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Report

Differential steroid hormone regulation of human glandular kallikrein (hK2) and prostate-specific antigen (PSA) in breast cancer cell lines

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Summary

We have investigated the steroid hormone regulation of human glandular kallikrein (hK2) and prostate-specific antigen (PSA) in the breast cancer cell lines BT-474, T-47D, MFM-223, MCF-7, ZR-75-1, MDA-MB-435, and BT-20. Using highly sensitive time-resolved fluorometric immunoassays, we were able to detect significant amounts of both kallikreins in tissue culture supernatants of BT-474, T-47D, and MFM-223 cells after hormonal stimulation. However, BT-474 cells produce much more hK2 than PSA, whereas the situation is reversed in T-47D cells. Furthermore, BT-474 cells produce, on absolute terms, about 500–1,000-fold more hK2 than T-47D cells. From all steroids tested, mibolerone, a synthetic non-metabolizable androgen, was the most potent stimulator for both kallikreins followed by the synthetic progestin norgestrel. Estradiol was able to induce production of small but significant amounts of hK2 and PSA in the BT-474 cell line, supporting the notion that there is a cross-talk between the estrogen and androgen hormone-receptor signaling pathways. MFM-223 is an androgen responsive cell line, devoid of other steroid hormone receptors, which is also capable of producing hK2 and PSA but at much lower amounts. MCF-7 and ZR-75-1 cell lines failed to produce any protein, even though they have similar steroid receptor content as the BT-474 and T-47D cell lines. This was also the case for MDA-MB-435, a cell line rich in androgen receptors. Our data suggest that the expression of the hK2 gene in breast cancer cell lines is mainly under the control of androgens and progestins, similarly to PSA. These cell lines may represent good models for studying the differential expression of these two genes and for identifying cellular factors (e.g. co-activators/co-repressors), which may modify the potency of expression after hormonal stimulation.

Abbreviations: PSA, prostate-specific antigen; hK2, human glandular kallikrein; DFP, diflunisal phosphate; ALP, alkaline phosphatase; AR, androgen receptor; PR, progesterone receptor; ER, estrogen receptor

Introduction

Prostate specific antigen (PSA) and human glandular kallikrein (hK2) belong to the human kallikrein gene family and share many biochemical and structural properties [1]. Recent studies indicate that hK2 may be a novel marker for prostate cancer, supplementing the well-established clinical value of PSA [2–4]. Both of these androgen-regulated kallikreins have been detected in extra-prostatic tissues, especially in female breast [5–9]. Accumulating evidence indicates that both kallikreins may play an important role in the physiology of normal and malignant breast tissue. PSA was found in breast cancer extracts and its presence was associated with presence of steroid hormone receptors [10]. Patients with PSA-positive cancer tended to have earlier stage disease [11]. In comparison to PSA-negative breast cancer, patients with PSA-positive tumors have longer relapse-free survival regardless of other clinical and pathological features, suggesting that PSA is a favorable prognostic marker in breast cancer [12]. Steroid hormone receptor-positive breast cancer cell lines, like T-47D, but not steroid hormone receptor-negative cell

lines like BT-20, have the ability to produce PSA *in vitro* after steroid hormone stimulation [13]. Fluids secreted by breast epithelial cells such as milk, breast cyst fluid, and nipple aspirate fluid, contain PSA [14, 15] and hK2 [8, 9]. Similarly, other studies identified the expression of hK2 in the breast carcinoma cell line T-47D after stimulation by steroid hormones [16]. This study was undertaken to investigate and compare the mode of regulation of hK2 and PSA genes in breast cancer cell lines, after steroid hormone stimulation.

Materials and methods

Cell lines

The following human breast cancer cell lines were obtained from American type culture collection (ATCC), Rockville, MD: BT-474, T-47D, ZR-75-l, BT-20, MCF-7, and MDA-MB-453. The MFM-223 cell line was a gift from Dr. R. Hackenberg.

Steroid compounds

All steroids were obtained from Sigma Chemical Co., St. Louis, MO. ICI 182,780 was purchased from Tocris Cookson, Inc., BalIwin, MO and RU 486 (mifepristone) from Roussell UCLAF, Paris, France. Stock solutions (10^{-2} or 10^{-3} M) were prepared in absolute ethanol. More dilute solutions were also prepared in the same solvent.

Stimulation experiments for protein determination

All cell lines were cultured in phenol-free RPMI medium (Gibco BRL, Gaithersburg, MD) supplemented with glutamine (200 mmol/l), bovine insulin (10 mg/l) and fetal calf serum (10%), at 37°C, 5% CO_2 in plastic culture flasks. Once confluent, the cells were transferred into 24-well microtiter plates (Corning no. 25280) using the same medium, but with substitution of charcoal-stripped fetal calf serum for the regular fetal calf serum. Stimulation was carried out with confluent cells and was initiated by adding 2 µl of each steroid dissolved in 100% ethanol and incubating for a certain period of time. Negative controls (only alcohol or nothing added) were included in each experiment. Tissue culture supernatants were collected for PSA and hK2 determination.

Time course studies

Cells were stimulated with each steroid at concentrations of 10^{-7} M and 10^{-9} M. Tissue culture supernatants (about 2 ml) were removed for PSA and hK2 analyses at days 1,3,5, and 7.

Dose-response studies

BT-474 and T-47D cells were stimulated with different concentrations of each steroid (10^{-7} to 10^{-12} M), incubated for 7 days and then the supernatants were harvested and assayed for PSA and hK2. Steroid dilution in each well was 1000-fold in all experiments as we added 2 µl of steroid solution in 2 ml of tissue culture medium. In these studies we always refer to the final concentrations of the tested steroids.

Blocking studies

BT-474 and T-47D cells were stimulated with either steroid alone (norgestrel, DHT or estradiol) at a concentration of 10^{-9} M, blocker alone (ICI 182,780, mifepristone, 4-OH-tamoxifen or RU 56,187) at 10^{-7} M, or with both blocker and steroid together. In wells with both blocker and steroid, blocker was added first (10^{-7} M), then incubated for 1 h prior to addition of steroid (10^{-9} M). Following a 7 day incubation, supernatants were harvested and assayed for PSA and hK2. Percentage blocking was calculated by comparison to the same experiment without blocker. Any agonist activity of the blocker was subtracted.

Immunoassays

The determination of PSA levels in all culture supernatants was performed by an ultrasensitive time-resolved immunofluorometric assay as described elsewhere [17]. A new, time-resolved immunofluorometric assay, recently developed in our laboratory, was used to measure serum hK2 concentrations [18]. Briefly, the hK2 assay uses a mouse monoclonal anti-hK2 capture antibody (coded G586, supplied by Hybritech and raised against recombinant hK2), a biotinylated mouse monoclonal detection antibody (coded 8311, Diagnostic Systems Laboratories Inc. Webster, TX, USA) and alkaline phosphatase-labeled streptavidin. The ALP activity was measured by adding the substrate diflunisal phosphate (DFP), incubating for 10 min and then adding a Tb^{3+}-EDTA developing solution. The fluorescence was measured on a Cyberfluor 615 Immunoanalyzer (MDS Nordion, Kanata, ON, Canada). A full description of the method and its evaluation has been published elsewhere [18]. The PSA immunoassay does not cross-react with hK2 and the hK2 immunoassay does not cross-react with PSA [18].

Stimulation experiments for mRNA determination

Confluent BT-474 cells were stimulated with steroids at a final concentration of 10^{-7} M and then harvested at 1, 2, 4, 8, and 24 h after stimulation for total RNA extraction.

Reverse transcriptase polymerase chain reaction

Total RNA was extracted from the cells using Trizol reagent (Gibco BRL) following the manufacturer's instructions. The purity and quantity of the extracted RNA was checked spectrophotometrically at 260 and 280 nm. Two micrograms of total RNA were reverse transcribed using oligo dT primers and Superscript II reverse transcriptase (Gibco BRL). The final volume was 20 µl. Two microliters of cDNA were then amplified, with a pair of 5′, and 3′ primers for either PSA or hK2, using Taq polymerase. The primers used for PSA and hK2 have been published previously by Deguchi et al. [19] and Hsieh et al. [16] respectively, and their sequences are as follows: PSA-1: 5′-TGC-GCA-AGT-TCA-CCC-TCA-3′ and PSA-2: 5′-CCC-TCT-CCT-TAC-TTC-ATC-C-3′; hK2-l: 5′-GGT-GGC-TGT-GTA-CAG-TCA-TGG-AT-3′ and hK2-2: 5′-CAG-AAA-GCA-CAG-GTC-AGT-AGG-AC-3′. The cycling conditions for PSA were 95°C for 5 min, followed by 30 cycles of 94°C for 30 s, 58°C for 30 s and 72°C for 30 s and a final extension at 72°C for 7 min. The cycling conditions for hK2 were 95°C for 5 min, followed by 35 cycles of 94°C for 1 min, 56°C for 1 min and 72°C for 1 min and a final extension at 72°C for 10 min. Equal amounts of PCR products were electrophoresed on 1% agarose gels and visualized by ethidium bromide staining.

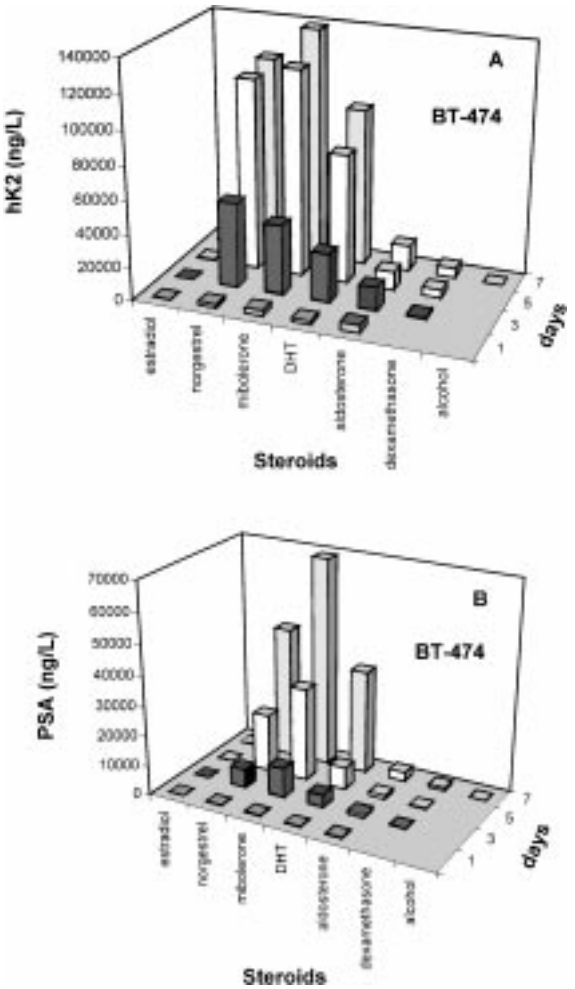

Figure 1. hK2 (A) and PSA (B) concentration in BT-474 cell line tissue culture supernatants after a single 10^{-7} M stimulation with various steroids and collection of the supernatants at 1, 3, 5, and 7 days post-stimulation.

Results

In BT-474 cells, we investigated which steroids stimulate the production of hK2 and PSA, as well as the time-course of protein accumulation into the tissue culture supernatants. We stimulated the cells with each steroid (estradiol, norgestrel, mibolerone, dihydrotestosterone (DHT), aldosterone and dexamethasone) at two concentrations (10^{-7} M and 10^{-9} M) and collected the supernatants at days 1, 3, 5, and 7 after stimulation. hK2 (Figure 1a) is produced at slightly higher amounts than PSA (Figure 1b). Overall, mibolerone and norgestrel are the strongest stimulators for both kallikreins, followed by DHT. The corticoids aldosterone and dexamethasone possessed a rather weak stimulatory activity, compared to the above steroids, while estradiol induced a small but significant hK2 and PSA production after day 5. Already in day 1, both hK2 and PSA were detectable after stimulation with norgestrel, mibolerone, DHT or aldosterone. In general, the production of hK2 was saturable at day 5 while for PSA we saw a further increase between days 5 and 7 (Figure 1).

For BT-474 cells, dose-response experiments were carried out to determine the lowest concentration of stimulating steroids which could still induce production of hK2 and PSA. Some data are presented in Figure 2. Norgestrel, mibolerone, and DHT were still active at concentrations down to at least 10^{-11} M, for both proteins. At levels higher than 10^{-9} M the

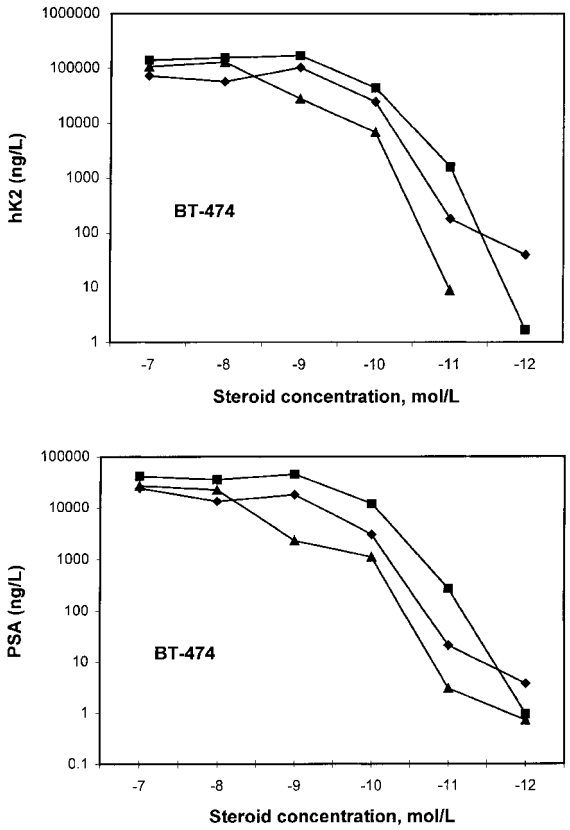

Figure 2. hK2 (A) and PSA (B) concentration in BT-474 cell line tissue culture supernatants 7 days after stimulation with norgestrel (♦), mibolerone (■), and DHT (▲) at different concentrations. DHT, dihydrotestosterone.

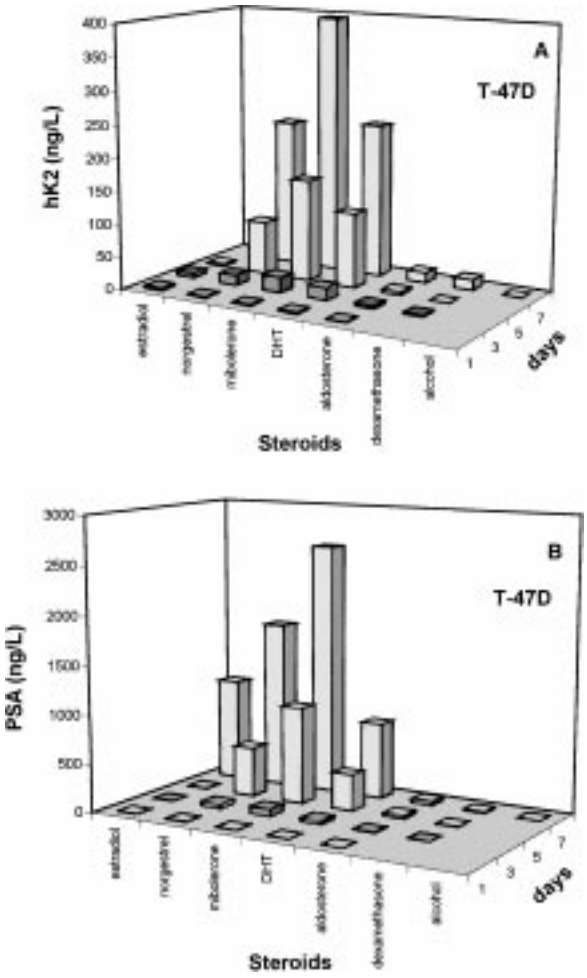

Figure 3. hK2 (A) and PSA (B) concentration in T-47D cell line tissue culture supernatants after a single 10^{-7} M stimulation with various steroids and collection of the supernatants 1, 3, 5, and 7 days post-stimulation.

response was saturated. Aldosterone was clearly a weaker stimulator, producing traces of hK2 and PSA at 10^{-10} M, but significantly inducing at concentrations $\geq 10^{-9}$ M. Dexamethasone and estradiol exerted a very weak stimulatory activity at concentrations 10^{-10} to 10^{-9} M, being significantly more potent only at 10^{-7} M for hK2 and 10^{-8} M for PSA.

In order to further elucidate the mechanism of steroid hormone regulation of hK2 and PSA in BT-474 cells, we conducted blocking experiments. Mifepristone (an antiprogestin) was able to block completely the stimulatory action of norgestrel on the production of both kallikreins, while RU 56,187 (an antiandrogen) also had a significant blocking effect on norgestrel. The anti-estrogens 4-OH-tamoxifen and ICI 182,780 did not show any significant blocking effects on the action of norgestrel. Both mifepristone and RU 56,187 blocked completely the action of DHT (data not shown).

The time course of steroid hormone stimulation of hK2 and PSA proteins was also examined in T-47D breast cancer cells. Traces of hK2 and PSA were detectable from the third day after stimulation, while the levels of the protein secreted into the culture medium were significantly increased further on the fifth and seventh day (Figure 3a). Mibolerone, DHT and norgestrel were, again, the most potent stimulators for hK2 and PSA production. Aldosterone and dexamethasone showed a very weak activity on the seventh day after the initial stimulation. Estradiol had no stimulatory effect on hK2 but did stimulate some PSA production on the seventh day (Figure 3b). The dose-response effect of the steroids on hK2 and PSA production by the breast cancer cell line T-47D

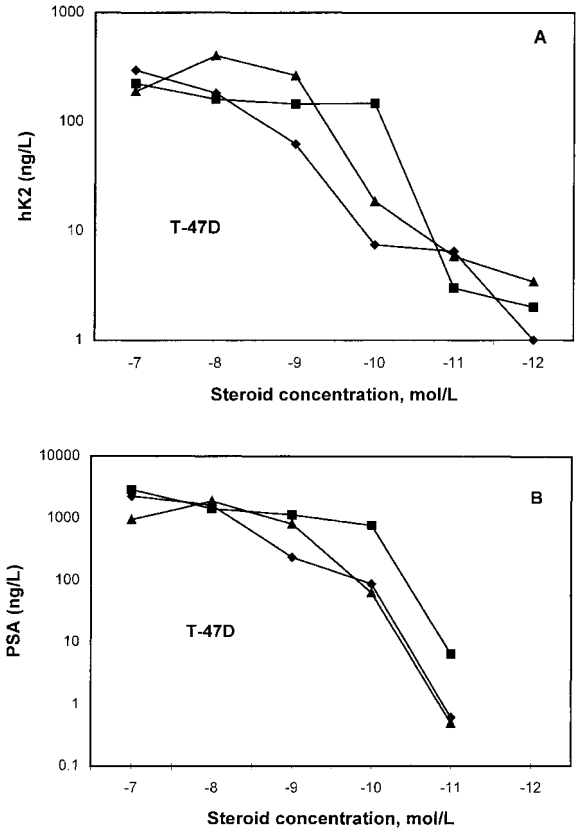

Figure 4. hK2 (A) and PSA (B) concentration in T-47D cell line tissue culture supernatants 7 days after stimulation with norgestrel (♦), mibolerone (■), and DHT (▲) at different concentrations. DHT, dihydrotestosterone.

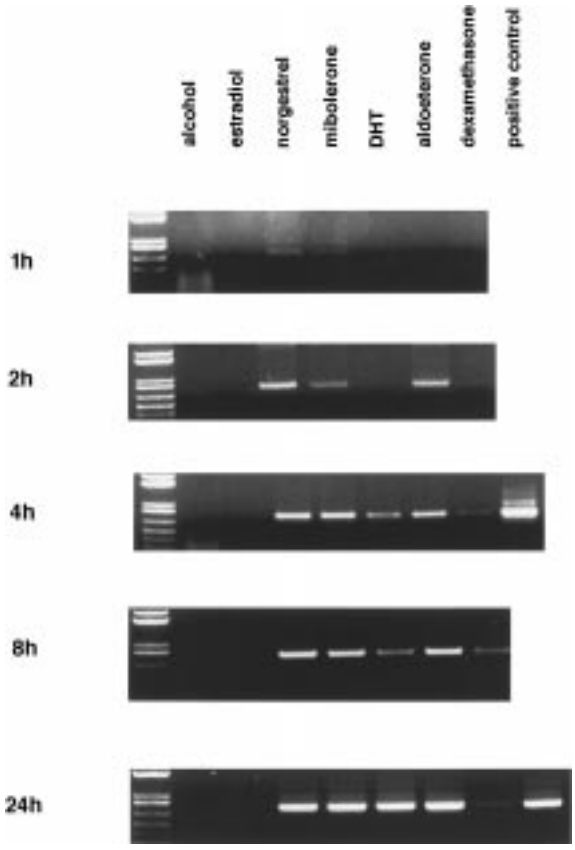

Figure 5. RT-PCR of hK2 mRNA extracted from BT-474 cells at 1, 2, 4, 8, and 24 h post-stimulation with various steroids. DHT, dihydrotestosterone. The length of the PCR product is 820 bp.

is shown in Figure 4. The expression of hK2 was induced more strongly by mibolerone, at levels of hormone 10^{-10} M, while the stimulatory effects of norgestrel and DHT were weaker but also apparent at this concentration. None of the rest of the steroids displayed any stimulatory activity. After 7 days of treatment, both androgens and the progestin were able to induce significant PSA expression at final concentration 10^{-11} M, whereas the glucocorticoid had a minor stimulatory effect only at 10^{-7} M. The mineralcorticoid and the estrogen had no effect. Blocking experiments confirmed the blocking activity of mifepristone on norgestrel for both of the kallikreins. The anti-estrogen ICI 182,780 blocked significantly the stimulatory effect of norgestrel on PSA production, but not on hK2 production. Norgestrel was also blocked partially by RU 56,187, mainly on its action on hK2 regulation. DHT was completely blocked by the anti-androgen RU 56,187 and by mifepristone, while the anti-estrogen ICI 182,780 produced a significant reduction on DHT action. We observed that 4-OH-tamoxifen induced production of PSA and especially of hK2, when incubated with DHT (synergistic phenomenon).

MFM-223 cells were also found to produce low but significant amounts of hK2 and PSA. In contrast to the BT-474 and T-47D cell lines, DHT was the strongest stimulator for the production of both kallikreins in MFM-223 cells, followed by mibolerone. Norgestrel was also a potent stimulator, while the estrogen and the corticoids showed no stimulatory activity. Time course experiments showed that both kallikreins were present at roughly equal levels and they were detectable in the medium even one day after treatment with their concentrations increasing till the seventh day (data not shown). Dose-response experiments revealed that only mibolerone could still induce production of hK2 and PSA down to a concentration of 10^{-11} M (data not shown). Both DHT and norgestrel were blocked effectively (>80%) by the RU 56,187 and by mife-

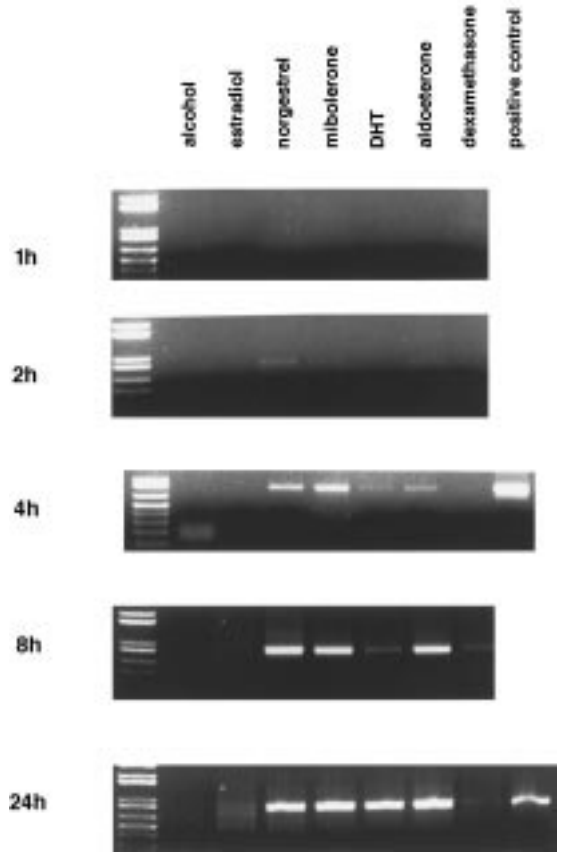

Figure 6. RT-PCR of PSA mRNA extracted from BT-474 cells at 1, 2, 4, 8, and 24 h post-stimulation with various steroids. DHT, dihydrotestosterone. The length of the PCR product is 754 bp.

pristone. ICI 182,780 had no blocking effect on the stimulatory action of the two steroids.

In order to study the kinetics of the hK2 and PSA mRNA regulation in BT-474 cells after stimulation by different steroid hormones, time course experiments were conducted. hK2 mRNA was detected 2 h after stimulation with norgestrel, mibolerone, and aldosterone. At 4 h, hK2 mRNA was also detectable in cells stimulated by DHT, while 8 h post-stimulation the effects of dexamethasone were also apparent (Figure 5). PSA mRNA appeared 1 h after stimulation with norgestrel and mibolerone. The induction of aldosterone and dexamethasone was apparent 2 h post-stimulation, while DHT-stimulated cells produced detectable mRNA after 4 h (Figure 6). The mRNAs of both kallikreins increased with time in all cases and persisted for at least 24 h after stimulation.

Several other human breast cancer cell lines (ZR-75-1, BT-20, MCF-7, MDA-MB-453) were tested for expression of hK2 and PSA in the presence or absence of steroid stimulation. None of these cells produced significant amounts of either hK2 or PSA.

Discussion

Mounting evidence suggests that PSA, a steroid hormone regulated gene, may have a significant effect on the pathophysiology of malignant breast tissue, and so could hK2, due to the striking similarities between these two kallikreins [1, 5, 6]. Consequently, the establishment of breast cancer cell lines which are able to produce sufficient amounts of these proteins, would be an important tool in the investigation of the molecular mechanisms that control the expression of these genes *in vitro* and *in vivo*. The elucidation of these mechanisms could facilitate a better understanding of the pathophysiology of steroid hormone-dependent breast tumors.

The BT-474 breast cancer cell line is a steroid receptor-positive cell line, which produces large amounts of PSA and hK2 upon induction with androgens and progestins (reported here for the first time). hK2 was found to be produced in slightly higher amounts than PSA, when induced by different steroids (Figure 1). Among all the steroids tested, mibolerone, and norgestrel were the most potent stimulators of both kallikreins. Mibolerone (7a, 17a-dimethyl-19-nortestosterone) is a synthetic androgen, stable to metabolic conversion, which has been shown to also stimulate progestin specific activity at nM or pM levels. These effects seem to be mediated by the progesterone receptor (PR), since they are completely abolished by antiprogestins [20]. In our experiments, mibolerone was able to induce hK2 and PSA protein production at concentrations, down to 10^{-11} M. Norgestrel, a synthetic progestin, was also a strong stimulator for kallikrein production, at concentrations as low as 10^{-12} M, indicating that its action is likely mediated via high affinity binding to PR receptor. These results support our previous hypothesis that PSA production is mediated independently by either the PR or the AR [13]. This is likely also the case for hK2. Dihydrotestosterone (DHT), the reduced form of the physiological androgen testosterone, was able to induce hK2 and PSA production at levels as low as 10^{-10} M. Aldosterone, a natural mineralocorticoid, and dexamethasone, a synthetic glucocorticoid, were capable of hK2 and PSA regulation only weakly and at concentrations $\geq 10^{-9}$ M and $\geq 10^{-8}$ M, respectively. These results suggest that these corticoids have some

capability to regulate both kallikreins through their cognate receptors. Finally, estradiol was able to induce the secretion of small but significant amounts of hK2 and PSA at concentrations around 10^{-7} M. Since an estrogen response element has not been found in the promoter region of these kallikrein genes, we do not support the view that estradiol induces their transcription through the ER. Mutated ARs are believed to mediate induction of PSA production by estradiol in prostate cancer cells [21] but we did not examine this possibility in BT-474 cells. Furthermore, another group [22] has found that estradiol can activate androgen-regulated genes in the presence of AR and the AR co-activator ARA_{70}. This is direct evidence to support the 'tripartite receptor system' concept [23] and indicates that receptor specificity (estradiol binding to AR) and biological diversity (estradiol activates AR targeted genes) of the steroid hormone family can be conferred at the level of cofactors. Thus, it is reasonable to suggest that BT-474 cells may contain co-activators that allow transcriptional regulation of the PSA and hK2 genes by estradiol via AR or other receptors.

Mifepristone, an antiprogestin with antiglucocorticoid and antiadrogenic activity, has weak agonistic activity [13], but was also a strong antagonist, blocking completely the action of norgestrel (100%) and of DHT (85–100%) for both kallikreins. RU 56,187, an antiandrogen with high affinity for the androgen receptor, was able to block completely the action of DHT, as expected, but also blocked effectively (65–70%) norgestrel's activity, indicating that a significant portion of the progestin's stimulatory action is likely mediated through the androgen receptor. This also becomes evident by the finding that norgestrel induced PSA and hK2 production in the MFM-223 cells, which lack sufficient concentrations of PRs [24]. Estradiol, even though it has a small stimulatory effect on hK2 and PSA production, it blocks, at high concentrations, the action of DHT, either by competing for androgen binding to AR [13] or by forming an active complex with the ER, which can modulate the AR transcriptional activity [25] or by consumption of factors of the transcriptional machinery (squelching).

With T-47D cells, our results confirmed the data previously reported [13, 16]. Briefly, androgens, progestins, glucocorticoids and mineralocorticoids were able to induce significant PSA and hK2 expression, while estradiol and 4-OH tamoxifen had no stimulatory effect. However, in T-47D cells, significantly more PSA than hK2 accumulated in media with time, and this was verified repeatedly by a series of experiments, in contrast to previous reports [16]. Although both BT-474 and T-47D breast tumor cell lines have roughly the same content of steroid receptors, they show significantly different mode of regulation of PSA and hK2 secretion, suggesting that there are likely cell-specific differences (e.g., levels of co-factors and co-repressors) determining the potency of response of these cell lines.

Table 1. ER, PR and AR expression in six human breast cancer cell lines

Cell line	Receptors levels*		
	ER	PR	AR
BT-474	+	+	+
T47-D	+	+++	+
MCF-7	++	+	+
ZR-75- 1	+	+	++
MDA-MB-453	−	−	+++
BT-20	−	−	−
MFM-223**	−	+++	

*Determined by radioligand binding. [Adapted and modified from ref 26].
**Determined with the charcoal dextran method [24].

This conclusion is also supported by the results from the other cell lines tested. None of them produced any significant amounts of either hK2 or PSA, with the exception of the MFM-223 cell line, even though the MCF-7 and the ZR-75-1 cells possess all types of steroid receptors (Table 1). It is of interest that the MDA-MB-453 cell line failed to produce any amounts of either PSA or hK2 after stimulation with androgens (mibolerone, DHT). MDA-MB-453 cells express high levels of AR mRNA and protein, whereas expression of ER or PR mRNA and protein could not be detected. Evidence that the AR is functional is provided by the observation that androgens induce the activity of endogenous androgen-responsive genes (e.g. fatty acid synthetase, gross cystic disease fluid protein 15 and the prolactin receptor) [26]. Thus, it appears that the MDA-MB-453 cell line shares the same characteristics with the MFM-223 cells (androgen responsive, lack of sufficient amounts of other steroid receptors), however, it does not produce either PSA or hK2. This argues in favor of the presence of cell-specific factors, necessary for the expression of these genes. We believe that our cell lines may represent a good model for identifying co-factors that are important in the regulation of PSA and hK2 genes.

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