

ABSTRACT

The regulation of gene expression by steroid hormones plays an important role in the normal development and function of many organs, as well in the pathogenesis of endocrine-related cancers. However, clinical data suggest that combined testosterone and estrogen treatments on post-menopausal women increase the risk of breast cancer. Experiments have shown that many, if not all kallikreins are under steroid hormone regulation, particularly in breast cancer cell lines. Their implication as prognostic and diagnostic markers has been well-documented. Thus, we investigated the effect of combined hormone stimulation with DHT and 17 β -estradiol on the ductal carcinoma cell line BT474. This cell line has been shown to be sensitive to both, androgens (secreting PSA), and estrogens (secreting a number of kallikreins including KLK10, 11, and KLK14). We found that PSA expression was downregulated upon combined hormone stimulation, confirming reports that estrogen can antagonize and block the activity of the androgen receptor. Upon analysis of estrogen sensitive kallikreins, all showed to be synergistically enhanced in their expression 3- to 4-fold, upon joint hormone treatment versus individual hormone stimulation. The enhancement is dependent upon the action of androgens as treatment with the androgen receptor antagonist Cyproterone Acetate normalized the expression of KLK10, 11, and KLK14 to estrogen-stimulation levels. The synergistic effects between estrogens and androgens on estrogen-sensitive genes may have implications on the role of the kallikreins in breast cancer progression.

INTRODUCTION

Steroid hormones play a critical role in breast cancer development and have been associated with an increase epithelial cell proliferation and in turn facilitating malignant transformation. In particular, two sex hormones that have been very well characterized both *in vitro* and *in vivo*-estrogen and progesterone. The serum concentrations of these hormones together with their respective receptors are also used as epidemiological markers in assessing breast cancer risk. Studies have also shown that the direct action of these steroid hormones on different breast tissue is dependent upon their specific receptors. Another category of sex hormones that has been extensively studied in breast cancer in human and mice are androgens. Androgens have been shown to have both stimulatory and inhibitor actions on the growth of several breast cancer cell lines. However, their etiological role in breast cancer has been unclear. Unclear is whether the action of androgens is direct through their cognate receptor or via their metabolism into estrogen-like byproducts by aromatase activity. Also, recent studies suggest that subnormal levels of androgens may adversely affect a women's health, while on the other hand other studies indicate that supranormal levels may also have adverse effects on the female reproductive system including abnormal growth and tumorigenesis. The latter studies have been gaining more attention due to the increased use of androgens for various therapeutic purposes, specifically in hormone replacement therapies (HRT) of postmenopausal women. Recently, there have been several studies that have associated endogenous elevated serum levels of estrogen and free testosterone hormone with breast cancer risk. This increased risk is of particular significance in postmenopausal women receiving HRT.

By far, the gene whose regulation by steroid hormones has been most thoroughly studied is the human tissue kallikrein gene, Prostate-Specific Antigen (PSA). The PSA gene possesses three androgen response elements (ARE-I, ARE-II, and ARE-III). ARE-I and ARE-II were identified in the upstream promoter region (-170 bp and -400 bp), functionally tested and found to be active in LNCaP, a prostate cancer cell line. ARE-III was found at -4,316 bp, which induced a dramatic increase in PSA transcription, in comparison to ARE-I and ARE-II. AREs have been found in other genes, including other members of the kallikrein gene family.

All 15 kallikrein genes show differential expression patterns in many cancers at the mRNA and protein levels and many kallikreins have been examined as prognostic indicators in breast cancer including, PSA, KLK5, 6, 10, and KLK14. Previous studies have found that there is a close association between steroid hormone stimulation of breast cancer cell lines and coordinated kallikrein gene expression. However, it has never been examined if the expression profiles would change upon multiple hormone stimulations. Therefore, would significant changes in kallikrein gene expression be of clinical importance within the context of HRT with estrogen and testosterone and increase breast cancer risk? Thus, we examined a number of estrogen hormone regulated kallikrein genes in the breast cancer cell line BT474, to determine if combined hormone stimulations can act synergistically to enhance kallikrein gene expression.

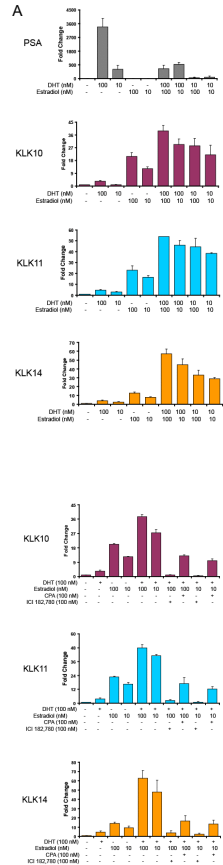


Figure 2. Hormone receptor antagonist treatment of joint hormone stimulated cells. Jointly hormone stimulated BT474 cells were treated with a single dose of 100 nM final concentration of either IC1182780 (estrogen antagonist) or cyproterone acetate (CPA - androgen antagonist). After 6 days, the cell supernatants were collected and ELISAs were carried out for KLK10, 11 and KLK14. The enhanced expression of these kallikreins is estrogen-dependent as CPA treatment abolishes all synergistic effects of combined hormone treatments.

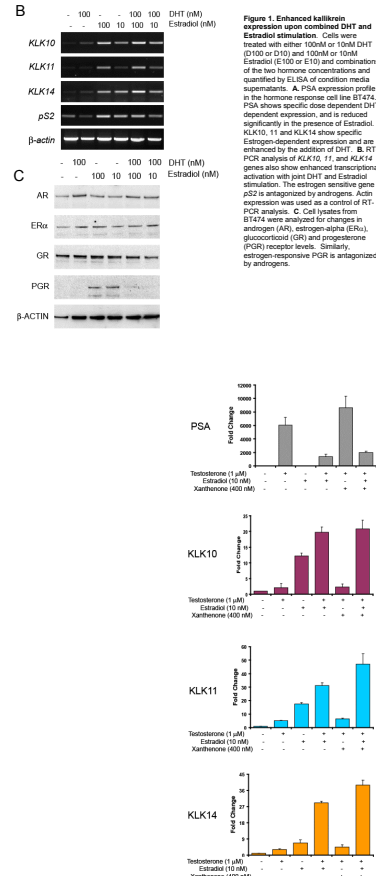


Figure 3. Synergistic KLK expression is not a result of aromatization. Although DHT is a non-aromatizable androgen, similar KLK enhancement was observed with combined testosterone-estradiol stimulation. The stimulations were also carried out in the presence of the aromatase inhibitor 4-(dimethylamino)-1- α -methyl-3-methyl-1,4-dioxane (Xanthone).

RESULTS

Figure 1. Enhanced kallikrein expression upon combined DHT and Estradiol stimulation. Cells were treated with either 100nM or 100nM DHT (D100 or D10) and 100nM or 10nM Estradiol (E100 or E10) and combinations of the two hormone concentrations and quantified by ELISA of condition media supernatants. A. PSA expression profile in the hormone response cell line BT474. PSA shows specific dose-dependent DHT dependent expression, and is reduced significantly in the presence of Estradiol. KLK10, 11 and KLK14 show specific Estradiol-dependent expression and are enhanced by the addition of DHT. B. RT-PCR analysis of KLK10, 11, and KLK14 genes also show enhanced transcriptional activation with joint DHT and Estradiol stimulation. The estrogen sensitive gene pS2 is antagonized by androgens. Actin expression was used as a control of RT-PCR analysis. C. Cell lysates from BT474 were analyzed for changes in androgen (AR), estrogen alpha (ER α), glucocorticoid (GR) and progesterone (PGR) receptor levels. Similarly, estrogen responsive PGR is antagonized by androgens.

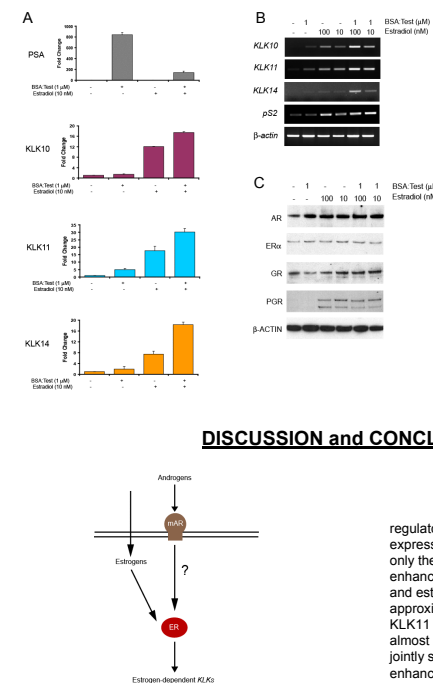


Figure 4. Enhanced KLK expression is via a membrane-bound androgen receptor. A. BT474 were stimulated with Testosterone conjugated to BSA (BSA-Test) together with 10nM estradiol and secreted levels of PSA, KLK10, 11 and KLK14 were measured by ELISA. As BSA-Test cannot be internalized, the action of the androgen must occur via a membrane bound androgen receptor. BSA-Test is capable of stimulating PSA and similarly is antagonized by estrogens. The estrogen sensitive kallikreins 10, 11, and 14 are similarly enhanced in their expression with BSA-Test and estradiol. B. RT-PCR analysis indicates that the enhanced expression of KLK10, 11, and KLK14 is a transcription event. However, pS2 expression is not antagonized by the action of BSA-Test. C. Western blot analysis of steroid hormone receptors also indicate that PGR is also no longer antagonized by BSA-Test.

DISCUSSION and CONCLUSIONS

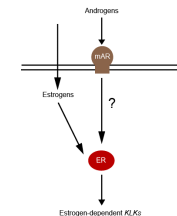


Figure 5. Schematic diagram of androgen action via a membrane-bound androgen receptor.

We have identified and characterized a novel regulatory pathway for hormone-dependent KLK gene expression in breast cancer cells. It appears, so far, that only the estrogen-sensitive kallikreins are synergistically enhanced in their expression upon combined androgen and estrogen hormone stimulation. KLK10 is enhanced approximately two-fold by DHT (Figure 1). Whereas, KLK11 and KLK14 expression levels are enhanced by almost 3- and 4-fold respectively, when BT474 cells are jointly stimulated with estradiol and DHT. These enhanced changes in kallikreins levels are specific to androgen and estradiol stimulations as combined stimulations with the glucocorticoid dexamethasone did not show any effect (data not shown). The increase in secreted kallikrein protein levels observed in the condition media is also a transcriptional event as RT-PCR analysis of KLK10, 11, and KLK14 mRNA expression show a pattern of increased transcript levels upon joint hormone stimulation. The enhanced KLK gene expression is dependent upon the action of the androgen receptor as treatment with the androgen antagonist cyproterone acetate abolished any synergistic hormone activity and returned KLK levels to near estrogen-only levels (Figure 2). Other estrogen-sensitive genes such as pS2, PGR, c-fos and c-myc (data not shown for c-fos and c-myc) do not show a similar expression profile when BT474 is jointly stimulated with androgens and estrogens. However, there are many other estrogen-sensitive genes that have not been characterized for changes in their expression profiles in this study.

The action of the enhanced expression is not a result of either aromatization of androgens (Figure 3) or a binding of the androgen receptor to AREs upstream of these kallikreins, but rather through a membrane-bound androgen receptor (Figure 4). It has been reported that a membrane-bound androgen receptor can stimulate PSA secretion. However, the ability of a membrane-bound androgen receptor to influence the activity of the estrogen receptor is a unique regulatory pathway in hormone-dependent gene expression for the kallikreins. We are currently investigating whether the membrane-bound androgen receptor can activate intracellular signaling pathways such as RAS/MEK/ERK or PI3K/AKT can have been shown to both be activated by androgens and also influence estrogen receptor activity.

As the kallikreins have been shown to be of prognostic value for detection and monitoring breast cancer progression and therapeutic efficacy, the important link between HRT for post-menopausal women and increase breast cancer risk is of more significance with our findings of the synergistic actions of the androgens on estrogen responsive genes.