Molecular Mechanisms of Protein Kinase A Signaling Pathway on Estrogen Receptor Action in Breast Cancer

Submitted by
Mariam Al-Dhaheri

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Date of Defense:
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Major Advisor
Brian G. Rowan, Ph.D.

Academic Advisory Committee
Kam Yeung, Ph.D.
Sonia Najjar, Ph.D.
Manohar Ratnam, Ph.D.
Han-Fei Ding, Ph.D.

Senior Associate Dean, College of Graduate Studies
Michael S. Bisesi, Ph.D.
Molecular Mechanisms of Protein Kinase A Signaling Pathway

Effect on Estrogen Receptor Action in Breast Cancer

Mariam Hamad Al-Dhaheri

University of Toledo

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DEDICATION

I dedicate this work to my husband, Mohamed, who has provided me of love, support, care and inspiration, and to my family and friends.
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INTRODUCTION

Endogenous estrogen, a sex steroid hormone, has pleotropic physiological effects on many different tissues. These tissues include classical targets such as the genital tract and breast tissue and non-classical targets that include bone, brain, the cardiovascular system and the immune system (Diel, 2002). An imbalance of estrogen levels may result in pathological disorders that affect human health. Generally, estrogen mediates and regulates its physiological effects through binding to the estrogen receptor (ER). The ER belongs to the nuclear receptor super family of ligand-dependent transcription factors. The ER primarily resides in the nucleus where, upon ligand binding, its major function is the regulation of gene expression (McKenna and O'Malley, 2002). Among ER target genes are genes involved in the regulation of the cell cycle and cellular growth and differentiation (Basu and Rowan, 2005; Charpentier et al. 2000; Foster et al. 2001).

There are two types of ERs, ERα and ERβ, both the product of two separate genes (Hall et al. 2001). In the unliganded state, ER binds Hsp90 and immunophilins that maintain the receptor in an inactive conformation. Upon estrogen binding, several changes occur including Hsp90 dissociation, homo- or hetero-dimerization and phosphorylation of ER at specific sites (Klinge, 2000; Nilsson et al. 2001). Classically, activated ER is recruited as a dimer to estrogen response element (ERE) containing promoter that initiates the assembly of the transcriptional machinery (Chen et al. 1999b; Huang et al. 2005). Alternatively, ERα either acts as a transcriptional coactivator for other transcriptional proteins, such as SP-1 and AP-1, or binds to half site EREs and interacts with other transcriptional proteins to form a transcriptional initiator complex (Cerillo et al. 1998; Kushner et al. 2000; Stoner et al. 2004). The ER is a phosphoprotein
that can be phosphorylated through crosstalk with cellular kinases (Lannigan, 2003) that may activate ER independent of ligand (ligand-independent activation).

It has been found that uncontrolled ER signaling can initiate and promote the growth of breast cancer (Mills et al. 2003; Pietras, 2003). Breast cancer is the second leading cause of cancer death among women in the United States. Approximately 70% of breast cancers are classified as estrogen receptor positive (ER+). Selective estrogen receptor modulators (SERMs), such as tamoxifen, were developed to suppress the undesirable ER action in breast cancer by competing with endogenous estrogens for binding to the ER (Jordan, 2003; MacGregor and Jordan, 1998). Tamoxifen therapy for breast cancer has been approved as both adjuvant therapy following surgery, and as a chemopreventative agent for women at high risk of developing breast cancer. Although tamoxifen is initially effective in ER positive breast tumors, more than one third of ER positive patients do not respond to tamoxifen, and virtually all patients develop resistance to tamoxifen during the course of treatment. In addition, tamoxifen exhibits estrogen-like effects in the uterus resulting in uterine pathologies and an increased risk of development of endometrial cancer (Anzai et al. 1989; Kleinman et al. 1996; Sakamoto et al. 2002).

Protein kinase A (PKA) is a cellular kinase that is activated through the cAMP signaling pathway and until recently, PKA was known as the major effector for the cAMP pathway. cAMP also activates the guanine exchange factor (GEF/Epac) pathway, a non-PKA signaling pathway (de Rooij et al. 1998; Kawasaki et al. 1998). The PKA is a dimer of two regulatory (R) subunits and two catalytic (C) subunits. The activated catalytic subunits phosphorylate PKA target proteins at Serine/Threonine residues. The PKA crosstalks with the ER signaling pathway and its effect in mammary carcinomas has
been established (Fontana et al. 1987; Fujimoto and Katzenellenbogen, 1994; Marsaud et al. 2003; Zivadinovic et al. 2005). However, there are conflicting results regarding the effect of PKA in ER signaling and estrogen dependent growth of breast cancer. Fujimoto and Katzenellenbogen (1994) showed that in MCF-7 breast cancer cells transiently transfected with ERE containing reporter genes, activation of PKA promoted the agonist activity of both estrogen and tamoxifen. In contrast, Marsaud et al. (2003) showed that inactivation of PKA by H-89 enhances estrogen agonist activity in MCF7 cells stably transfected with an ERE containing promoter. In addition, inhibition of PKA by H-89 promoted estrogen dependent growth of MCF7 cells (Zivadinovic et al. 2005).

In this dissertation, the mechanisms involved in PKA regulation of the ligand dependent and ligand-independent activation of ER in tamoxifen sensitive and tamoxifen resistant breast cancer have been thoroughly investigated. This includes the effect of PKA on (1) estradiol dependent transcription of specific ER target genes, (2) ERα recruitment to cMyc and pS2 promoters, (3) estradiol binding, (4) Hsp90 association with ERα, (5) ERα phosphorylation, and (6) breast cancer cell growth. A related study identified differentially expressed novel proteins in T47D breast cancer cell treated with estradiol (pure agonist) or SERMs of varying estrogenicity including 4-hydroxytamoxifen (partial agonist/antagonist) or acolbifene (pure antagonist).

A study of PKA regulation of the ligand dependent and the ligand independent transcriptional activity of ER in breast cancer cells in this study yielded new mechanisms by which PKA regulates ER action. Most importantly, this study identified for the first time, PKA inhibition of estrogen dependent growth of breast cancer cell via gene selective changes in ER regulated gene expression. These findings support the concept of
activation of PKA signaling as a therapeutic target to counter the proliferative effects of estrogen in breast cancer.
LITERATURE

Breast Cancer

According to the American Cancer Society, cancer is the second cause of death in the United States representing 22.7% of all mortality cases in 2006. Breast cancer is the most common type of malignant disease and the second leading cause of cancer-related death of women in the United States. Breast cancer accounts for 30% of total cancer cases in the United States (Greenlee et al. 2000). Approximately 70% of breast cancer cases are ER positive and responsive to estrogen-dependent progression of the disease. ER-negative breast cancer is predominantly a result of deficient ER expression (Roodi et al. 1995). Currently, the SERM tamoxifen is used as adjuvant therapy for breast cancer patients and as a chemopreventative agent for women at high risk of breast cancer disease (Osborne, 1998; Osborne et al. 2000). However, almost 50% of breast cancer patients either have intrinsic tamoxifen resistance or acquire resistance during the course of therapy.

Nuclear Receptors

Nuclear receptors are part of an evolutionary related superfamily of transcriptional factors that are induced by ligands. Known ligands include sex steroids (estrogen, progesterone, and androgen), adrenal steroids (glucocorticoids and mineralocorticoids), thyroid, retinoid hormones, and vitamin D, in addition to a group of metabolic ligands (Nagy and Schwabe, 2004). Nuclear receptors regulate the expression of a diverse set of genes that are involved in regulation of different biological processes
(Kato et al. 2005). Nuclear receptors are broadly classified into two major groups, type I and type II, based upon the residence of the receptors in the absence of ligand (Chen et al. 2006). Unliganded type I receptors (steroid receptors including the ER) are present in an inactive complex with heat shock proteins and immunophilins and do not play a role in repression of basal transcription (McKenna and O'Malley, 2002). Unliganded type II receptors are tightly bound to hormone responsive elements (HRE) in the promoter of target genes and repress basal gene transcription.

**Estrogen Receptor**

Estrogen is a sex steroid hormone that is synthesized in the granulose layer of the ovarian follicles through converting cholesterol to estrogen by aromatases in several metabolic steps:

Cholesterol → pregnenolone → progesterone → 17-OH progesterone

    dihydrotestosterone

    tetrosterone ← androstenedione

    estradiol

Classically, there are three types of estrogen with 17β-estradiol the most potent due its high affinity to the ER. This estrogen is de novo synthesized in the ovary and termed E2. Estrone (E1) is synthesized in both the adipose tissue and in the adrenal gland, while estriol (E3) is the main estradiol metabolite in the periphery. The E3 also is produced in high amounts by the placenta during pregnancy. However, both E1 and E3 are less potent than E2 and the latter significantly drops at postmenopause age while E1
levels remain the same due to the continuous production of E1 by the adrenal gland and adipose tissue (Ruggiero and Likis, 2002).

Estrogen mediates its action through binding to its cognate receptor ER. There are two forms of the ER, ERα and ERβ (Kuiper et al. 1997; Mosselman et al. 1996; Walter et al. 1985) both the product of separate genes. Like many nuclear receptors, ER consists of six domains, A-F. The A/B domain located at the N-terminus contains the ligand-independent activation domain 1 (AF-1). The C domain contains two zinc finger motifs involved in DNA binding, where both ERα and ERβ share the highest sequence homology (84-97%). The D domain contains the nuclear localization signal. The E/F domain contains the ligand binding site, the dimerization site and the ligand-dependent activation domain (AF-2) (Nilsson et al. 2001; Hewitt and Korach, 2002) (Figure 1).

![Figure 1. Schematic Diagram of ERα and ERβ. Adapted from Klinge C. (2000)](image-url)
Estrogen receptor is expressed in the mammary gland, ovary, uterus, prostate, the pituitary gland as well as in bone and the cardiovascular system. In normal tissues, the ER regulates the growth and differentiation of reproductive tissues (Diel, 2002), where ER expression and function are essential for the development of the reproductive system. The ERα and ERβ deficiency in the uterus causes complete infertility for female mice (Hewitt and Korach, 2003). The ER expression level is determined by several molecules including hormones, growth factor, and cellular kinases (Pinzone et al. 2004). However, estrogen deficiency may also result in several pathological disorders such as osteoporosis (Carbonell et al. 2005), arterosclerosis (Rubanyi et al. 2002), and Alzheimers disease (Li and Shen, 2005). In addition, uncontrolled ER signaling can result in a variety of endocrine-related tumors such as breast cancer and endometrial cancer (Mills et al. 2003; Pietras, 2003; Shang, 2006). Regulation of the ER signaling pathway is a complex process (Katzenellenbogen et al. 2000) that involves ER subtype (Hall and McDonnell, 1999), ligand-ER conformation (Warnmark et al. 2003), DNA response element (Loven et al. 2001) homo versus hetero-dimerization (Li et al. 2004; Pettersson et al. 1997), coregulators (Hall and McDonnell, 2005; McDonnell and Norris, 2002), and cellular kinases/phosphatases (Ikeda et al. 2004; Marsaud et al. 2003).

Nuclear Receptor Coregulators

Nuclear receptor coregulators, both coactivator and corepressors (Hong and Privalsky, 2000; Ko et al. 2002; Rowan et al. 2000b; Zhou et al. 2001) regulate the transcriptional activation and repression activity of nuclear receptors (Edwards, 2000). Coactivators interact with nuclear receptors at conserved interaction domains called
nuclear receptor boxes (NR box) which contain LXXLL (where L is leucine and X is any amino acid) motif. The p160 family is a well characterized class of coactivators including steroid receptor coactivator-1 SRC-1, TIF-2 [GRIP1, NcoA-2, SRC-2] and RAC3 [ACTR, AIB1, P/CIP, TRAM, SRC-3]. The main function of these coactivators is to interact with ligand-bound nuclear receptors to recruit histone acetyltransferases and methyltransferases to specific promoter sequence that facilitate the transcription process of the target gene (Glass et al. 1997; Xu and Li, 2003). At least 50 other coactivators have been identified to date.

Nuclear receptor corepressors are key regulators of the transcriptional activity of type II of nuclear receptors and antagonist bound type I of nuclear receptor. This group of coregulators interacts with a complex of proteins that can interact with nuclear receptors and represses the transcriptional machinery. Nuclear receptor corepressor (N-CoR) and silencing mediator of retinoic and thyroid hormone receptors (SMRT) are related corepressors that interact with nuclear receptors through nuclear receptor interaction domains (NRID). The NRID contains the conserved motif L-X-X-X-I-X-X-X-I/L that is similar to the NR box coactivator recognition motif but is predicted to form an extra helical turn compared with the NR box. Both N-COR and SMRT contain three independent repressor domains (RI, RII, and RIII) that interact with a battery of histone deacetylases (HDAC) and other transcriptional proteins (Jepsen and Rosenfeld, 2002; Misiti et al. 1998). The N-COR plays an important role in defining the antagonist character of tamoxifen. In N-COR null mouse embryo fibroblast, the loss of N-CoR reverses the tamoxifen antagonist action and induces the ligand to act as an agonist. Re-
expression of exogenous N-COR in these cells restores the tamoxifen antagonist action (Jepsen et al. 2000).

Mechanism of ER Activation

The ER is activated through several mechanisms. In the classical ligand dependent pathway of ER signaling, estrogen binding initiates the dissociation of ER from Hsp90 (Devin-Leclerc et al. 1998), dimerization (Tamrazi et al. 2002) and interaction with estrogen response elements (EREs) on the promoter of estrogen target genes (Gruber et al. 2004; Klinge, 2001; Loven et al. 2001). Liganded ER recruits a set of transcriptional coactivators with associated histone acetylation activity (McDonnell and Norris, 2002) that facilitates transcriptional activation (Klinge, 2000; Shang et al. 2000) (Figure 2). In an alternate pathway for ER activation ER regulates transcription not through direct interaction with DNA, but through binding to other transcription factors at the promoter including Sp-1 proteins (Bruning et al. 2003; Safe, 2001; Stoner et al. 2004), AP-1 proteins (Kushner et al. 2000; Paech et al. 1997), GATA-1 (Davis and Burch, 1996) and NFKappaB (Cerillo et al. 1998). Although estrogen induced the transcriptional activity of ER target genes, estrogen also was found to repress genes in breast cancer cells (Frasor et al. 2004). However, the mechanisms of ER repression of ER target genes are not fully understood.

In addition to ligand dependent activation, ER also may be activated through crosstalk with various signal transduction pathways (ligand-independent) (Coleman and Smith, 2001; Weigel and Zhang, 1998). Several kinases and stimuli, such as MAPK,
The Classical Pathway of ER action

![Diagram of the Classical Pathway of ER action]

Figure 2. Model of Estrogen Dependent Activation of ER Target Gene

PKA, Src, leptin, EGF, Rho GDI (Guanine nucleotide dissociation inhibitor), cyclin A-Cdk2 complex, P38α-SAPK2a complex, PKC, and AKT were reported to activate the ligand independent activity of ER and induce estrogen target gene expression (Catalano et al. 2004; Chen et al. 2002; Feng et al. 2001; Joel et al. 1995, 1998b; Lee and Bai, 2002; Marquez et al. 2001; Schreihofer et al. 2001; Shah and Rowan, 2005; Su et al. 2002; Sun et al. 2001; Tremblay et al. 1999). It is widely accepted that crosstalk between kinases and ER cause direct or indirect phosphorylation of ER at specific sites (Joel et al. 1998a; Le Goff et al. 1994; Rogatsky et al. 1999) and facilitate estrogen receptor interaction with coactivators (Dutertre and Smith, 2003; Kato et al. 2005; Su et al. 2002; Tremblay et al. 1999) that in turn initiates the assembly of the transcriptional machinery and induce ER
transcriptional activity. It has been reported that ER can dimerize in the absence of ligand (Salomonsson et al. 1994).

**Estrogen Receptor Phosphorylation**

Estrogen receptor undergoes different posttranslational modifications including phosphorylation (Lannigan, 2003), sumoylation (Sentis et al. 2005), ubiquitination (Valley et al. 2005), and acetylation (Kim et al. 2006; Wang et al. 2001). Phosphorylation of ER is the well established posttranslational event that regulates ER action. To date, there are eight identified phosphorylation sites of ERα, and two identified phosphorylation sites in ERβ (Tremblay et al. 1999). Most of ERα identified phosphorylation sites are located within the A/B domain of ERα (Figure 3) although evidence suggests there are as yet, unidentified sites in the E/F domain that remain to be identified. ERα known phosphorylation sites are S104, S106, S118, S167 (Joel et al. 1995, 1998a; Le Goff et al. 1994; Rogatsky et al. 1999; Simoncini et al. 2000), S236 (Chen et al. 1999a), S305 (Michalides et al. 2004; Wang et al. 2002), T311 (Lee and Bai, 2002), and Y537 (Arnold et al. 1995). The ERβ known phosphorylation sites are S106 and S124 (Tremblay et al. 1999).

There is growing evidence showing that estrogen treatment of breast cancer cells activates (1) PKA signaling that phosphorylates CREB, (2) PI3K signaling that phosphorylates AKT and SRF, (3) MAPK signaling that phosphorylates ERK1/ERK2 and ELK-1, (4) calcium calmodulin-dependent kinase IV that phosphorylates p65 (subunit of NFκB), and (5) Src signaling (Migliaccio et al. 1996 Li et al. 2006; Zhang and Trudeau, 2006; Zhang et al. 2004). Activation of different signaling pathways by
estrogen treatment represents the non genomic effect of ER and contributes to the genomic effect of ER, where the activated signaling pathways phosphorylate both ER as well as transcriptional coactivators (Rowan et al. 2000a).

It has been shown that S104 and S106 of ERα are phosphorylated by estrogen treatment and by cyclin A-Cdk2 complex. S118 is a MAPK consensus site and is phosphorylated by estrogen treatment and by activating MAPK signaling. S167 is AKT consensus site that can be activated by activating AKT and MAPK signaling as well as estrogen treatment. S236 and S305 are PKA consensus sites that are phosphorylated by estrogen treatment and by activating PKA, P21-kinase 1 signaling. The direct kinase that induce T311 phosphorylation is not known, however, T311 is induced by estrogen treatment (Al Dhaheri and Rowan, 2006) and by P38-SAPK complex. Y537 is a Src consensus site that is activated by Src kinase as well as by estrogen treatment. For ERβ both S106 and S124 are located on the A/B domain and are MAPK phosphorylation sites.

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**Figure 3. Schematic Presentation of ERα Phosphorylation Sites**

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**Estrogen Receptor Target Genes**

The ER is transcriptional factor that activates the transcription of genes involved in different cellular processes (Basu and Rowan, 2005; Carroll and Brown, 2006; Frasor et al. 2004; Sanchez et al. 2002) in particularly the cell growth and differentiation of the female reproductive system. Most importantly, ER target genes are varied in promoter context. Some ER target gene promoters contain perfect ERE sequences, 5’CAGGTCANNTGACCTG-3’, such as vitellogenin A1, A2, B1, and B2 (Klein-Hitpass et al. 1988), whereas other target gene promoters contain imperfect ERE (differ in 1-3 nucleotides in ERE consensus site), such as pS2 (trefoil factor 1), oxytocin and cathepsin D (Augereau et al. 1994; Berry et al. 1989; Jakowlew et al. 1984; Krishnan et al. 1994; Richard and Zingg, 1990; Safe, 2001; Stack et al. 1988; Sun et al. 2005). Furthermore, other ER target gene promoters contain half ERE palindromic elements such as lactoferrin and heat shock protein 27 (Lee et al. 1995; Porter et al. 1996). In addition, ER can activate genes that contain estrogen like elements and GC rich sequences, as in the case of PR (progesterone receptor), c-fos (Petz et al. 2004; Petz and Nardulli, 2000; Schultz et al. 2005; Weisz and Rosales, 1990), and cMyc (Dubik and Shiu, 1988, 1992; Sanchez et al. 2002). Additionally, ER can activate some ER target genes [cyclin D1 and IGF I (insulin-like growth factor I)] through interaction with other transcriptional factors (Altucci et al. 1996; Sabbah et al. 1999; Shang and Brown, 2002; Umayahara et al. 1994).

Several ER target genes such as cMyc, cyclin D1, pS2 and PR are well characterized for regulation by estradiol and SERMs. Both cyclin D1 and cMyc are key mediators of estrogen induced cell growth (Butt et al. 2005; Foster et al. 2001). Cyclin
D1 is involved in the G1/S phase transition of breast cancer cells (Caldon et al. 2006), while cMyc contributes to accelerating cell proliferation and growth rate (Hanson et al. 1994; Nass and Dickson, 1997). In clinical samples, ER positive breast cancer tumors displayed high mRNA levels of cMyc, Cyclin D1 and Insulin-like growth factor 1 receptor (Tian et al. 2005). In normal tissue, pS2 protein is highly expressed in the gastrointestinal tissue, but not in breast, uterus, testis, muscle, kidney, brain, spleen, liver, pancreas, or heart tissue. However, in malignant tissue, pS2 is highly expressed in breast, ovary, uterus, prostate, pancreas, colon, and lung tissue (Henry et al. 1991; Ribieras et al. 1998). The exact function of pS2 in tumor progression is unclear. However, pS2 is a prognostic factor for breast cancer (May and Westley, 1997; Ribieras et al. 1998).

Paradoxically, PR plays a critical role in the development of the mammary glands and breast cancer (Conneely et al. 2003; Cui et al. 2005).

Strategies to Control Estrogen Receptor Signaling in Breast Cancer

To control the proliferative effect of estrogen receptor in mammary glands, antiestrogens were developed. There are two types of antiestrogen: type 1, or SERMs, and type 2, or pure antiestrogens (MacGregor and Jordan, 1998). The main difference between SERMs and pure antiestrogens is that SERMs are non-steroidal analogues that display estrogentic-like activity in specific tissues and antiestrogenic activity in other tissue (Meegan and Lloyd, 2003), with the exception of acolbifene (EM-652), which represents a pure SERM (Labrie et al. 2001). In contrast, pure antiestrogens are steroidal analogues of estrogen that exhibit antiestrogenic effects in all tissues (Meegan and Lloyd, 2003). In general, antiestrogens mimic estrogen and compete for binding to the estrogen receptor. Tamoxifen is a first generation SERM that is currently used in the clinic as
adjuvant therapy for ER positive breast cancer. Fulvestrant (ICI, 182, 780, or faslodex) is a well-established pure antiestrogen (steroidal analogue) that has currently completed clinical trials (Dowsett et al. 2005; Elkak and Mokbel, 2001; Vergote and Abram, 2006).

In addition to antiestrogens, other strategies to control ER signaling in breast cancer cells are under clinical investigation (Ariazi et al. 2006). These strategies include aromatase inhibitors that were developed to block estrogen synthesis and control circulating estrogen levels (Buzdar et al. 2006). Currently, letrozole (femara), an aromatase inhibitor, are approved for clinical use for breast cancer control (Dunn and Keam, 2006). Signal transduction inhibitors (STIs), were initially designed to overcome tamoxifen resistance and control cellular signaling that is associated with tamoxifen resistance (Johnston et al. 2003; Newby et al. 1997; Pasapera Limon et al. 2003). The well characterized STIs include gefintinib (Iressa) that controls EGFR signaling in breast cancer tissue and currently in phase II clinical trials. Lapatinib (GW572016) controls EGFR/HER2 signaling, and is at clinical phase II trials. Everolimus (RAD-001) controls the mammalian target of rapamycin (mTOR) signaling and is currently at clinical phase III trials of investigation (Johnston et al. 2003; Johnston, 2005). Further, Hsp90 binding drugs destabilize and deplete steroid hormone receptors in breast cancer cells are currently under investigation (Bagatell et al. 2001).

Selective Estrogen Receptor Modulators (SERMs)

Selective estrogen receptor modulators exhibit tissue selective estrogenic and antiestrogenic activity with the goal of blocking estrogen action in some tissues (e.g., breast and endometrium in cancers of these tissues, (Jordan, 2003; Jordan, 2004)) while
maintaining the beneficial effects of estrogen in other non-target tissues (e.g., bone, cardiovascular system, (Bagdade et al. 1990; Barrett-Connor et al. 1999; Jordan, 2003; Ryan et al. 1991). Tamoxifen (triphenylethylene) is a non-steroidal analogue of estrogen and the well-established SERM that successfully block ER action in breast tissues (MacGregor and Jordan, 1998). In a clinical setting, approximately 50% of ER positive breast cancer patients initially respond favorably to tamoxifen although the majority of these patients develop resistance during the course of therapy (Jordan, 2003). In addition to tamoxifen, several generations of SERMs were developed, that includes raloxifen (Barrett-Connor, 2001), idoxifene (Nuttall et al. 1998), and acolbifene (EM-652) (Labrie et al. 2001, 2004).

**Mechanism of Tamoxifen Action on ER**

The major mechanism by which tamoxifen suppresses breast cancer growth is as an ER antagonist action (Muller et al. 1998). Tamoxifen also has an apoptotic action that may contribute to its efficacy (Cameron et al. 2000; Mandlekar and Kong, 2001). The agonist/antagonist activity of tamoxifen is influenced in part by ER subtype, tissue, the complement of coregulators expressed, and the nature of the ER-responsive promoter. In general, tamoxifen is a partial agonist for ERα but not ERβ on ERE-dependent genes (Barkhem et al. 1998; Dutertre and Smith, 2000; Hall and McDonnell, 1999; McInerney et al. 1998). In contrast, tamoxifen may act as an agonist for both ERα and ERβ on AP-1 containing reporter genes (Paech et al. 1997).
Tamoxifen Action in Breast Tissue

Figure 4. Model of Tamoxifen Antagonism of ER Action in Breast Cancer Tissue.

Tamoxifen antagonist action in breast cancer is regulated, in part, by corepressor interaction with tamoxifen-ER complex which recruits proteins containing histone deacetylase activity (Figure 4) (MacGregor and Jordan, 1998). By recruiting corepressors instead of coactivators, tamoxifen represses ER target genes and inhibits estrogen proliferative effect in breast cancer (Park and Jordan, 2002).

Tamoxifen Resistance

About 30-50% of ER positive breast cancer patients either develop resistance to tamoxifen or do not respond (Girault et al. 2006). Tamoxifen resistance is a complex phenomenon comprised of several mechanisms that involve genomic and non-genomic
factors (Gururaj et al. 2006). EGFR and HER2/neu crosstalk with ER (Gee et al. 2005; Osborne and Schiff, 2003), reduced IGF receptor I α expression (Brockdorff et al. 2003), constitutively activated MAPK signaling in endometrial cancer cells (Hayashi et al. 2003), and oxidative stress (Schiff et al. 2000) are all associated with tamoxifen agonist activity. Both ERα (Schafer et al. 2002) and N-COR (Girault et al. 2003) play an important role in tamoxifen resistance. Almost 25% of tamoxifen resistant tumors result from loss of ER expression (Kuukasjarvi et al. 1996). Loss of ER function due to mutations is another resistance mechanism, although this is a rare event (Roodi et al. 1995). The ER crosstalk with other cellular signaling pathways, such as EGFR signaling, increases the phosphorylation of the AF-1 domain that activates the ligand-independent activity of ER (Joel et al. 1998b) that is associated with the agonist activity of tamoxifen (McInerney and Katzenellenbogen, 1996).

In addition to ERα, N-COR expression level is also associated with tamoxifen resistance and a predictive marker for tamoxifen response (Girault et al. 2003). McDonnell (Huang et al. 2002) identified a negative regulatory surface within ERα where a corepressor may regulate the agonist/antagonist activity of tamoxifen. Importantly, the functional interaction between N-COR and nuclear receptors is regulated by diverse signaling pathways in which the PKA signaling pathway disrupts the functional interaction between N-COR and tamoxifen-occupied ERα receptor (Lavinsky et al. 1998) and modulates the agonist/antagonist activity of tamoxifen (Fujimoto and Katzenellenbogen, 1994). Moreover, activating PKA signaling causes conformational arrest of ERα and tamoxifen resistance (Michalides et al. 2004).
Potential Crosstalk Between ER and PKA Signaling Pathway

The PKA is a holoenzyme composed of two regulatory subunits and two catalytic subunits which phosphorylates target proteins at serine/threonine residues. There are two isoforms of the regulatory (R) subunit, RI (PKAI) and RII (PKAII), and three isoforms of the catalytic subunit (α, β and γ). PKAI and PKAII are differentially expressed and the ratio of subunit expression has been correlated with cell differentiation and neoplastic transformation. In general, PKAI is more highly expressed in cancerous cells, whereas PKAII is more highly expressed in differentiated cells (Brandon et al. 1997; Chin et al. 2002; Cho-Chung et al. 1995). Cyclic adenosine 3’, 5’-monophosphate (cAMP)-dependent pathways govern many endocrine and neural functions. Elevated intracellular cAMP binds to the regulatory subunit of PKA causing phosphorylation of the catalytic subunit of PKA. The phosphorylated catalytic subunits then translocate to the nucleus and phosphorylate transcriptional proteins that regulate gene expression. The cAMP response element binding protein (CREB) and the cAMP response element modulator (CREM) are well-established transcriptional factors that are regulated by PKA activation (Sassone-Corsi, 1998). The PKA is activated through stimulation of cAMP. Several cAMP analogues were developed to stimulate the action of cAMP, such as 8-bromo-cAMP and 8-chloro-cAMP, however, cAMP can be elevated and stimulated by some toxins and herbs. Cholera toxine (CT) (Lubran, 1988) and forskolin (Insel and Ostrom, 2003) have the ability to stimulate cAMP, while 3-isobutyl-1-methylxanthine (IBMX) is a phosphodiesterases inhibitor that inhibits cAMP degradation (von der, 1989), which elevate cAMP levels.
In addition to PKA, cAMP activates Epacs (protein exhibits guanine nucleotide exchange (GEF) activity) (Figure 5) (de Rooij et al. 1998; Kawasaki et al. 1998). Crosstalk between cAMP and other signaling pathways were the subject of several reviews (Robinson-White and Stratakis, 2002; Stork and Schmitt, 2002). To dissect cAMP effect, in physiological concentrations, a specific analogue of cAMP named 8CPT-2Me-cAMP (Enserink et al. 2002) has the ability to activate Epac signaling but not PKA signaling.

![cAMP Signaling Pathways](image)

Figure 5. Proposed Model for cAMP Signaling in Endocrine Cells Adapted, with modification, from Richards, 2001; Robinson-White and Stratakis, 2002.
The PKA signaling pathway regulates activity of several nuclear receptors and coregulators. Activated PKA decreases the interaction between N-COR and antagonist bounded-progesterone receptor (Wagner et al. 1998) and stimulates activity of both the mineralocorticoid receptor and vitamin D receptor (Massaad et al. 1999; Murayama et al. 2004). Activated PKA phosphorylates GATA-3 and liver receptor homolog (LRH)-1 activate the transcriptional activity of aromatase PII promoter and LRH-1 promoter in breast cancer cells (Bouchard et al. 2005). The PKA signaling pathway also is involved in both ligand-dependent and ligand-independent action of ER (El Tanani and Green, 1997; Fujimoto and Katzenellenbogen, 1994; Lamb et al. 2000; Le Goff et al. 1994; Schreihofer et al. 2001). However, there are controversial results regarding PKA effect on ER in breast cancer cells. Fujimoto and Katzenellenbogen (1994) showed that activation of PKA in MCF-7 breast cancer cells alters the agonist/antagonist balance of tamoxifen and promotes estrogen dependent transcription. In contrast, another study showed that inhibiting PKA signaling by H-89 enhances estrogen dependent transcription (Marsaud et al. 2003). Importantly, activation of the PKA signaling pathway protects ERα from estrogen-induced degradation and promotes ER protein stability (Marsaud et al. 2003; Tsai et al. 2004).

To date there are two well-characterized PKA phosphorylation sites in ERα. The PKA phosphorylates ERα at serine 236 within the DNA binding domain (DBD) and this phosphorylation inhibits ER dimerization in the absence of the ligand (Chen et al. 1999a). The PKA also phosphorylates ERα at S305 which blocks ERα acetylation and enhance ERα transcriptional activity (Cui et al. 2004).
The PKA pathway may crosstalk with other signal transduction pathways to enhance and/or repress ER activity by increasing phosphorylation of ER and coregulators. Activation of the EGFR signaling pathway has an important role in activating ligand-independent signaling of ER. Interestingly, PKA directly interacts with EGFR (Tortora et al. 1997) and can contribute to the ligand-independent activation of ER. With regard to AP-1 promoters in which ERα may indirectly regulate transcription through binding Jun/Fos proteins, it is of interest to note that PKA has been shown to activate AP-1 containing promoters (Hershko et al. 2002).

PKA-dependent phosphorylation of coregulators also may be important in modulating tamoxifen activity. In neural stem cells, activation of the PI3K/AKT pathway results in phosphorylation of N-COR at serine 401 causing temporal redistribution of N-COR in the cytoplasm (Hermanson et al. 2002). Interestingly, elevation of cAMP activates AKT through the Epac signaling pathway (Mei et al. 2002). Elevated cAMP also induces phosphorylation of SRC-1 at two sites that enhance ligand-independent activation of chicken progesterone receptor (Rowan et al. 2000a). In addition, PKA signaling negatively regulates the activity of HDAC 8 (Lee et al. 2004) which reduces the gene repression effect of HDACs. In this scenario, PKA may relieve the repressive effect of N-COR by negatively regulating the activity or interaction between HDAC and N-COR.

The PKA activation significantly induces the expression of IV-1, pS2 and WISP-2 (Wnt-1 inducible signaling pathway protein 2) genes, all of which are estrogen-induced genes in MCF-7 cells (El Tanani and Green, 1996; Inadera, 2003). In MCF7 cells, both
estradiol and 8-bromo-cAMP induce the expression of pS2 mRNA, while 4-OH-tamoxifen inhibited the expression of pS2. The inhibitory effect of 4-OH-tamoxifen is reversed by co-incubation with 8-bromo-cAMP (Chalbos et al. 1993).

PKA and Cancer Therapy

Although the activated PKA signaling pathway significantly contributes to activation of a set of transcriptional factors and transcriptional coregulators that are involved in promoting cell growth, elevating cAMP also suppresses cancer cell growth. Activated PKA inhibited both ER positive and ER negative breast cancer cell growth as well as repressed estrogen dependent growth of ER positive breast cancer cell line (Fontana et al. 1987; Katsaros et al. 1988; Zivadinovic et al. 2005). Activated PKA also inhibited a variety of fibroblast, leukemic, gliomas, lung and colon cancer cells (Ally et al. 1989; Chen et al. 1998; Cho-Chung et al. 1989; Tagliaferri et al. 1988).

It has been proposed that the balance between PKA regulatory subunit I (RI or PKAI) and PKA regulatory subunit II (RII, or PKAII) plays a critical role in tumor progression and differentiation (Cho-Chung and Nesterova, 2005). The PKAI is highly expressed in tumor and cancerous tissue, while PKAII is highly expressed in normal and differentiated tissues (Brandon et al. 1997; Chin et al. 2002; Cho-Chung et al. 1995) and the ratio of PKAI/PKAII may define the ability of PKA to inhibit cell growth. In fact, stimulated cAMP has the ability to bind two different sites on each regulatory subunit with different affinity (Rannels and Corbin, 1980). cAMP analogues displayed different affinity to these binding sites (Beebe et al. 1984; Rannels and Corbin, 1980). 8-Cl-cAMP
discriminates between PKAI and PKAII binding sites and showed selective modulation of PKAI, and PKAII expression level, in which 8-Cl-cAMP downregulates PKAI level and upregulates PKAII expression level that was correlated with inhibition of colon cancer cell growth (Ally et al. 1988). Selective modulation of PKAI expression by 8-Cl-cAMP qualifies this analogue for clinical investigation (phase I) (Cho-Chung and Nesterova, 2005). In contrast, another study showed that the anti-proliferative effect of 8-Cl-cAMP in breast cancer cells was not correlated with its effect on PKAI expression level (Lamb and Steinberg, 2002).

The PKAI expression level was associated with increased proliferation rate in normal and malignant breast tissue (Miller, 2002), and has been proposed as a therapeutic target for breast cancer (Yang et al. 2002). Several studies support the use of anti-sense PKAI as a novel anticancer agent. The potential anti-tumor effect of antisense PKAI involves suppression of the mitogenic signaling pathway, induction of apoptosis and antiangiogenesis (Cho-Chung et al. 2002; Cho-Chung, 2004; Tortora and Ciardiello, 2002; Tortora and Ciardiello, 2003). Co-incubation of non-inhibitory dose of selective cyclooxygenase-2 inhibitor with EGFR tyrosine kinase inhibitor ZD1839 and PKA-I antisense inhibits the growth of human colon cancer xenografts in nude mice (Tortora et al. 2003). Furthermore, the anti-sense PKAI α inhibits the growth of ovarian cancer cells (Alper et al. 1999).
PKA exhibits selective modulation of estradiol dependent transcription in breast cancer cells that is associated with decreased ligand binding, altered ERα promoter interaction and changes in receptor phosphorylation.

Abbreviated title: PKA modulates estradiol transcription.

Mariam H. Al-Dhaheri¹ and Brian G. Rowan¹, ²*

¹Department of Biochemistry and Cancer Biology, University of Toledo, Toledo, Ohio
²Current address: Department of Structural and Cellular Biology, Tulane University School of Medicine and the Louisiana Cancer Research Consortium, New Orleans, Louisiana, USA.

*Corresponding Author:  Brian G. Rowan, Ph.D.  Department of Structural & Cellular Biology, SL49.  Tulane University School of Medicine and the Louisiana Cancer Research Consortium, 1430 Tulane Avenue, New Orleans, Louisiana 70112.  Phone: 504-988-1365, Fax: 504-988-1687
e-mail: browan@tulane.edu

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Abstract

Inhibition of protein kinase A (PKA) promotes estrogen-dependent growth of MCF7 breast cancer cells although the mechanisms by which PKA regulates estrogen receptor (ER) function remain unclear. In this study elevation of cAMP by Forskolin/IBMX (F/I) suppressed estradiol-dependent MCF7 and T47D breast cancer cell growth but not tamoxifen-resistant MCF7-LCC2 cells. Although F/I induced ligand independent activation of ERα, F/I also decreased estradiol-dependent reporter gene transcription. Overexpression of PKA or PKA inhibitor (PKI) demonstrated that F/I effects on repression of estradiol action occurred through the PKA pathway. 8CPT-2Me-cAMP, a selective inducer of non-PKA signaling did not alter ER-dependent transcription. In contrast to F/I effects on reporter genes, F/I exhibited gene specific effects on endogenous, ER-regulated genes. F/I enhanced estradiol induction of pS2 and cMyc but repressed estradiol induction of cyclin D1 mRNA and protein in MCF7 cells. To explore likely mechanisms by which F/I regulated ER, experiments examined estradiol binding, Hsp90 interaction, promoter recruitment and ERα phosphorylation. F/I decreased estradiol binding and increased Hsp90 association with ERα. Chromatin immunoprecipitation revealed that F/I recruited ERα to both pS2 and cMyc promoters at earlier times than estradiol and F/I shifted estradiol recruitment of ERα to earlier time points. F/I induced a unique ERα phosphorylation profile (increase in serine 305 and decrease in serine 118 phosphorylation) that was distinct from estradiol and estradiol+F/I. Taken together, F/I signaling through PKA selectively regulates estradiol-dependent genes in breast cancer that is associated with reduced ligand binding and changes in promoter interaction and ERα phosphorylation.
Introduction

The estrogen receptor (ER) is a ligand-dependent transcription factor involved in normal growth and differentiation of mammary tissue. However, uncontrolled ER signaling can initiate and promote the growth of breast cancer. Regulation of the ER signaling pathway is a complex process that involves ER subtype ligand-ER conformation, DNA response element, homo versus hetero-dimerization, coregulators, and cellular kinases/phosphatases among other events (for review see (1-4)).

In the classical, ligand dependent activation of ER, estrogen binding increases ER phosphorylation at specific sites (4) that facilitate ER dimerization and direct interaction with estrogen response elements (EREs) in the promoter of estrogen target genes (5). Liganded ER recruits a set of transcriptional coactivators with associated histone acetyltransferase activity that facilitates transcriptional activation. At some promoters, ER regulates transcription not through direct interaction with DNA but through binding to other transcription factors at the promoter including Sp-1 (6;7), AP-1 (8;9), GATA-1 (10) and NFKappa B (11). In addition to ligand dependent activation, ER may also be activated in the absence of ligand by crosstalk with signal transduction pathways that is associated with changes in receptor and coregulator phosphorylation (12-18).

Protein Kinase A (PKA), a holoenzyme composed of two regulatory subunits and two catalytic subunits, phosphorylates substrate proteins on serine/threonine residues. Cyclic adenosine 3’, 5’-monophosphate (cAMP)-dependent pathways govern many endocrine and neural functions. Elevated intracellular cAMP binds to the regulatory
subunit of PKA causing phosphorylation of the catalytic subunit of PKA. The phosphorylated catalytic subunit then translocates to the nucleus and phosphorylates transcription factors such as CREB and CREM (19). In addition to activating PKA signaling, cAMP also activates Epac (Exchange protein directly activated by cAMP), a pathway that exhibits guanine nucleotide exchange (GEF) activity (20;21).

PKA signaling promotes ligand independent activation of ER$\alpha$ and regulates ligand-dependent ER$\alpha$ activation (1;18;22-24). Fujimoto and Katzenellenbogen (1994) showed that activation of PKA in MCF7 breast cancer cells alters the agonist/antagonist balance of anti-estrogens. PKA phosphorylates ER at serine 236 within the DNA binding domain (DBD) and this phosphorylation inhibits ER dimerization in the absence of estradiol (15). Activation of the PKA signaling protects ER$\alpha$ from estrogen-induced degradation and promotes ER protein stability (25). In addition, PKA phosphorylates ER$\alpha$ at S305 that is associated with tamoxifen resistance (26) and reduced ER$\alpha$ acetylation (27).

Activated PKA inhibits both human breast cancer proliferation (28) and estrogen regulated cell growth (29) in which ER$\alpha$ contributes to cAMP level and activity (28;29). However the mechanisms by which PKA inhibits ER$\alpha$-positive breast cancer growth and affects both ligand dependent and ligand independent activation of ER$\alpha$ in breast cancer remain unclear. Here we demonstrate gene specific regulation of ER-dependent genes by F/I that may underlie F/I suppression of breast cancer proliferation. We further describe the molecular signaling pathways and mechanisms that occur during ligand independent activation by F/I and F/I suppression of estradiol action in breast cancer cells.
Results

cAMP signaling through PKA decreases estradiol activation of ER–dependent promoters

Forskolin is a well known inducer of cAMP signaling in tamoxifen-sensitive MCF7 breast cancer cells (30). To measure the effect of F/I on ER transcriptional activity, MCF7 cells was transfected with the ER-dependent ERE\textsubscript{2}e1b-luciferase (ERE-TATA-luc) or ERE\textsubscript{2}TK-luciferase (ERE-TK-luc) reporter genes. F/I induced ligand independent activation of ERE-TATA-luc (Figure 1A, bar 4) and ERE\textsubscript{2}TK-luciferase (Figure 1B, bar 4) in MCF7 cells. Although the magnitude of the ligand independent activation by F/I relative to estradiol activation varied among experiments, the ligand independent activation by F/I was consistently and quantitatively less than estradiol activation. This variability of the ligand independent activation was likely due to subtle changes in intracellular kinase/phosphatase activity present during different experiments. Remarkably F/I reduced full reporter activation by estradiol for both the ERE-TATA-luc and the ERE-TK-luc reporters (Figures 1A, 1B, bars 5). 4-(OH)-tamoxifen did not activate either promoter (Figures 1A, 1B bars 3) and repressed the basal transcriptional activity of these ERE containing promoters. Basal reporter activity could be the result of residual estrogen present in the charcoal stripped FBS and/or the result of ER\textalpha ligand independent activation by growth factors present in the medium.
Elevation of cAMP by F/I can activate both PKA and the Epac effectors of cAMP signaling (20). To determine whether the F/I suppression of estradiol-dependent reporter activation occurred through the PKA or Epac pathways, ER-dependent reporter activity was measured following selective activation/inhibition of PKA or the Epac signaling pathways. Overexpression of PKA or PKI was used to measure PKA effects on reporter activity. Incubation of cells with 8CPT-2Me-cAMP, a specific activator of the Epac signaling pathway, was used to measure Epac effects on reporter activity.

Overexpression of PKA, but not PKI, significantly repressed estradiol activation of ERE-TK-luc (Figure 2A). In these experiments tamoxifen antagonist action was assessed by the ability of tamoxifen to repress estradiol activation of the reporter gene (Figure 2A, bar 2 versus bar 4). Overexpression of PKA completely repressed estradiol activation of the reporter (Figure 2A, bar 2 versus bar 5) and enhanced the tamoxifen repression of estradiol activation (Figure 2A bar 4 versus bar 6). In contrast, overexpression of PKI had only modest effects on estradiol activation (Figure 2A, bar 2 versus bar 7) and did not alter tamoxifen repression of estradiol activation (Figure 2A bar 4 versus bar 8).

Similar results as those described in Figure 2A were found with the ERE-TATA-luc reporter (data not shown).

Epac signaling is a separate pathway downstream of elevated cAMP. In contrast to the PKA pathway, activation of the Epac pathway by 8CPT-2ME-cAMP had no significant effect on estradiol-induced reporter activation in MCF7 cells (Figure 2B). Examination of another tamoxifen-sensitive breast cancer cell line (T47D) revealed that overexpression of PKA in T47D cells decreased estradiol-dependent reporter activation
(Figure 2C bar 2 versus bar 5) while overexpression of PKI actually increased estradiol-dependent reporter activation (Figure 2C bar 2 versus bar 6). Similar to MCF7 cells, 8CPT-2Me-cAMP had no significant effect on estradiol dependent reporter activity in T47D cells (data not shown).

MCF7-LCC2 cells are resistant to tamoxifen antagonism of estradiol reporter activation (Figure 2D bar 2 versus bar 4). Interestingly, overexpression of PKA repressed both estradiol reporter activation and estradiol + tamoxifen reporter activation in MCF7-LCC2 cells (Figure 2D bars 2 and 4 versus bars 5 and 6). In contrast, overexpression of PKI had no significant effect on estradiol alone (Figure 2D bar 2 versus bar 7) and only partially blocked estradiol + tamoxifen reporter activation in MCF7-LCC2 cells (Figure 2D bar 4 versus bar 8). Incubation of cells with 8CPT-2Me-cAMP exhibited effects similar to that found with overexpression of PKI in MCF7-LCC2 cells (data not shown). To confirm the functional activity of the PKA and PKI expression vectors and of F/I treatment, MCF7 cells were cotransfected with the cAMP and PKA-activated CRE-luciferase (CRE-luc) reporter. Overexpression of PKA (Figure 2E) or incubation of cells with F/I (Figure 2F) resulted in significant activation of the CRE-luc reporter. In contrast, PKI reduced basal CRE-luc reporter activity (Figure 2E). Incubation of cells with 8CPT-2Me-cAMP showed no significant effect on reporter activity (Figure 2F). Nonspecific effects of F/I on general transcription was assessed by transfection of MCF7 cells with the constitutive RSV-Luc reporter. F/I, at the concentrations used in this study, had no effect on RSV-Luc activation (data not shown).
The results presented above are consistent with F/I regulation of ER-dependent reporter gene activity occurring through selective activation of PKA, but not Epac signaling.

**Gene-selective effects of F/I on ER-dependent transcription.**

The above data indicated that elevation of cAMP induced ligand independent activation of ER and also suppressed full estradiol activation of ER-dependent reporter genes. To more comprehensively assess F/I effects on ER-regulated gene expression, four well-characterized ER-regulated genes in MCF7 cells were measured using real time RT-PCR. F/I exhibited distinct differences in regulation of cMyc, pS2, progesterone receptor (PR) and cyclin D1 mRNA and this regulation was markedly different from F/I effects on the ER-dependent reporter genes. F/I alone induced the expression of pS2, PR and cMyc mRNA in MCF7 cells (Figures 3A, 3B, and 3C, respectively). However in stark contrast to F/I effects on the ERE-TATA-luc and ERE-TK-luc reporters, F/I in combination with estradiol actually elevated mRNA for pS2 and cMyc above that of estradiol alone (Figures 3A and 3C, open □ versus closed □). The relative effects of F/I or F/I + estradiol on cMyc mRNA corresponded to similar changes observed with cMyc protein levels (Figure 3D). Although all treatments (F/I; F/I+estradiol and estradiol) resulted in a rapid induction of cyclin D1 mRNA at 2 hours, only estradiol treatment resulted in induction of cyclin D1 protein (Figure 3F, lane 2) as previously reported (31-33). However, the rapid and transient induction of cyclin D1 mRNA at 2 hours by F/I and F/I + estradiol was not sufficient to induce cyclin D1 protein. Notably, only estradiol treatment resulted in a reproducible and significant induction of cyclin D1 mRNA at 18
hours treatment. It is likely that the sustained induction of cyclin D1 mRNA by estradiol at later time points was responsible for the increased cyclin D1 protein. F/I induction of ER target gene mRNA was ER-dependent since both 4-hydroxytamoxifen (Figure 3G) and ICI 182,780 (data not shown) significantly reduced F/I induction of cMyc mRNA. F/I induction of PR, cyclin D1 and pS2 mRNA was also blocked by 4-hydroxytamoxifen and ICI 182,780 (data not shown).

The differences between F/I effects in reporter gene assays versus endogenous gene expression is probably related to fundamental differences between the artificial and natural promoters. The ERE-e1b reporter gene assay represents a simple ERE-driven promoter that is not subject to regulation by other transcription factors. In contrast, endogenous genes are subject to more complex regulation by other transcription factors. While endogenous genes represent a more realistic appraisal of the overall effects of F/I on gene expression, reporter gene analysis has utility in assessing direct effects of F/I on ERα transcriptional activity. Reporter gene assays were used in conjunction with endogenous gene expression to assess overall effects on gene expression.

**F/I rapidly recruits ERα to pS2 and cMyc promoters and alters estradiol-ERα cyclic recruitment.**

Estradiol-activated ERα can bind directly to ERE sequences in promoters or may interact with DNA indirectly through protein:protein interactions with other transcription factors (e.g. AP-1 and SP1 proteins) (4). ERα is recruited to promoters in a cyclic
fashion involving successive rounds of recruitment and dissociation (34-36). The ERα recognition sequences in the cMyc and pS2 promoters have been well characterized. ERα is recruited to the ERE region of the pS2 promoter (37) (Figure 4A) and ERE and AP-1 regions of the cMyc promoter (38) (Figure 4B). To measure the effect of activated cAMP on ERα association with cMyc and pS2 promoters, quantitative ChIP assays were performed as previously described by our laboratory (13). Incubation of MCF7 cells with F/I for 45 min. significantly increased the association of ERα with ERE sequences in the pS2 promoter (Figure 4C closed bar) and with the ERE/AP1 sequences in the cMyc promoter (Figure 4C open bar). In contrast, treatment with estradiol or F/I + estradiol did not recruit ERα to these promoters at this early (45 min.) time point (Figure 4C).

However at longer incubation periods (2 hrs) incubation with estradiol, but not F/I nor F/I + estradiol, significantly increased ERα association with the pS2 promoter (Figure 4D closed bar) and with the cMyc promoter (Figure 4D open bars). To further examine time-dependent ERα recruitment to the cMyc promoter and to determine the relative differences between F/I and estradiol, time course recruitment of ERα to the cMyc gene was assessed. ERα regulates cMyc transcription through both ERE and AP-1 sequences that are in close proximity to one another. Consequently ChIP assays for the cMyc promoter assessed combined ERα recruitment to ERE and AP1 sequences. ERα recruitment to the cMyc promoter was significantly increased by F/I at 1hr, F/I + estradiol at 1.5hr, and estradiol at 2hrs (Figure 4E bars •, closed □ and open □, respectively). A similar trend was observed for the ERE sequence of the pS2 promoter (data not shown).
These preceding set of data reveal several features about F/I versus estradiol effects on ERα promoter recruitment in MCF7 cells. F/I, but not estradiol, induces a rapid recruitment of ERα to pS2 and cMyc promoters. Promoter recruitment by estradiol is delayed (2 hours) but this delay is shifted to earlier time points (1.5 h) by the addition of F/I to estradiol treatments.

**F/I reduces estradiol binding to ERα and is accompanied by increased association of ERα with Hsp90.**

To examine possible mechanisms by which PKA represses estradiol-dependent transcription of selected genes, the effect of F/I on estradiol binding in intact cells was examined in MCF7 (Figure 5A), MCF7-LCC2 (Figure 5B) and T47D cells (Figure 5C). F/I significantly reduced estradiol binding by 45% in all cell lines. Since ERα is expressed approximately four fold higher than ERβ in MCF7 cells (Figure 5D), the reduced estradiol binding by F/I was due predominantly to effects on ERα. ERβ protein expression in MCF7 cells was low compared to ERα (Figure 5D) as previously reported (39) and detection by Western blot using crude cellular extract was weak. To confirm expression of ERβ in MCF-7 cells, ERβ was immunoprecipitated from cells followed by detection by Western blot (Figure 5E). Notably, decreased ligand binding by F/I was not the result of reduced ERα protein since F/I as well as estradiol did not significantly alter ERα protein levels at 2 hours incubation and earlier (refer to Figure 7B). Interestingly, F/I had no effect on estradiol binding to purified ERα (Figure 5F) indicating that other cellular processes, and not direct F/I interaction with ERα, were required for reduced
ligand binding. Although reduced ligand binding may contribute to F/I antagonism of estradiol activation of cyclin D1 and the ERE-TATA-luc and ERE-TK-luc reporters, the reduced ligand binding by F/I was not sufficient to suppress estradiol induction of cMyc, pS2 and PR.

Since Hsp90 dissociates from ER upon ligand binding (40) it was of interest to determine whether reduced ligand binding by F/I altered Hsp90 interaction with ERα. F/I significantly increased Hsp90 association with ERα in MCF7 cells (Figure 6A). As a control we assessed ligand binding and Hsp90 interaction in HeLa cells, an ER negative cell line in which ERα is activated by F/I (Figure 6B) as previously reported (16). In HeLa cells transfected with ERα expression vector, F/I had no effect on estradiol binding (Figure 6C). In parallel, F/I did not alter the Hsp90 interaction with ERα in HeLa cells (Figure 6D). These data suggest that increased association of Hsp90 with ERα may be related to the selective attenuation of estradiol binding by F/I in MCF7 cells and may further lead to selective gene activation by F/I (currently under investigation).

**F/I alters the phosphorylation profile of ERα**

ERα is phosphorylated on eight residues (Figure 7A) and receptor phosphorylation is known to regulate multiple receptor functions including nuclear translocation, ligand binding, ER dimerization and transcriptional activation (3;41). To identify F/I-induced changes in ERα phosphorylation that may contribute to altered gene expression, phospho-specific antibodies to each ERα phosphorylation site were
developed (42) and used to profile ERα phosphorylation in MCF7 cells. The major effect of F/I on ERα phosphorylation was the induction of S305 phosphorylation, reduction of S118 phosphorylation and suppression of the estradiol-induced S118 phosphorylation (Figure 7B-D). Other effects of F/I and estradiol on ERα phosphorylation were less pronounced although consistent: estradiol induced phosphorylation at sites S104, S106, S118, S167, S236, S305, T311, and Y537 (Figure 7B lane 2); F/I suppressed estradiol induction of S118, S236 and Y537 (Figure 7B lane 2 versus lane 4). F/I repression of estradiol-induced phosphorylation likely contributed to F/I-regulated decreases in ligand binding and estradiol-dependent gene activation. The effects of F/I and F/I + estradiol on S167 phosphorylation were inconsistent (data not shown). F/I and estradiol effects of ERα phosphorylation were not the result of changes in ERα protein level. Although previous reports showed that estradiol significantly decreased ERα protein in MCF7 cells by 4 hrs and inhibition of PKA by H-89 decreased ligand-free ERα protein in MCF7 cells (43), at incubation periods used in the present study (2 hours and earlier) estradiol and F/I did not alter ERα protein levels (Figures 5-7).

F/I resulted in a distinct ERα phosphorylation profile (decreased S118 and increased S305 phosphorylation) that was markedly different from vehicle or estradiol-induced phosphorylation. S118 is a MAPK consensus site that is phosphorylated following estradiol treatment and via crosstalk with ERK1/ERK2 signaling (3; 17). PKA phosphorylates ERα at S305 (26) Further experimentation investigated the involvement of S305 and S118 phosphorylation in F/I action. Since F/I decreased basal S118 phosphorylation, the effect of F/I on ERK1/ERK2 signaling was examined by Western
blot analysis using phospho-specific antibodies to activated ERK1/ERK2.

Activation/inhibition of ERK1/ERK2 by PKA is cell line and tissue dependent (44-47). Overexpression of PKA in MCF-7 cells decreased basal phosphorylation of ERK1/ERK2 (Figure 8A, lane 2). In addition, incubation of MCF-7 cells with F/I resulted in rapid decrease in ERK1/ERK2 phosphorylation (Figure 8A lanes 4-7). These data suggest that in MCF-7 breast cancer cells, PKA repressed S118 phosphorylation via reduced ERK1/ERK2 activation.

Mutation of S118 to alanine results in reduced ligand dependent and ligand independent activation of ERα (13;48;49) and therefore F/I suppression of S118 phosphorylation likely contributes to F/I effect on reducing estradiol activation of selected genes. However ligand independent activation of ERα by F/I would not likely be explained by suppression of S118 phosphorylation. To investigate the involvement of S305 in F/I action, ERα wild type or ERαS305A mutant were overexpressed in MCF7 cells and ERE-TK-luc reporter activity was measured. F/I induced ligand independent activation of wild type ERα and decreased estradiol-dependent activation of ERE-TK-luc (Figure 8B). With overexpression of ERαS305A, there was reduced ligand independent activation by F/I compared to overexpression of wild type ERα and F/I did not significantly reduce estradiol activation of the reporter. This suggests that S305 contributes to both F/I ligand independent activation of ERα and to the F/I-dependent attenuation of estradiol activation of the reporter.
In summary F/I, estradiol and estradiol + F/I resulted in three distinct ERα phosphorylation profiles in MCF7 cells. These profiles likely represent three different ERα conformations that are important for differences in time-dependent recruitment of ERα to pS2 and cMyc promoters and define the gene selective action of F/I and estradiol.

**F/I suppresses MCF7 cell growth**

MCF7 cells are dependent upon estradiol for proliferation (30) and forskolin exhibits an antiproliferative effect in MCF7 cells through activation of PKA signaling (29). The effect of F/I on estradiol-dependent growth of several ERα positive breast cancer cell lines was measured at different times. At 72 hrs estradiol significantly induced growth of MCF7 (Figure 9A, closed □) and T47D cells (Figure 9B, closed ■) but did not promote growth of tamoxifen-resistant MCF7-LCC2 cells (data not shown) as previously reported (50). 10μM F/I alone decreased growth of MCF7 (Figures 9A, open □) and T47D cells (Figure 9B open □) but had no effect on MCF7-LCC2 cells (data not shown). Similar results were observed at lower concentrations of F/I (1 and 5μM) for MCF-7 cells (data not shown). Remarkably, F/I significantly inhibited the estradiol-dependent growth of MCF7 and T47D cells (Figures 9A and 9B, closed ▲, respectively).
Discussion

Previous studies using reporter gene analysis indicate that ligand independent activation of ER amplifies estradiol action and disrupts the agonist/antagonist balance of tamoxifen, the end result being promotion of breast cancer cell proliferation (23;51). Similar to MAPK (18), PKA induced ligand independent activation of ERα (18) although activated PKA also inhibited breast cancer cell growth (28;52) and estrogen-induced cell growth (29). The present study investigated F/I activated signaling pathways leading to regulation of ERα transcriptional activity in breast cancer cells. F/I induced ligand independent activation of ERα but decreased estradiol activation of ERα-dependent reporter genes specifically through PKA signaling. In contrast to general effects on reporter genes, F/I had gene selective effects on endogenous ER-regulated transcription in which F/I induced PR, cMyc and pS2 but repressed cyclin D1 expression. Associated with the gene selective effects of F/I was decreased estradiol binding, increased ERα association with Hsp90, rapid recruitment of ERα to cMyc and pS2 promoters and a unique ERα phosphorylation profile that was distinct from basal and estrogen-induced phosphorylation. F/I and activation of PKA suppressed breast cancer cell growth and inhibited estradiol-dependent cell growth in accordance with a previous study showing that inhibition of PKA signaling promoted estradiol-dependent cell growth (29). Possibly, the inhibition of cyclin D1 by F/I and PKA is involved in PKA suppression of estradiol-dependent cell growth.
Elevation of cAMP by F/I resulted in ERα ligand independent activation in MCF7, T47D and MCF7-LCC2 breast cancer cell lines but, unexpectedly, F/I decreased ERα activation by estradiol for selected genes. Dissecting the cAMP effect on estradiol dependent reporter activity revealed that the PKA signaling pathway attenuated estradiol-dependent reporter activity in all cell lines and also reversed tamoxifen resistance in MCF7-LCC2 cells. Unlike PKA, activating Epac signaling (20) by 8CPT-2Me-cAMP treatment had no significant effect on ERα activation and only partially reversed tamoxifen resistance in MCF7-LCC2 cells. To our knowledge, this is the first report that separates the Epac pathway from effects on ERα signaling in breast cancer cells following elevation of intracellular cAMP.

Previous studies demonstrated conflicting results for PKA effects on ER-dependent reporters. Inhibition of PKA by H-89 significantly stimulated ligand independent activation of ERα and enhanced estradiol-dependent transcriptional activity in MCF7 cells stably transfected with an ERE-luc reporter (43). In contrast, Katzenellenbogen and co-workers (23) reported that overexpression of PKA or treatment with cholera toxin (CT)/IBMX induced estradiol dependent activity of (ERE)2-TATA-CAT but not ERE-TK-CAT. In addition, PKA or CT/IBMX treatment altered tamoxifen antagonism but had no significant effect on ligand independent activation of ERα in MCF7 cells. Data presented here using endogenous ER-regulated genes regulated by different promoters provide evidence that the conflicting data with reporter genes are likely related to the gene specific effects of F/I.
Although F/I attenuated estradiol activation of reporter genes, F/I demonstrated a selective activation of endogenous genes containing complex promoters. F/I had no effect on estradiol-dependent transcription of PR and exhibited an additive effect on estradiol-dependent transcription of cMyc and pS2 mRNA. Similar results with pS2 were reported by El Tanani and Green (53) in which elevation of cAMP by (CT)/IBMX exhibited an additive effect on estradiol-dependent transcription of the pS2 and LIV-1 genes in MCF7 cells. In the present study, F/I induced cyclin D1 mRNA at early time points similar to results with estradiol treatment. However at later time points F/I repressed estradiol induction of cyclin D1 mRNA with the end result being suppression of estradiol-induced cyclin D1 protein. The differences in the ER-regulated promoter sequences in each of these genes may provide clues to the F/I selective repression of cyclin D1 but not cMyc, PR or pS2. (the role of specific ERE sequences in ER promoter interaction is reviewed in (54)). Estradiol regulates cMyc transcription through ERE and AP-1 sequences (38), PR transcription through ERE, AP-1 and SP1 sequences (55;56) and pS2 transcription through ERE, SP1 and SP3 sequences (37;57). In contrast, although cyclin D1 contains a partial and imperfect ERE (58), ER regulates cyclin D1 predominantly through a cAMP response element (CRE) in MCF7 cells (31). In addition, Nadella and Kirsch (59) provided clear genetic evidence that cyclin D1 expression was regulated by PKA signaling. ATF-2 binding to the CRE element is crucial for activation of the cyclin D1 promoter and it is possible that F/I treatment disrupts this interaction. Although Sabbah and colleagues (31) reported that forskolin had no effect on cyclin D1 promoter activity in MCF7 cells, the effect of forskolin or F/I on ATF-2 binding to the CRE was not investigated. It was reported that PKA inhibits the
cyclin D1 promoter by phosphorylation of CREB that binds to the CRE (32). CREB may compete with ATF-2 for binding to the CRE and prevent cyclin D1 activation by ER.

There are likely both direct and indirect effects of F/I on ERα. F/I induction of ER target genes were ER dependent in which both 4-hydroxytamoxifen or ICI, 182, 780 were able to reverse F/I induction of ER target genes. F/I reduced ERα ligand binding and increased association of ERα with Hsp90. Some of these effects of F/I on ER function are likely related to direct effects of F/I on altering ERα phosphorylation at serines 305 and 118. However these data do not exclude the possibility that F/I regulates ER function through indirect mechanisms.

This is the first report to demonstrate ligand independent recruitment of ERα to pS2 and cMyc promoters induced by F/I. Recruitment of ERα by F/I was rapid and, similar to estradiol, occurred in a cyclic fashion. However a clear correlation between F/I recruitment of ERα to promoters and levels of mRNA induction could not be made. It will be of interest to profile the proteins associated with ERα at each promoter during both ligand independent activation and ligand dependent activation to determine whether different protein complexes contribute to selective gene activation by F/I and PKA signaling (currently under investigation).

It is widely accepted that ligand binding is regulated by both Hsp90 association and nuclear receptor phosphorylation (4;41; (60-62). In the absence of ligand, Hsp90 interacts with the ligand binding domain of ERα (63) and dissociates upon ligand binding.
leading to tight association of ERα with the nuclear compartment (60). In the present study, F/I increased Hsp90 association with ERα that was accompanied with a decrease in ligand binding. As shown in HeLa and MCF7 cells, the F/I effect on ligand binding was correlated with Hsp90 association with ERα. This suggests that F/I induces conformational changes in ERα and/or Hsp90 that decrease estradiol binding and increase Hsp90 association (the effect of estradiol or F/I + estradiol on Hsp90 association with ERα could not be examined because estradiol results in tight association of ERα with nuclear structures thereby preventing extraction for co-immunoprecipitation assays). Remarkably, decreased estradiol binding and increased association of ERα with Hsp90 by F/I did not prevent ERα recruitment to promoters suggesting that an ERα-Hsp90 complex may be present at the promoter as previously suggested for the GR-Hsp90 complex (64).

F/I, estradiol and F/I + estradiol resulted in three distinct ERα phosphorylation profiles. In general, ligand independent activity of ERα results from changes in AF-1 (A/B) domain phosphorylation (4), S118 phosphorylation, a MAPK site that is enhanced by estradiol (2), is required for full ERα transcriptional activity by estradiol (13;17). S118 phosphorylation was dramatically reduced by F/I and F/I + estradiol compared to vehicle. It is likely that activation of PKA by F/I in MCF7 cells blocks MAPK phosphorylation. In this context, PKA suppression of MAPK could also reduce steroid receptor coactivator 1 (SRC-1) regulation of ER (46) since SRC-1 contains several MAPK consensus sites that are required for full coactivator activity (63; (65)). S305 is contained within a PKA consensus sequence located at the interface of the hinge (D)
domain and the ligand binding (E/F) domain and within the nuclear localization region (63). A previous study showed that forskolin results in phosphorylation of S305 in U2OS osteosarcoma cells (26) and Cos-1 cells transfected with wild type ERα (42). The present study demonstrates the involvement of S305 in the ligand independent activity as well as in repressing estradiol-dependent transcription of ERE-luciferase containing promoter. The dual effect of PKA on ERα phosphorylation in MCF7 cells (induction of S305 and suppression of S118 phosphorylation) may explain both the ligand independent activity (induction of S305) and suppression of estradiol dependent activity (suppression of S118), at least for the simple promoters present in the reporter genes.

Interestingly, F/I did not induce phosphorylation of S236, a PKA consensus site (15), but instead modestly inhibited estradiol dependent phosphorylation of S236 as well as Y537. S236 phosphorylation prevents ERα dimerization in the absence of estradiol (15). Y537 phosphorylation regulates ligand binding and transcriptional activity (60;61). Similar to suppression of S118 phosphorylation, F/I inhibition of S236 and Y537 would be consistent with the reduced estradiol binding and reduced ligand dependent activation observed here. A similar effect of forskolin was reported by Blok et al. (66) where forskolin blocked androgen binding to its cognate receptor by dephosphorylating androgen receptor. Remarkably, F/I in combination with estradiol resulted in a unique and distinct phosphorylation profile compared to estradiol or F/I alone (phosphorylation of S104, S106, S305 and T311). S104 and S106 can be phosphorylated by estradiol binding or by cross talk with cyclin A/CDK2 signaling (67) and these sites potentiate estradiol action and induce ligand independent activation of ERα (67). T311 is
phosphorylated by a P38α/SAPK2a complex and regulates ERα nuclear localization (68).

It is widely believed that ERα posttranslational modifications, including phosphorylation, affect chromatin remodeling and ERα promoter occupancy (69). Therefore, the differences in ERα phosphorylation profiles represented by the three different treatment regimens (estradiol, F/I, or estradiol + F/I) likely result in distinct receptor conformations that impact the dynamic association of ERα with ER target promoters. F/I regulated dual phosphorylation events (induction of S305 phosphorylation and repression of S118 phosphorylation) that were correlated with rapid recruitment of ERα to pS2 and cMyc promoters. Recently, Kumar and co-workers (70) found that mutation of S118 to alanine (mimic dephosphorylation) and mutation of S305 to glutamic acid (mimic phosphorylation) inhibited estradiol-dependent transcription. This dual mutant is very similar to the ERα phosphorylation pattern induced by F/I and reproduces the F/I suppression of estradiol activation of the reporter. F/I + estradiol treatment resulted in three additional phosphorylation events (phosphorylation of S104, S106 and T311) and was associated with a delay in ERα promoter recruitment compared to F/I alone whereas estradiol alone resulted in phosphorylation of eight ERα phosphorylation sites and was associated with further delay in promoter recruitment.

F/I alone inhibited growth of ERα positive, tamoxifen-sensitive MCF7 and T47D breast cancer cells but not ERα positive, tamoxifen-resistant MCF7-LCC2 cells. Furthermore, F/I blocked estradiol dependent growth of tamoxifen sensitive breast cancer
cells. Previous studies reported an antiproliferative effect of cAMP in MCF7 cells in which ERα contributed to cAMP levels and activity (28;29) and ERα predicted the antiproliferative effect of cAMP (29). However, activated cAMP also suppressed growth of ERα-negative MDA-MB-231 breast cancer cells (28) suggesting an ERα independent pathway for cAMP growth suppression. de Cremoux and colleagues (71) characterized tamoxifen-resistant sublines of MCF7 cells and found that both ERα and PR mRNA were decreased and ERβ mRNA was increased in MCF7-LCC2 cells compared to parental, tamoxifen-sensitive MCF7 cells. In addition, the differences in the bcl-2/bax ratio and overexpression of transforming growth factor β (TGFβ) in MCF7-LCC2 cells possibly contributed to resistance (72). It is likely that combined changes in ERα expression, TGFβ expression and the bcl-2/bax ratio contribute to the inability of F/I to have an antiproliferative effect on MCF7-LCC2 cells.

It is likely that the inhibition of cyclin D1 by F/I contributes to suppression of estradiol dependent growth of breast cancer cells. Typically, estradiol regulates cell cycle progression from the G0-G1 phase by induction of cMyc and cyclin D1 followed by activation of Cdk4 and Cdc25A enzymatic activity in a time dependent manner (73). It is possible that the antiproliferative effect of PKA is mediated, in part, by inhibition of cyclin D1 that in turn blocks cells at G0-G1. F/I induction of cMyc alone would not be sufficient for cells to progress through the G1 phase.

A model for estradiol and F/I effects on ERα activation in breast cancer cells is presented in Figure 10. Estradiol treatment dissociated the Hsp90-ERα complex and
induced all ERα phosphorylation sites resulting in transcription of reporter genes and endogenous genes (cMyc, pS2, PR and cyclin D1) and breast cancer cell growth (Figure 10A). In contrast, F/I (Figure 10B) increased Hsp90-ERα association, decreased estradiol binding, induced S305 and repressed S118 phosphorylation. This was accompanied by ligand independent activation of ERα and decrease of estradiol-dependent reporter activity in a PKA dependent manner. However, the F/I effect on estradiol-dependent transcription was gene specific resulting in inhibition of cyclin D1 expression and inhibition of breast cancer cell growth. Crosstalk between PKA and estradiol signaling (Figure 10C) resulted in a unique phosphorylation profile and also inhibited estradiol induction of cyclin D1 expression that may inhibit estradiol-dependent growth of breast cancer cells (29).
Materials and Methods

Reagents:

17\(\beta\)estradiol (E2) and 4-hydroxytamoxifen (4-(OH)-Tam) were purchased from Sigma (St. Louis, MO). Forskolin, Coleus forskohlii and 3-isobutyl-1-methylxanthine (IBMX) were purchased from Calbiochem (San Diego, CA). 8CPT-2Me-cAMP was purchased from Tocris (Ellisville, MO). Cyclin D1 (DCS-6), cMyc (C-8), ER\(\alpha\) (D12) and actin (C-11) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). ER-P-S104, ER-P-S106, ER-P-S118, ER-P-S167, ER-P-S236, ER-P-S305, ER-P-T311, and ER-P-Y537 antibodies were provided by Bethyl Laboratories (Montgomery, TX). ER\(\alpha\) (VP-E613) antibody, peroxidase labeled anti-mouse and peroxidase labeled anti-rabbit were purchased from Vector Laboratories (Burlingame, CA). ER\(\beta\) (14C8) antibody was purchased from GeneTex (San Antonio, TX). Protein A Sepharose beads (CL-4B) were purchased from Amersham Biosciences (Piscataway, NJ). Protein A magnetic beads were purchased from New England Biolabs (Ipswich, MA).

Cell Culture:

MCF7 ER\(\alpha\) positive tamoxifen-sensitive breast cancer cells and HeLa cells were maintained in phenol red free DMEM media containing 10% Fetal bovine serum (FBS), 1% Penicillin/streptomycin (P/S), and 2% Glutamine. T47D ER\(\alpha\) positive tamoxifen-sensitive breast cancer cells were maintained in phenol red free RPMI media containing 10% FBS, 1% P/S and 5\(\mu\)g/ml insulin. MCF7-LCC2 ER\(\alpha\) positive tamoxifen-resistance breast cancer cells were maintained in phenol red free IMEM media containing 5%
stripped FBS (FBS were incubated with dextran coated charcoal to remove steroids). All cells were incubated at 37°C and 5% CO₂.

**Luciferase Assay:**

0.5x10⁶ of MCF7, T47D, MCF7-LCC2 or HeLa cells were plated in each well of 6 well plates in medium containing 5% stripped FBS for 48 hrs. Cells were transfected with ERE₂e1b-firefly luciferase, ERE₂TK-firefly luciferase or CRE-firefly luciferase by using Fugene 6 (Roche) for 24 hrs. In experiments in which overexpression of PKA, PKI, wild type ERα or ERαS305A was performed, ERαS305A (obtained from Dr. Rakesh Kumar, University of Texas M.D. Anderson Cancer Center), pFC-PKA, RSV-PKI (obtained from Dr. Mario Ascoli, University of Iowa), ERα-pCR3.1 or empty vector were transfected along with the reporter constructs. Cells were incubated with vehicle, 17-βestradiol (10⁻⁸M), 4-hydroxytamoxifen (4-(OH)-Tam) (10⁻⁷M), Forskolin (10μM) + IBMX (100μM) (F/I), or 8CPT-2Me-cAMP for 18 hrs. Media were replaced with 400μl of 1X SDS lysis buffer, incubated for 1 hr at -70°C and firefly luciferase activity was measured using the Luciferase Assay System Kit from Promega (Madison, WI). Relative luciferase units (RLU) were normalized to the total protein content of each sample. Constitutive renilla luciferase (1-phRL-null reporter vector (Promega) and pCMV-β-gal reporters were markedly activated by F/I and/or overexpression of PKA and PKI precluding normalization of ERE₂e1b-firefly luciferase and ERE₂TK-firefly luciferase assays to transfection efficiency (data not shown).

**Real time RT-PCR:**
Real time RT-PCR was performed as previously described by our laboratory (13;74). Briefly, MCF7 cells (4x10^6) were plated in 10 cm dishes in phenol red free DMEM containing 5% stripped FBS for 3 days followed by incubation with vehicle, E2 (10^{-8}M), F/I (10μM/100μM) (F/I), 4-(OH)-Tam (10^{-7}M) or combination for 2, 6, 12, 18, or 24 hrs. Cells were harvested and total RNA was extracted using the Trizol (Invitrogen)/chloroform method. Total mRNA was adjusted to 200ng/reaction and reverse transcribed using the TaqMan Reverse Transcription Kit (Applied Biosystems, Foster City, CA). cDNA was measured by real time RT-PCR as previously described (13;74;75). The following primers and probes were used.

Progestosterone receptor (PR): FWD-5’CTATGCAGGACATGACAAACAA3’, REV-5’TGCTCTCGCCTAGTTGATTAAG3’,
Probe-5’/56FAM/CCTGACACCTCCAGTTTTGCTGACAG/3BHQ-1/-3’.

GAPDH: FWD-5’CAACGGATTTGGTGTTGATCGG3’, REV-5’GCAACAATATCCACTTTACCAGAGTT3’,
Probe-5’/56FAM/CGCCTGGTACAGGCTGCT/3BHQ-1/-3’.

cMyc: FWD-5’CGTCTCCACACATCAGCACAA-3’, REV-5’TCTTGGCACAGGACATGCTT-3’,
Probe-5’/56FAM/ACGCGAGAGCCTCCCTCCACT/3BHQ-1/-3’

pS2: FWD-5’CGTGAAAGAATGTGGTTTT-3’

Cyclin D1: FWD-5’TGGGTCTGTGCATTTCTGGTT-3’, REV-5’GCTGGAAACATGCCGGTTAC-3’

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Probe-5'/-56FAM/ CGGCGCTTCCCAGCACCAA/3BHQ-1/-3’

The CT value of the gene of interest was normalized to GAPDH and calibrated to vehicle treatment CT value to obtain the fold difference.

**Western Blot:**

MCF7 cells (4x10⁶) were plated in 10 cm dishes in phenol red free DMEM medium containing 5% charcoal stripped FBS for 3 days. Fresh medium was replaced before treatment and cells were incubated with vehicle, E2 (10⁻⁸M), 4-(OH)-Tam (10⁻⁷M), F/I (10µM/100µM) (F/I) or combination of F/I + ligand for 2 or 24 hrs. To detect ERK1/ERK2 phosphorylation, cells were either transfected with empty vector or PKA expression vector, or incubated with F/I for 5, 10, 15 or 30 min. Cells were harvested in PBS and cell pellets lysed with high salt lysis buffer (0.4 M NaCl, 5 mM sodium fluoride, 10 mM Tris pH 8, 2 mM EDTA, 2 mM EGTA, 1 mM sodium orthovanadate, 0.1% Triton x100, 10 mM β-mercaptoethanol, 5 mM of β-glycerophosphate, protease inhibitor mix, 0.1 mM PMSF) for 10 min. on ice, and cellular debris removed by centrifugation at 20,000 x g for 10 min. SDS loading buffer was added to the total cellular extract and samples were boiled for 2 min. Proteins were separated by 10% SDS-PAGE analysis and transferred to nitrocellulose membrane. Protein expression and ERα phosphorylation was determined by Western blotting with specific antibodies listed above and expression signals were obtained by enhanced chemiluminescence (ECL). Protein expression was normalized to β-actin levels. ERα and ERK1/ERK2 phosphorylation was normalized to the total ERα and ERK1/ERK2 protein expression, respectively. Relative quantification
of ERα and ERβ in MCF-7 crude cell extract by Western blot was determined by comparison of signal intensity to a known amount of purified ERα and ERβ.

**Quantitative Chromatin Immunoprecipitation (ChIP) assay:**

Quantitative ChIP assays were applied as previously described by our laboratory with little modification (13;76). MCF7 cells (10x10⁶) were plated in 15 cm dishes in phenol red free DMEM medium containing 5% stripped FBS for 3 days. Medium was replaced before cells were incubated with vehicle, E2 (10⁻⁸M), F/I (10μM/100μM) or combination of E2 and F/I for 45 min, 1 hr, 1.5 hr and 2 hrs. Chromatin and proteins were cross linked with formaldehyde (final concentration 1%) for 15 min. at 37°C. Cells were washed with PBS, harvested and lysed in SDS lysis buffer (1%SDS, 10mM EDTA pH 8.0, 50mM Tris-HCl pH 8.0) that contained protease inhibitors and phosphatase inhibitors. Cells were incubated in lysis buffer for 10 min followed by sonication on ice (four times, 10 sec each, with 40 sec intervals). Cellular debris was removed by centrifugation of samples at 20,000 x g rpm for 10 min and the total cellular extract was diluted 10 fold in ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2mM EDTA pH 8.0, 16.7mM Tris-HCl pH 8.0, 167mM NaCl) that contained protease inhibitors and phosphatase inhibitors. 10% of the diluted samples were frozen at -70°C for use as input. Samples were pre-cleared with 2 μg of normal mouse IgG (Santa Cruz), 3μg of sheared salmon sperm DNA and 100μl of protein A Sepharose beads in PBS (1:1) for 2 hrs at 2°C. Beads were removed by centrifugation at 1500 x g for 5 min and samples were incubated with 2 μg of ERα (D-12) antibody for 18 hrs at 4°C with rotation. Negative
control samples were incubated with normal mouse IgG. 3μg of sheared salmon sperm DNA and 5μl of Protein A magnetic beads were added and samples were incubated for 2 hrs at 4°C with rotation. Beads were separated and washed 2X with ChIP washing buffer (0.1%SDS, 1% Triton X-100, 2mM EDTA pH 8.0, 20mM Tris-HCl pH 8.0, 150mM NaCl) and 1X with TE buffer (10mM Tris-HCL pH 8.0 and 1mM EDTA pH 8.0). Protein-chromatin complexes were eluted and reverse cross-linked by incubating beads with elution buffer (1%SDS, 0.1mM NaHCO3) at 65 °C for 12 hrs (input samples were also incubated at this step). The DNA was purified with QIAquick PCR purification kit (QIAGEN, Chatsworth, CA) and re-suspended in 50μl of ddH2O. ChIP was measured by applying validated primers and probes for real time RT-PCR for pS2 and cMyc as previously described (13). The CT value of the sequence of interest was normalized to input samples and calibrated to the vehicle treatment CT value to obtain the fold difference.

**Ligand binding in intact cells:**

MCF7, MCF7-LCC2, T47D cells (0.5x10⁶/well) were plated in 6 well plates for three days in medium containing charcoal stripped FBS. Medium was replaced and cells were pretreated with vehicle or F/I for 1 hr. Cells were incubated with 1.5 nM of estradiol, [2,4,6,7-3H(N)] (Perkin Elmer (20μCi), Boston, MA) in the presence or absence of 1.5 μM of E2 (cold) for 1 hr. Cells were washed and incubated with 700 μl of absolute ethanol for 20 min at room temperature. Samples (500μl) were collected and radioactivity was measured and normalized to the total protein amount. HeLa cells were
plated in 10 cm dishes in phenol red free DMEM containing stripped FBS for 2 days, cells were transfected with ERα (500μg) for 24 hrs, harvested and re-plated (1x10^6) in 6 well plates for 1 day. Ligand binding was performed as described above.

**In vitro ligand binding:**

Purified ERα (500ng) was pre-incubated with F/I, vehicle or ICI 182,780 (10^-9M) for 1 hr in TESH buffer (10 mM Tris, 1.5 mM EDTA, 1 mM dithiothreitol, pH 7.4) on ice. 1μM estradiol, [2,4,6,7-3H(N)] was added with or without 10 mM E2 (cold) and samples were incubated on ice for 1 hr. Dextran-coated charcoal (DCC) slurry (1.0 g charcoal, 0.05 g dextran T-70 in 100 ml of TESH buffer) was added for 30 seconds and DCC was separated by immediate centrifugation at 5000 x g for 5 min at 4 °C. Supernatant was collected and radioactivity was measured.

**Immunoprecipitation of ERβ:**

MCF7 cells were plated in 15 cm dishes in phenol red free DMEM containing 5% charcoal stripped FBS for 3 days. Cells were washed with PBS, harvested and cell pellets lysed with high salt lysis buffer for 15 min on ice. Cellular debris was removed by centrifugation at 20,000 x g for 10 min. Protein A Sepharose beads were incubated with ERβ antibody for 2 hrs at room temperature and washed twice with PBS. Total cellular extract was diluted with PBS and incubated with beads for 18 hrs at 4 °C with rotation. Beads were washed twice with PBS, SDS loading buffer was added to the beads and boiled for 2 min. Immunoprecipitated ERβ was detected by Western blot as described above.
Co-immunoprecipitation of ERα and HsP90:

MCF7 and HeLa cells were plated in 10 cm dishes in phenol red free DMEM containing 5% stripped FBS for 2 days. HeLa cells were transfected with hERα (500μg) for 24 hrs using Fugene 6. Cells were incubated with vehicle or F/I for 2 hrs and harvested. Cells pellet were incubated with HEMG buffer (10 mM HEPES, 3 mM EDTA, 20 mM sodium molybdate, 5% glycerol, pH 7.4, containing protease and phosphatase inhibitors) on ice for 10 min and ruptured using a dounce homogenizer (60 strokes). Debris was removed by centrifugation at 20,000 x g for 30 min at 4 °C. 2 μg of ERα (D-12) antibody was incubated with protein A Sepharose beads (in PBS) for 3 hrs at room temperature. Beads were washed 3X in PBS and added to the total cellular extract. Samples were incubated at 4 °C for 18 hrs with rotation. Beads were washed with CO-IP buffer (10 mM Tris-HCl, 3mM EDTA, 10% glycerol, 50 mM NaCl, 20 mM sodium molybdate, pH 7.4) 3X, 5 min. each with rotation. The ER-protein complex was eluted by boiling beads in SDS loading buffer for 5 min. Samples were subjected to standard Western blot analysis with Hsp90α/β (F8) antibody (Santa Cruz). To normalize the Western blot signals, blots were stripped by incubating membrane in stripping buffer (2% SDS, 100mM 2-β-mercaptoethanol, 62.5 mM Tris HCL pH 6.8) at 55 °C for 30 min with rotation. Membranes were re-probed with ERα antibody (Vector laboratories).

MTT assay:

MCF7, MCF7-LCC2 or T47D cells (20x10³ cells/well) were plated in 24 well plates in medium containing 5% stripped FBS for 24 hrs. Cells were incubated with
vehicle, E2 (10⁻⁶M), F/I (1μM/100μM; 5μM/100μM; or 10μM/100μM), or combination of E2 and F/I in triplicate for 24hrs, 48hrs, or 72hrs. Treatments were repeated after 24 hrs and 48 hrs. 5X buffer (50 mg/10 mls of 1X PBS) for MTT reagent (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl tetrazolium, MP Biomedicals, Aurora, OH) was prepared. 100 μl of MTT buffer (final concentration 1X) was added to each well and plates were incubated for 20 min. at 37 °C. Medium was replaced with 300 μl DMSO and plates were incubated for 15 min. at room temperature with shaking. 200 μl of each sample was transferred to 96 well plates and optical density was measured at 595 nM. Cell growth was calculated as % of vehicle treatment.

**Statistical Analysis:**

Data are expressed as mean ± S.D. p. values were calculated using Anova Dunnett’s T Test and Independent T Test. p. values <0.05 was considered significant.

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Figure legends

Figure 1. F/I induced ligand independent activation of ERα and decreased estradiol-dependent transcription in MCF7 cells. A and B. MCF7 cells were plated in 6 well plates (0.5x10^6/well) for 24 hrs in phenol red free DMEM containing 10% FBS that had been treated with charcoal to remove endogenous steroids. Each well was transfected with ERE-TATA-Luc (500ng) (A) or ERE-TK-luc (500ng) (B) for 24 hrs, followed by incubation with estradiol (E2; 10^{-8}M), 4-(OH)-tamoxifen (10^{-7}M) or F/I (forskolin, 10μM; IBMX, 100 μM) for an additional 18 hrs. Cell extracts were prepared and standard luciferase reporter activity was measured as described in the Materials and Methods. Data were normalized to the total protein amount of each sample. * represents a significant difference compared to vehicle treatment. † represents a significant difference compared to estradiol treatment. A p value <0.05 was considered significant. Data are representative of results from at least three separate experiments.

Figure 2. F/I decreased estradiol activation of ERα via PKA signaling and reversed tamoxifen resistance in MCF7-LCC2 cells. MCF7 (A, B, E, and F), T47D (C) and MCF7-LCC2 (D) cells were plated in 6 well plates (0.5x10^6/well) in medium containing 5 % charcoal stripped FBS for 24 hrs. Each well was transfected with ERE-TK-Luc (500ng) (A, B, C, D) or CRE-Luc (500ng) (E and F) along with empty vector, PKA or PKI expression vector for 24 hrs. Cells were incubated with estradiol (10^{-8}M), 4-(OH)-tamoxifen (10^{-7}M), F/I (forskolin, 10μM; IBMX, 100 μM), or 8CPT-2Me-cAMP (10μM) as indicated for an additional 18 hrs. Cell extracts were prepared and standard luciferase reporter activity was measured as described in the Materials and Methods. Data were
normalized to the total protein amount of each sample. * represents a significant
difference compared to vehicle treatment. † represents a significant difference compared
to estradiol treatment. A p value <0.05 was considered significant. Data are
representative of results from at least three separate experiments.

Figure 3. Gene-selective effects of F/I on ER-dependent transcription. MCF7
cells (4x10^6) were plated in 10 cm dishes for three days in phenol red free DMEM media
containing 5% charcoal stripped FBS. Cells were incubated with estradiol (10^-8M), F/I
(forskolin, 10μM; IBMX, 100 μM), 4-(OH)-tamoxifen (10^-7M) or combinations for the
indicated times or for 2 hours in G. Cells were harvested, and RNA was extracted and
reverse transcribed to cDNA. Gene expression of pS2 (A), PR (B), cMyc (C and G), and
cyclin D1 (E) were measured by real time RT-PCR normalized to GAPDH. For protein
expression, MCF7 cells were incubated with estradiol (10^-8M), 4-(OH)-tamoxifen (10^-7
M), F/I or combination of ligand + F/I for 24 hrs. Cells were harvested and total cellular
extract was prepared in high salt lysis buffer. cMyc (D) and cyclin D1 (F) protein
expression was measured by Western blot normalized to β actin. * represents a
significant difference compared to vehicle treatment. A p value <0.05 was considered
significant. Data are representative of results from at least three separate experiments.

Figure 4. F/I rapidly recruits ERα to pS2 and cMyc promoters and alters estradiol
time dependent cycling of ERα. A and B represent a schematic map of ERE and AP-1
sequence in the pS2 and cMyc promoters. MCF7 cells (10x10^6) were plated in 15 cm
dishes in phenol red free DMEM containing 5% charcoal stripped FBS for 3 days. Cells
were incubated for 45 min. (C), 2 hrs (D), and for the indicated time (E) with estradiol (10^{-8}M), F/I (forskolin, 10\mu M; IBMX, 100 \mu M) or F/I + estradiol and cells were prepared for quantitative chromatin immunoprecipitation with antibody to ER\alpha as described in the Materials and Methods. DNA was purified and ER target promoter sequence of pS2 and cMyc were amplified by the real-time PCR method. Data were normalized to the input values. * represents a significant difference compared to vehicle treatment. A \textit{p} value <0.05 was considered significant. Data are representative of results from at least three separate experiments.

Figure 5. F/I blocks estradiol binding to ER\alpha in MCF7, MCF7-LCC2 and T47D cells. MCF7 (A), MCF7-LCC2 (B) and T47D (C) cells (0.5\times 10^6/well) were plated in 6 well plates for 3 days and pre-treated with F/I (forskolin, 10\mu M; IBMX, 100 \mu M) for 1hr. Cells were incubated with 1.5nM of 3H labeled estradiol in the presence or absence of 1.5\mu M of cold estradiol for 1 hr. Cells were washed with PBS and incubated with absolute ethanol for 20 min. at room temperature. Samples were collected and radioactivity was measured and normalized to the total protein amount. D. ER\alpha and ER\beta in crude cellular extract of MCF7 cells was detected by Western blot and quantified based on the signal of the purified ER\alpha and ER\beta (50ng). E. MCF7 cells were plated in a 15 cm dish for three days, harvested, and total cellular extract was prepared. ER\beta antibody were incubated first with Protein A Sepharose beads for 2hrs, the beads were washed 2X with PBS and then incubated with the total cellular extraction at 4^\circ C for 18hrs with rotation. Beads were washed 3X with PBS/0.1\% Triton X 100 and ER\beta was
eluted by boiling 5 min. in SDS loading buffer. ERβ was detected by Western blot. F. Purified ERα (500ng) was pre-incubated with FI, vehicle or ICI 182,780 (10^{-9}M) for 1 hr. 1μM of \(^{3}\text{H}\)estradiol was added with or without 10mM of cold estradiol and incubated on ice for 1hr. DCC slurry was added for 30 seconds and DCC was separated by centrifugation. Supernatant samples were collected and radioactivity was measured in CPM. * represents a significant difference compared to vehicle treatment. A p value <0.05 was considered significant. Data are representative of results from at least three separate experiments.

Figure 6. F/I-regulated decrease in estradiol binding is accompanied by increased association of Hsp90 with ERα in MCF7 cells. A. MCF7 cells (4x10^6) were plated in 10cm dishes for 3 days in phenol red free DMEM medium containing 5% stripped serum. Cells were incubated with vehicle or F/I (forskolin, 10μM; IBMX, 100 μM) for 2 hrs, cells were harvested and lysed in HEMG buffer. ERα (D12) antibody was incubated with protein A Sepharose beads for 2 hrs at room temperature. Beads were added to the cellular extract and incubated at 4°C for 18 hrs. Beads were collected, washed and ER was eluted by adding SDS loading buffer and boiling samples for 5min. Samples were subjected to Western blot analysis by immunoblotting with Hsp90 antibody and the signal was normalized to ERα expression. B, HeLa cells (0.5x10^6/well) were plated in 6 well plates and each well was transfected with ERE-TK-luc (500ng) along with ERα expression vector for 24hrs followed by incubation with vehicle, estradiol (E2)(10^{-8}M), or F/I (forskolin (10μM) + IBMX (100 μM)) for an additional 18hrs. Luciferase activity
was measured as described in Materials and Methods and data were normalized to the total protein amount of each sample. C. HeLa cells (4x10^6) were plated in 10 cm dishes for 24 hrs and transfected with ERα expression vector for 24 hrs. Cells were harvested, (1x10^6), and then were re-plated in 6 well plates for 24 hrs and ligand binding was measured as described in Materials and Methods. D, HeLa cells (4x10^6) were plated in 10cm dishes and transfected with ERα expression vector (500ng) for 24 hrs. Cells were treated with vehicle or F/I for 2 hrs and CO-IP was applied as described above in (A). * represents a significant difference compared to vehicle treatment. A p value <0.05 was considered significant. Data are representative of results from at least three separate experiments.

Figure 7. F/I-regulated changes in estradiol-dependent ERα phosphorylation. A. Schematic presentation of hERα phosphorylation sites. B. MCF7 cells (4x10^6) were plated in 10 cm dishes in phenol red free DMEM medium containing 5% stripped FBS for 3 days and incubated with (E2)(10^{-8}M), F/I (forskolin, 10μM; IBMX, 100 μM) or combination of E2 and F/I for 2 hrs. Cells were harvested, lysed in high salt buffer and subjected to Western blot analysis by using the indicated phosphoantibody. Phosphorylation of ERα was normalized to ERα expression level. C and D. Western blot signals were quantified for S118 phosphorylation (C) and S305 phosphorylation (D) following different treatments. Phosphoantibody signal was normalized to the total ERα protein expression. * represents a significant difference compared to vehicle treatment. A p value <0.05 was considered significant. Data are representative of results from three separate experiments.
Figure 8. F/I regulation of ERK1/ERK2 phosphorylation and role of S305 phosphorylation in F/I regulation of ERα. A. MCF7 cells (4x10^6) were plated in 10 cm dishes for three days in phenol red free DMEM media containing 5% charcoal stripped FBS. Cells were transfected with empty or PKA expressing vector for 48 hrs or incubated with F/I (forskolin, 10μM; IBMX, 100 μM) for the indicated times. Cells were harvested and total cellular extract was prepared in high salt lysis buffer. ERK1/ERK2 phosphorylation was detected by Western blot normalized to the total ERK1/ERK2 protein expression. B. MCF7 cells were plated in 6 well plates (0.5x10^6/well) for 24 hrs in phenol red free DMEM containing 10% FBS that had been treated with charcoal to remove endogenous steroids. Each well was co-transfected with ERE-TK-luc (500ng) along with wild type ERα (25ng) or ERαS305A vector (25ng) for 24 hrs, followed by incubation with E2 (10^-8M), F/I (forskolin, 10μM; IBMX, 100 μM) or combination for an additional 18 hrs. Cell extracts were prepared and standard luciferase reporter activity was measured as described in the Materials and Methods. Data were normalized to the total protein amount of each sample and expressed as % of vehicle treatment for each expressing vector. * represents a significant difference compared to vehicle treatment. † represents a significant difference compared to estradiol treatment. A p value <0.05 was considered significant. Data are representative of results from three separate experiments.

Figure 9. F/I suppressed MCF7 and T47D breast cancer cell growth. MCF7 (A) or T47D (B) cells (20x10^3) were plated in 24 well plates in medium containing 5%
charcoal stripped FBS. After 24 hrs cells were incubated with vehicle, E2 ($10^{-8}$M), F/I (Forskolin 10μM; IBMX, 100 μM) or combination in triplicate with one additional treatment at 48 hrs. Cell growth was measured by MTT assay at the indicated time points as described in the Materials and Methods. * represents a significant difference compared to vehicle treatment. † represents a significant difference compared to 48 hr time point. A p value <0.05 was considered significant. Data are representative of results from at least three separate experiments.

Figure 10. Model for F/I regulation of ERα gene expression in breast cancer cells. A. Estradiol dependent activation of ERα in breast cancer cells. Estradiol binds to ERα causing dissociation of Hsp90 and phosphorylation of ERα. Activated ERα induces the transcription of ER target genes and promotes breast cancer cell growth. B. cAMP dependent activation of ERα in breast cancer cells. PKA induces S305 phosphorylation and increases Hsp90 association with ERα that results in selective gene expression. Inhibition of cyclin D1 is associated with decrease in tamoxifen-sensitive breast cancer cell growth. C. Cross talk between PKA and estradiol signaling results in phosphorylation of ERα at S104, S106, S305 and T311 that is associated with decreased ligand binding. Cross talk between PKA and estradiol also inhibited cyclin D1 expression that is likely involved in PKA inhibition of estradiol dependent cell growth.
Figure 1.

A.

ERE-TATA — LUC

B.

ERE-TK — LUC
Figure 2.

A. MCF7

B. MCF7

C. T47D

D. MCF7-LCC2

E. MCF7

F. MCF7
Figure 3.

A. p52

B. PR

C. cMyc

D. cMyc

E. Cyclin D1

F. Cyclin D1

G. cMyc
Figure 4.

A. pS2

B. cMyc

C. 45 min treatment

D. 2 hr treatment

E. cMyc

76
Figure 5.

A. MCF7  
B. MCF7-LCC2  
C. T47D  

D.  

E. Purified ERα  

F. Purified ERα
Figure 6.
Figure 7.

A.

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B.

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C.

D.

![Graph showing data comparison]

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79
Figure 8.

A.

B.

E.
Figure 9.

A. MCF7 cells

B. T47D cells
Figure 10.
Application of phosphorylation site-specific antibodies to measure nuclear receptor signaling: characterization of novel phosphoantibodies for estrogen receptor α.

Mariam H. Al-Dhaheri¹ and Brian G. Rowan².

¹Department of Biochemistry and Cancer Biology, Medical University of Ohio, Toledo, Ohio and ²Department of Structural and Cellular Biology, Tulane University School of Medicine and the Louisiana Cancer Research Consortium, New Orleans, Louisiana, USA.

*Corresponding Author:
Brian G. Rowan, Ph.D.
Department of Structural & Cellular Biology, SL49. Tulane University School of Medicine. Louisiana Cancer Research Consortium. 1430 Tulane Avenue. New Orleans, Louisiana 70112
Phone: 504-988-1365
Fax: 504-988-1687
email: browan@tulane.edu

“Technique”

Abbreviations: Estrogen receptor α (ERα), 17β-estradiol (E2), Protein Kinase A (PKA), Serine (S) Alanine (A), Threonine (T), Tyrosine (Y).

Key words and molecules: phosphorylation, polyclonal antibody, phosphoantibody, nuclear receptor, estrogen receptor, in vitro phosphorylation, kinase, phosphatase, validation.

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Abstract

An understanding of posttranslational events in nuclear receptor signaling is crucial for drug design and clinical therapeutic strategies. Phosphorylation is a well-characterized posttranslational modification that regulates sub cellular localization and function of nuclear receptors and coregulators. Although the role of single phosphorylation sites in nuclear receptor function has been described, the contribution of combinations of multiple phosphorylation sites to receptor function remains unclear. The development of phosphoantibodies to each phosphorylation site in a nuclear receptor is a powerful tool to address the role of phosphorylation in multiply phosphorylated receptors. However, phosphoantibodies must be rigorously validated prior to use. This review describes the general methodology for design, characterization and validation of phosphoantibodies using the example of eight phosphoantibodies raised against phosphorylation sites in estrogen receptor α (ERα).
Introduction

Phosphorylation is the well-established posttranslational event that regulates sub cellular localization, dimerization, DNA binding, coregulator interaction and transcriptional activity of nuclear receptors [Orti et al. 1992]. Understanding the role of phosphorylation in nuclear receptor function is limited by receptor expression levels, intracellular phosphatase activity, and low stoichiometry and/or rapid turnover of some phosphorylation sites.

Several approaches may be used to study previously identified phosphorylation sites in nuclear receptors (reviewed in [Rowan et al. 2003]). Cells may be labeled in vivo with $^{32}$P orthophosphate followed by immunopurification and phosphopeptide mapping. Similarly, purified receptor may be phosphorylated in vitro with different kinases and individual sites assessed by phosphopeptide mapping [Rowan et al. 2003]. However these approaches are labor intensive, time consuming, and require radioactive material and large amounts of purified receptor. Mass spectrometry is another approach to study protein phosphorylation [Garcia et al. 2005]. This non-quantitative approach requires large amounts of purified receptor and is limited primarily by costly instrumentation and the need for a highly skilled operator.

The phosphoantibody approach to study receptor phosphorylation overcomes several limitations inherent in other approaches and provides the additional benefit of allowing rapid assessment of combinations of receptor phosphorylation sites by Western blotting (for review see [Rowan et al. 2003]). The advantages of the phosphoantibody approach
include; 1) a highly sensitive assay that can detect phosphorylation in crude extracts containing low receptor levels and/or low stoichiometry of phosphorylation without the need for purified receptor; 2) the ability to profile total receptor phosphorylation and to quantify relative levels of each phosphorylation site; 3) immunoprecipitation of receptors phosphorylated at specific sites to investigate the recruitment of phosphoproteins to promoters (chromatin immunoprecipitation (ChIP)) and to identify phosphorylation sites involved in specific protein-protein interaction (co-immunoprecipitation); 4) identification of phosphorylation sites associated with real time dynamics of receptor subcellular localization and recycling and other cellular processes (immunofluorescence; flow cytometry sorting); 5) the ability to monitor changes in the total receptor phosphorylation profile during disease processes such as the progression from benign to malignant cancer (immunohistochemistry).

The phosphoantibody approach has been widely applied to characterize nuclear receptor activity and subcellular localization [Wang et al. 2002]. Glucocorticoid receptor (GR) GR-P-S203 and GR-P-S211 phosphoantibodies have been used by immunoblotting to study GR activation by different ligands and by indirect immunofluorescence to investigate the subcellular localization of the active GR. Progesterone receptor (PR) phosphospecific antibodies to sites S400 [Pierson-Mullany and Lange, 2004], S162, S190 and S294 [Narayanan et al. 2005] were used by immunoblotting to study the ligand independent activation of PR and to correlate PR transcriptional activity and cell-cycle progression. Androgen receptor (AR) phosphoantibody to site S81 was used by immunoblotting to study the mechanism of ligand-dependent arrest of the AR in
subnuclear foci [Black et al. 2004]. Phosphoantibodies to ERα sites S118 and S167 [Shah and Rowan, 2005; Chen et al. 2002] were used by immunoblotting to study crosstalk between ERα and several kinases. In addition, these phosphoantibodies have been used by immunohistochemistry to detect ERα phosphorylation in human breast tumors [Murphy et al. 2004; Yamashita et al. 2005].

The following section describes six key steps in development and characterization of phosphoantibodies to study nuclear receptor phosphorylation (Figure 1). The first three steps include preparation, purification and initial testing of phosphoantibodies and are generally performed by commercial vendors. The remaining three steps to validate and confirm specificity of the phosphoantibody for specific phosphorylation sites are performed by the investigator in the laboratory and are the major focus of this review.

1-Peptide design and immunogen preparation

The synthetic phosphopeptide used for immunization is recommended to be 10-20 amino acids in length and conjugated with a suitable carrier that will solicit a strong immune response and produce a large quantity of the antibody (reviewed in [Eisele et al. 1999; Frank, 1984; Harlow and Lane, 1988]. The most widely used carriers in antibody production are keyhole limpet hemacyanin (KLH) and bovine serum albumin (BSA) [Harlow and Lane, 1988]. It is important to be aware of the conjugated carrier used during immunization to anticipate any false positives that may occur during antibody testing. For example if the conjugated carrier is BSA a false positive may arise if BSA was used as a blocking agent for Western blotting. Immunogenicity of the peptide-protein
carrier can be verified by an enzyme linked immunosorbent assay (ELISA) to identify the most appropriate protein carrier dose.

2-Producing hyper-immune serum

For broad specificity it is recommended that phosphoantibodies be prepared as polyclonal antibodies (for review see [Leenaars and Hendriksen, 2005; Lipman et al. 2005; Burns, 2005; Harlow and Lane, 1988]. Rabbits are the host of choice to avoid self-recognition of the immunogen and since rabbits provide high amounts of sera. The immunogen is mixed with adjuvant prior to immunizing animals. The immunogen mixture is injected subcutaneously and re-administered at day 14 and 44 post immunization. At day 54 sera is collected by bleeding of the marginal ear vein. Bleeding is repeated on day 60 and then every 4 weeks until the antibody titer has declined. A booster dose of immunogen should be administered to re-enhance animal immunity and produce more sera [Burns, 2005]. After each bleeding, antibody titer should be measured by ELISA.

3-Affinity Purification

Following harvest of the hyper-immune sera the phosphoantibody can be enriched by an affinity purification against the phosphorylated peptide [Harlow and Lane, 1988]. The affinity purification is validated by ELISA screening against the phosphopeptide in which phosphoantibody purity should exceed 95%.

Numerous commercial suppliers will produce custom, affinity purified phosphoantibodies (Bethyl Laboratories, Invitrogen, Global Peptide, ABGENT, Open Biosystems and others).
4-Phosphoantibody specificity assessed by receptor phosphorylation site mutations

Affinity purified phosphoantibody must be validated as phosphorylation site specific. In mammalian cells that do not express the receptor of interest, wild type receptor expression plasmids or plasmids containing phosphorylation site mutations (Serine (S) to Alanine (A), Threonine (T) to A, Tyrosine (Y) to Phenylalanine (F)) should be transfected in cells to achieve high level protein expression. Total cell extracts from wild-type and phosphorylation site mutant transfected cells should be prepared for Western blotting with the phosphoantibody and peptide-competed phosphoantibody to assess specificity for the phosphorylation site. High receptor levels may be required to measure phosphorylation sites that exhibit low stoichiometry. For other sites that require an activation event, cells may first need to be incubated with ligand, growth factor, kinase activator, etc. prior to detection of the specific phosphorylation with a phosphoantibody.

The ideal phosphoantibody should recognize receptor from cells transfected with wild type but not the site-specific mutant protein. However, depending on cell context, protein expression and stoichiometry, some phosphoantibodies may fail to detect a signal in total cellular extract. If this occurs, enrichment of receptor by immunoprecipitation with a receptor-specific antibody is recommended prior to Western blotting with the phosphoantibody. It is critical that immunoprecipitation be carried out over a brief period (no more than a few hours) since dephosphorylation may occur over longer periods. Regardless, phosphatase inhibitors should always be included in lysis buffers whether receptor is immunoprecipitated or not.
A critical step when first using phosphoantibodies for Western blotting is to empirically determine the optimal phosphoantibody concentration that will; 1) exhibit reactivity with wild type but not phosphorylation site mutant receptor, and : 2) not result in high general background on Western blots. In our experience some phosphoantibodies exhibit specificity for wild type and not phosphorylation site mutant receptor only at high antibody dilutions. The appropriate antibody dilution must be determined empirically using serial dilutions of the affinity purified antibody in Western blot analysis. We have found that some affinity purified phosphoantibodies (stock concentration of 1 mg/ml) require dilution as high as 1:10,000 to eliminate non-specific reactivity with the phosphorylation site mutant receptor.

5-Absence of phosphoantibody reactivity with de-phosphorylated receptor

A second step for validation of phosphoantibodies is to confirm the antibody does not react with receptor that has been de-phosphorylated. In this context the hyper-phosphorylated form of the purified receptor should be incubated in the presence or absence of λ phosphatase followed by Western blotting with the phosphoantibody. Phosphatase treatment should prevent reactivity of the phosphoantibody with the receptor.

6-In vitro receptor phosphorylation to measure phosphoantibody reactivity

In some cell/tissue contexts that lack a specific kinase or have inactive kinase, some phosphoantibodies may not react with receptor due to absence or very low stoichiometry
of phosphorylation at a particular site. In this scenario *in vitro* phosphorylation of purified receptor with a kinase known to phosphorylate the specific site can be used prior to incubation with λ phosphatase. Phosphoantibody reactivity with the purified protein should increase following *in vitro* phosphorylation and signal should be lost following λ phosphatase treatment. A kinase that is not specific for the site in question should be used as a control for the *in vitro* phosphorylation.

**Reactivity and specificity: phosphoantibodies to ERα as an example**

In this study, phosphoantibodies developed against eight different phosphorylation sites of ERα were characterized (Figure 2). ERα is phosphorylated upon ligand binding and/or crosstalk with kinases. Although there are eight identified phosphorylation sites in ERα, (S104, S106, S118, S167, S236, S305, T311, and Y537 (for review see [Lannigan 2003; Michalides et al. 2004]), only phosphoantibodies against S118 and S167 have been widely applied for receptor functional studies. Phosphoantibodies directed against S104, S106 and Y537 are also commercially available although application of these antibodies to receptor functional studies is limited. This may be due to lower stoichiometry of phosphorylation at these sites, rapid turnover of tyrosine phosphorylation sites and/or poorer affinity of these antibodies compared to the S118 and S167 phosphoantibodies.

**Reagents**

**Antibodies:**

Bethyl Laboratories (Montgomery, TX) provided complimentary phosphoantibodies:

ERα ER-P-S104 (Cat. # BL1637), ER-P-S106 (Cat. # BL1638), ER-P-S118 (Cat. #
BL1641), ER-P-S167 (Cat. # BL1643), ER-P-S236 (Cat. # 1645), ER-P-S305 (Cat. # 1665), ER-P-T311 (Cat. # BL1667) and ER-P-Y537 (Cat. # BL1647). Antibody D12 for ERα immunoprecipitation (Cat. # sc-8005) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). ERα antibody clone 6F11 for Western blots of total ERα (Cat. # VP-E613), peroxidase conjugated anti-mouse (Cat. # PI-2000) and peroxidase conjugated anti-rabbit antibodies (Cat. # PI-1000) were purchased from Vector Laboratories (Burlingame, CA).

Purified ERα, phosphatase and kinases:

Baculoviral expressed ERα (Cat. # P2187) was purchased from Invitrogen (Carlsbad, CA). λ protein phosphatase (Cat. # P0753S) and active recombinant full length human CDK2-cyclin A complex expressed in E. coli (Cat. # P6025S) were purchased from New England Biolabs (Ipswich, MA). Baculoviral expressed, active recombinant full length human protein kinase A (PKA) catalytic subunit β (Cat. # PPK-448) was purchased from Stressgen (San Diego, CA). Baculoviral expressed, active recombinant full length human Src kinase (Cat. # 14-326) and active recombinant human full length p38α/SAPK2a kinase expressed in E. coli (Cat. # 14-587) were purchased from Upstate (Charlottesville, VA).

Methods

Cell culture and transfection:

COS-1 cells were maintained in DMEM phenol red free medium containing 10% Fetal Bovine Serum (FBS), 2% glutamine and 1% penicillin/streptomycin. Cells were plated in
15 cm dishes for 48 hrs in DMEM phenol red-free medium containing 5% FBS that was treated with dextran-coated charcoal to remove endogenous steroids. Wild type or phosphorylation site mutant ERα constructs (6 µg) were transiently transfected in cells for 48 hrs using Fugene 6 (Roche, Indianapolis, IN). Cells were incubated with 17β-estradiol (E2) (10 nM) or Forskolin (10 µM) + IBMX (100 µM) (F/I) for 2 hrs. Cells were harvested and cellular pellet were incubated in high salt lysis buffer (10 mM Tris pH 8.0, 0.4 M NaCl, 2 mM EDTA pH 8.0, 2 mM EGTA, 10 mM β-mercaptoethanol, 0.1% Triton X100, 1 mM sodium orthovanadate, 20 mM β-glycerophosphate, 25 mM sodium flouride, 0.1 mM PMSF) containing protease inhibitor mixture (Sigma, St. Louis, MO) for 10 min in ice.

**Immunoprecipitation of ERα:**
Protein A sepharose beads (Amersham Biosciences, Piscataway, NJ) were re-swelled in phosphate buffer saline (PBS) and incubated with ERα antibody (D12, Santa Cruz) by rotating the beads at room temperature for 2 hrs. Beads were washed three times with PBS followed by incubation with the total COS-1 cellular extract for 3 hrs at 4°C with rotation. Beads were washed three times with PBS followed by addition of 5X SDS-PAGE loading buffer. Beads were incubated at 100 °C for 5 min to elute ERα. Samples were electrophoresed by SDS-PAGE and phosphorylation of ERα was detected by Western blotting using site specific phosphoantibodies. Phosphoantibody signals obtained by Kodak Image analysis of Western blots were normalized to total ERα level by incubating the membrane in stripping buffer (2% SDS, 100 mM β-mercaptoethanol, 62.5 mM Tris HCl pH 6.8) at 55 °C for 30 min with rotation and re-probing the
membrane by Western blot with antibody against total ERα (clone 6F11, Vector Laboratories).

λ Phosphatase analysis:
200 ng purified ERα was incubated with 200ng λ phosphatase in reaction buffer (50mM Tris-HCl, 100 mM NaCl, 2 mM MnCl₂, 2mM dithiothreitol (DTT), 0.1 mM EGTA, 0.01% Brij 35, pH 7.5) for 1 hr at 30°C. To terminate the reaction, 5X SDS-PAGE loading buffer was added and samples were incubated at 100 °C for 5 mins followed by SDS-PAGE and Western blots using site specific phosphoantibodies.

In vitro phosphorylation:
Purified ERα (200 ng) was incubated with the site specific kinase or kinase complex, ATP, and kinase buffer for 1 hr at 30 °C in the presence or absence of λ phosphatase. Kinases used were: CDK2-cyclin A (100 ng) (kinase buffer 50 mM Tris-HCl, 10 mM MgCl₂, 1 mM EGTA, 2 mM DTT, 0.01% Brij 35, pH 7.5); PKA (100 ng) or inactive PKA (PKA incubated at 100 °C for 2 mins) (kinase buffer 20 mM MOPS pH 7.0, 1 mM sodium orthovanadate, 25 mM β-glycerophosphate, 1 mM EGTA, 1 mM DTT, 7 mM MgCl₂); P38α/SAPK2a (100 ng) (kinase buffer 25 mM Tris-HCl pH 7.5, 0.02 mM EGTA); and Src kinase (100 ng) (kinase buffer 8 mM MOPS pH 7.0, 0.2 mM EDTA). In vitro phosphorylation reactions were terminated by addition of 5X SDS-PAGE loading buffer and incubation of samples at 100 °C for 5 mins. Following SDS-PAGE, phosphorylation of S104, S236, S305, T311, and Y537 were detected by Western blotting with phosphoantibodies.
**Results**

The initial step for characterization of phosphoantibodies is to prove the antibody is site specific. For this purpose wild type ERα and site mutant ERα constructs were separately transfected into ERα-negative COS-1 cells. Cells were then incubated with 17β-estradiol (10⁻⁸M) or Forskolin (10µM) + IBMX (100µM) for 2 hrs and cell extracts prepared for Western blotting. Phosphorylation sites with low stoichiometry or antibody with low affinity may not detect receptor in crude cellular extracts. For this reason ERα was first immunoprecipitated with a separate ERα antibody (D12, Santa Cruz) and then the immunopurified receptor was subjected to Western blotting with the phosphoantibodies. ERα-P-S104 (Figure 3A), ERα-P-S106 (Figure 3B), ERα-P-S118 (Figure 3C), ERα-P-S167 (Figure 3D), ERα-P-S236 (Figure 3E), ERα-P-S305 (Figure 3F), ERα-P-T311 (Figure 3G) and ERα-P-Y537 (Figure 3H) detected wild type ERα but not phosphorylation site ERα mutants indicating that antibodies were site specific. For some phosphoantibodies, serial dilutions were prepared to determine the optimal antibody concentration for detection of wild type, but not mutant ERα. At high phosphoantibody concentration (2 ug/ml) the ERα-P-S167 antibody recognized both wild-type ERα and mutant S167A (data not shown). This nonspecific interaction with mutant S167A was absent when the antibody concentration was decreased to 0.25 ug/ml (Figure 3D).

Specificity of the antibodies was verified by λ phosphatase treatment of the hyper-phosphorylated purified ERα or by *in vitro* phosphorylation of ERα with specific kinases. Phosphoantibodies to sites S106, S118 and S167 recognized purified, baculoviral
expressed ERα indicating that kinase pathways that phosphorylate these sites are conserved in insect Sf9 cells (Figure 4 A-C, lane 1). A similar conservation between mammalian and Sf9 insect cells for receptor phosphorylation was found with baculoviral expressed progesterone receptor [Beck et al. 1996]. Purified ERα was incubated with λ phosphatase for 1 hr at 30°C and the phosphorylation of S106, S118, and S167 was assessed by Western blotting with the phosphospecific antibodies. λ phosphatase treatment of ERα resulted in loss of Western blot signal with phosphoantibodies to sites S106 (Figure 4A), S118 (Figure 4B) and S167 (Figure 4C).

Unlike phosphoantibodies to S106, S118, and S167, phosphoantibodies to sites S104, S236, S305, T311 and Y537 exhibited no reactivity with purified ERα (data not shown). The antibody specificity was assed by in vitro phosphorylation of ERα with CDK2-cyclin A, PKA, p38α/SAPK2a and Src kinases followed by Western blotting with phosphoantibodies to sites S236, S305, T311 and Y537.

CDK2-cyclin A significantly induced the phosphorylation of site S104 (Figure 5A, lane 2) and subsequent λ phosphatase treatment (Figure 5A, lane 3) abolished the phosphorylation as assessed by Western blotting with the ERα-P-S104 antibody. In the absence of kinase, incubation of purified baculoviral ERα with kinase buffer and ATP also resulted in phosphorylation of site S104 (Figure 5A, lane 1) suggesting that purified, baculoviral ERα may be contaminated with a co-purifying kinase.
Incubation of ERα with active PKA, but not inactive PKA, resulted in phosphorylation of ERα at S236 (Figure 5B, lane 2 versus lane 1) and S305 (Figure 5C, lane 2 versus lane 1). *In vitro* phosphorylation of ERα with P38α/SAPK2a complex resulted in T311 phosphorylation (Figure 5D, lane 3) and this phosphorylation was lost with subsequent λ phosphatase incubation (Figure 5D, lane 2). Incubation of ERα with Src kinase resulted in phosphorylation of ERα at Y537 (Figure 5E, lane 2) and this phosphorylation was markedly reduced with subsequent λ phosphatase incubation (Figure 5E, lane 3).

**Discussion**

Although there are several different methods to characterize changes in receptor phosphorylation, the phosphoantibody approach is particularly useful for simultaneously measuring each phosphorylation site. In this report the general steps in characterizing and validating phosphoantibodies are described using the example of phosphoantibodies raised against eight phosphorylation sites in ERα. Validation of each phosphoantibody was conducted in two main steps, first the site specificity of the phosphoantibody were validated by expressing the wild type or the site mutant constructs in ERα negative cell lines. Second, the phosphoantibody specificity were validated either by incubating the hyper-phosphorylated purified ERα with λ phosphatase or by performing *in vitro* phosphorylation with specific kinase or kinase complex that phosphorylate each phosphorylation site.
All the eight phosphoantibodies were site specific, where only the wild type ERα, but not site mutant ERα, showed reactivity with the phosphoantibody. However, phosphoantibodies reacts differently with the hyper-phosphorylated purified ERα. Only phosphoantibodies for the S106, S118 and S167 cross react with the hyper-phosphorylated purified ERα. This may have occurred because these sites are not phosphorylated in insect Sf9 cells due to either the absence of kinase activity or the presence of phosphatases that block ERα phosphorylation at these sites. Therefore, validation of the specificity of phosphoantibody for the sites S106, S118 and S167 was performed by incubating the hyper-phosphorylated purified ERα with λ phosphatase. In contrast, validation of the specificity of phosphoantibody for the sites S104, S236, S305, T311 and Y537 was performed by *in vitro* phosphorylation.

CDK2-cyclin A is a specific kinase complex previously shown to phosphorylate both S104 and S106 of ERα [Rogatsky et al., 1999]. Because of the close proximity of S104 and S106, it is possible that phosphorylation of one site may be required for phosphorylation of the adjacent site. This possibility is currently under investigation. Serine 236 and Serine 305 are located within consensus sequences for PKA and phosphorylation of these sites by PKA has been demonstrated [Chen et al., 1999; Michalides et al., 2004]

The direct kinase that phosphorylates ERα at T311 has not been identified. Lee and Bai [Lee and Bai, 2002] reported that T311 is phosphorylated by a p38 MAPK kinase
complex that contained both p38α and SAPK2a. Since T311 does not lie within a consensus sequence for MAPK phosphorylation (the T residue is not followed by a proline residue) it is highly unlikely that p38 directly phosphorylates T311. A more likely explanation is that the kinase that directly phosphorylates T311 is a kinase that copurified with p38 in the present and previous study. Y537 is present within a Src kinase consensus sequence and is phosphorylated by Src kinase in vitro [Arnold and Notides, 1995; Arnold, et al. 1995].

In summary, development of phosphoantibodies to assess the functional role of nuclear receptor phosphorylation is a powerful approach that has distinct advantages over other more labor intensive and costly approaches. However, rigorous validation experiments must precede use of phosphoantibodies to ascertain both reactivity with the phosphorylated receptor and absence of cross reactivity with non-phosphorylated receptor. Phosphoantibody specificity is determined using several complimentary approaches including expression of wild type and mutant receptor in cells, phosphatase treatment of receptor, and in vitro phosphorylation of receptor. Using phosphoantibodies that are specific for each identified phosphorylation sites will permit investigators to simultaneously measure the entire profile of receptor phosphorylation during studies that probe nuclear receptor mechanisms of action.

Acknowledgments: This work was supported by National Institutes of Health Grant RO1 DK06832 (to B.G.R.) and by a Department of Defense Breast Cancer Research Program Idea Award (DAMD17-02-1-0531) and Career Development Award
We thank Drs. Simak Ali (Imperial College, UK), Rakesh Kumar (University of Texas M.D. Anderson Cancer Center) and Wenlong Bai (University of South Florida) for the plasmids ERαS236A, ERαS305A and ERαT311A, respectively. We thank Drs. Abeer El-Gharbawy and Aninda Basu for technical assistance.

Reference List


receptor in subnuclear foci alters phosphorylation and coactivator interactions. 


Figure legends

Figure 1. Steps in phosphoantibody production and validation.
Phosphopeptide design, rabbit immunization, phosphoantibody production and affinity purification (steps 1-3) are performed by commercial vendors. Phosphoantibody specificity experiments (steps 4-6) are performed by the investigator.

Figure 2. Schematic representation of ERα phosphorylation sites.
ERα phosphorylation sites that have been confirmed in previous studies by in vivo and/or in vitro phosphorylation are shown.

Figure 3. Immunoprecipitation of wild type and mutant ERα and Western blotting with phosphoantibodies.
COS-1 cells were plated in DMEM media containing 5% FBS treated with charcoal coated dextran to remove the endogenous steroids. Cells were transfected with wild type ERα (A-H) or ERα phosphorylation site mutants ERαS104A (A), ERαS106A (B), ERαS118A (C), ERαS167A (D), ERαS236A (E), ERαS305A (F), ERαT311A (G), or ERαY537A (H). Cells were incubated with 17β-estradiol (A-D, G, H) or F/I (E, F) for 2 hrs, followed by preparation of cell extracts for immunoprecipitation. Total ERα was immunoprecipitated with ERα antibody (D12, Santa Cruz) and phosphorylation of ERα was detected by Western blot using site specific phosphoantibodies. The immunoblot was stripped and re-probed by Western blot with antibody to total ERα (Clone 6F11, Vector Laboratories).
Figure 4. Phosphatase treatment of purified ERα and Western blotting with phosphoantibodies.

Purified ERα (200ng) was incubated in the presence or absence of phosphatase λ (200ng) at 30 °C for 1 hr and phosphorylation of ERα was detected by Western blotting using ERα-P-S106 (A), ERα-P-S118 (B), or ERα-P-S167 (C) phosphoantibodies.

Figure 5. *In vitro* phosphorylation of purified ERα and Western blotting with phosphoantibodies.

Purified ERα (200 ng) was incubated with or without CDK2-cyclin A complex (100 ng) (A), PKA (100 ng) (B, C), P38α/SAPK2a complex (100 ng) (D), or Src (100 ng) (E) in the presence or absence of λ phosphatase (200 ng) (A, D, E) for 1 hr at 30°C. PKA was inactivated by incubating the kinase at 100°C for 2 min. ERα phosphorylation was detected by Western blot analysis using ERα-P-S104 (A), ERα-P-S236 (B), ERα-P-S305 (C), ERα-P-T311 (D), or ERα-P-Y537 (E) phosphoantibodies.
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**Figure 4**
## Figure 5

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Title: Identification of novel proteins induced by estradiol, 4-hydroxytamoxifen and acolbifene in T47D breast cancer cells.

Mariam H. Al-Dhaheri¹, Yatrik M. Shah¹, Venkatesha Basrur², Steven Pind³, and Brian G. Rowan¹,4∗.

¹Department of Biochemistry & Cancer Biology, Medical University of Ohio, Toledo, OH. ²Program in Bioinformatics & Proteomics/Genomics, Medical University of Ohio, Toledo, OH. ³Department of Biochemistry and Medical Genetics, the University of Manitoba, Canada. ⁴Current address; Department of Structural and Cellular Biology/School of Medicine, Tulane University, New Orleans, LA.

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∗Corresponding author: Brian G. Rowan. Current address: Department of Structural and Cellular Biology, School of Medicine, Tulane University, 1430 Tulane Ave, New Orleans, LA 70112; Tel: 504-988-1365; Fax: 504-988-1657; Email: browan@tulane.edu

Running Title: 4-hydroxytamoxifen regulated proteins in T47D cell

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Abstract

Tamoxifen is currently used as adjuvant therapy for estrogen receptor (ER) positive breast cancer patients and as a chemopreventative agent. Although ER is a predictive marker for tamoxifen response, ER status fails to predict tamoxifen response in a significant number of patients highlighting the need to identify new pathways for tamoxifen sensitivity / resistance. To identify novel proteins induced by tamoxifen in breast cancer cells sensitive to tamoxifen growth inhibition, two-dimensional (2D) gel electrophoresis was used to profile proteins in T47D breast cancer cells. Six proteins were identified that were differentially regulated by 17β-estradiol, 4-hydroxytamoxifen and the pure antagonist Acolbifene (EM-652); calreticulin, synapse associated protein 1 (SYAP1), CD2 antigen binding protein 2 (CD2BP2), nucleosome assembly protein 1 like 1 (NAP1L1), D-3-phosphoglycerate dehydrogenase (3-PHGDH) and pyridoxine 5’phosphate oxidase (PNPO). At the mRNA level, these ligands differentially regulated expression of mRNAs encoding the identified proteins in T47D and MCF7 cells but had no effect on mRNA in ERα-negative MDA-MB-231 breast cancer cells. These novel SERM-regulated proteins may a potential ER target proteins that could participate in new or existing pathways for sensitivity or resistance to SERMs.

Keywords: breast cancer, 17β-estradiol, 4-hydroxytamoxifen, acolbifene
**Introduction**

Breast cancer is the most common type of malignant disease and is the second cause of cancer-related death among women in the United States \[1\]. Approximately 70% of breast cancer patients express estrogen receptor (ER) and are responsive to estrogen-dependent progression of the disease. ER belongs to the nuclear receptor super family of ligand-dependent transcription factors. Classically, ER is activated upon estrogen binding that initiates a proliferative action on breast cancer. There are two types of ER, ERα and ERβ, both the products of separate genes and with tissue specific expression and function. The N-terminal domain of ER contains activation function-1 (AF-1) that activates transcription in a ligand independent manner. The central region of the receptor contains the DNA binding function and the C-terminal ligand binding domain contains an activation function-2 (AF-2) that is ligand dependent \[2\].

Selective estrogen receptor modulators (SERMs) are estrogen analogs that selectively regulate estrogen action in different tissues \[3\] by repressing estrogen action to various degree in some tissues and exhibits estrogen-like effect in other tissues. Certain SERMs such as tamoxifen block estrogen proliferative effects on breast carcinomas by competing for ER binding. Tamoxifen is a first generation SERM that is approved as an adjuvant therapy for breast carcinomas \[4\] and as a chemopreventative for women at high risk for development of breast cancer \[4,5\]. Upon receptor binding, tamoxifen alters the ER conformation thereby preventing interaction with coactivators and promoting the interaction with corepressors. This results in a transcriptionally inactive complex that blocks ER induction of genes important for cell proliferation \[6\].
Tamoxifen effects on ER-dependent transcription are promoter, cell and ER subtype specific. Tamoxifen displayed a partial agonist activity with ERα and full antagonist activity on ERβ\(^\text{[7]}\). Tamoxifen functions as an antagonist on promoters containing estrogen response element (ERE) sequences but functions as an agonist on promoters containing AP-1 sequences\(^\text{[8]}\). Tamoxifen antagonizes estrogen action in breast cancer tissue and exhibits estrogen like effect in endometrial tissue\(^\text{[9]}\). The relative agonist / antagonist activity was correlated with tamoxifen effect on ER turnover\(^\text{[10]}\). Unlike estradiol, in breast cancer tissue tamoxifen stabilized ERα and ERβ protein but in Ishikawa endometrial cancer cells tamoxifen, similar to estradiol induced ERα, but not ERβ degradation\(^\text{[9,10]}\).

Despite the therapeutic efficacy of tamoxifen approximately 50 % of breast cancer patients that exhibit ER-positive tumors do not respond to tamoxifen. The precise mechanism of tamoxifen resistance is unclear but is associated with several factors. Almost 25 % of tamoxifen-resistant tumors result from loss of ER expression\(^\text{[11]}\). Less frequent is loss of ER function due to mutations\(^\text{[12]}\). ER crosstalk with other signaling pathways such as EGFR alters ERα phosphorylation contributing to ligand-independent ER activation\(^\text{[13]}\) that is associated with tamoxifen agonist activity\(^\text{[14]}\). Reduced levels of IGF receptor Iα\(^\text{[15]}\), constitutively activated MAPK signaling in endometrial cancer cells\(^\text{[16]}\) and oxidative stress\(^\text{[17]}\) have been associated with tamoxifen agonist activity. In addition, reduced expression of the corepressor N-COR is associated with tamoxifen resistance and may also be a predictive marker for tamoxifen response\(^\text{[18]}\).
Several potential biomarkers have been examined in breast cancer to predict tamoxifen response [19-23]. Among these biomarkers ERα and the HER2 / neu oncogene have proven effective in predicting therapeutic efficacy. Unfortunately tamoxifen resistance often develops in ERα positive tumors [24] and the HER2 / neu oncogene has given conflicting results as a predictor of tamoxifen efficacy [22]. In general, current biomarkers have been successful in evaluating the progress of the disease but not in predicting tamoxifen sensitivity. The mechanisms for tamoxifen sensitivity and resistance in breast cancer are not clearly defined and may involve unidentified proteins and pathways. In the present study, we set out to use a proteomic strategy to identify novel tamoxifen induced proteins in T47D breast cancer cells. By focusing on proteins that are regulated by tamoxifen and other SERMs using two-dimensional (2D) protein electrophoresis and mass spectrometry we sought to identify proteins that may participate in novel pathways of tamoxifen sensitivity and resistance. Furthermore, these proteins could themselves serve as novel tumor targets for therapy and tumor image analysis. By using the 2D protein electrophoresis and mass spectrometry approach we eliminated the problems inherent with the gene array approach (discordance between mRNA and protein.


Materials and Methods

Cell culture

T47D (tamoxifen-sensitive, ERα-positive) breast cancer cells were maintained in phenol red-free RPMI 1640 medium containing insulin (5 μg / ml), fetal bovine serum (FBS) (10 %), and penicillin / streptomycin (1 %). MCF7 (tamoxifen-sensitive, ERα-positive) and MDA-MB-231 (tamoxifen-resistant, ERα-negative) breast cancer cell lines were maintained in phenol red-free DMEM medium containing glutamine (2 %), FBS (10 %), and penicillin / streptomycin (1 %). Cells were cultured at 37 °C in a 5 % humidified incubator. After reaching 80 % of confluency, the cells were removed by incubation with trypsin, and subsequently plated under the specified conditions described below.

MTT assay

T47D cells were plated in 24 well plates (6000 cells / well) and cultured for 24 hrs in phenol red-free RPMI 1640 containing 2 % FBS that had been incubated with dextran coated charcoal to remove endogenous steroids (charcoal stripped FBS). Cells were incubated with vehicle, 17β-estradiol (10⁻⁸ M), 4-hydroxytamoxifen (10⁻⁷ M) or acolbifene (10⁻⁷ M) for 9 days with fresh medium replacement containing vehicle, 17β-estradiol or SERMs every two days. After 9 days, medium was replaced with 0.5 mL of MTT reagent (1 mg / ml of 2, 5-diphenyl tetrazolium bromide in PBS) for 1 hr. The MTT reagent was replaced with 200 μL DMSO per well. The plates were read at a wavelength of 595 nm.

Transfection and Luciferase assay

T47D cells were plated in 6 well plates (0.5 * 10⁶ / well) and cultured for 48 hrs in phenol red-free RPMI 1640 medium containing 2 % charcoal stripped FBS. Cells were
transfected with 500 ng of ERE2e1b-luciferase reporter using Fugene 6 (Roche) for 24 hrs. Following transfection, cells were incubated with 4-hydroxytamoxifen (10^{-7} M), acolbifene (10^{-7} M), or 17\beta-estradiol (10^{-8} M) for 18 hrs. Reporter luciferase activity was measured by the standard luciferase assay system (Promega, Madison, WI). Relative luciferase values were normalized to the total protein content.

**Real time RT-PCR**

T47D, MCF7 and MDA-MB-231 breast cancer cells were plated in 10 cm dishes with medium containing 5 \% charcoal stripped FBS until cells reached 90 \% of confluency. Medium was replaced with RPMI or DMEM FBS-free medium for 12 hrs prior to addition of 4-hydroxytamoxifen (10^{-7} M), acolbifene (10^{-7} M), 17\beta-estradiol (10^{-8} M), or vehicle for an additional 12 hrs. Cells were rinsed once with PBS, scraped from the plates and total RNA was extracted using the Trizol (Invitrogen) / chloroform method. Total RNA was adjusted to 50 ng / \mu l and 200 ng RNA was reverse transcribed using the TaqMan Kit Reagents (Applied Biosystems, Foster City, CA). Reverse transcription was performed at 25 °C for 10 min, 48 °C for 30 min, and 95 °C for 5 min. cDNA was quantified by real time RT-PCR as previously described \cite{9,25}. Values were calculated using the Comparative CT method outlined in the GeneAmp 5700 user manual. The original amount of target gene was calculated as 2^{-\Delta\Delta CT}, where \Delta\Delta CT is the threshold CT value of the gene of interest normalized to GAPDH and calibrated to the vehicle treatment CT value to obtain the fold difference.

**Preparation of the total cellular extract for 2D electrophoresis**

T47D cells (8*10^6 cells) were plated in 15 cm dishes and cultured for 48 hrs in RPMI medium containing 2 \% charcoal stripped FBS. The medium was replaced with FBS-free
medium containing 4-hydroxytamoxifen (10^{-7} M), acolbifene (10^{-7} M), 17β-estradiol (10^{-8} M), or vehicle and cells were cultured for an additional 24 hrs. Following treatment, cells were rinsed once with PBS and scraped from the plates in TEN buffer (10 mM Tris, pH 8.0, 1 mM EDTA, pH 8.0 and 150 mM NaCl). Cells were centrifuged at 1500 x g for 10 min at 4 °C and the cell pellet was lysed in urea IEF buffer [urea, (8 M), thiourea (2 M), CHAPS (4 %), TBP (5 mM), immobilized pH gradient IPG buffer 3-10 non linear (NL) carrier ampholytes (0.5 %)]. The lysate was incubated at room temperature for 1 hr followed by centrifugation at 100,000 x g for 30 min. The supernatant was used for 2D gel electrophoresis.

2D gel electrophoresis

2D electrophoresis was performed as previously described by our laboratory \cite{9}. Briefly, one mg of the total cellular protein extract in a final volume equal to 350 μl was applied to an 18 cm 3-10 NL IPG strip (Amersham-Pharmacia Biotech, Piscataway, NJ) for 24 hrs and subjected to isoelectric focusing in the first dimension using a Multiphor II Isoelectric Focusing System (Amersham) programmed with a voltage gradient increasing to 3500 V for a total of 19.5 hrs at room temperature. The IPG strips were equilibrated for 10 min in SDS PAGE Equilibration Buffer 1 (50 mM Tris-HCl, pH 8.8, 6 M urea, 30 % glycerol, 2 % SDS, 1 % DTT, and a trace of bromophenol blue), then in Equilibration Buffer 2 (50 mM Tris-HCl, pH 8.8, 6 M urea, 30 % glycerol, 2 % SDS, 4 % iodoacetamide, and a trace of bromophenol blue) for an additional 10 min. The equilibrated IPG strips were placed on the top of a second dimension SDS PAGE (12.5 %) gel and the sample was electrophoresed at 10 mA for 1 hour, then 18mA for 15 hrs with liquid cooling to reduce heat. Gels were stained using the Colloidal Blue Staining
Kit (Invitrogen) for 16 hrs and rinsed with distilled water for 10 min and then for 5 hrs (two times). Followed staining, gels were scanned with a Personal Densitometer SI, Molecular Dynamics (Amersham Biosciences) and images were analyzed by Image Master 2D Elite, version 4 (Amersham-Pharmacia Biotech INC). Any protein spot that exhibited a consistent change with 17β-estradiol or SERMs treatment vs. vehicle from at least three separate experiments was excised from the gel for identification by mass spectrometry sequencing. The optical density of the selected spots was measured by using the Image mater 2D database software (Amersham-Pharmacia Biotech INC) and normalized to the spot density of seven reference spots that exhibited no change with 17β-estradiol or SERMs treatment vs. vehicle.

Protein Identification

Coomassie stained gel spots corresponding to proteins of interest (determined as described above) were destained with 30 % methanol for 3 hrs at room temperature. In gel proteolysis with modified, sequencing grade trypsin (Promega, Madison, WI) was carried out essentially as previously described [26]. Peptides were resolved using an Aquasil C18 reverse phase column (15 μm tip x 75 μm id x 5 cm Picofrit column, New Objectives, Woburn, MA) on a MAGIC 2002 HPLC system with pre-column splitting capability (Michrom Bioresources, CA). Mobile phase consisted of 1 % acetic acid / acetonitrile gradient system (5-75 % acetonitrile over 35 min followed by 95 % acetonitrile wash for 5 min) at a flow rate of 250 nl / min. Eluent (peptides) was directly introduced into an ion-trap mass spectrometer (LCQ-Deca XP Plus, ThermoFinnigan) equipped with a nano-spray source. The mass spectrometer was set for analyzing the positive ions and operated on double play mode in which the instrument was set to
acquire a full MS scan and a collision induced dissociation (CID) spectrum on the most abundant ion from the full MS scan (relative collision energy ~30 %). Dynamic exclusion was set to collect two CID spectra on the most abundant ion and then exclude it for 2 min. An initial database search of CID spectra was carried out against a subset of indexed human database using TurboSEQUEST (BioworksBrowser v 3.0, ThermoFinnigan). Results of such an analysis were manually verified and any un-interpreted CID spectra were searched using the MS-Tag provision of Protein Prospector (http://prospector.ucsf.edu) against human / mouse protein in the non-redundant or Swiss Port database.

**Western Blot Analysis**

T47D breast cancer cells were incubated with 17β-estradiol or SERMs for 24 hrs and total cellular extract was prepared as described above. 0.25 or 50 μg of each total cellular extract was separated by SDS-PAGE. Standard Western blot analysis was performed with primary antibodies against calreticulin (Stressgen, Victoria, Canada) and phosphoglycerate dehydrogenase [27]. The membrane signal was developed by enhanced chemiluminescence (ECL) and exposed to Kodak BioMax XAR film. ECL signal was measured by Kodak Digital Science 1D image analysis software station 440 systems.

**Data Analysis**

The optical spot intensity of the identified protein was normalized to the intensity of seven reference spots that consistently exhibited similar band intensity among the various treatment groups. These reference spots were distributed in different pH ranges of the developed 2D gel and stained with different degrees of intensity (high, medium and low). The statistical analysis ANOVA with repeated measures [28] was applied, and $P < 0.05$
was considered significant. To analyze real-time RT-PCR results and Western blot results, data were first normalized to GAPDH message or actin protein, respectively, and the fold difference values were calculated with the vehicle treated group set to a value of 1. One-way ANOVA-Dunnett’s T-Test was applied to the fold difference values and $P < 0.05$ was considered significance.
Results

Anti-estrogenic activity of SERMs in T47D breast cancer cells

We sought to identify and compare novel ER-regulated proteins in T47D breast cancer cells incubated with ligands that ranged from purely estrogenic (17β-estradiol), mixed agonist/antagonist (4-hydroxytamoxifen) to purely antiestrogenic (acolbifene) activity. T47D is an estrogen-responsive breast cancer cell line that expresses both ERα and ERβ. Both 4-hydroxytamoxifen and acolbifene exhibit anti-estrogenic effects on cell growth and well-characterized markers of ER-regulated gene expression in T47D cells (Figure 1). 17β-estradiol significantly induced the ERE2e1b-luciferase reporter whereas both 4-hydroxytamoxifen and acolbifene did not alter reporter activity (Figure 1A) and both these ligands antagonized estradiol activation of the reporter (data not shown).

Endogenous expression of the progesterone receptor, an ER-regulated gene involved in breast cancer progression, was induced by 17β-estradiol. However, 4-hydroxytamoxifen showed no significant effect and acolbifene repressed progesterone receptor expression (Figure 1B). Finally, 17β-estradiol, but not 4-hydroxytamoxifen or acolbifene, stimulated T47D cell growth (Figure 1C). These results established the presence of a functional ER signaling pathway and confirmed previous findings of tamoxifen and acolbifene antiestrogenic effects in T47D cells.

Identification of 17β-estradiol, 4-hydroxytamoxifen and acolbifene-regulated proteins in T47D breast cancer cells.
Total protein extract from T47D cells was profiled by 2D electrophoresis following 24 hrs of incubation with vehicle, 17β-estradiol, 4-hydroxytamoxifen, or acolbifene (Figure 2). Six novel proteins were identified by Nano-LC-Tandem-mass-spectrometry (Table 1). Colloidal coomassie staining of 2D gels indicated that 17β-estradiol and both SERMs induced calreticulin (Figure 3A) where normalized spot density was significantly higher compared with the vehicle incubated cells. To validate ligand effects on calreticulin expression Western blot analysis was applied. Only 4-hydroxytamoxifen significantly induced the expression of calreticulin in T47D cells (Figure 3B), albeit not to the degree that was observed from normalized spot density on the 2D gels. This discrepancy may be due to high basal calreticulin expression in cells coupled with a highly sensitive calreticulin antibody that, taken together, limit quantitative comparison by the Western blot procedure. We found a similar limitation when comparing quantified spot density from 2D / Coomassie stained gels with quantitative Western blots in a related study [9].

To further confirm protein inductions we measured mRNA level for each identified protein by real-time RT-PCR. All ER ligands significantly induced calreticulin expression after 12 hrs incubation (Figure 3C). This is correlated with the results from the 2D-coomassie stained gels and suggests that mRNA accumulation is likely the major mechanism for ligand induction of the calreticulin protein. However it is not yet known whether mRNA accumulation was the result of increased mRNA expression and / or reduced mRNA turnover.

A second protein spot that migrated close to calreticulin on the 2D gel was significantly induced by 4-hydroxytamoxifen (Figure 4A). This spot consists of three co-migrating
proteins; CD2 antigen, cytoplasmic tail binding protein 2 (CD2BP2), nucleosome assembly protein 1 like 1 (NAP1L1), and synapse associated protein 1 (SYAP1). Currently there are no available antibodies to these proteins. Gene expression analysis revealed that both 17β-estradiol and 4-hydroxytamoxifen significantly induced the expression of CD2BP2 (Figure 4B). Only 4-hydroxytamoxifen significantly induced NAP1L1 gene expression (Figure 4C). SYAP1 gene expression was not affected by 17β-estradiol or SERMs (data not shown). These data suggest, as with calreticulin, that 4-hydroxytamoxifen induction of CD2BP2 and NAP1L1 proteins in T47D cells is regulated, in part, by mRNA accumulation. With regard to SYAP1, this protein may simply be present as a co-migrating protein that is not affected by 4-hydroxytamoxifen treatment. Alternately the protein could actually be increased by 4-hydroxytamoxifen through mechanisms other that mRNA accumulation. Although 17β-estradiol induced CD2BP2 mRNA there was no change in the protein optical spot density from 2D gels.

Pyridoxine 5’ phosphate oxidase (PNPO) was induced in T47D cells by 17β-estradiol and 4-hydroxytamoxifen (Figure 5A). No antibody is available for PNPO. PNPO gene expression was also induced by 17β-estradiol and 4-hydroxytamoxifen in T47D cells (Figure 5B). This suggests that both 17β-estradiol and 4-hydroxytamoxifen regulate PNPO protein in T47D cells by increasing mRNA accumulation.

D-3-phosphoglycerate dehydrogenase (3-PHGDH) was identified as a faint 4-hydroxytamoxifen induced protein spot on 2D gels from T47D extract (Figure 6A). Western blot analysis revealed a weak but reproducible induction of 3-PHGDH by 4-
hydroxytamoxifen in T47D cells (Figure 6B). Both 17β-estradiol and 4-
hydroxytamoxifen significantly induced 3-PHGDH mRNA (Figure 6C). This suggests
that 4-hydroxytamoxifen likely up-regulates 3-PHGDH protein by increasing mRNA
levels. Although 17β-estradiol significantly increased 3-PHGDH mRNA, there was no
significant effect on 3-PHGDH protein as assessed by 2D gels and by Western blot
(Figure 6A-B).

Effect of ERα content and cell context in protein expression

To correlate ERα content and cell context to the proteins identified above, we
investigated two additional well-characterized breast cancer cell lines; MCF7 (ERα and
ERβ positive) and MDA-MB-231 (ERα negative and not responsive to estrogen or 4-
hydroxytamoxifen). In contrast to T47D cells, 17β-estradiol or SERMs had no effect on
the identified proteins in MDA-MB-231 cells (data not shown). This suggests an
association between the induction of these proteins and ERα expression. Since tamoxifen
functions as a partial agonist with ERα but not ERβ [7], and most of the identified genes
were induced by both 4-hydroxytamoxifen and 17β-estradiol, this further suggests an
ERα requirement for tamoxifen induction of these proteins. This ERα requirement likely
explains why there was no effect of ligands on protein induction in MDA-MB-231 cells.
Although MCF7 cells share the similar property with T47D cells of being sensitive to the
antiestrogenic effects of 4-hydroxytamoxifen and acolbifene, MCF7 cells exhibited
marked differences in mRNA regulation of the identified proteins. Only PNPO and 3-
PHGDH were regulated by 17β-estradiol and 4-hydroxytamoxifen in MCF7 cells
Calreticulin, SYAP1, CD2BP2 and NAP1L1 were not regulated by 17β-estradiol and SERMs (data not shown). This may be due to specific differences between MCF7 and T47D cells or specific time course requirements for each gene. Both 17β-estradiol and 4-hydroxytamoxifen induced 3-PHGDH gene expression in MCF7 cells (Figure 6D). In contrast only 17β-estradiol consistently and significantly induced PNPO mRNA in MCF7 cells but only to a modest degree (Figure 5C).
Discussion

ER regulates expression of a wide range of genes involved in cell proliferation and apoptosis [32]. Despite the effectiveness of tamoxifen in antagonizing estrogen action in ER-positive breast cancer tissues tamoxifen may exhibit a mixed agonist / antagonist activity in breast cancer tissue that contributes to tamoxifen resistance in breast cancer patients. ER and other biomarkers have been successful in predicting progression of the disease but have limited success in predicting tamoxifen response in ER-positive breast cancer patients. This highlights a need to identify proteins that may be involved in novel mechanisms of tamoxifen sensitivity and resistance in breast cancer.

Total protein profiling of T47D extracts identified six novel proteins that were differentially regulated by SERMs. Calreticulin was induced by all ligands. 4-hydroxytamoxifen exhibited a similar effect as 17β-estradiol in inducing PNPO protein expression. In contrast, 4-hydroxytamoxifen significantly induced both 3-PHGDH and CD2BP2 at both the protein and mRNA level whereas 17β-estradiol induced these two genes at the mRNA level only. Finally, only 4-hydroxytamoxifen induced NAP1L1 protein and mRNA. Accumulation of mRNA was the likely mechanism by which proteins were induced. There are two major mechanisms that lead to mRNA accumulation; an increase in mRNA expression and / or reduction mRNA turnover. Future studies will determine the mechanisms that resulted in mRNA accumulation in this study.
Acolbifene (EM-652) is the active metabolite of EM-800, a third generation SERM that has higher affinity for ER than 17β-estradiol and 4-hydroxytamoxifen \(^{33}\). Unlike tamoxifen, acolbifene inhibits AF-1 and AF-2 transactivation of ER and has anti-estrogenic effects in endometrial tissue \(^{29}\). In fact, tamoxifen causes some stimulation of the proliferation of human breast cancer cells in vitro and tumors in vivo while acolbifene is free of any stimulatory effect in these two tissues \(^{29}\). EM-800 has exhibited significant efficacy in overcoming tamoxifen resistance in breast cancer patients \(^{34}\). In a previous study we found discordance between acolbifene vs. 4-hydroxytamoxifen-regulated proteins in the Ishikawa endometrial cancer cell line in which acolbifene, but not tamoxifen, exhibited anti-estrogenic activity \(^{9}\). It was therefore possible that tamoxifen and acolbifene would result in a similar pattern of protein induction in T47D breast cancer cells in which both ligands are antiestrogenic. Despite the similar antiestrogenic action of these ligands acolbifene did not reproduce the effects of tamoxifen on protein and mRNA induction with the exception of calreticulin which was induced by all ligands at the mRNA level. The functional effect of acolbifene in inhibiting both AF-1 and AF-2 activity of ER is likely a major mechanism contributing to differences between 4-hydroxytamoxifen and acolbifene in protein and gene regulation. This highlights the gene specific nature of different SERMs regardless of whether each ligand exhibits identical action on the more general properties of cell growth and ER-regulated gene expression.

Calreticulin was the only protein that was induced by all ligands at both the protein and mRNA level in T47D cells. Calreticulin mediates nuclear receptor export \(^{35-37}\).
resulting in suppression of receptor transcriptional activity. In addition both calreticulin and its N-terminal fragment, vasostatin, inhibit angiogenesis \[38\]. Differential calreticulin expression in breast and other epithelial cancer cells has been previously reported. Calreticulin was downregulated in MCF7 cells resistant to melphalan treatment compared to MCF7 cells sensitive to melphalan \[39\]. In prostatic epithelial cells, calreticulin expression was regulated by androgen \[40\] and by Enable (fungicide that is classified as an endocrine disruptive chemical (EDC)) in MCF7 cells \[41\]. Calreticulin protein level was increased in infiltrating ductal carcinoma of the breast \[42\]. Calreticulin has been proposed as a potential tumor marker for bladder cancer \[43\] that may have diagnostic value \[44\].

Calreticulin may exhibit dual functions in regulation of ER\(\alpha\) dependent breast cancer growth and metastasis. The ability of calreticulin to inhibit ER\(\alpha\) transcriptional activity in breast cancer would likely be beneficial in retarding estradiol-dependent proliferation. However calreticulin may also prevent ER\(\alpha\) dependent inhibition of breast cancer metastasis. ER\(\alpha\) expression in MDA-MB-231 breast cancer cells inhibited invasiveness in the presence and absence of ligand and calreticulin reversed the ligand-independent inhibition of invasion \[37\]. The finding in this report that calreticulin was induced by all ligands suggests a negative feedback on ER\(\alpha\) function in primary tumor cells that may serve to suppress further ER\(\alpha\) dependent proliferation. However the relevance of calreticulin induction to breast cancer metastasis remains unclear.
CD2BP2 and NAP1L1 were identified as two co-migrating proteins on 2D gels that were induced by 4-hydroxytamoxifen in T47D cells. CD2BP2 interacts with the cytoplasmic tail of CD2 antigen \[45\textsuperscript{,}46\] resulting in IL-2 production in CD2 cross-linked Jurkat cells \[45\]. It has been shown that IL-2 treatment inhibits the growth of estrogen-dependent MCF7 cells but not estrogen-independent, ER\(\alpha\)-negative MDA-MB-231 cells \[47\]. A therapeutic role for IL-2 in breast cancer has also been reported \[48\textsuperscript{,}49\]. Induction of CD2BP2 may indirectly enhance the anti-proliferative effect of 4-hydroxytamoxifen by activating IL-2 signaling. Although 17\(\beta\)-estradiol induced CD2BP2 mRNA, the protein spot on the 2D gels was not induced by 17\(\beta\)-estradiol. 4-hydroxytamoxifen significantly induced NAP1L1 in T47D cells. To date the exact function of NAP1L1 is not clear although it is speculated to be involved in regulating DNA replication, gene expression, and cell growth \[50\textsuperscript{,}52\]. SYAP1, the third protein that co-migrated in the 2D protein spot, was not altered by 4-hydroxytamoxifen in T47D cells suggesting that SYAP1 mRNA level is not regulated by 4-hydroxytamoxifen treatment or probably this protein was not affected by the different ligands. NAP1L1 was proposed as a genetic marker for small intestinal carcinoid malignancy \[53\].

Both 4-hydroxytamoxifen and 17\(\beta\)-estradiol exhibited the similar property of inducing PNPO protein and mRNA in T47D cells. These data mirror the finding that 4-hydroxytamoxifen is more similar to estradiol in regulation of gene expression in MCF7 cells when compared to raloxifene and ICI 182,780 \[32\]. PNPO oxidizes pyridoxal to form pyridoxal 5\'phosphate, the functionally active form of vitamin B6. Remarkably vitamin B6 inversely modulates the transcriptional activity of multiple members the
steroid receptor super family including ER. Estrogen-induced ER transcriptional activity was reduced 30% by vitamin B6 and ER activity was enhanced 85% following vitamin B6 deficiency\textsuperscript{[54]}. In addition, higher plasma levels of vitamin B6 may reduce the risk for developing breast cancer\textsuperscript{[55]}. In this context, induction of PNPO may indirectly enhance 4-hydroxytamoxifen sensitivity in T47D cells and/or serve as a negative feedback of estrogen action through elevation of vitamin B6. To date, there are no available reports suggesting PNPO as a tumor marker although the activity of PNPO is decreased in some types of human neuroblastoma\textsuperscript{[56]}

4-hydroxytamoxifen significantly induced both 3-PHGDH protein and mRNA while 17\textsuperscript{β}-estradiol induced 3-PHGDH mRNA but not protein in T47D cells. 3-PHGDH is an enzyme involved in de novo biosynthesis of serine\textsuperscript{[57]}. Snell and colleagues suggested that 3-PHGDH is required for serine biosynthesis, a precursor of nucleotide biosynthesis. 3-PHGDH activity is increased in human colon carcinoma\textsuperscript{[58]} and indirectly involved in promoting cancer progression\textsuperscript{[58]-[60]}

17\textsuperscript{β}-estradiol and SERMs exhibited differences in regulation of the proteins in T47D, MCF7 and MDA-MB-231 breast cancer cells. These cell lines differ in their morphology and tumorigenicity. T47D cells are estrogen-dependent for growth and express both ER\textsubscript{α} and ER\textsubscript{β} in addition to progesterone, androgen and glucocorticoid receptors. MCF7 cells are also estrogen-dependent for growth, express ER\textsubscript{α} and ER\textsubscript{β} but are more differentiated compared to T47D cells. MDA-MB-231 cells are estrogen-independent for growth, ER\textsubscript{α} negative but ER\textsubscript{β} positive, and are less differentiated compared to T47D
cells. As mentioned above, the absence of 17β-estradiol and SERMs effects on the identified proteins in ERα-negative MDA-MB-231 cells suggests an association of ligand effects on the proteins with ERα content in cells. However when comparing ERα-positive T47D and MCF-7 cells there were marked differences in 17β-estradiol and SERMs induction of the identified proteins. Only PNPO and 3-PHGDH exhibited a similar pattern of induction by 17β-estradiol and 4-hydroxytamoxifen in both cell lines suggesting the presence of cell specific factors that influence ligand action in different tissues. PNPO protein and gene expression was induced with both 4-hydroxytamoxifen and 17β-estradiol in T47D cells. However only 17β-estradiol significantly induced PNPO gene expression in MCF7 cells. In contrast to PNPO, 3-PHGDH induction by 17β-estradiol and SERMs was identical in both MCF7 and T47D cell lines indicating a possible connection to ER signaling.

In general, the present study identified similar properties between 17β-estradiol and 4-hydroxytamoxifen and marked differences between 4-hydroxytamoxifen and acolbifene in regulation of specific proteins in different cell lines (Table 2). Katzenellenbogen and colleagues [32] profiled gene expression regulated by 17β-estradiol, ICI 182,780 (ICI), raloxifene (Ral) and trans-hydroxytamoxifen (TOT) in MCF7 cells by using Affymetrix GeneChip microarrays that contains about 12000 genes. They found that both TOT and Ral but not ICI exhibited partial agonist activity on some of estradiol regulated genes. Interestingly, ICI, Ral and TOT regulated some genes that were not regulated by 17β-estradiol although these genes were not identified in the present study. This could be due
to the differences in SERMs concentration, duration of treatment, cell context and/or
due to the GeneChip microarray that was limited to 12000 genes. Regardless, genes
identified in the previous study were not confirmed at the protein level. It is widely
accepted that the relative agonist/antagonist action of SERMs is defined by cell and
promoter context and different SERMs may exhibit similar or diverse effect on the same
gene [8]. SERMs also exhibit diverse effects on ER protein turnover. For example 4-
hydroxytamoxifen and raloxifene stabilize ERα protein [61] whereas ICI 182,780 induces
proteosome-dependent ERα degradation in MCF7 cells [62].

It is important to note several caveats in the 2D gel approach to identify ligand-induced
proteins. 2D gel analysis is optimized to detect the more abundant proteins in a cell.
Ligand regulation of less abundant proteins that may be critical for cell physiology would
not be detected by 2D analysis. Related to this limitation was that ligand-induced changes
quantitated in this study were only two-fold or less. Although a two-fold change in
protein level would likely have significant impact on cell physiology, protein changes
greater than two fold were not observed. One potential reason for no greater than two-
fold protein changes may be residual estrogen compounds in the cell culture medium that
might attenuate the maximal effect of any exogenous ligands. Another potential
limitation of 2D gel analysis was that ligand induced changes in calreticulin and 3-
PHGDH as measured by Western blot analysis were not quantitatively reflective of
ligand-induced changes recorded by optical spot density analysis. Although Western blot
analysis for calreticulin and 3-PHGDH provided a qualitative confirmation of optical spot
density results the induction of these proteins by ER ligands was quantitatively less than
measured with optical spot density analysis. This disparity may be related to the different sensitivity of the two assays in which 2D gel analysis is optimized to quantitate changes in more abundant proteins whereas Western blot analysis is more sensitive and optimized for less abundant proteins. An alternate explanation is that antibody epitopes in the proteins may be masked (e.g. by ligand-induced posttranslational modifications) and therefore ligand-induced changes in proteins by Western blot may be quantitatively less than changes recorded by optical spot density analysis. In this regard it should be noted that the quantitative changes in mRNA were more reflective of the quantitative changes measured by optical spot density. Another limitation to the present study was that proteins downregulated by 17_ estradiol or SERMs were not identified. This could be due, in part, to the broad pH range used during isoelectric focusing and/or the limited sensitivity of coomassie blue staining. Silver staining of proteins while more sensitive than coomassie staining, is not optimally compatible with the downstream processing of samples for MS sequencing. Finally, additional studies are required to address the functional significance of the identified proteins for ER action, and cellular growth/differentiation of breast cancer cells. Despite these limitations, differential protein profiling by 2D gel analysis has been used successfully to identify proteins associated with diverse functions including drug resistance, activation of specific signaling pathways, cancer progression and metastasis [42,63–66]. This approach remains an attractive alternative to gene array analysis for assessing ligand effects on ER signaling.
Acknowledgements

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Table 1. Proteins identified in T47D cells by Nano-LC-Tandem-MS. Each protein spot was digested with sequencing-grade, modified trypsin (Promega) overnight at 37 °C. Peptides were extracted with 60 % acetonitrile: 0.1 % TFA. Two μl of the concentrated sample was separated on a reversed phase column, then the eluent was directly introduced into an ion-trap mass spectrometer (LCQ-Deca XP Plus, Finnigan) equipped with a nano-spray source. The mass spectrometer was operated on a double play mode in order to acquire a full MS scan and a collision induced dissociation (CID) spectrum on the most abundant ion from the full MS scan. CID spectra were either manually interpreted or searched against an appropriate non-redundant database using TurboSEQUEST. m = Oxidized methionine (met. Sulfoxide), q = pyroglutamine.

Table 2. Estradiol and SERM regulation of the proteins identified in T47D cells

Figure 1. Anti-estrogenic activity of 4-hydroxytamoxifen and acolbifene in T47D breast cancer cells. A. T47D cells were plated in 6 well plates and cultured for 2 days in RPMI medium containing 2 % charcoal stripped FBS. Cells were transfected with ERE2e1b-luciferase reporter for 24 hrs followed by incubation with vehicle, 17β-estradiol (E2) (10-8 M), 4-hydroxytamoxifen (4-(OH)-tam) (10-7 M), or acolbifene (ACOL) (10-7 M) for 18 hrs. Luciferase activity was measured in cell extracts and normalized to total protein amount. B. T47D cells were plated in 10 cm dishes until cells reached 90 % confluency. Cells were then incubated with vehicle, E2 (10-8 M), 4-(OH)-tam (10-7 M), or ACOL
(10-7 M) for 6 hrs. Total RNA was extracted, reverse transcribed and progesterone receptor mRNA was measured by real-time RT PCR normalized to GAPDH expression.

C. T47D cells were plated in 24 well plates and incubated with vehicle, E2 (10-8 M), 4-(OH)-tam (10-7 M), or ACOL (10-7 M) for 9 days. Cell growth was measured by the MTT assay as described in Materials and Methods. One way ANOVA was applied and * indicates significant difference at $P$ value = 0.05.

Figure 2. Protein profiling of T47D breast cancer cells. T47D cells were incubated with vehicle, E2 (10-8 M), 4-(OH)-tam (10-7 M) or ACOL (10-7 M) for 24 hrs in FBS-free RPMI medium. Cell extract was prepared with 8 M urea IEF buffer. One mg of cellular extract was subjected to 2D gel analysis. The gel was stained with colloidal blue for 12 hrs at room temperature. Stained gels were imaged by a Personal Densitometer SI scanner. The boxes indicate the regions on the 2D gel where proteins that were identified by mass-spectrometry had migrated. Box 1 contains calreticulin and the protein spot containing three co-migrating proteins; CD2BP2, NAP1L1 and SYAP. Box 2 contains 3-PHGDH, while Box 3 contains PNPO.

Figure 3. 4-hydroxytamoxifen induction of calreticulin in T47D cells. A. 2D gel image of the calreticulin protein spot from T47D cells incubated for 24 hrs in FBS-free medium containing vehicle, E2 (10-8 M), 4-(OH)-tam (10-7 M), or ACOL (10-7 M). The optical density of the calreticulin spot (arrow) was measured and normalized to 7 reference spots. ANOVA with repeated measure statistical analysis was applied (inset table) where numbers represent the mean optical spot density from three independent experiments ±
S.E. B. Western blot analysis of T47D cells incubated with vehicle, E2 (10^{-8} M), 4-(OH)-tam (10^{-7} M), or ACOL (10^{-7} M) for 24 hrs in FBS-free medium. Cells were harvested, lysed in 8M urea extraction buffer and 0.25 μg of the total cellular extract was prepared for Western blotting using antibody to calreticulin. Band intensities were quantified and normalized to β-actin levels. C. T47D cells were incubated with vehicle, E2 (10^{-8} M), 4-(OH)-tam (10^{-7} M), or ACOL (10^{-7} M) for 12 hrs and expression of calreticulin was measured using real-time RT-PCR normalized to GAPDH expression. One way ANOVA was applied to the results. * indicates a significant difference at $P$. value = 0.05.

Figure 4. Induction of a protein spot containing three co-migrating proteins in T47D cells. A. 2D gel image of a protein spot containing CD2BP2, NAP1L1, and SYAP1 proteins from T47D cells incubated with vehicle, E2 (10^{-8} M), 4-(OH)-tam (10^{-7} M), or ACOL (10^{-7} M) for 24 hrs in FBS-free medium. A normalized optical density of the indicated spot (arrow) displayed in inset table where ANOVA with repeated measure statistical analysis was applied where numbers represent the mean optical spot density from three independent experiments ± S.E. B and C. T47D cells were incubated with vehicle, E2 (10^{-8} M), 4-(OH)-tam (10^{-7} M), or ACOL (10^{-7} M) for 12 hrs and expression of CD2BP2 (B) and NAP1L1 (C) was measured using real-time RT-PCR normalized to GAPDH expression. One way ANOVA was applied to the results. * indicates a significant difference at $P$. value = 0.05.
Figure 5. Induction of PNPO expression by 17β-estradiol and 4-hydroxytamoxifen in T47D cells. A. 2D gel image of the PNPO protein spot from T47D cells incubated with vehicle, E2 (10\(^{-8}\) M), 4-(OH)-tam (10\(^{-7}\) M), or ACOL (10\(^{-7}\) M), in FBS-free medium for 24 hrs. A normalized optical density of the indicated spot (arrow) displayed in inset table where ANOVA with repeated measure statistical analysis was applied where numbers represent the mean optical spot density from three independent experiments ± S.E. N.D.; not detected. B and C. T47D (B) and MCF7 (C) cells were plated in medium containing 5% charcoal stripped FBS until cells reached 90% confluency. Cells were incubated with vehicle, E2 (10\(^{-8}\) M), 4-(OH)-tam (10\(^{-7}\) M), or ACOL (10\(^{-7}\) M) for 12 hrs and PNPO expression was measured by real-time RT-PCR normalized to GAPDH expression. One way ANOVA was applied. * indicates a significance difference at \(P.\) value = 0.05.

Figure 6. Induction of 3-PHGDH by 17β-estradiol and 4-hydroxytamoxifen in T47D and MCF7 cells. A. 2D gel image of the 3-PHGDH protein spot from T47D cells incubated with vehicle, E2 (10\(^{-8}\) M), 4-(OH)-tam (10\(^{-7}\) M), or ACOL (10\(^{-7}\) M) in FBS-free medium for 24 hrs. A normalized optical density of the indicated spot (arrow) displayed in inset table where ANOVA with repeated measure statistical analysis was applied where numbers represent the mean optical spot density from three independent experiments ± S.E N.D.; not detected. B. Western blot analysis of T47D cells incubated with vehicle, E2 (10\(^{-8}\)M), 4-(OH)-tam (10\(^{-7}\)M), or ACOL (10\(^{-7}\) M) for 24 hrs in FBS-free medium. Cells were harvested, lysed in 8M urea extraction buffer and 10 µg of the total cellular extract was prepared for Western blotting using antibody to 3-PHGDH. Band intensities were

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quantified and normalized to β-actin levels. C & D. T47D (C) and MCF7 (D) cells were incubated with vehicle, E2 (10⁻⁸ M), 4-(OH)-tam (10⁻⁷ M), or ACOL (10⁻⁷ M) for 12 hrs and expression of 3-PHGDH was measured using real-time RT-PCR normalized to GAPDH expression. One way ANOVA was applied. * indicates a significant difference at $P$. value = 0.05.
Table 1. Proteins identified in T47D cells by Nano-LC-Tandem-MS.

<table>
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Table 2. Estradiol and SERM Regulation of the proteins identified in T47D cells.

<table>
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<th>Acolbifene</th>
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<td>↑</td>
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<td>Synapse associated protein 1</td>
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</table>
Figure 1

A. ERE₁₀e 1b-Reporter

B. Progesterone Receptor mRNA

C. Cell Growth
Figure 2
Figure 3

A. 2D gel image of calreticulin

B. Western blot analysis for calreticulin

C. Calreticulin mRNA expression

<table>
<thead>
<tr>
<th>Normalized optical spot density</th>
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<th>ACOL</th>
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<td></td>
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Figure 4

A. 2D gel image

![2D gel image with Vehicle and E2 conditions]

Vehicle  | E2
---|---
4-(OH)-tam | 4-(OH)-tam

Normalized optical spot density

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B. CD2BP2 mRNA expression

![CD2BP2 mRNA expression graph]

C. NAP1L1 mRNA expression

![NAP1L1 mRNA expression graph]
Figure 5

A. 2D gel image of PNPO in T47D cells.

B. PNPO mRNA expression in T47D cells.

C. PNPO mRNA expression in MCF7 cells.

Normalized optical spot density

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Figure 6

A. 2D gel Image of 3-PHGDH In T47D cells.

Normalized optical spot density

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<th>Vehicle</th>
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</table>

B. Western blot analysis of 3-PHGDH in T47D cells.

C. 3-PHGDH mRNA expression in T47D cells.

D. 3-PHGDH mRNA expression in MCF7 cells.
SUMMARY

The main objective of this study was to identify the molecular mechanisms in which PKA signaling pathway regulate ERα function in breast cancer cells. Comprehensive molecular techniques were utilized to characterize PKA regulation of both the ligand dependent as well as the ligand independent activation of ERα in breast cancer cells. MCF7, a well established ERα positive, tamoxifen-sensitive breast cancer cell line was used as an in vitro model system. Forskolin/IBMX (F/I) was used to induce PKA activity and PKA or PKI expression plasmid was used to alter PKA activity in cells. Two other ERα positive breast cancer cell lines were used, T47D tamoxifen-sensitive and MCF7-LCC2 tamoxifen-resistance cell lines also were used to characterize the effect of PKA signaling in ERα action. Two different ERE containing reporters indicated that F/I induced the ligand independent activity of ERα and repressed estradiol-dependent transcription in all cell lines. The F/I effect on ERα action was PKA dependent, in which PKA, but not PKI nor 8CPT-2Me-cAMP repressed estradiol-dependent transcription. This is the first study that identifies PKA signaling as the major pathway for cAMP effects on ER action in breast cancer cells while excluding effects of cAMP Epac signaling.

Profiling specific ERα endogenous target gene expression revealed that F/I effects on ERα transcriptional activity was gene specific and F/I effects on ERE-reporter gene assays is not a good indices for cAMP effect on ER signaling. The F/I increased estradiol-dependent transcription of cMyc and pS2 but repressed estradiol-dependent transcription of cyclin D1. It is likely that gene promoter context plays a major role in F/I
regulation of ER target gene. In addition to profiling endogenous ER target gene expression, ERα recruitment to pS2 and cMyc promoters was measured by the chromatin immunoprecipitation assay (ChIP). For the first time, this study shows that ligand independent activation by F/I recruited ERα to the cMyc and pS2 promoters. In fact F/I induced a rapid cyclic ERα association/dissociation that was distinct from recruitment to promoters by estradiol. The F/I shifted estradiol-induced ERα recruitment to promoters to an earlier time point. Since F/I repressed estradiol dependent transcription from ERE containing promoters, the F/I effect on estradiol binding was analyzed. The F/I blocked estradiol binding in all cell lines. Interestingly, F/I suppression of estradiol binding was correlated with an increase in Hsp90 association with ERα. The F/I repression of estradiol binding to its cognate receptor and increase of Hsp90 association to ERα is a novel finding that suggests a specific ERα conformation induced by F/I that may be involved in defining ERα cycling time on ER target promoters and regulation of estradiol-dependent transcription.

The ERα is a phosphoprotein and currently there are eight identified phosphorylation sites in ERα. The ERα phosphorylation regulates ERα ligand binding, dimerization, localization and transcriptional activity. To profile ERα phosphorylation, eight novel phosphoantibodies were characterized. All phosphoantibodies were site and phospho-specific. The F/I, F/I+estradiol and estradiol treatments resulted in three distinct phosphorylation profiles that are likely representative of three distinct ERα conformations. Importantly, F/I repressed estradiol induction of S118 phosphorylation and induced S305 phosphorylation. The F/I inhibition of S118 phosphorylation was likely due to F/I
repression of ERK1/ERK2 activity. Induction of S305 phosphorylation by F/I was shown to be important for the ligand independent activation of ERα as well as in repressing estradiol dependent transcription. This is the first study to profile total ERα phosphorylation using phosphoantibodies to each ERα phosphorylation site and to profile ERα phosphorylation in response to elevated cAMP through F/I. Profiling multiple phosphorylation sites may provide insight for ERα conformations under different stimuli.

Although F/I induced cMyc gene expression, F/I also repressed cyclin D1, a key cell cycle protein. Therefore, F/I effects on cell growth and estradiol dependent growth of breast cancer cells was explored. The F/I inhibited tamoxifen-sensitive breast cancer cell growth but not tamoxifen-resistant breast cancer cell growth. Importantly, F/I repressed estradiol-dependent breast cancer cell growth. These results highlighted a possible link between F/I regulation of cyclin D1 and estradiol-dependent breast cancer.

Finally, in a related study two-dimensional analyses were performed to profile protein expression in T47D cells incubated with estradiol (full agonist), 4-hydroxytamoxifen (mixed agonist/antagonist) and acolbifene (pure antagonist) for 24 hrs. Six novel, differentially expressed proteins were identified (calreticulin, synapse associated protein, CD2 antigen binding protein 2, D-3-phosphoglycerate dehydrogenase, and pyridoxine 5’ phosphate oxidase). 4-hydroxytamoxifen and estradiol share higher similarity in regulation of these identified proteins compared to acolbifene. Increase of mRNA accumulation was the probable mechanism in which 4-hydroxytamoxifen, estradiol and acolbifene upregulated these protein. However, it is not clear if increase in mRNA accumulation is due to an increase in mRNA level or decrease in mRNA turnover.
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ABSTRACT

Estrogen receptor α (ERα) is a transcriptional factor that promotes breast cancer growth by inducing a set of genes that regulate cell cycle and proliferation. ERα is activated by estrogen binding and/or by crosstalk with different signaling pathways.

Here, PKA regulation of ERα action was investigated in tamoxifen-sensitive MCF7 and T47D, and tamoxifen-resistant MCF7-LCC2 breast cancer cells. Forskolin/IBMX (F/I), which elevates cAMP, induced ligand-independent activation of ERα but repressed estradiol activation of ERα in PKA-dependent manner as assessed by estrogen response element (ERE)-reporter gene assays. However, F/I exhibited gene specific effects on endogenous ER-regulated genes. F/I enhanced estradiol induction of pS2 and cMyc that was correlated with rapid and cyclic ERα recruitment to the respective promoters as assessed by chromatin immunoprecipitation assays. In contrast, F/I repressed estradiol induction of cyclin D1 mRNA and protein that was correlated with inhibition of estradiol-dependent breast cancer growth. To identify potential mechanisms by which F/I regulates ERα action, estradiol binding, Hsp90 interaction with ERα and ERα phosphorylation were examined. F/I blocked estradiol binding to ERα in all cell lines that was correlated with an increase in Hsp90 association with ERα. Importantly, F/I induced a unique ERα phosphorylation profile (inhibition of serine 118 phosphorylation and increase serine 305 phosphorylation) that was distinct from phosphorylation profiles regulated by estradiol and estradiol + F/I. F/I inhibition of serine 118 phosphorylation was correlated with a decrease in ERK1/ERK2 phosphorylation. Serine 305
phosphorylation was required for ligand-independent activation of ERα by F/I as well as the F/I suppression of ERα activation by estradiol.

Development of tamoxifen resistance in breast cancer and the paucity of biomarkers that predict tamoxifen sensitivity illustrate an urgent need to identify novel proteins that regulate tamoxifen sensitivity and/or serve as surrogate markers of tamoxifen response. Two dimensional protein analysis and mass spectrometry were applied to identify proteins induced by tamoxifen in tamoxifen-sensitive T47D cells. Six novel tamoxifen-regulated proteins were identified (calreticulin, synapse-associated protein, CD2 antigen-binding protein-2, D-3-phosphoglycerate dehydrogenase, and pyridoxine 5’-phosphate oxidase). Tamoxifen regulation or proteins occurred at the mRNA level. These proteins could serve as markers for tamoxifen sensitivity in breast cancer cells.