itself occupies the coactivator binding site, rendering ERα inactive (4, 13, 14, 15). In contrast, the NTD contains a hormone-independent coactivator interface AF1 (3). In a nonligand-bound state, helix 12 is highly mobile, and upon binding of an agonist it takes a more fixed position, stabilizing the conformation of ERα. Coactivators also bind to the AF1 domain of ERα, in a ligand-independent manner (4). Structural changes of ER can influence coregulator binding and hence potentially the response to ligands (4). However, in the presence of hormone antagonists, the ER undergoes a conformational change that facilitates co-repressor binding (5). The balance between activators and repressors may be a key concept for understanding the ER-regulated gene expression (5).

The NTD contains the majority of phosphorylation sites, which are phosphorylated by a wide range of kinases (2, 3, 16). Many of these sites identified in receptors isolated from hormone-treated cells contain serine-proline motifs (Ser-Pro) which can be recognized by the peptidyl prolyl isomerase (Pin1), which facilitates in protein folding (2, 3, 16). Net phosphorylation of the receptors also increases in response to hormone (2). Thus, in addition to inherent change of charge, phosphorylation of these sites can result in the isomerization and subsequent alteration of receptor structure (3).

Receptors respond differently in the presence or absence of hormones. For instance, ERα is predominantly nuclear in the absence of hormone, whereas in response to hormone treatment it undergoes nuclear localization (2). Without the hormone, several of the nuclear receptors, including the ER, are unable to bind to corepressors (5). In the classical mechanism of estrogen action, estrogens diffuse into the cell and bind to the receptor, which is located in the nucleus. This nuclear estrogen-ER complex binds to the ERE sequences directly or indirectly through protein-protein interactions with activator protein 1 (AP1) or specificity protein 1 (SP1) sites in the promoter region of estrogen-responsive genes, resulting in recruitment of coregulatory proteins to the promoters, increased or decreased mRNA levels and associated protein production, and a physiological response (1). This classical, or genomic, mechanism typically occurs over the course of hours. In contrast, estrogen can act more quickly (within seconds or minutes) via nongenomic mechanisms through other non-ER plasma membrane-

associated estrogen-binding proteins, resulting in cellular responses such as increased levels of Ca^{2+} or nitric oxide (NO), and activation of kinases (1).

Phosphorylation and Cell Signaling

SRs and their associated cofactors can be phosphorylated on multiple sites by a wide range of kinases, which regulate various functions such as protein stability, hormone sensitivity, DNA binding, subcellular localization, and protein interactions (3). These functions can determine the timing, specificity, and extent of SR target gene regulation (3). The specific response of SRs to the cognate ligands is largely influenced by the cellular context including the levels of active kinases and phosphatases in addition (3, 17, 18). Thus, at least some aspects of tissue specific actions of SRs are controlled by cell signaling pathways (3).

SRs exhibit an increase in receptor phosphorylation and, in the classical pathway, form homodimers that bind to response elements on DNA and recruit a series of coactivator complexes that modify chromatin to facilitate transcription of target genes (3). Although the majority of identified phosphorylation sites are serines (Ser), a few threonines (Thr) and tyrosines (Tyr) also have been identified (3) (Appendix B). In general, phosphorylation of serine residues in the AF1 domain of ERα appears to influence the recruitment of coactivators, resulting in enhanced ER-mediated transcription (19). Phosphorylation of sites within or outside the AF1 region may affect the AFα dependent binding of cofactors as well (4). Site-specific SR phosphorylation has

been studied extensively in ERα and to a lesser extent in ERβ (3). In the case of the ER, ER-mediated gene transcription is tissue and cell-specific and can be coordinately regulated by nongenomic signaling (5). In total, 19 phosphorylation sites have been identified in ERα thus far, and most sites contain a Ser-Pro motif (3, 4). This however, is not the case for Ser 167, which does not reside in a Ser-Pro motif (4).

There is the potential that phosphorylation not only causes a change in charge, but results in isomerization of the peptide bond, substantially altering receptor structure (2). Phosphorylation of the ER may change the three-dimensional structure of the protein. Unfortunately, thus far no full-length ERα has been crystalized (4). Due to the inability to study the crystalized structure, it is complicated to characterize the structural changes upon ligand binding or PTMs, such as phosphorylation (4). Furthermore, a conformational change due to phosphorylation could have consequences for the action of estrogens and antiestrogens (4).

In addition to phosphorylation, ERα can undergo other PTMs, which include acetylation, ubiquitination, and sumoylation (20, 21). However, relatively little is known about the function and regulation of any of the PTMs that ERα can potentially undergo and even less is known about their relevance *in vivo* (20). Phosphorylation plays a major role in the regulation of receptor stability, although the mechanisms for regulation appear to be receptor specific (2). Multifaceted mechanisms underlying estrogen action have been identified, including: multiple ERs and variants; multiple subcellular localization sites; multiple transcription coactivators and corepressors; multiple PTMs; multiple levels of cross talk with other signaling pathways; and multiple levels of control

of ER expression, including proteasomal-mediated degradation (20). Nevertheless, the studies support the idea that specific phosphorylation events within SRs can influence receptor localization, stability, dimerization, and transcriptional activity (3). The phosphorylation of ERα can affect DNA binding, for example, by inhibiting dimerization of the receptor, and can influence ERα activity by changing the binding of coactivators or the orientation of components of the transcription factor complex (4).

Most of the phosphorylation changes studied to date have been done in breast cancer, especially in the resistance to tamoxifen. The three sites studied in this research project were Serine 104/106 (Ser 104/106), Serine 118 (Ser 118), and Serine 167 (Ser 167). Interestingly, within the A/B domain of ERα, often only small effects on transcriptional function were observed when one site, e.g. Ser 104, Ser 106, and Ser 118 was mutated to eliminate phosphorylation (20, 22). The results illustrated that when the three sites were mutated, they appeared to be additive, giving an approximately 50% reduction in transcriptional activity (20). This showed that combinations of phosphorylation sites affected activity more than individual sites alone (20, 23, 24). Additionally, and more importantly, lack of phosphorylation of all of these sites does not eliminate estrogen-induced ERα transcriptional activity (20). Furthermore, other data suggests that it is the combination of phosphorylation sites within ERα rather than any one individual site that may be important for mediating effects of any individual kinase (20). This concept illustrates that combinations of PTMs of ERα rather than individual sites may be of primary importance in affecting function and response to endocrine therapies is emerging (20, 25, 26).

Ser 118 is one of the most reported phosphorylation sites of ERα, in part because phosphorylation of this site causes reduction in mobility of SDS-PAGE gels, and thus the level of phosphorylation at this site could be detected before the development of site specific phosphoantibodies (3, 4). Phosphorylation, at least at Ser 118, has been suggested to be involved in protein turnover via a proteasome-mediated mechanism (20, 27, 28). However, proteasome-mediated turnover of steroid receptors has been shown to be essential for the dynamic and cyclical nature of receptor occupancy on target gene promoters, which is in turn critically important for transcriptional activity (20, 29).

Different pathways are responsible for the phosphorylation of different sites. Estrogen induces mitogen-activated protein kinases (MAPK) signaling, which is essential for cell proliferation (5). Ser 118 primarily deals with the MAPK-ERK1/2 pathway, an important enzyme activated by growth factor receptor pathways, which can phosphorylate Ser 118 in a ligand-independent manner *in vitro* and *in vivo* (20, 23, 30). Ser 118 phosphorylation by MAPK-ERK1/2 increases binding of coactivator SRC3 and renders ERα hypersensitive to estrogen (4, 31, 32). This occurs upstream of the receptor, where the MAPK-ERK1/2 pathway can be activated by insulin-like growth factor (IGF) stimulation. This activation induces the phosphorylation of ERα Ser 118 and results in ERα activation and enhanced response to estrogen (4). Likewise, estrogen and epidermal growth factor (EGF) can induce the extracellular signal-regulated kinase 1 and 2 (MAPK-ERK1/2) pathway, which also leads to Ser 118 phosphorylation of ERα (4).

Interestingly, estrogen treatment is the most powerful stimulator of phosphorylation at Ser 118 and it is independent of MAPK-ERK1/2 (20, 35).

Furthermore, the Ser 118 site has been shown to be important for the direct binding to and activation/repression of a subset of endogenous ERα target genes (3). In addition to target gene induction, Ser 118 has been reported to be important for both ligand-dependent dimerization of ERα and ERα-mediated RNA splicing (3). While MAPK-ERK1/2 is the most common pathway, phosphorylation of Ser 118 can also occur through IkappaB kinase complex alpha (IKKα), cyclin-dependent kinase 7 (CDK7), a subunit of transcription factor II H, protein kinase B (PKB, which is also induced by EGF and IGF), and glycogen synthesis kinase-3 beta (GSK3β), which stabilizes ERα without ligand and modulates ERα transcriptional activity upon ligand binding (4, 19, 30, 33, 34, 35). This is especially common in breast cancer cell lines where there is a resistance to tamoxifen; however, the clinical relevance of Ser 118 in its resistance to tamoxifen is still unresolved (4).

Phosphorylation of Ser 118 affects the binding of coactivators in the presence of tamoxifen, which reduces binding to DNA when ERα is tamoxifen bound, decreasing the affinity for tamoxifen (4, 36). When Ser 118 was phosphorylated in a tamoxifen-resistant cell line, MAPK-ERK1/2 activity was found to be elevated and phosphorylation of Ser 118 was increased (4, 32). Activation of the MAPK-ERK1/2 pathway results in Ser 118 phosphorylation, but it also induces a bypassing of the ER pathway, thereby rendering tumors hormone-independent (4). Phosphorylation of Ser 118 has been associated with a more differentiated phenotype, good prognosis, and better response to tamoxifen,

which is supported by other studies (4, 37). Most importantly, these studies reported that the Ser 118 phosphorylation had no effect on the progression of disease or survival without tamoxifen treatment, thereby emphasizing that Ser 118 phosphorylation is a clear predictive marker for response to tamoxifen in these studies (4).

The predictive and prognostic value of phosphorylated Ser 118 was assessed in a randomized controlled trial of no systemic treatment versus two years of adjuvant tamoxifen therapy (20, 37). Improved recurrence-free survival was found in those patients whose tumors expressed high levels of phosphorylated Ser 118 (20, 38). In addition, there are data to support the view that combinations of phosphorylated Ser 118 with known biologically relevant biomarkers such as progesterone receptors (PR) may further improve the prediction of prognosis and response to endocrine therapy (20, 38). Such data supports the combined use of biologically relevant markers for the improved prediction of therapy response (20, 38).

Like Ser 118, Ser 167 is phosphorylated by MAPK-ERK1/2 and PKB (4, 19, 39, 40). In addition, it is also phosphorylated by protein 90 ribosomal s6 kinase (p90Rsk), mammalian target of rapamycin (mTOR/p70S6K), ribosomal s6 kinase (Rsk), and casein kinase II, upon estrogen binding of ERα (19, 4, 39, 40, 41). In the case of casein kinase II, it has been suggested that ligand-bound ERα undergoes a conformational change that exposes the Ser 167, making this residue available for phosphorylation (3). Overexpression of the epidermal growth factor receptor (EGFR) induces Ser 167 phosphorylation, increases binding of ERα to DNA, enhances the binding of coactivator SRC3 to ERα in the presence of estrogen, and consequently enhances transcription (4).

Increased phosphorylation at Ser 167 via PKB has been associated with poor clinical outcome in breast cancer patients treated with tamoxifen, with high risk for relapse and decreased overall survival, which would imply that phosphorylation of Ser 167 is associated with a worse disease outcome (4, 19, 20, 42). Comparing the levels of phosphorylated Ser 118 and 167 expression in primary breast tumors related to secondary tumors from 10 patients after relapse, a study found that there was increased levels of both phosphorylated Ser 118 and 167 on the secondary versus the primary tumors, although this was statistically significant only for phosphorylated Ser 118 (20, 42). This corresponds with the research performed on a set of 75 primary breast carcinomas of patients with metastatic breast cancer who received first-line endocrine treatment after relapse, and those staining high for phosphorylated Ser 167 relapsed later (4). This statistic changed with endocrine treatment, where metastases responded well to endocrine treatment and phosphorylated Ser 167 correlated with longer survival after relapse (4). This implies that phosphorylation of Ser 167 is a predictive marker for a good response to endocrine therapy (4).

Furthermore, in contrast to what would have been expected from laboratory model systems, higher expression of either phosphorylated Ser 167 and/or Ser 118 is most often, but not always, associated with a better clinical outcome in patients on tamoxifen therapy (20, 38, 42, 43). High levels of phosphorylation for Ser 167 expression is the better predictor of benefit from tamoxifen and also suggests that both of these phosphorylation sites either alone or in combination in primary breast tumors may be useful biomarkers of endocrine therapy response (19, 20). Either phosphorylated Ser

118 or Ser 167 were found to be associated with the parameters of less aggressive and more differentiated tumors as well as an intact estrogen-responsive signaling pathway (20). Moreover, in vitro, phosphorylated Ser 167 reduces sensitivity to tamoxifen, keeping in mind that clinical data of Ser 167 phosphorylation are conflicting (4).

Tamoxifen resistance has been associated with several kinase pathways, which occurs upstream of ERα (4). Pathways include activation of the protein kinase A (PKA), MAPK-ERK1/2, GSK-3, IKKα, CDK7, mTOR/p70S6K, and p21-activated kinase 1 (PAK-1) signaling pathways (4). These kinases induce phosphorylation of ERα or of its coregulators (4). Dependent on the pathway and the phosphorylation sites involved, tamoxifen response can be affected either directly through ERα modification or by activation of other signaling pathways (4). Phosphorylation of Ser 118 is described as an example of this: an activated MAPK-ERK1/2 pathway phosphorylates Ser 118, but possibly induces tamoxifen resistance through the MAPK-ERK1/2 pathway itself, rather than ER signaling (4).

A third site that known to be resistant to tamoxifen is Ser 104/106. This site is also phosphorylated by ERα and signaling pathways. Most functional studies of Ser 104/106 have also included the Ser 118 site (4). Serine residues 102, 104, and 106 at the N-terminal AF1 of ERα are phosphorylated by GSK-3 and MAPK-ERK1/2 pathways (4). These modifications lead to ligand-independent transcription of ERα and to an agonistic activity of tamoxifen (4). Ser 104 and Ser 106 can also be phosphorylated by the CDK2/cyclin A complex; and cyclin A has been reported as a predictive marker for

tamoxifen resistance in breast cancer patients (4, 44). Because of this, tamoxifen resistance is likely to occur when Ser 104/106 is phosphorylated (4).

Abnormalities- Breast Cancer

Estrogen and phosphorylation of the three sites (Ser 104/106, Ser 118, and Ser 167) has been studied most extensively in ER-positive breast cancer. ERα is the main target of endocrine therapies because the nuclear hormone receptor is a master regulator of gene expression and proliferative of breast cancer cells (6). Approximately 70% of human breast tumors express ERα and depend on estrogen for growth; therefore, endocrine therapy has become the most important treatment option for women with ER-positive breast cancer (6, 19). Advanced-stage breast cancers often lack SRs and/or are resistant to endocrine therapies (45). PRs are key markers for steroid hormone dependence and indicators of disease prognosis in breast cancer; their loss signals development of the aggressive tumor prototype associated with acquisition of enhanced sensitivity to growth factors (45, 46, 47). Many breast cancer patients, especially those in advanced stages, with tumors expressing high levels of ER are unresponsive to endocrine therapy; and all patients with advanced disease eventually develop resistance to the therapy (19). An urgent issue is the discovery of prognostic methods to identify those patients who need additional adjuvant therapy, such as signal transduction inhibitors or chemotherapy, for ER-positive early breast cancer (19, 48, 49).

The regulation of the cellular level of ERα is key to the effectiveness of endocrine therapies in breast cancer, and an understanding of its underlying mechanism is critical for the identification of novel drug targets for the design of combinatorial therapies (6). Treatment options for tumors rendered positive for ER include drugs such as selective ER modulators/antiestrogens (such as tamoxifen) and aromatase inhibitors, which are quite effective and have relatively few side effects (6). Over the last 30 years, tamoxifen has been the antiestrogen of first choice (4, 50, 51). However, about half of the recurrences in ER-positive breast cancer do not respond to tamoxifen (4, 50, 51). This is due to either acquired resistance or to intrinsic insensitivity to tamoxifen (4). From experimental studies, many different mechanisms have been suggested to explain resistance, including activation of kinase pathways or inactivation of retinoblastoma protein (pRb), a tumor suppressor protein that is dysfunctional in breast cancer (4). These changes in activation render the tumor cell independent of the ER pathway for its proliferation (4). Tamoxifen stimulates the growth of osteoblasts (bone formation), while it inhibits ERα-positive breast tumor cells (4). These two opposing effects of tamoxifen on cell growth can be explained by the fact that tamoxifen is a partial antagonist, acting as an agonist under particular conditions (4, 52). Tamoxifen resistance is often attributed to a direct effect on ERα; tamoxifen may acquire agonistic properties for transactivation of ERα (4, 12). Therefore, a molecular understanding of the underlying mechanism of tamoxifen resistance could result in markers that specify how a patient will respond to endocrine therapy (4).

Another cell cycle regulator is cyclin D1, which forms a complex with other regulatory subunits whose activity is required for G1/S transition in the cell cycle. In breast cancer cells, cyclin D1 protein is often upregulated (5). It can function as a bridge and recruit steroid receptor coactivators to the ER and stimulate transcriptional activation in the absence of estrogen (5). The binding of cyclin D1 to the ERα promoter increases expression because BRCA1 (breast cancer 1, early onset), a repressor of ERα transcription, is unable to bind when the promoter is occupied by cyclin D1 (5). ERα is lost in a breast cancer cell line by direct binding of a transcription factor, Snail – a repressor of E-cadherin, with DNA-regulatory regions along the ERα promoter (5). Loss of ERα signaling leads to altered transforming growth factor β (TGF-β) signaling in that breast cancer cell line (5). TGF-β regulates cell growth, therefore altering this signaling contributes to breast cancer.

Two current hypotheses exist to explain the relationship between breast cancer and estrogen (1,53). The first, binding of estrogens to the ER stimulates proliferation of mammary cells, increasing the target cell number within the tissue, and the increase in cell division and DNA synthesis elevates the risk for replication errors (1). This may result in the acquisition of the detrimental mutations that disrupt normal cellular processes such as apoptosis, cellular proliferation, or DNA repair (1).

In the second hypothesis, estrogen metabolism leads to the production of genotoxic by-products that could directly damage DNA, again resulting in point mutations (1). There is evidence that estrogen may act through both mechanisms to initiate and/or promote mammary cancer (1). Several sequence variations or single-

nucleotide polymorphisms (SNPs) in the ERα gene (ESR1) have been identified that are associated with either an increased or decreased risk of breast cancer (1). The best-characterized SNPs of ESR1 are the *PvuII* and *XbaI* restriction site polymorphisms, both of which are located in the first intron (1, 54, 55). The *PvuII* is associated with increased breast cancer risk, as well as risk for other diseases in which estrogen is implicated (1, 56). Both cell culture and animal model studies indicate that the ER is involved in the mammary gland development and mammary cancer (1). Studies in ERα knockout mice demonstrate that ERα is required for normal mammary gland development (1, 57).

Exploring the effects of phosphorylation on Ser 118 and Ser 167 has led to an increase or decrease in overall survival. Murphy et al. (19, 38) reported that in 45 human breast tumor biopsies, phosphorylation of ERα Ser 118 correlated with active MAPK-ERK1/2. Because MAPK-ERK1/2 is located downstream of human epidermal growth factor receptor 2 (HER2), it is possible that phosphorylation of ERα Ser 118 is in part caused by HER2-MAPK-ERK1/2 signaling in breast cancer (19). On the other hand, phosphorylation of ERα Ser 167 seems to be controlled by different mechanisms (19). Phosphorylation of Ser 118 and Ser 167 was previously analyzed using immunohistochemistry (IHC) in primary breast tumor specimens from 75 metastatic breast cancer patients who received first-line treatment with endocrine therapy on relapse (19, 42). The results indicated that patients whose primary breast tumors showed high phosphorylation of Ser 167, but not Ser 118, responded significantly to endocrine therapy and had a better survival than other patients, suggesting that phosphorylation of Ser 167 frequently occurs via estrogen-dependent signaling in

human breast cancer (19). The study concluded that for the first time it has been demonstrated that low phosphorylation of Ser 118 and high phosphorylation of Ser 167 affects survival in ER-positive breast cancer and could be helpful in distinguishing patients who are likely to benefit from endocrine therapy alone from those who are not (19).

Transcription and Ubiquitination

The regulation of gene expression by transcription via nuclear receptors such as ERα is critical because it controls the phenotypic properties and diverse biological functions of target cells. This regulation is conducted in two ways, classical and indirect. In the classical way, an estrogen-bound ER dimerizes (forms the protein structure), binds to the ERE, and transcribes the gene that lies within its proximity (4). The ER can also regulate transcription of genes in an indirect manner by binding to other transcription factors: AP1, SP1, or activated NFkB (4). When these interactions occur, transcription of the AP1, SP1, or NFkB-dependent genes also becomes dependent on ERα (4). The indirect method involves the receptor interacting with other transcription factors to bind DNA by tethering to regulate target gene expression (3).

Co-repressors and coactivators function widely in transcriptional regulation (5). Recent evidence suggests that the recruitment of coactivators and co-repressors to the promoter of ER target genes can be affected by the binding of estrogen (ligand), antiestrogens, and the ERE along the DNA (5). The co-repressors repress gene

transcription by blocking access of other factors to DNA regulatory regions (5). They operate by recruiting histone deacetylases (HDACs), allowing them to bind more tightly to DNA, and interfere with transcriptional initiation (5). Activation of one receptor can trans-repress the activity of another receptor by depleting a common coactivator pool, and overexpression of these limiting factors can reverse this trans-repression or squelching phenomenon (59, 60, 61, 62).

In contrast, nuclear receptor coactivators (NRCoA) are molecules that interact with ligand-bound nuclear receptors and serve to facilitate the efficient transcriptional regulation of target genes (59, 63, 64, 65, 66, 67, 68, 69, 70). Histone acetyltransferase (HAT) adds acetyl groups to DNA, allowing the histones to unbind and leaving the DNA free for transcription. HAT activity was the first enzymatic activity attributed to coactivators (59). It has been proposed that coactivators are able to enhance gene transcription either by acting as a bridge between the activated nuclear receptor and general transcription factors (GTFs) and/or as catalytic enzymes, which may covalently modify histones, GTFs, receptors, coactivators, and other proteins (59). The SR coactivators have been shown to contain HAT activity that may contribute to their ability to enhance receptor-mediated gene transcription (59). Recently, ATPase, methyltransferase, and ubiquitin-conjugation and ubiquitin-ligase activities have also been detected in coactivators (59). Furthermore, it has been proposed that when assembled at the promoter of hormone-responsive genes, the concert of HAT, methyltransferase, ATPase, and bridging activities contributed by coactivators stimulate

transcription through nucleosome remodeling and/or covalent modification of other components of the transcriptional complex (59).

Ubiquitination is a PTM where ubiquitin is attached to a substrate protein and can affect proteins in many ways. These affects include signaling for the cells' degradation via the proteasome, altering their cellular location, affecting their activity, and promoting or preventing protein interactions. This pathway is the major system in eukaryotic cells for selective degradation of short-lived regulatory proteins, such as ERα, controlling the levels of target proteins and/or compositions of multiprotein complexes in cells by targeted protein degradation (59). During the ubiquitin-proteasome pathway, the highly conserved 76-amino acid ubiquitin protein is covalently attached to target proteins, which are then degraded by the 26S proteasome (59).

The conjugation of ubiquitin to target proteins involves three consecutive steps mediated by activities of the sole E1 ubiquitin-activating enzyme (UBA), multiple E2 ubiquitin conjugating enzymes (UBCs), and multiple E3 ubiquitin-protein ligases (UBLs), respectively (59) (Appendix C). The initial step activates ubiquitin via the UBA enzyme in an ATP-dependent reaction. The next step maintains the high-energy linkage by transferring ubiquitin from the UBA enzyme to any one of a number of UBC enzymes (59). Finally, UBC enzymes transfer ubiquitin covalently to target proteins either directly or in conjunction with a UBL enzyme that defines target specifically (59). This occurs when the first ubiquitin molecule binds to the ε-amino group of lysine residues of the target protein (59). In succeeding reactions, a polyubiquitin chain is synthesized by transferring activated ubiquitin to lysine of the ubiquitin moiety previously linked to the

target protein (59). Finally, the polyubiquitinated target protein is degraded by the 26S proteasome, a large multisubunit protease that resides both in the nucleus and cytoplasm (59).

SRs action can be limited through the ubiquitination process, where the receptors can be ubiquitinated, exported to the cytoplasm, and undergo proteasome-mediated degradation (3). NRCoA was originally thought to exist based upon the fact that the different receptors compete for a limited pool of accessory proteins that are required for maximal gene transcription (59). However, recent identification of the ubiquitin-proteasome pathway components as coactivators link this pathway to nuclear receptor-mediated gene transcription (59). These studies demonstrate that the ubiquitin-conjugating enzyme, UBC9, the E3 ubiquitin-protein ligases, E6-associated protein (E6-AP) and receptor potentiation factor 1/reverse Spt phenotype 5 (RPF1/RSP5), interact with nuclear receptors and modulate their transcriptional activity (59).

The coactivators of the ubiquitin-protease pathway are arranged in different places along the ubiquitin-proteasome protein degradation system, harboring enzymatic activities such as ubiquitin conjugation, ubiquitin-protein ligation, and ATPase activities (59). There are two possibilities regarding how the enzymes operate in transcriptional activity. The first is the enzymes of the ubiquitin-proteasome pathway exert a positive effect on transcription by promoting degradation of negative regulators of gene transcription (59). The second, these enzymes may be employed in the obligate turnover of positively acting factors such as receptors, GTFs, and coactivators (59).

Consistent with the second possibility, estrogen receptors, along with progesterone, glucocorticoid, retinoid, and thyroid receptors are ubiquitinated and degraded through the ubiquitin-proteasome pathway (59).

Ubiquitin-proteasome activity was shown to be required for the transcriptional activities of most members of the nuclear hormone superfamily, but not other nonnuclear receptor transcription factors (59). Furthermore, RNA polymerase II has also been shown to be ubiquitinated, indicating that protein turnover is an integral part of gene transcription (59). Microscopic analysis has also revealed the presence of proteasome subunits at the loci of hormone-responsive genes (59). These observations all imply that ubiquitin-proteasome-mediated protein degradation is an important component in eukaryotic gene transcription (59).

The process of proteasomal-mediated ubiquitination is illustrated in the cellular turnover of ERα and the identification of S-phase kinase-associated protein 2 (Skp2) (6). Skp2 is an F-box protein (FBP) and a substrate recognition component of the Skip, Cullin, F-box (SCF) ubiquitin ligase complex, which is overexpressed in many cancers, including breast cancer (6). The protein functions as a novel E3-ubiquitin ligase that regulates ubiquitination and the turnover of ERα upon specification by the p38 mitogen-activated protein kinase (p38MAPK)-mediated phosphorylation of the receptor while positively regulating the functional activity of this receptor (6). Scientists have observed that ERα and the E3 ubiquitin ligase Skp2 appear to be inversely correlated (6). This concept was confirmed while observing the half-life of ERα which was shortened from 4 hours to 45

min with overexpressed Skp2, implying SCF^{Skp2} E3-ligase to be a regulator of ERα protein turnover (6).

T Cells

T cells, also called T lymphocytes, are an integral part of the immune system. Peripheral autoimmune T cells recognize dominant self-antigens, which is a property of all healthy immune systems. The number of T cells is indicative of a healthy individual and a count that is too low or too high is a sign of a disease, such as HIV or lupus. Human T cells express both ER subtypes, and the use of receptor-specific ligands indicates that the receptors are functional (17, 71). Furthermore, it had been shown that estrogen increases two markers of T-cell activation (17, 72, 73).

Protein kinase C (PKC) is a family of enzymes involved in controlling the function of other proteins through the phosphorylation of amino acid residues on these proteins. PKC is thought to be the mediator of the phosphorylation events that occur after treatment of cells with phorbol 12-myristate 13-acetate (PMA) (74). This occurs in conjunction with ionomycin, which is used to raise the intracellular level of calcium.

The cluster of differentiation (CD3) T cell co-receptor is a protein complex composed of four distinct chains. The chains associate with a molecule known as the T cell receptor (TCR) to generate activation signal in T lymphocytes. Stimulation of T cells with antibodies to the CD3/T-cell receptor complex causes turnover of phosphatidylinositol to form inositol trisphosphate (IP3) which can mobilize calcium from cytoplasmic stores (74). Diacylglycerol (DAG) is a second messenger molecule used

in signal transduction and lipid signaling in biological cells. It is a product of the phospholipid phosphatidylinositol 4,5-bisphosphate (PIP2) by the enzyme phospholipase C (PLC), which produces IP3 in the same reaction.

This signal transduction pathway can lead to T-cell activation when other required signals are provided by accessory cells: interleukin 1, a group of 11 cytokines which plays a central role in the regulation of immune and inflammatory responses; as well as anti-Tp44 and anti-CD5 monoclonal antibodies (Mabs), which bind to the antigen on the surface of the T cell protein (74). The increase in cytoplasmic Ca^{2+} in normal T cells after anti-CD3 stimulation was sensitive to inhibition by pertussis toxin, supporting the conclusion that this activation pathway relies on phospholipase C-dependent formation of IP3 and DAG (12).

There is evidence that anti-CD3 causes cytoplasmic calcium levels to increase through two mechanisms: to control a membrane potentially sensitive calcium gate and to cause mobilization of cytoplasmic calcium through PKC-mediated hydrolysis of PIP2 (74). The calcium increase after CD3 stimulation was only partially inhibited by ethylene glycol tetraacetic acid (EGTA) (related to EDTA), which binds to calcium and prevents adjoining of cadherins; but was totally inhibited by pertussis toxin (74). Pertussis toxin ribosylates (attaches a ribose or ribosyl group to a molecule) and thus inactivates Gi (inhibits the production of cAMP from ATP) and other GTP-binding proteins (G proteins) that regulate signal transduction, including phospholipase C activation in neutrophils (74).

While CD3 activates T cells, T cells themselves can activate different cell signaling pathways, such as MAPK-ERK1/2 signal transduction pathway through the stimulation of T cells with antibodies against the T cell receptor complex. Anti-CD3 antibody and PMA activate MAPK signaling through ERK1/2 and affect T-cell responsiveness by altering the levels of transcription factor activity (17). MAPK-ERK1/2 signaling controls enzymes that modulate DNA methylation which in turn exerts direct effects on gene expression (17). The altering of target gene transcription also occurs when estrogen binds to its receptors. However, estrogen can also act through the plasma membrane and rapidly stimulate second messengers including calcium flux and kinase activation (17, 75). ERK activation by estrogen has been reported in tumor cell lines, vascular endotheium and osteocytes (17). The importance of this rapid signaling at the plasma membrane for subsequent cell function has been shown by increased proliferation, survival and migration of target cells (17).

Abnormalities – Systemic Lupus Erythematosus

Systemic lupus erythematosus (SLE) is a prototypical systemic autoimmune disease characterized by multiple organ damage, high titers of antibodies, and various clinical manifestations (76). The characteristic manifestation is the butterfly rash that erupts on the cheeks of the face. SLE affects an estimated 1.5 million people in the United States, with 16,000 new cases reported every year. Of those cases, there is a 10-15 times higher frequency of SLE in women during childbearing years, probably due to

an estrogen hormonal effect; and several studies have suggested that gender differences in lupus susceptibility are mediated by sex hormones, consistent with the fact that 90% of SLE sufferers are females (76). Furthermore, there is an increased risk of developing SLE in postmenopausal women who received estrogen hormone replacement therapy (76).

It is well established that ERs stimulate the proliferation of a variety of cell types including cells belonging to the immune system (5). Estrogen is found to be a potential contributor to the development of biased autoimmune disease, such as SLE (5). Overexpression of cell-cycle-regulatory proteins may lead to abnormal estrogen-dependent gene regulation (5). ERα is an epigenetically regulated gene, and T cells from SLE patients have decreased total genomic methylation compared with age-matched controls, predicting an increase on transcription. This is consistent with a report suggesting ERα transcripts are higher in circulating T cells of SLE patients compared with normal T cells (5). This is reversed at the protein level, however, where ERα is lower in the T cells of some SLE patients compared with the amount of T cells from normal individuals (5).

Low ERα levels in SLE T cells could result from increased turnover in the protein, in which protein degradation via the ubiquitin-proteasome system has emerged as a major regulator of nuclear receptor transcription (5). The 20S proteasome β subunit low molecular mass polypeptide 2 (LMP2) is necessary for ER-dependent transcription and cell-cycle progression (5). It is thought that inappropriate assembly and turnover of the ERα complex could underpin the aberrant estrogen-dependent gene regulation (5). This

could contribute to global deregulation of gene expression, since SLE T cells are known to be deficient in protein phosphorylation (5). Results suggest that the T cells from SLE patients with inactive or mild disease respond to estrogen by suppressing phosphorylation, but as disease activity increases, the suppressive effect is lost (5).

The primary cause of disease onset is suggested to occur from defects in T cell-mediated signaling that leads to hyper-responsive B cells, increased cytokine production and the breakdown of immunological tolerance (17). Estrogen increases the production of cytokines and immunoglobulins in circulation, and is reported to enhance the proliferation of T cells and macrophages and to directly stimulate the expression of genes in mouse B cells that allows some autoreactive cells to escape apoptosis (5).

Cytokine networks have been studied in pregnant women, and are found to be involved in sex hormones. These networks regulate the level of sex hormones both systematically and locally, especially in the reproductive organs (76). There are two types of cytokines that estrogens mediate: T helper type 1 (Th1) and T helper type 2 (Th2). Estrogens inhibit cell-mediated immune response via Th1 cytokines and induce antibody production via Th2 cytokines. Low levels of estrogens and interleukin-6 (IL-6) are thought to be responsible for low activation of the humoral immune response, which then leads to the lower disease activity observed over the same period in SLE patients (76). This is suggested from research preformed on SLE patients and healthy controls in their third trimester of pregnancy, which found that IL-6 progressively increases in maternal circulation in healthy individuals during pregnancy, but low levels of IL-6 have been reported during the third trimester of pregnancy in SLE patients (76).

Proposed Project

The intent of this research was to identify whether there are alterations on three specific phosphorylation sites (Ser 104/106, Ser 118, Ser 167) of the ERα between resting and activated T cells. Based on previous work conducted in the laboratory, we know that the MAPK-ERK1/2 pathway phosphorylates T cells, and the pathway is abnormal in SLE T cells. The purpose of this study was to see if there were differences in the three sites in healthy volunteer women to get a baseline before conducting this study on SLE patient.

The research was conducted by analyzing ERα in human T cell extracts using phospho-specific antibodies and chemiluminescent detection on Western blots. Statistical analyses was completed to show the significance in the changes of phosphorylation.

CHAPTER II

MATERIALS AND METHODS

Study Participants

Person	Age	Ethnicity	Estradiol/Estrogen Levels (pg/mL)
1	22	Caucasian	298.45
2	22	Caucasian	313.44
3	36	Caucasian	112.14
4	32	Caucasian	267.67
5	35	Caucasian	300.72
6	18	African American	309.23
7	23	Indian	124.61
8	27	Indian	334.38
9	20	Caucasian	161.36
10	19	Caucasian	160.01

Table 1

Table 1. The study participants were 10 healthy female volunteers. It was important that they were not on birth control that regulated their estrogen hormone levels and had a regular menstrual cycle. There was no age requirement as long as they had regular menstrual cycles and their estradiol were in the normal range. The plasma estradiol/estrogen levels showed to be within the normal range for women with regular menstrual cycles.

T Cell Separation

80 mls of blood was drawn from the female control volunteers 0sing BD Vacutainer K2 EDTA (K2E) Plus Blood Collection Tubes and diluted about 1:1 with 1X PBS (phosphate buffered saline), making sure the tubes were mixed well before collecting and after diluting. The diluted blood was layered over 12 ml of Histopaque 1077 and centrifuged for 20 minutes in a centrifuge at 1600 rpm at 22°C. Plasma (1.5 ml) was collected for future use, stored at -80°C, and the rest of the platelets and most of the medium was discarded. The white blood cell layer was carefully collected into a clean tube and the volume was brought up to 50 ml with medium (1X PBS). The cells were washed at 1900 rpm for 10 minutes and the medium was poured off. The pellet was resuspended and 10 ml of medium was added. The volume was brought up to 30 ml with medium and washed again before being centrifuged at 1600 rpm for 10 minutes and the wash medium was poured off. While the cells were being centrifuged, lysis

buffer was prepared by adding 1 ml of H-Lyse buffer into 9 ml of sterile water and mixing well. Lysis buffer (10 ml) was added to the cells and incubated at room temperature for 10 minutes. The ammonium chloride in the solution disrupted the osmolality of the red blood cells (RBCs) but not the T cells, causing lysis of only the RBCs. The volume was brought up to 50 ml with 1X wash buffer and centrifuged the cells at 1900 rpm for 10 minutes.

The R&D System Human T Cell Enrichment Column allows for negative selection of $CD3^+$ T cells. The column has mononuclear cell suspensions, which allow for B cells and monocytes to bind to glass beads coated with anti-Ig and Ig respectively. The column was prepared by equilibrating the column and the 1X Column Wash Buffer at room temperature. After it was at 22°C, the column was placed in the column rack before removing the top cap followed by the bottom cap. The fluid in the column was allowed the drain into a waste receptacle before the column was washed with 6 ml of Column Wash Buffer. Once the column had been washed, the waste receptacle was replaced with a sterile 15 ml polypropylene tube. After cells are centrifuged, the wash and lysis buffer was poured off and the cell pellet was resuspended in 1 ml of column Wash Buffer. The cell suspension was added to the column and the filter was removed for proper draining. Once the cells had drained down to the white filter, they were incubated at room temperature for 10 minutes. The cells were eluted from the column using 4 aliquots (2 ml each) of Column Wash Buffer. The collected T cells were centrifuged at 250 xg for 5 minutes. The T cells were now pure and ready for use. The cells were either activated for 4 hours or lysed and stored at -80°C.

Pharmacological Activation of Human T Cells

The T cells were activated by the addition of phorbol 12-myristate 13-acetate (PMA) at 1 mg/ml in sterile dimethyl sulfoxide (DMSO). The stock was diluted 1:100 so 10 µl of stock was added to 990 µl of serum free culture medium with glutamine to make 10 µg/ml. Stock (1 µl) was added to 1 ml of culture medium for a final concentration of 10 ng/ml.

For the ionomycin, the stock was made up of 1 mg/ml in sterile culture medium or RPMI medium. The stock was diluted 1:10 for a concentration of 0.1 µg/µl. Ionomycin (5 µl) was added per ml of serum free medium with glutamine for a final concentration of 0.5 µg/ml. PMA and ionomycin enhance the activation of PKC and causes an influx of Ca^{2+}.

Immunoprecipitation Using Antibodies and Protein A/G PLUS Slurry

After the T cells were either activated or the resting T cells were pelleted, they were resuspended in 1 ml of PBS and transferred to 1.5 ml eppendorf tube. The tubes were centrifuged at 8000 rpm for 5 minutes and the medium was removed before 500 µl of lysis solution (10 mM tris-HCl, pH 7.5, 30 mM sodium pyrophosphate, 50 mM sodium chloride, 50 mM sodium fluoride, 5 mM EDTA, 1% Triton x-100, 1 mM sodium orthovnadate) was added and the cells were resuspended. The tube was placed on ice for 30 minutes before it was centrifuged for 10 minutes in a microcentrifuge at 4°C at

2,500 rpm to get rid of cellular debris. The protein was transferred to a fresh tube on ice and the concentration was determined using a NanoDrop Lite Spectrophotometer. This worked by placing 2 µl of the sample onto the metal plate after blanking and selecting the protein µg/µl. It quantifies the concentration by wavelengths, in which different wavelengths give different concentrations. In this case, the spectrophotometer read the absorbance of the protein at 280 nm wavelength. The µl needed for 100 µg of protein was obtained by dividing 100 by the number it gave you from the spectrophotometer. Protein (100 µg) was combined into a chilled Eppendorf and volume was brought up to 100 µl with lysis solution.

ERα antibody (1 µl) was added to the Eppendorf tube and nutated at 4°C for 1 hour (Appendix D). Protein A/G PLUS slurry (20 µl, Santa Cruz, sc-2003) was added to the tube and left overnight on the nutator. The tubes were centrifuged at 2,500 rpm for 5 minutes at 4°C, and the supernatant was removed into a fresh tube and saved. The tubes were washed 3 times with 1 ml of PBS-0.1 M NaCl each time and microcentrifuged at 2,500 rpm for 5 minutes at 4°C between washes, with the supernatant removed each time.

Western Blot

Making the gel

The lower gel was made by adding 11.9 ml of distilled water to 10 ml of 30% acrylamide, then 7.5 ml of lower gel buffer (4x). Sodium dodecyl sulfate (SDS, 10%, 300

µl) and 300 µl of ammonium persulfate (10%) was added to the beaker or flask. Tetramethylethylenediamine (TEMED, 30 µl) was placed in the solution and poured in between two plates that have been cleaned with detergent and ethanol. A thin layer of water was added to the top of the layer to aid in polymerization. The gel was set aside for 15 minutes to ensure polymerization. Once the layer has polymerized, the water was discarded and the upper gel was ready to be added.

The upper gel was made by adding 3 ml of 30% acrylamide to 11.7 ml of distilled water, followed by 5 ml of upper gel buffer (4x). SDS (200 µl, 10%) and 200 µl of ammonium persulfate (10%) were added to the flask. TEMED (20 µl) was placed in the flask or beaker and the solution was poured on top of the lower gel. The comb was added and the gel aside was set for an hour to polymerize completely. After the gel had set up, it was placed in an apparatus where 1X reservoir buffer was added to the chamber.

Preparing the samples

After the samples had been washed with PBS-0.1 M NaCl 3 times and the sample dye (2 ml of 1 M Tris—HCl at a pH of 6.8, 4.6 ml of 50% glycerol, 1.6 ml of 10% SDS, 0.4 ml of 0.5% bromphenol blue, and 0.4 ml of β-mercaptoethanol) was added, the samples were heated at 95°C for 5 minutes and centrifuged for 30 seconds to one minute at 16,873 xg. The Precision Plus Protein Kaleidoscope molecular weight ladder and the samples were added to the desired wells and the apparatus was attached to a power

source where 60 volts was applied until the samples had left the wells, once that happened it was turned up to 100 volts. The gel ran for 3 to 4 hours or until the dye had reached the bottom of the lower gel.

Membrane Transfer

The plates were disassembled from the apparatus and one glass plate was removed. The gel was soaked in Transblot buffer for 5 minutes on the plate before a dry Whatman no. 2 filter paper, cut to the size of the Scotchbrite pad, was placed onto the gel. The paper was rubbed to adhere gel to paper and the filter paper was peeled back along with the gel. The nitrocellulose sheet was prewet in the transblot buffer (2 M Tris pH 8.3, glycine, methanol, 10% SDS, deionzed water) and placed onto the gel, and with a gloved hand,air bubbles trapped between the gel and filter were rubbed out. The molecular weight markers were marked with a pen and a prewet Whatman filter paper the size of the pads was placed on top of the nitrocellulose before any air bubbles were rubbed out. The Whatman filter sandwich was closed into the gel holder and placed into the electrophoresis tank that was filled with transblot buffer. The tank was attached to a power supply and set at 12 volts where it was left overnight.

Chemiluminescent Detection of the Receptor

ER-α

The membrane was blocked for 1 day in 100 ml of Super Block Buffer (Thermo Scientific 37517) containing 500 µl 10% Tween. Afterwards, the membrane was reacted for 1 hour with shaking using 8 ml of a primary antibody (ER-α Santa Cruz MC-20) at a 1:1000 dilution. This was done by taking 8 µl of ER-α and placing it in 8 ml of block buffer. The membrane was placed in a sealed bag and the primary antibody was added before the end was sealed. The membrane was wash 4 times for 5 minutes eachin approximately 200 ml of Wash Buffer containing 5 ml of 10% tween in 1 liter of PBS. Afterwards, the membrane was incubated for 1 hour with shaking in 8 ml of secondary antibody specific for rabbit antibodies at 1:4000 dilution. This was done by taking 2 µl of goat anti-rabbit antibody (Thermo Scientific 32460) and placing it in 8ml of block buffer. The membrane was placed in sealed bag just like for the primary antibody. After one hour it was washed again the same way as the primary wash. The blot was incubated with a SuperSignal West Femto Maximum Sensitivity Substrate kit, using 5 ml Luminol solution and 5 ml of horseradish peroxidase buffer, measured in a small graduated cylinder and inverted to mix. The bag was placed on shaker for 5 minutes. The blot was placed in a gel documentation system for 30 seconds to 1 minute. Densitometry was analyzed using ImageJ.

Strip and reblock

The blot was stripped using 10 ml of stripping buffer (Thermo Scientific 21059) and placed on the shaker for 15 minutes. It was reblocked for 1 hour or overnight and reprobed with ER-α ser 104/106 (sc-12956), ER-α ser 167 (sc-101676), and ER-α ser 118 (sc-12915).

Statistics

The statistical analysis was conducted using a t-Test. The test compared the mean value of total ERα to the mean values of each phospho-specific site in both resting and activated conditions. A p value of less than 0.05 was considered to be statistically significant.

CHAPTER III

RESULTS

Experimental Control

The T47D cell line (85102201 SIGMA) was used as the positive control in the Western Blots. This is a human breast tumor cell line established from the pleural effusion of a ductal carcinoma of the breast of a 54-year-old female. The cells were grown in RPMI-1640 and glutamine, along with fetal bovine serum (FBS), pen-strep (penicillin streptomycin), and bovine insulin (insulin from bovine pancreas, Sigma-I6634). When the cells were first taken out of the liquid nitrogen, they were placed in 20% FBS, then after a day the cells were switched to 10% FBS and grown until the desired number was used or refrozen. The positive control helped establish errors in the technique of the blots early in the process.

Activation with PMA and Ionomycin

The extracted T cells were divided into two groups, resting and activated. The rationale behind activating half of the cells and determining if there was a

difference between the two groups was due to the fact that T cells are activated through the ERK pathway, which is known to be part of the immune process. This simulated how the cells would change in phosphorylation during an immune response or deficiency such as SLE. The T cells were extracted and activated with PMA (10 ng/ml) and ionomycin (0.5 μg/ml) for 4 hours.

The Ratio between ERα and the Phospho-Specific Sites in Resting and Activated T Cells

Early work (Table 2) shows the progression of technical skills and new antibody troubleshooting. The ratio data was accomplished from a gel documentation system instead of X-ray film like blots shown in early work. Tables 3-6 illustrate the phosphorylation of the four antibodies in resting T cells, while Tables 7-10 show the activated T cells in response to the four antibodies.

An analysis was performed on the blots using Image J and the mean value was collected from each phosphorylation for both resting and activated. Each phospho-antibody was divided by ERα to get a percentage in the amount of phosphorylation for a particular site compared to the entire receptor. In the resting T cells (Table 11), ERα Ser 104/106 was found to have the highest amount of phosphorylation and ERα Ser 118 had the lowest. This differed when the T cells were activated (Table 12), where again ERα Ser 104/106 had the highest amount of phosphorylation, but ERα Ser 167 had the lowest. Finally the mean values were graphed and standard error was added (Figures 1 and 2).

Measurement of Plasma Estradiol/Estrogen

The plasma estradiol levels were measured to ensure the estradiol in plasma from the control females were within normal range. The normal levels of estradiol range between 30 to 400 pg/ml. The range is broad because the level varies in time and is dependent on the woman's menstrual cycle. During the follicular phase, the levels range from 19 to 140 pg/ml. It increases during the preovulatory peak around 110 to 410 pg/ml. The range falls between 19 to 160 pg/ml during the luteal phase. It was important to make sure the women used in the study were within normal range since it was the hormone of interest.

Plasma was collected at the time of blood draw. Samples were analyzed by EIA using a kit ALPO (11-ESTHU-E01). The coefficient of variation was 5.85% across all samples (Table 1). The assay was conducted at the Kansas Intellectual and Development Disabilities Research Center at the University of Kansas Medical Center.

Statistics

The t-test found that for ERα Ser 118 and ERα Ser 167 in both resting and activated, the values were statistically significant with a $p = 0.006$ for resting ERα Ser 118, $p = 0.048$ for activated ERα Ser 118, $p = 0.050$ for resting ERα Ser 167, and $p = 0.05$ for activated ERα Ser 167. However, for ERα Ser 104/106 in both resting and activated, values were found not to be significant.

Data Used for Analysis

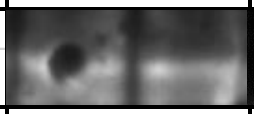
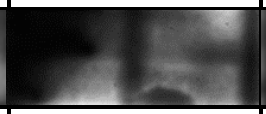
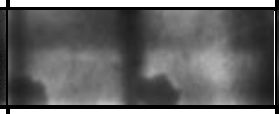
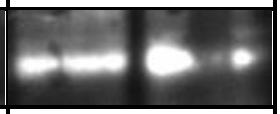
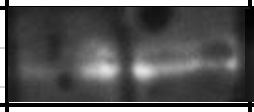
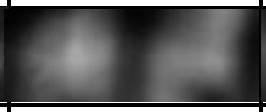
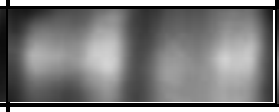
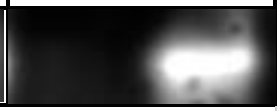
Samples	1	2	3	4
ERα Resting				
	5	6	7	8
ERα Resting				
	9	10		
ERα Resting				

Table 2

Table 2. Western blots of phosphorylated ERα in resting T cells. The 10 resting T cell samples in duplicate were reacted with the primary ERα antibody at a 1/1000 dilution before adding the secondary goat anti-rabbit antibody at a 1/4000 dilution. The phosphorylated ERα migrated to 66 kDa.

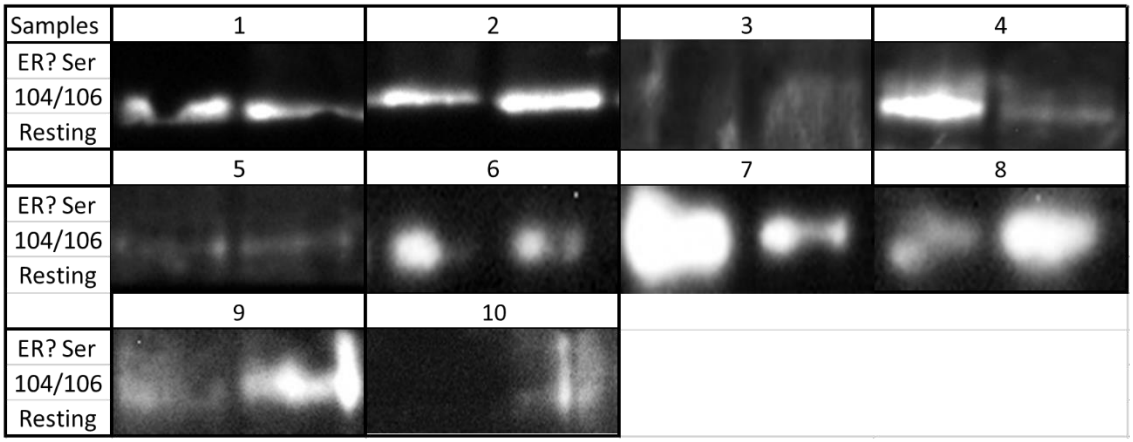


Table 3

Table 3. Western blots of phosphorylated ERα Ser 104/106 in resting T cells. The 10 resting T cell samples in duplicate were reacted with the primary ERα antibody at a 1/1000 dilution before adding the secondary goat anti-rabbit antibody at a 1/4000 dilution. The phosphorylated ERα Ser 104/106 migrated to 66 kDa.

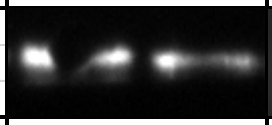
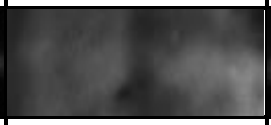
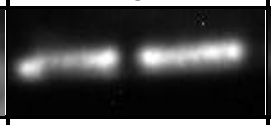
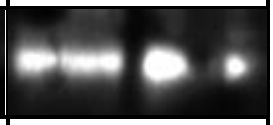
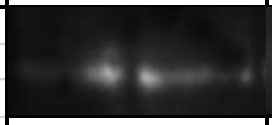
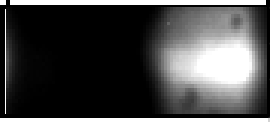
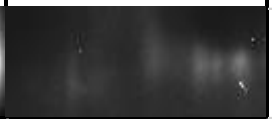
Samples	1	2	3	4
ERα Ser 118 Resting				
	5	6	7	8
ERα Ser 118 Resting				
	9	10		
ERα Ser 118 Resting				

Table 4

Table 4. Western blots of phosphorylated ERα Ser 118 in resting T cells. The 10 resting T cell samples in duplicate were reacted with the primary ERα Ser 118 antibody at a 1/1000 dilution before adding the secondary goat anti-rabbit antibody at a 1/4000 dilution. The phosphorylated ERα Ser 118 migrated to 66 kDa.

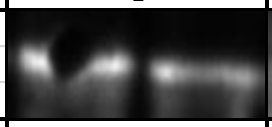
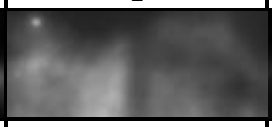
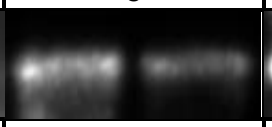
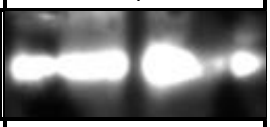
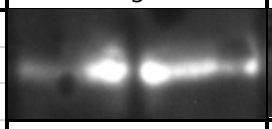
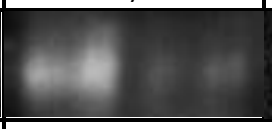
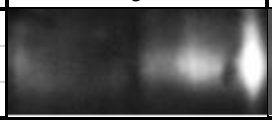
Samples	1	2	3	4
ERα Ser 167 Resting				
	5	6	7	8
ERα Ser 167 Resting				
	9	10		
ERα Ser 167 Resting				

Table 5

Table 5. Western blots of phosphorylated ERα Ser 167 in resting T cells. The 10 resting T cell samples in duplicate were reacted with the primary ERα Ser 167 antibody at a 1/1000 dilution before adding the secondary goat anti-rabbit antibody at a 1/4000 dilution. The phosphorylated ERα Ser 167 migrated to 66 kDa.

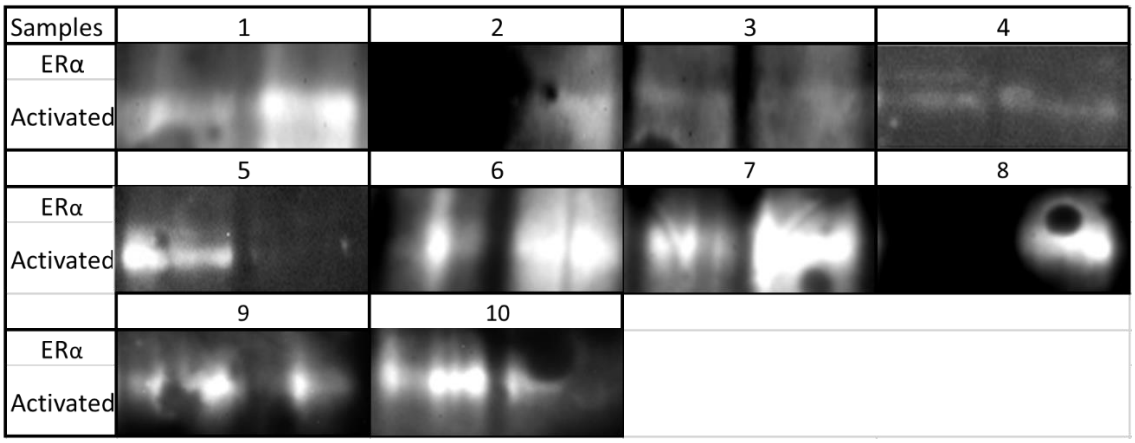


Table 6

Table 6. Western blots of phosphorylated ERα in activated T cells. The T cells were activated in PMA (10 ng/ml) and ionomycin (0.5 μg/ml) for 4 hours. The 10 activated T cell samples in duplicate were reacted with the primary ERα antibody at a 1/1000 dilution before adding the secondary goat anti-rabbit antibody at a 1/4000 dilution. The phosphorylated ERα migrated to 66 kDa.

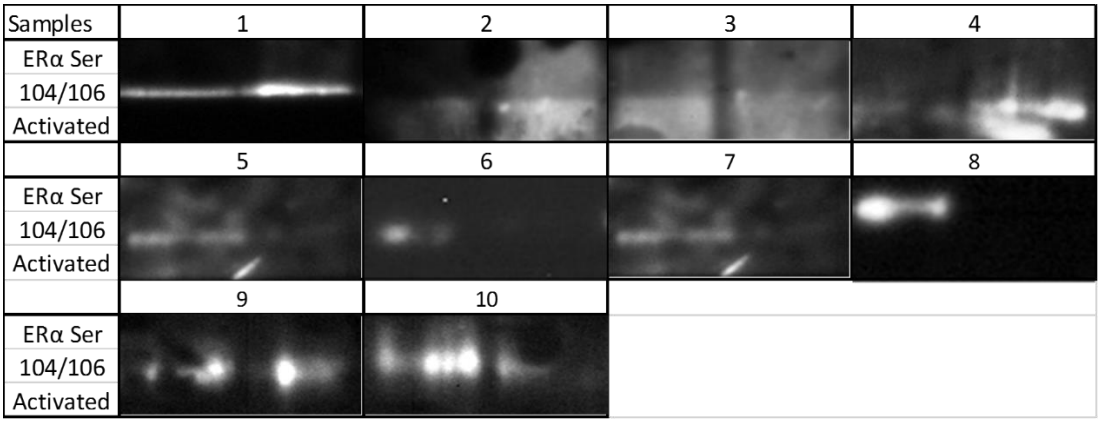


Table 7

Table 7. Western blots of phosphorylated ERα Ser 104/106 in activated T cells. The T cells were activated in PMA (10 ng/ml) and ionomycin (0.5 µg/ml) for 4 hours. The 10 activated T cell samples in duplicate were reacted with the primary ERα Ser 104/106 antibody at a 1/1000 dilution before adding the secondary goat anti-rabbit antibody at a 1/4000 dilution. The phosphorylated ERα Ser 104/106 migrated to 66 kDa.

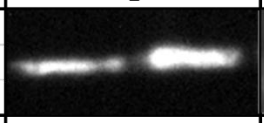
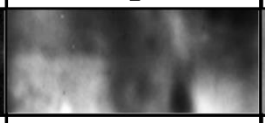
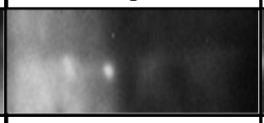
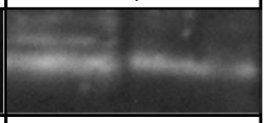
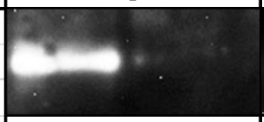
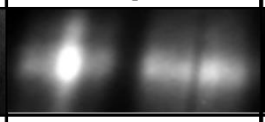
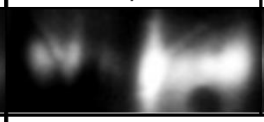
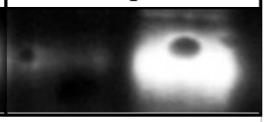
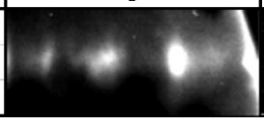
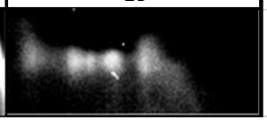
Samples	1	2	3	4
ERα Ser 118 Activated				
	5	6	7	8
ERα Ser 118 Activated				
	9	10		
ERα Ser 118 Activated				

Table 8

Table 8. Western blots of phosphorylated ERα Ser 118 in activated T cells. The T cells were activated in PMA (10 ng/ml) and ionomycin (0.5 µg/ml) for 4 hours. The 10 activated T cell samples in duplicate were reacted with the primary ERα Ser 118 antibody at a 1/1000 dilution before adding the secondary goat anti-rabbit antibody at a 1/4000 dilution. The phosphorylated ERα Ser 118 migrated to 66 kDa.

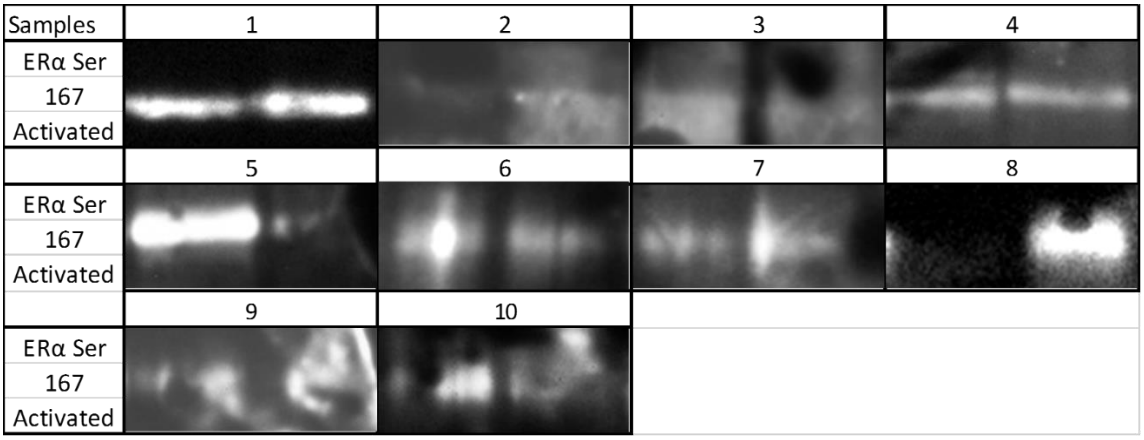


Table 9

Table 9. Western blots of phosphorylated ERα Ser 167 in activated T cells. The T cells were activated in PMA (10 ng/ml) and ionomycin (0.5 μg/ml) for 4 hours. The 10 activated T cell samples in duplicate were reacted with the primary ERα Ser 167 antibody at a 1/1000 dilution before adding the secondary goat anti-rabbit antibody at a 1/4000 dilution. The phosphorylated ERα Ser 167 migrated to 66 kDa.

	ERα	ERα Ser 104/106	ERα Ser 118	ERα Ser 167
Mean Expression Percentage	100%	89%	80%	86%

Table 10

Table 10 The percentage of phosphorylation of ERα and three sites compared with total ERα in the same samples. Data and mean values from 10 resting T cell samples in duplicate.

	ERα	ERα Ser 104/106	ERα Ser 118	ERα Ser 167
Mean Expression Percentage	100%	92%	88%	78%

Table 11

Table 11 The percentage of phosphorylation of ERα and three sites compared with total ERα in the same samples. Data and mean values from 10 activated T cell samples in duplicate.

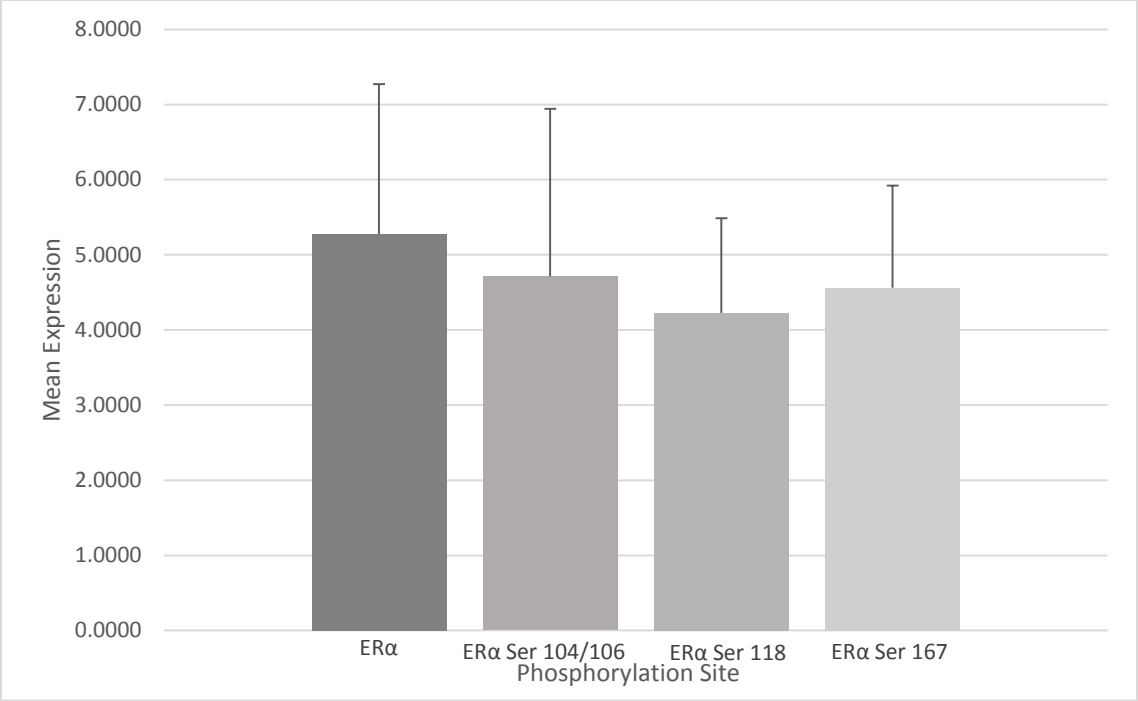


Figure 1

Figure 1 The greatest amount of phosphorylation was in ERα Ser 104/106, while the least amount of phosphorylation was in ERα Ser 118. Data are mean values from 10 resting T cell samples in duplicate ± SD. The standard deviations were 1.99, 2.23, 1.26, and 1.36 respectively.

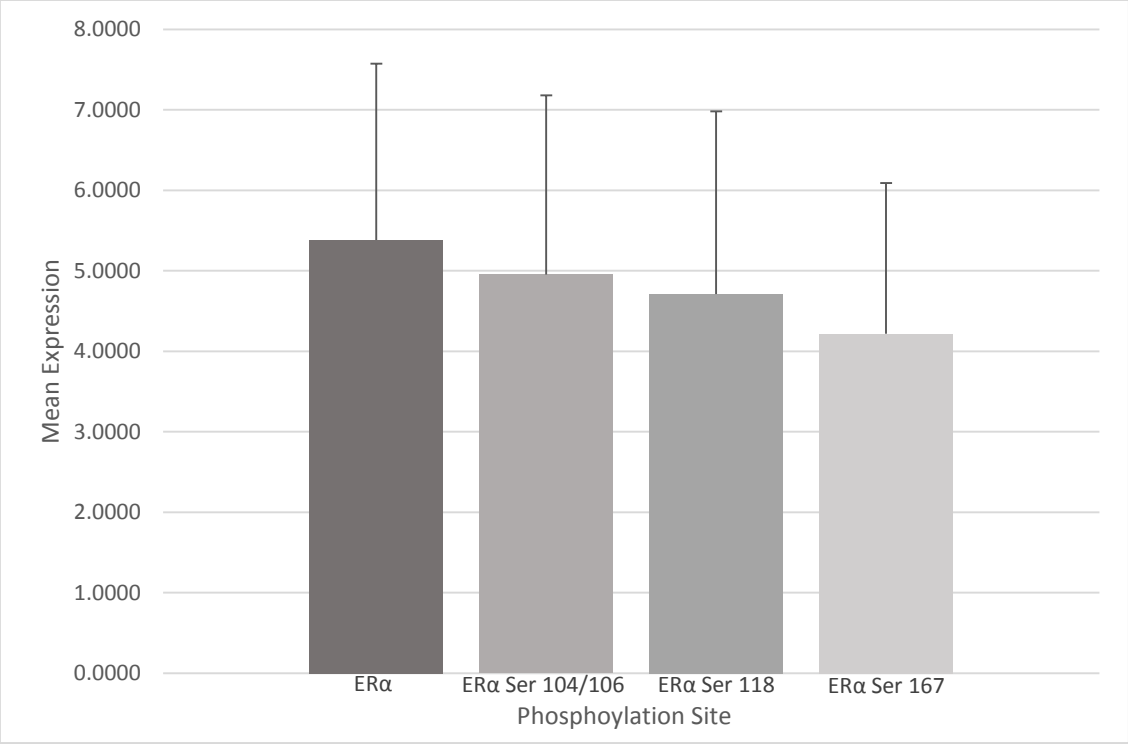


Figure 2

Figure 2 The greatest amount of phosphorylation was in ERα Ser 104/106, while the least amount of phosphorylation was in ERα Ser 167. Data are mean values from 10 activated T cell samples in duplicate ± SD. The standard deviations were 2.19, 2.23, 2.27, and 1.87 respectively.

CHAPTER IV

DISCUSSION

Western blot analysis was the main method used for studying the ERα because it is a protein. This method separates proteins by weight and a desired protein can be found on a gel with the addition of a size standard. Immunoprecipitation goes a step further and allows the extraction of the desired protein with the aid of the antibody for that protein and beads in the A/G slurry which pull the desired antibody-protein complex to the bottom of the tube while letting all the other protein be extracted in the supernatant. This extra step in the Western blot process grants more precise accuracy that the band being expressed is the protein desired and not one of many in the sample. The protein bound to the antibody is added to an acrylamide gel and a power source separates the protein vertically down the gel. Once the protein is embedded in the gel, it is transferred to a membrane, which allows multiple antibodies to be reacted with the membrane.

For this project, three sites on the ERα receptor (Ser 104/106, Ser 118, and Ser 167) were chosen to study. These sites were chosen based on a previous study performed in the laboratory looking at the ERK pathway in SLE T cells (17). The results

showed the response of T cells from patients with SLE to the effects of estrogen appeared divided into two groups. Approximately one-half of the SLE T cells showed decreased ERK ½ phosphorylation in response to estrogen (17). When the ratios of phosphorylated ERK ½ to total ERK based on the patient's disease activity, estrogen significantly decreased ERK ½ activation in the T cells from women with inactive or mild disease activity (17). The results suggest that the suppression of MAPK through ERK ½ phosphorylation is sensitive to estrogen in patients with inactive or mild disease activity, but the mechanism was not maintained when disease activity increased (17).

The three sites were phosphorylated from T cells obtained from ten healthy women in two conditions: resting and activated. I hypothesized that there would be a difference in phosphorylation of the three sites in resting conditions versus activated. Since the MAPK-ERK1/2 pathway is abnormal in lupus patients and it is responsible for activating T cells, it is thought the expression would be lower. Since no one has studied these particular sites in healthy individuals, a baseline needed to be obtained to see if there is a difference in resting versus activated before exploring the effects in SLE patients.

The results showed an increase in phosphorylation when the T cells were activated versus resting in both ERα Ser 104/106 and ERα Ser 118, but a decrease in resting versus activated in ERα Ser 167 (Tables 11 and 12). There was a significant difference in two of the three sites, ERα Ser 118 and Ser 167. The results were significant in both resting ($p=0.006$ and $p=0.050$ respectively) and activated ($p=0.048$

and p=0.05 respectively). This was not the case for ERα Ser 104/106, where in both resting and activated the results were not significant (p=0.12 and P=0.17 respectively).

Examining the results, I was not surprised by the P values. ERα Ser 118 gave the lowest p value, followed by ERα Ser 167. On the other hand Ser 104/106 gave the highest p value. I had to react the blot twice with the antibody with almost every Western blot prepared. There was either too much background to begin with or the exposure time had to be lengthened which caused more background to appear. Other times nothing would show up on the blot. This caused the antibody to have the most deviation in both conditions.

The estradiol/estrogen levels were obtained from serum samples in the control volunteers' blood to verify the levels were within the normal range given by the laboratory that tested the samples. Estradiol is the predominant form of estrogen during the reproductive years of a woman's life. 17β-estradiol (estradiol) exerts biological effects after binding to the receptors ERα and ERβ (77). The levels needed to be within range to ensure there was enough hormone in the sample for the antibody to bind. It also confirmed the levels were not too high.

Studying the role of estrogen and estradiol is critical to study in healthy individuals in order to know how it changes during diseases or abnormalities of human health. Two of the predominant diseases or abnormalities that estrogen has a role in is SLE and breast cancer. The disorder this laboratory is focusing on is SLE.

Steroid hormones facilitate the immune response, with estrogens as enhancers for humoral immunity (78). For SLE, estrogen are known to play an important role as a

mediator of SLE disease onset/perpetuation (78). The role of sex hormone concentrations at the level of inflammatory foci is valuable information needed to explain the modulatory effects exerted by these hormones on the immune-inflammatory reaction (78). There is a correlation in SLE patients between aromatase, the enzyme that produces estrogens, and IL-6 production, which causes inflammation. This aromatase activity, studied in the skin and subcutaneous tissue, varies inversely with disease activity; and furthermore, there is a significant direct correlation with estrogen levels in SLE patents (78). In addition, findings suggest there is an accelerated metabolic conversion of upstream androgen precursors to estrogen in SLE patents, and estrogen recognizes upstream precursors of different hormones (78). Furthermore, these findings may partially explain the abnormalities of peripheral estrogen synthesis in SLE, as well as the altered serum sex hormone levels and ratio (78). Taken all together, estrogen has a significant impact on SLE patients and finding out how this hormone interacts with the body in healthy individuals can lead to finding how it contributes to SLE.

Since this study has concluded that there are statistically significant differences in phosphorylation in resting versus activated in two of the three sites for healthy women, the next step is to test these antibodies on SLE patients. It is known that these three sites are phosphorylated through the MAPK-ERK1/2 pathway. The hypothesis is because the pathway is abnormal in SLE patients, there will be a greater difference in the resting versus activated ratio. It is thought there will be a significant change in expression of one or more of the three sites due to the abnormal MAPK-

ERK1/2 pathway in its role in the activation of T cells. This work has created a baseline for which the SLE patients can be compared to. If we can find there is a change in phosphorylation in one or more of these site, it will be the first step in treating SLE by changing one or more site on the ER without changing the entire receptor. I expect there will be a decrease in phosphorylation in the activated T cells compared with the controls due to the increase turnover of the T cells in SLE T cells. The loss in time between synthesis and degradation is also a loss of time the receptor has to carry out its action on the T cell, leading to a decrease in phosphorylation.

ABBREVIATIONS

ABBREVIATION	WORD
AF1	activation function 1
AF2	activation function 2
AP1	activator protein 1
BRCA1	breast cancer 1, early onset
CD3	cluster of differentiation
CDK7	cyclin-dependent kinase 7
DAG	diacylglycerol
DBD	DNA-binding domain
DMSO	dimethyl sulfoxide
E6-AP	E6-associated protein
EDTA	Ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EGTA	ethylene gycol tetraacetic acid
ER	estrogen receptor
ERα	estrogen receptor alpha
ERβ	estrogen receptor beta
ERE	estrogen response element
ERK1/2	extracellular signal-regulated kinase 1 and 2
FBP	F-box protein
FBS	fetal bovine serum
G1/S	gap 1/synthesis
GSK3β	glycogen synthesis kinase-3 beta
GTF	general transcription factor
H	hinge region
HAT	histone acetyltransferase
HDAC	histone deacetylase
HER2	human epidermal growth factor receptor 2
IGF	insulin-like growth factor
IHC	immunohistochemistry
IKKα	IkappaB kinase complex
IL-6	interleukin-6
IP3	inositol trisphosphate
K2E	K2 EDTA
LBD	carboxyl-terminal ligand binding domain
LMP2	low molecular mass protein 2
MABS	monoclonal antibodies
MAPK	mitogen-activated protein kinase
mTOR/p70S6K	mammalian target of rapamycin
NO	nitric oxide
NRCoA	nuclear receptor coactivator
NTD	amino-terminal domain

p38MAPK	protein 38 mitogen-activated protein kinase
p90RSK	protein 90 ribosomal s6 kinase
PAK-1	protein 21-activated kinase 1
PBS	phosphate buffered saline
PIP2	phospholipid phosphatidylinositol 4,5-bisphosphate
PKA	protein kinase A
PKB	protein kinase B
PKC	protein kinase C
PLC	phospholipase C
PMA	phorbol 12-myristate 13-acetate
PR	progesterone receptor
RBC	red blood cell
pRb	retinoblastoma protein
PRO	Proline
PTM	post-transcriptional modification
RPF1/RSP5	receptor potentiation factor 1/reverse Spt phenotype 5
RSK	ribosomal s6 kinase
SCF	skip, cullin, F-box
SDS	sodium dodecyl sulfate
SER	serine
Skp2	S-phase kinase-associated protein 2
SLE	systemic lupus erythematosus
SNP	single-nucleotide polymorphisms
SP1	specificity protein 1
SR	steroid receptor
TCR	T cell receptor
TEMED	tetramethylethylenediamine
TGF-β	transforming growth factor β
Th1	T helper type 1
Th2	T helper type 2
Thr	threronine
Tyr	tyrosines
UBA	ubiquitin-activating enzyme
UBC	ubiquitin conjugating enzymes
UBL	ubiquitin-protein ligases

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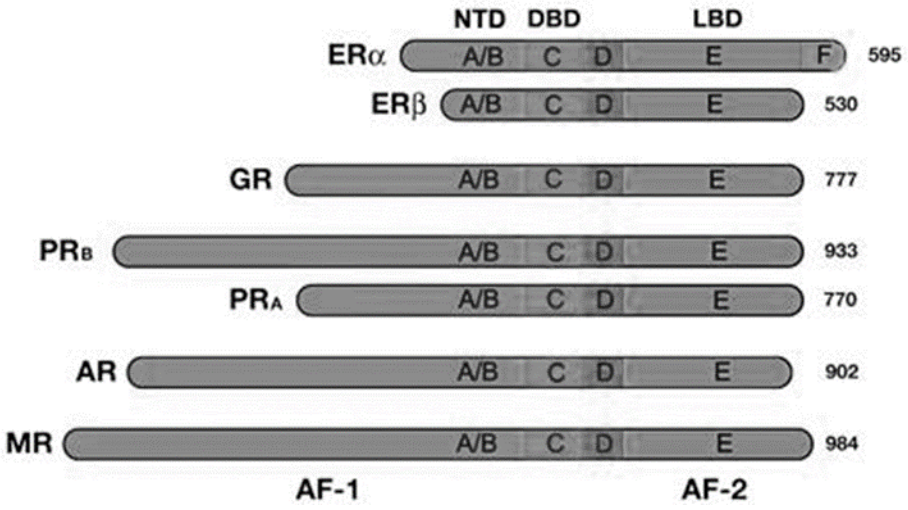
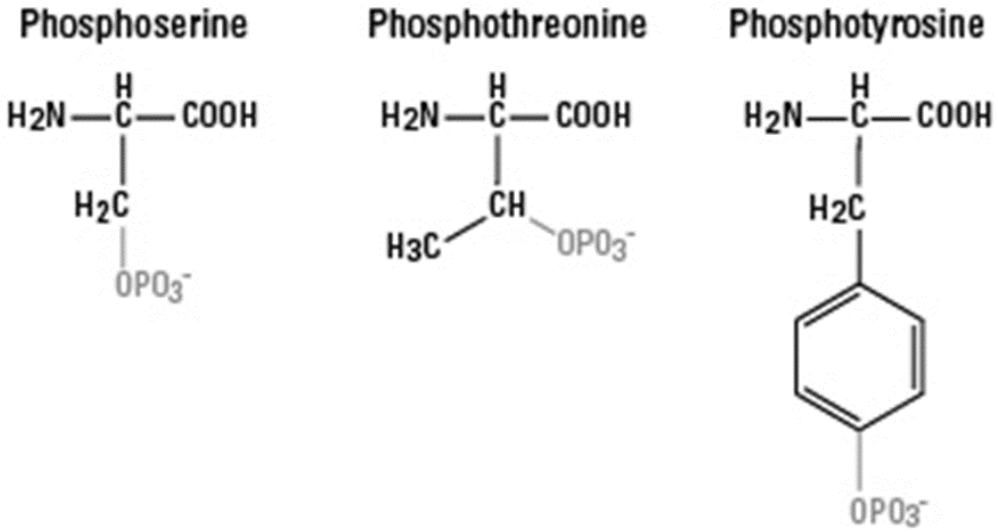
APPENDIX



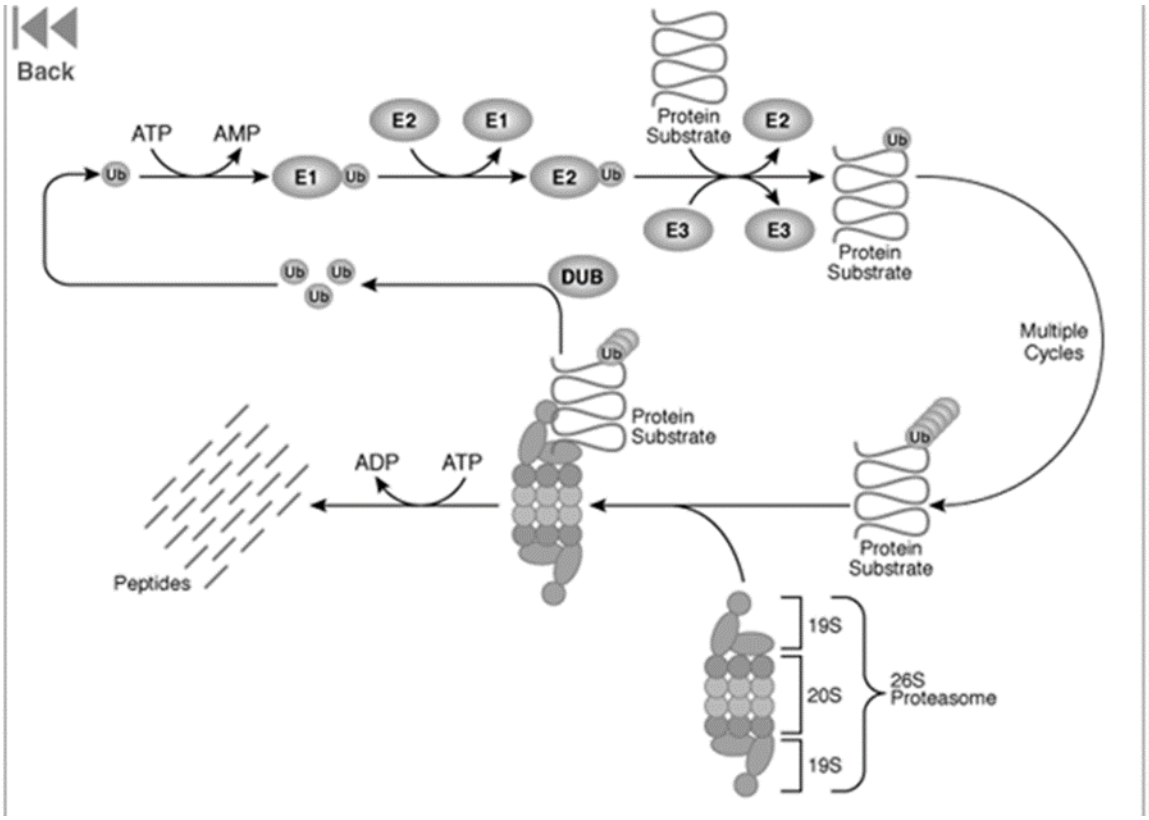
Appendix A

Steroid receptors share a common structure of carboxyl-terminal ligand binding domain (LBD), Amino-terminal domain (NTD), and a DNA-binding domain (DBD). The NTD contains the hormone independent coactivator interface AF1, where the majority of the phosphorylation sites are.



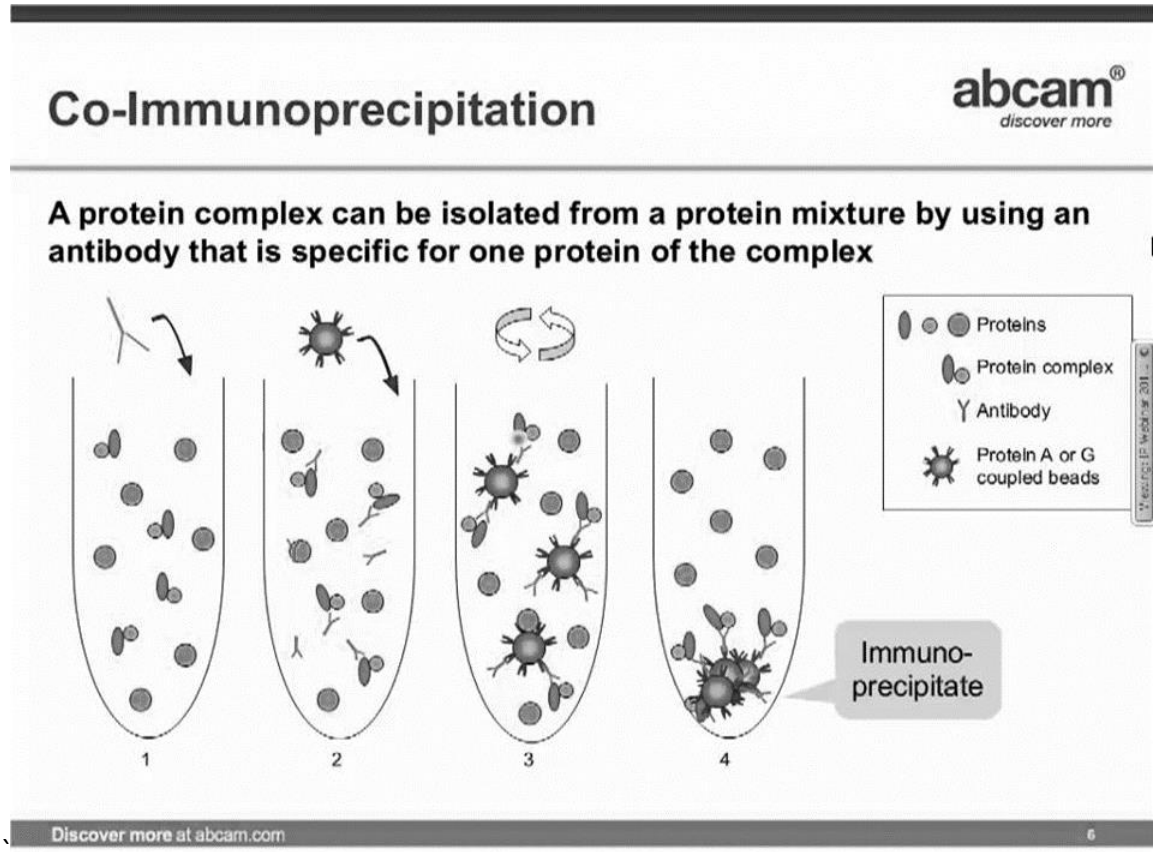
Appendix B

A total of 19 phosphorylation sites have been identified in ERα thus far. The majority of them are serine sites, and a few are threonines and and tyrosines.



Appendix C

The ubiquitination pathway allows for selective degradation of short-lived regulatory proteins, such as ERα, controlling the levels of target proteins and/or compositions of multiprotein complexes in cells by targeted protein degradation. It occurs in three steps: the initial step activates ubiquitin via the UBA enzyme (E1) in an ATP-dependent reaction. The next step maintains the high-energy linkage by transferring ubiquitin from the UBA enzyme to any one of a number of UBC enzymes (E2). Finally, UBC enzymes (E3) transfer ubiquitin covalently to target proteins either directly or in conjunction with a UBL enzyme that defines target specifically



Appendix D

Immunoprecipitation provides better accuracy for analyzing proteins. The first step is to add the protein to the desired antibody. After nutating for an hour, the protein A or G coupled beads are added. While on the nutator, the beads bind to the protein-antibody complex. The tubes are microcentrifuged, allowing the complex to be pulled to the bottom of the tube and the other protein to be extracted.