

Oestrogen receptor β is required for androgen-stimulated proliferation of LNCaP prostate cancer cells

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Abstract

The role of oestrogens in the development of prostate cancer is poorly understood. However, a large body of evidence has suggested that oestrogenic hormones may be involved in prostatic malignancy. The localization of oestrogen receptor β (ER β) in the secretory epithelium of the human prostate has raised the intriguing possibility that the action of oestrogen could be mediated, at least in part, by this receptor during the process of carcinogenesis. Hence, specific interference with oestrogen-activated and ER β -mediated transcriptional activity could open new issues in the endocrine manipulation of prostate tumours. In the present study, we provide new insights into the role of ER β in the context of an androgen-responsive prostate cancer cell line such as LNCaP, which was used as a model system together with steroid receptor negative HeLa cells. ER β and the mutated androgen receptor (AR) T877A did not discriminate between oestrogen- or androgen-induced transactivation, whereas ER β and AR transcriptional activity were inhibited only by the respective hormone antagonists ICI 182,780 and casodex. Furthermore, the nuclear localization of ER β evaluated by immunocytochemistry confirmed the promiscuous response to hormones in addition to the specific inhibitory action of antagonists. Interestingly, ICI 182,780 and an ER β antisense expression vector repressed the growth effects of both 17 β -oestradiol and 5 α -dihydrotestosterone, suggesting that ER β has a key role in the proliferation induced by these steroids in LNCaP prostate cancer cells. Thus our findings implicate ER β as a potential target for the treatment of prostate tumours.

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Introduction

Epidemiological and experimental studies have suggested that oestrogenic hormones are involved in prostatic malignancy (Taplin & Ho 2001, Weihua *et al.* 2001, and references therein), although their action remains to be completely understood. In a variety of mammals, including humans, pharmacological doses of oestrogens may induce proliferative alteration of the prostatic epithelium such as squamous metaplasia (Mawhinney & Neubauer 1979, Levine *et al.* 1991), and similar effects can also be promoted by

oestrogens in the regressed prostates of hypophysectomized or castrated dogs (Leav *et al.* 1978, Merk *et al.* 1986).

It has been demonstrated that the effects of oestrogens on target tissues are mediated by the oestrogen receptors α and β (ER α and ER β), which are ligand-inducible transcription factors (Kuiper *et al.* 1996). They present 10–15% homology in the N-terminal region, more than 90% homology in the DNA-binding domain and more than 60% homology in the ligand-binding domain, and share similar ligand-binding affinities (Mosselman *et al.* 1996). Both receptors are distributed in many of

the same organs; however, a distinct tissue and cell type localization, together with different levels of expression, have been reported (Enmark *et al.* 1997, Kuiper *et al.* 1997). For instance, in the prostate, ERα was localized in normal and malignant stroma (Schulze & Claus 1990, Brolin *et al.* 1992, Ehara *et al.* 1995), whereas ERβ was found to exhibit a progressive loss of expression in the secretory epithelium after the process of carcinogenesis (Horvath *et al.* 2001, Leav *et al.* 2001). ER-mediated gene expression (Carruba *et al.* 1994, Castagnetta *et al.* 1995, Lau *et al.* 2000) and cross-talk between ERs and other steroid receptors that trigger non-genomic proliferative pathways (Migliaccio *et al.* 2000, and references therein) have been demonstrated in several human prostate cancer cell lines. Interestingly, in our and other studies (Lau *et al.* 2000, Migliaccio *et al.* 2000, Maggiolini *et al.* 2002), the pure oestrogen antagonist ICI 182,780 inhibited the proliferation of human prostate cancer cells expressing ERβ. However, the mechanisms of action of oestrogens and antioestrogens in this system have not been fully clarified.

Herein, we provide new insights into the role of ERβ in the context of an androgen-responsive prostate cancer cell line, LNCaP, which was used as a model system together with steroid receptor negative HeLa cells. We demonstrate that ERβ and the mutated androgen receptor (AR) T877A do not discriminate between oestrogens or androgens in mediating transcription, whereas they are inhibited only by the respective hormone antagonists, ICI 182,780 and casodex. Most notably, ICI 182,780 and an ERβ antisense expression vector reverse the proliferative effects of both 17β-oestradiol (E2) and 5α-dihydrotestosterone (DHT), demonstrating that ERβ is involved in the growth response to these hormones in LNCaP prostate cancer cells.

Materials and methods

Reagents

DHT and E2 were purchased from Sigma (Italy), ICI 182,780 was purchased from Tocris Northpoint (UK), casodex was a gift from Astra-Zeneca (Italy), and Src inhibitor PP2 was purchased from Calbiochem (Italy).

Plasmids

Firefly luciferase reporter plasmids used were: XG46TL (Bunone *et al.* 1996) for the AR, GK1 for

the Gal4 fusion protein Gal93ERβ (Maggiolini *et al.* 2001), and XETL (Maggiolini *et al.* 2001), pS2 ERE and pS2ΔERE (both gifts from V. Giguère, Montreal, Canada) for ERβ. pS2 ERE contains the ~1050 bp pS2 promoter and PCR mutagenesis yielded the oestrogen response element (ERE) deletion construct, pS2ΔERE (Tremblay *et al.* 1997). The AR mutant T877A (a gift from F.S. French, Chapel Hill, NC, USA) was constructed by a two-step PCR method replacing the HindIII/BamHI fragment from the wild type in pCMVhAR (Tan *et al.* 1997). The *renilla* luciferase expression vector pRL-TK (Promega) was used as a transfection standard. The ERβ antisense plasmid contains, in reverse orientation, a 1170 bp fragment of the coding sequences of the human ERβ in the pIRESpuro2 vector (Clontech).

Cell culture

Human prostate cancer LNCaP cells (a gift from R. Baserga, Philadelphia, USA) were grown in RPMI 1640 medium without phenol red and supplemented with L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 U/ml) and 10% fetal calf serum (FCS). HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM) without phenol red and supplemented with L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 U/ml) and 10% FCS. Cells to be processed for immunoblotting and immunocytochemical staining were switched to medium without serum 1 and 3 days before treatments, respectively (see also Results).

Transfections and luciferase assays

Cells were transferred into 24-well plates with 500 µl regular growth medium/well the day before transfection. The medium was replaced with RPMI or DMEM lacking phenol red in addition to serum on the day of transfection, which was performed using the Fugene6 Reagent as recommended by the manufacturer (Roche Diagnostics), with a mixture containing 0.5 µg reporter plasmid, 2 ng pRL-TK and 0.2 µg plasmids expressing the AR or ER (where applicable). After 5–6 h, the medium was replaced with RPMI or DMEM lacking phenol red and supplemented with 1% charcoal stripped FCS; ligands were added at this point, and cells were incubated for 24 h. Luciferase activity was

then measured with the Dual Luciferase Kit (Promega) according to the manufacturer's recommendations. Firefly luciferase activity was normalized to the internal transfection control provided by the *renilla* luciferase activity.

Immunocytochemical staining

Cultured LNCaP cells were fixed in fresh paraformaldehyde (2% for 30 min). After removal of the paraformaldehyde, hydrogen peroxide (3% in methanol for 30 min) was used to inhibit endogenous peroxidase activity. Cells were then incubated with normal horse serum (10% for 30 min) to block the non-specific binding sites. Immunocytochemical staining was performed using a mouse monoclonal IgG primary antibody (Serotec, Oxford, UK) generated against the C-terminus of the human ER β (1:50 overnight at 4 °C). A biotinylated horse antimouse IgG (1:600) was applied for 60 min at room temperature, as the secondary antibody (Vector Laboratories, Burlingame, California, USA). Subsequently, antibody bindings were visualized by using the avidin–biotin–horseradish peroxidase complex (Vector Laboratories, Burlingame, California, USA) (1:100 for 30 min at room temperature) and 3,3'-diaminobenzidine tetrachloride dihydrate (Vector Laboratories) was used as detection system. After each step, cells were rinsed with Tris buffer saline (0.05 M Tris-HCl plus 0.15 M NaCl, pH 7.6) containing 0.05% Triton-X100. In control experiments, cells were processed, replacing the primary antibody with mouse serum (Dako) or by using a primary antibody pre-absorbed (48 h at 4 °C) with an excess of purified ER β protein (M-Medical, Milan, Italy).

Immunoblotting

LNCaP cells were grown in 10 cm dishes and exposed to ligands for 24 h before lysis in 500 μ l 50 mM HEPES (pH 7.5), 150 mM NaCl, 1.5 mM MgCl₂, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 1% SDS, and a mixture of protease inhibitors containing 1 mM aprotinin, 20 mM phenyl methylsulphonyl fluoride and 0.2 M sodium-orthovanadate. Equal amounts of whole-protein extract were resolved on a 10% SDS-polyacrylamide gel. Proteins were transferred to a nitrocellulose membrane, probed with the

antibodies against ER β (Serotec), AR and β -actin (both from Santa Cruz Biotechnology, Santa Cruz, California, USA), and revealed using the ECL System (Amersham).

Proliferation assays

For quantitative proliferation assays, 1×10^4 LNCaP cells were seeded in 24-well plates in regular growth medium. Once they had attached, cells were washed, transfected with 500 ng/well ER β antisense where applicable and incubated in medium without serum for 24 h. The next day the medium was changed and supplemented with 1% charcoal-stripped FCS. Ligands were added at this point; thereafter, medium was changed every day and treatments were renewed. On day 5 (after 3 days of treatment), cells were trypsinized and counted in a haemocytometer using the trypan blue exclusion method.

Statistical analysis

Statistical analysis was performed using analysis of variance followed by Newman–Keuls testing to determine differences in means. *P* values < 0.05 were considered significant.

Results

ER β and AR mutant T877A are cross-activated by E2 and DHT but not cross-inhibited by antihormones

The mutant AR T877A is expressed together with ER β , but not ER α , in LNCaP human prostate cancer cells (Lau *et al.* 2000), which have been used as a model for the androgen-sensitivity of prostate tumours (Webber *et al.* 1997). The mutation T877A influences the ligand-binding specificity of AR (Sack *et al.* 2001), allowing its transactivation by a variety of compounds including androgen antagonists and oestrogens (Elo *et al.* 1995, Kempainen & Wilson 1996, Tan *et al.* 1997, Grigoryev *et al.* 2000, Maggiolini *et al.* 2002). As for ER β , there are no available data regarding the transactivating properties of the natural ligand E2 or other steroids in the context of prostate cancer cell lines. Thus, as an initial step toward elucidating the transcriptional capability of ER β , we transiently transfected LNCaP cells with the ER reporter plasmid, XETL,

which carries the *firefly* luciferase coding sequences under the control of an ERE upstream of the thymidine kinase promoter. Likewise, cells were cotransfected with the expression vector of *renilla* luciferase to provide an internal control and, in most experiments as indicated, with a plasmid encoding ER β to boost the response of the endogenous receptor (Fig. 1). Luciferase activity of cells receiving vehicle was set as onefold induction, upon which the results of treatments were calculated.

Figure 1 shows that the endogenous and transfected ER β were able to respond to both E2 and DHT, and that activation was inhibited by the oestrogen antagonist ICI 182,780 but not by the androgen antagonist casodex or the Src inhibitor PP2. Next, we investigated whether ER β could induce the transcriptional activity of an ERE-containing natural promoter such as the pS2 promoter (Berry *et al.* 1989). Figure 2 demonstrates that E2 and DHT induced an efficient receptor transactivation, again reversed only by the anti-oestrogen ICI 182,780. The response was clearly mediated by the ER, as deletion of the ERE abolished the response to both hormones (Fig. 2B). Results similar to those with DHT were also obtained using the synthetic androgen, R1881 (data not shown).

To assess the potential of E2 and DHT, and of antihormones, for modulating transcription via AR, we transiently transfected LNCaP cells with the reporter plasmid, XG46TL, which carries firefly luciferase sequences under the control of an androgen response element (ARE) upstream of the thymidine kinase promoter. Figure 3 reveals that E2 and DHT were direct AR inducers, as the transactivation was inhibited by the androgen antagonist casodex but not by the oestrogen antagonist ICI 182,780 or the Src inhibitor PP2. Thus E2 and DHT are cross-activators of ER β and the AR mutant T877A expressed in LNCaP cells, whereas ICI 182,780 and casodex inhibit the transactivation of such receptors in a specific manner.

To provide further evidence that ER β is directly activated by E2 and DHT, the reporter plasmids XETL and pS2 ERE were transfected together with the ER β expression vector in steroid receptor negative HeLa cells (Fig. 4A). The results were similar to those obtained in LNCaP cells, confirming the potential effect of hormones and

antagonists on ER β -mediated transcriptional activity. Nuclear receptors such as ERs contain two main transcription activation functions (AF): the N-terminal AF1 and the C-terminal, hormone binding domain (HBD)-associated AF2, which is known to be agonist dependent (Kumar *et al.* 1987). A chimeric protein consisting of the heterologous DNA binding domain of the yeast transcription factor Gal4 and the ER β -HBD was able to respond to E2 and DHT in a transient expression assay (Fig. 4B). Also in this context, transcription was inhibited by ICI 182,780 but was not influenced by casodex (Fig. 4B).

Finally, using HeLa cells we verified the ability of DHT and E2 to transactivate directly the AR mutant T877A that was specifically inhibited by casodex (Fig. 4C). In agreement with the findings of a previous study (Panet-Raymond *et al.* 2000), coexpression of AR and ER β resulted in no significant change in receptor activation by either oestrogen or androgen (data not shown).

E2 and DHT induce nuclear localization of ER β in LNCaP cells

The genomic actions of steroid hormones are mediated by steroid receptors, which in their unliganded form reside associated with molecular chaperones in the cytoplasm, the nuclear compartment, or both (Beato *et al.* 1995, Pratt & Toft 1997). For ER α , mainly localized in the nucleus even in the absence of hormone, ligand binding leads to conformational changes that result in receptor activation and transcriptional regulation of target genes (Tsai & O'Malley 1994).

To provide evidence that E2 and DHT are able to induce the nuclear localization of the activated endogenous ER β expressed in LNCaP cells, we analysed its subcellular distribution by immunocytochemistry after a short (1 h) treatment with ligands.

ER β antibodies are now available from a number of commercial and private sources; however, considerable variability in specificity and sensitivity has been reported among the results of different studies (Skloris *et al.* 2002, and references therein). To verify the specificity of the antibody used (see Materials and methods), we transfected the steroid-receptor negative HeLa cells with an expression plasmid encoding ER β , which revealed a strong nuclear immunodetection not observed in

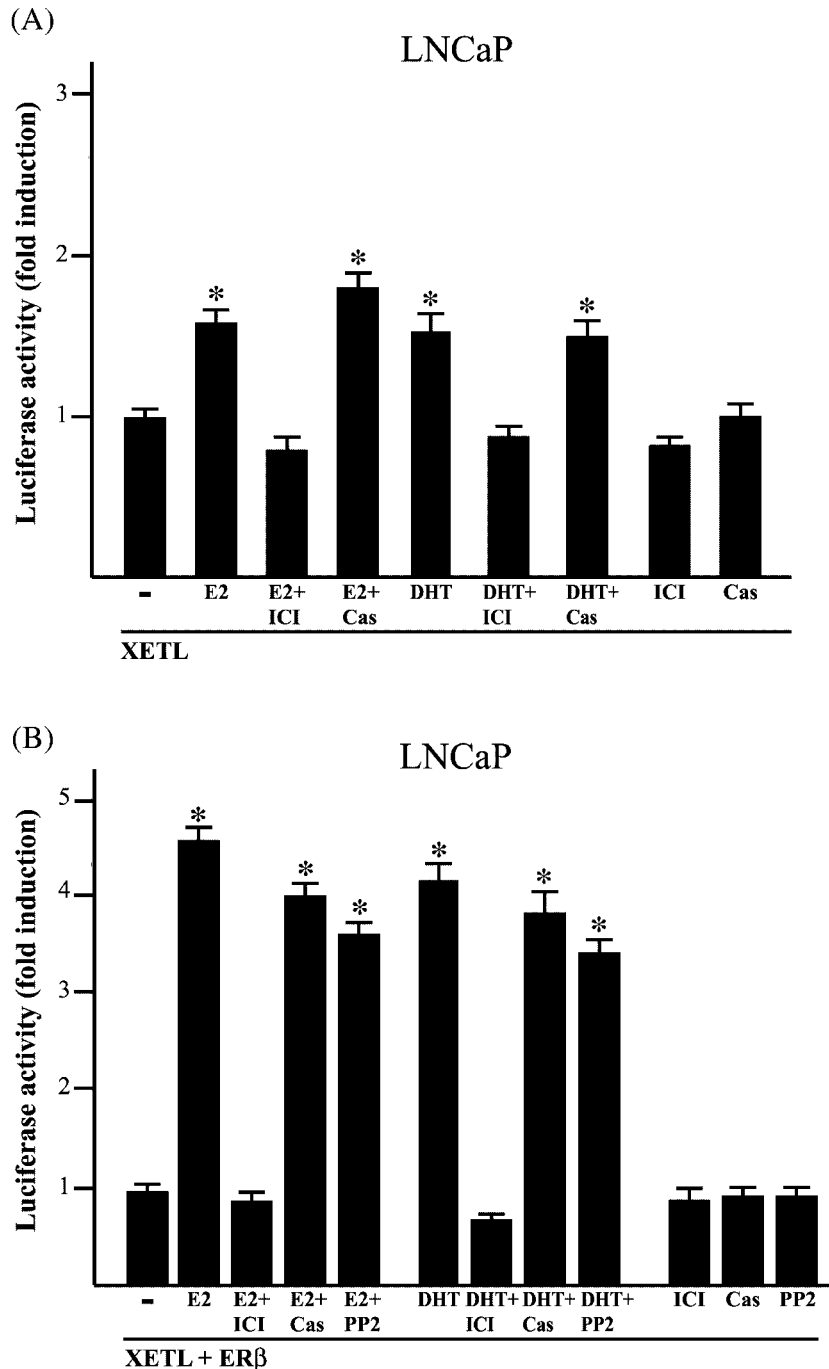


Figure 1 The activation of ERβ by E2 and DHT in LNCaP cells is inhibited only by ICI 182,780 (ICI). (A) The reporter plasmid XETL was transfected to evaluate the response of endogenous ERβ after a 24 h exposure to 10 nM E2 and DHT and 1 μM ICI 182,780 and casodex (Cas). Luciferase activity of cells receiving ethanol alone (-) was set as onefold induction, upon which treatments were calculated. (B) The response to hormones and antagonists was assayed as in (A), except that XETL was co-transfected with an expression plasmid encoding ERβ. The Src inhibitor PP2 was used at a concentration 5 μM. Each data point represents the mean±s.d. of three independent experiments performed in triplicate. *P<0.05 compared with ethanol alone (-).

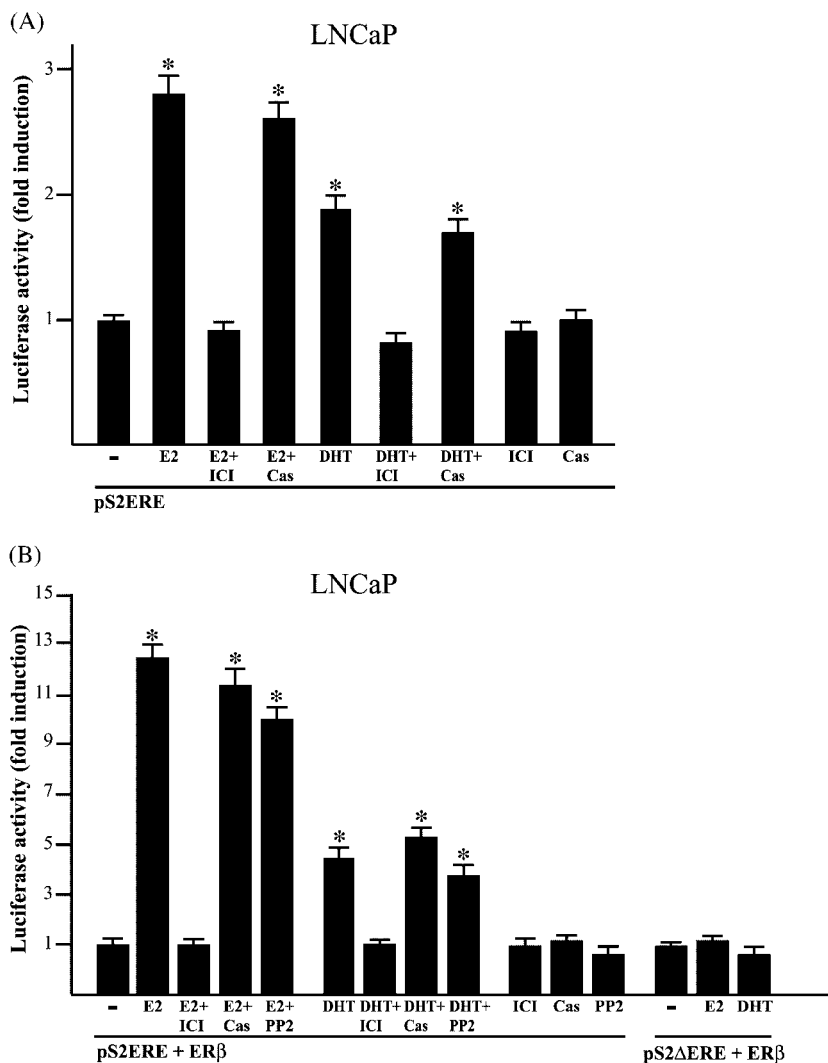


Figure 2 The ERE sequence is required for the activation of ER β by E2 and DHT in LNCaP cells. (A) An intact pS2 promoter (pS2 ERE) was transfected to evaluate the response of endogenous ER β after a 24 h exposure to 10 nM E2 and DHT and 1 μ M ICI 182,780 and casodex (Cas). Luciferase activity of cells receiving ethanol alone (-) was set as onefold induction, upon which treatments were calculated. (B) The response to hormones, antagonists and the Src inhibitor PP2 was assayed as in (A), except that the reporter gene pS2 ERE or the deleted pS2 promoter without ERE (pS2 Δ ERE) were co-transfected with an expression plasmid encoding ER β . Each data point represents the mean \pm s.d. of three independent experiments performed in triplicate. * P <0.05 compared with ethanol alone (-).

cells transfected with the vector alone (Fig. 5A). Then we turned to the LNCaP cells, which required 3 days of serum deprivation in order to achieve complete abolition of detectable ER β immunoreactivity (Fig. 5B; see comments below). This basal condition was essential to reveal the

hormone-specific activity. In fact, the addition of E2 or DHT produced a strong staining intensity exclusively in the nuclear compartment, as demonstrated by the representative immunoreactions in Fig. 5B. In contrast, no signals were observed either when the anti-ER β antibody was

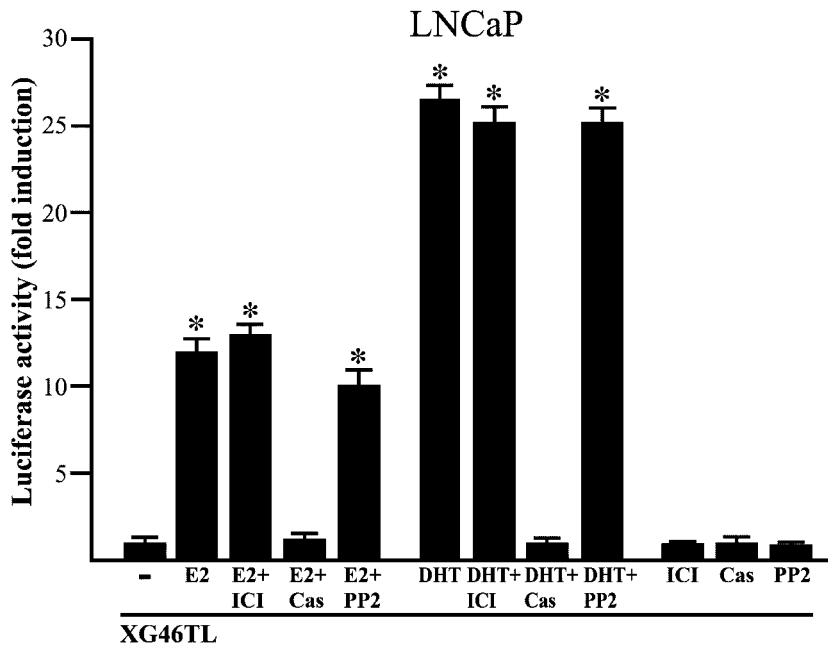


Figure 3 The activation of AR mutant T877A by E2 and DHT is inhibited only by casodex (Cas) in LNCaP cells. Transfections were performed with the reporter gene XG46TL to evaluate the response of the AR mutant T877A after a 24 h exposure to 10 nM E2 and DHT, 1 μ M ICI 182,780 and Cas and 5 μ M Src inhibitor PP2. Luciferase activity of cells receiving ethanol alone (-) was set as onefold induction, upon which treatments were calculated. Each data point represents the mean \pm s.d. of three independent experiments performed in triplicate. * P <0.05 compared with ethanol alone (-).

replaced by irrelevant mouse IgG or when the primary antibody preabsorbed with an excess of receptor protein (data not shown). Interestingly, ICI 182,780, but not casodex, reversed the immunoreactive signals elicited by both hormones in accordance with their ability to transactivate ER β in a direct manner (Fig. 5B).

Expression of ER β is up-regulated by E2 and DHT in LNCaP cells

In view of the ability of E2 and DHT to induce the nuclear accumulation and the transcriptional activity of ER β , we aimed to examine the effects of hormones on the expression of ER β protein in LNCaP cells. Figure 6 shows that a 24 h exposure to either steroid significantly enhanced the protein content of endogenous ER β . It is of note that, in the immunocytochemistry assay, the basal ER β signal was silenced only after 3 days of serum-deprived conditions. The ER β up-regulation by agonists observed in the present investigation and

in a recent study (Power & Thompson 2003) parallels that of the AR mutant T877A that resulted from the stabilization of the ligand-receptor complex (Krongard *et al.* 1991, Zhou *et al.* 1995, Yeap *et al.* 1999, Maggiolini *et al.* 2002), and further correlates the activity of both receptors in LNCaP cells. Likewise, an increased expression of ER α in response to E2 has been reported in Ishikawa endometrial carcinoma cells (Robertson *et al.* 2002), whereas it has largely been demonstrated that ER α is down-regulated by agonists in breast cancer cells (Santagati *et al.* 1997, Maggiolini *et al.* 1999, 2001).

ICI 182,780, casodex and ER β antisense block the E2- and DHT-induced proliferation of LNCaP cells

Having established that DHT and E2 are cross-agonists for both the AR mutant T877A and ER β whereas casodex and ICI 182,780 are specific androgen and oestrogen antagonists,

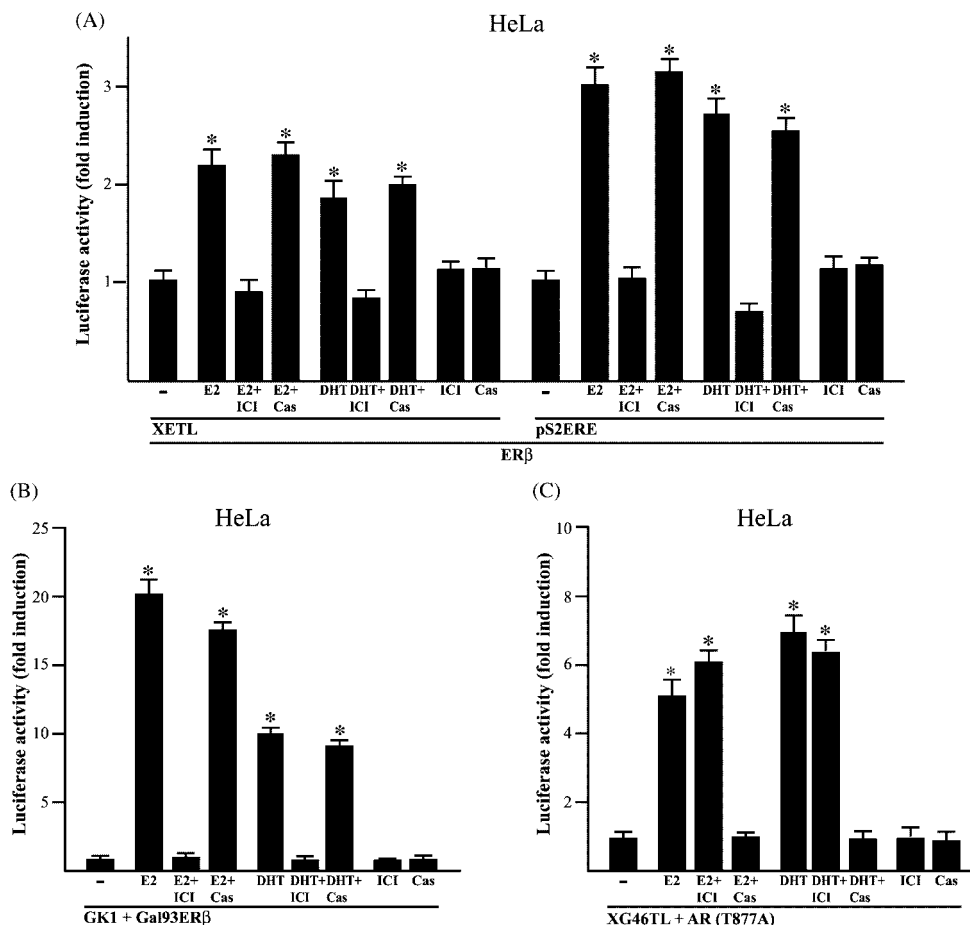


Figure 4 E2 and DHT are agonists for both ER β and AR (T877A), whereas ICI 182,780 and casodex (Cas) are specific antagonists in HeLa cells. (A) The reporter plasmids XETL or pS2 ERE were co-transfected with an expression plasmid encoding ER β to evaluate the response of a 24 h exposure to 10 nM E2 and DHT and 1 μ M ICI 182,780 and Cas. Luciferase activity of cells receiving ethanol alone (-) was set as onefold induction, upon which treatments were calculated. (B) The Gal4 reporter gene GK1 and the fusion protein Gal93ER β , consisting of the Gal4 DNA binding domain and the ER β hormone binding domain, were co-transfected to evaluate the response of hormones and antagonists as in (A). (C) The reporter gene XG46TL and the AR mutant T877A were co-transfected to evaluate the response of hormones and antagonists as in (A). Each data point represents the mean \pm s.d. of three independent experiments performed in triplicate. * P <0.05 compared with ethanol alone (-) for same reporter plasmid.

respectively, we attempted to evaluate the physiological interplay of hormones and antihormones in a complex response such as cell proliferation. Hence, we treated LNCaP cells with 10 nM DHT or E2 in combination with 1 μ M casodex or ICI 182,780. Medium and treatments were renewed every day for 3 days and then cells were trypsinized and counted. The growth response was then expressed as percentage of cells after treatments in

respect to those treated with ethanol used in the same volume as for compounds tested (Fig. 7A). Both DHT and E2 significantly stimulated cell proliferation, correlating with their ability to activate the AR mutant T877A. Interestingly, cell growth promoted by hormones was inhibited by either casodex or ICI 182,780. Note that only ICI 182,780 functions as an ER β antagonist in transactivation assays. The repression of LNCaP

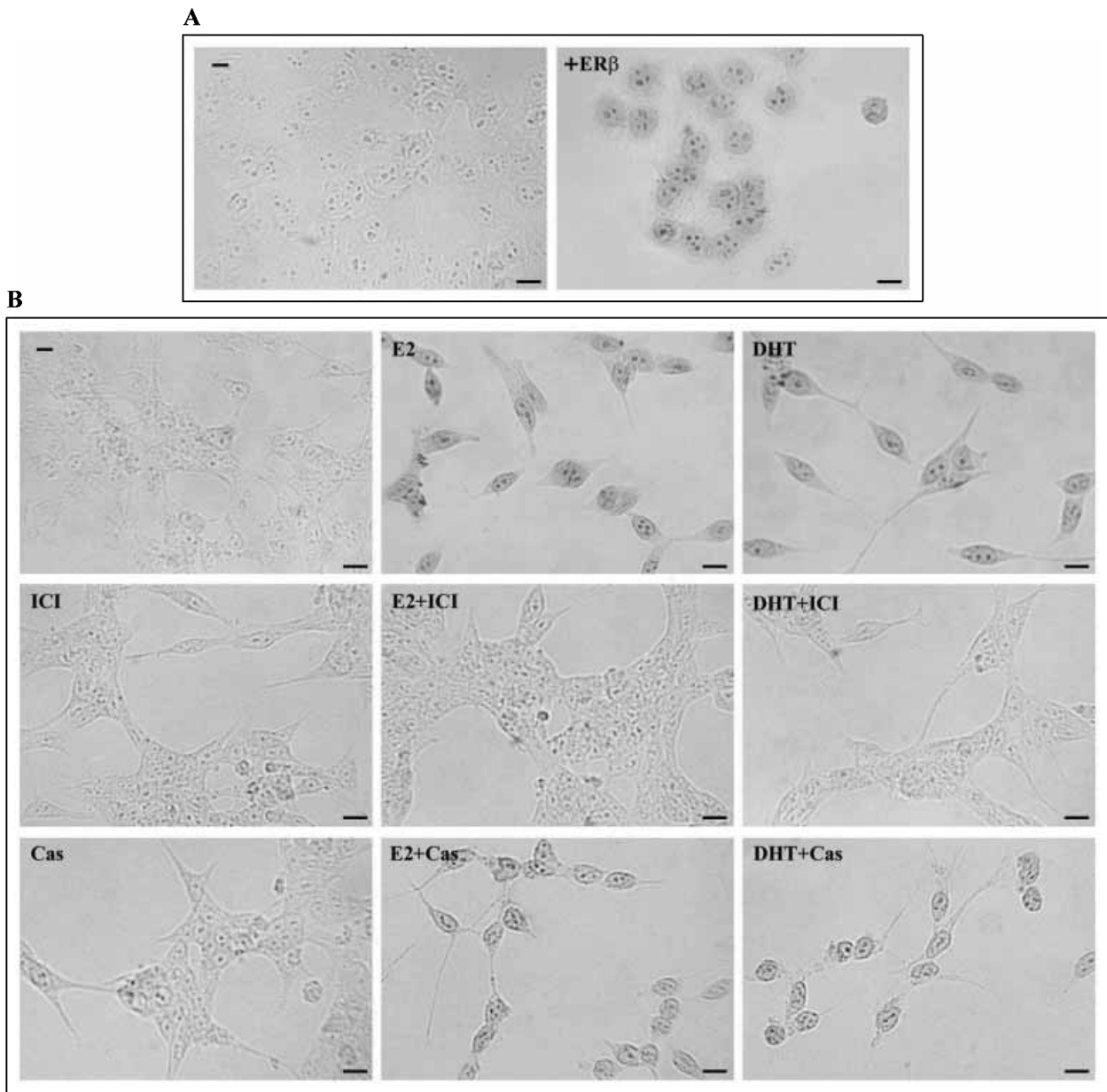


Figure 5 The nuclear compartmentalization of ER β induced by E2 and DHT in LNCaP cells is inhibited by ICI 182,780 but not by casodex (Cas). (A) Steroid receptor negative HeLa cells were transfected with an empty vector (-) or with an expression plasmid encoding ER β (+ER β) and treated 1 h with 10 nM E2 to verify the specificity of the antibody used. (B) LNCaP cells were treated with ethanol (-) or 10 nM E2 and DHT and 1 μ M ICI 182,780 and Cas for 1 h. Each experiment is representative of at least 10 tests. Bar represents 5 μ M.

proliferation by ICI 182,780, which displayed no effect on the transcriptional activation of AR by hormones, prompted us to utilize a different method to analyse the role of ER β in the growth of these androgen-responsive prostate cancer cells. Hence, we transfected LNCaP cells with an ER β

antisense expression vector to block expression of ER β . The ER β antisense construct effectively reduced ER β protein concentrations and inhibited only the growth of LNCaP cells stimulated by E2 or DHT (Fig. 7B, C), providing further evidence for an involvement of ER β in the proliferative

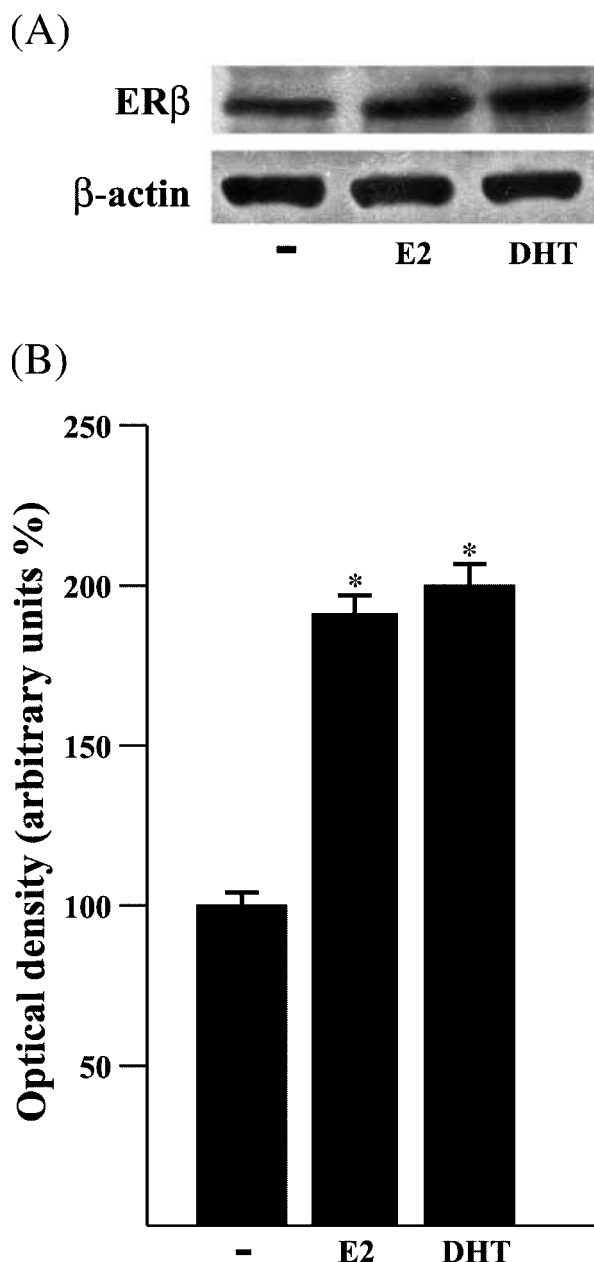


Figure 6 Immunoblot of ERβ from LNCaP. (A) Cells were treated for 24 h with ethanol (-) or with 10 nM E2 and DHT, and 70 μg total protein lysate was used per lane to evaluate ERβ protein levels. β-Actin served as loading control. (B) Quantitative representation of data of three independent experiments including that of (A) after densitometry and correction for values of cells treated with vehicle. * $P < 0.05$ compared with ethanol alone (-).

response to both hormones. Of note, the expression of AR was not changed by the ERβ antisense expression plasmid (Fig. 7B).

Discussion

Our results provide evidence for a proliferative role of ERβ in LNCaP cells, which have been used extensively as a model for androgen-responsive prostate cancer (Webber *et al.* 1997). These cells express a point mutation of the AR that allows it to respond to both androgens and oestrogens. Both types of hormone are also able to stimulate the proliferation of these cells and, therefore, it had been assumed that the proliferative stimulus of oestrogen was solely attributable to its ability to activate the AR mutant. To our surprise, either antagonist is able to block the proliferative stimulus of either steroid. These and additional findings call for a fundamental reinterpretation of the oestrogenic stimulation of LNCaP cell proliferation. We conclude that oestrogens stimulate the proliferation of these cells by activating both ERβ and AR. This suggests that the evaluation of receptor status in prostate tumours should include ERβ in addition to AR. At least for a subset of AR⁺ ERβ⁺ prostate tumours, antioestrogen treatment such as ICI 182,780 should be considered in addition to androgen ablation therapies.

We have shown that the mutated AR T877A and even ERβ were transactivated by DHT and E2 both in LNCaP cells and in HeLa cells that were engineered to express both receptors. Interestingly, such a promiscuous response of ERβ recalls previous studies on the role of androgens as potential agonists for ERα (Ekena *et al.* 1998, Maggiolini *et al.* 1999), indicating, furthermore, that the two ER isoforms exhibit similar ligand-dependent AF2 activity (Cowley & Parker 1999, McInerney *et al.* 1998). In contrast, the AF1 response of ERβ is weaker than that of ERα (Cowley & Parker 1999) and, as a consequence, ERα activation greatly exceeds that of ERβ when both AF1 and AF2 are active in a particular cell or promoter context (McInerney *et al.* 1998, Hall & McDonnell 1999, Delaunay *et al.* 2000). As observed in the present study, E2 and DHT cross-activated ERβ and the mutated AR; however, the transcriptional activity of each receptor could be reversed only by the specific inhibitors ICI 182,780 and casodex, respectively. This reinforces the concept previously proposed (Yeh *et al.* 1998) that the interaction between the agonist/antagonist receptor complex and cofactors may be essential for steroid hormone function and selectivity. Of

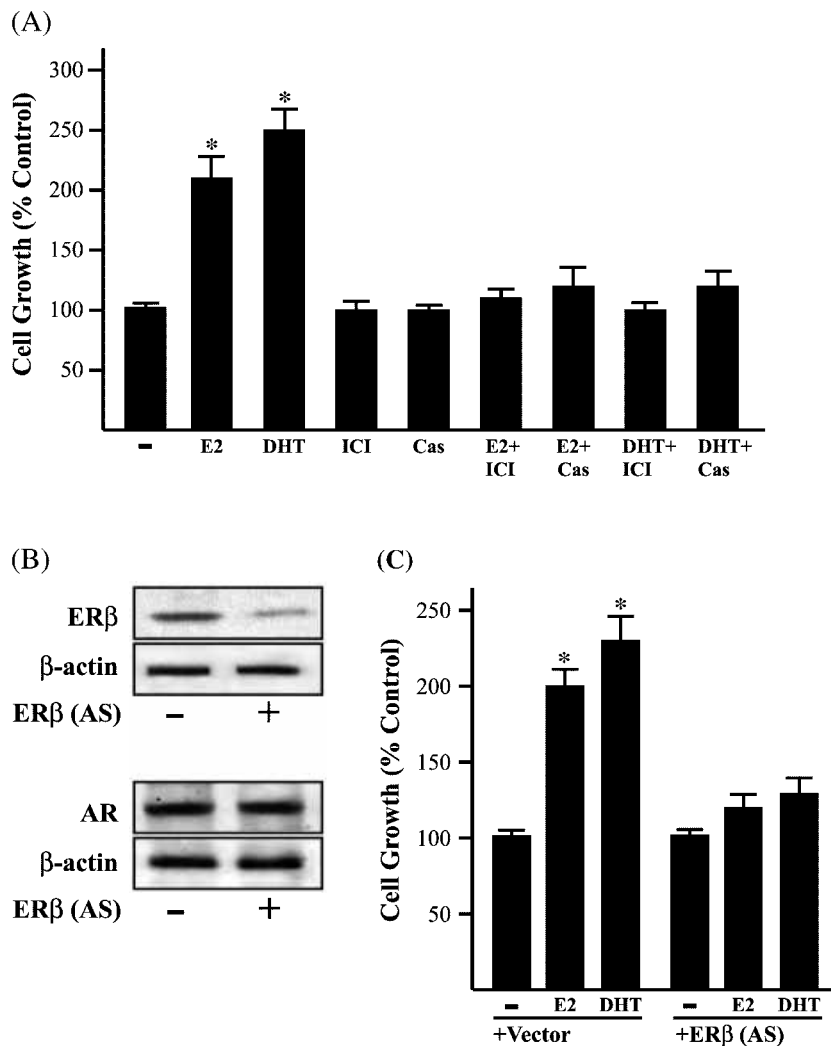


Figure 7 The proliferative effects of E2 and DHT on LNCaP cells are reversed by ICI 182,780, casodex (Cas) and ER β antisense (AS). (A) 10^4 cells were seeded in 24-multiwell plates and after 24 h of serum deprivation the medium was changed with 0.1% charcoal-stripped FCS. At this point cells were treated with ethanol (-), 10 nM E2 and DHT, 1 μ M ICI 182,780 and Cas. Medium and treatments were renewed every day for 3 days and the next day the cells were trypsinized and counted. The growth response is expressed as percentage of cells after treatments with respect to those treated with ethanol alone used in the same volume as for treatments. (B) Immunoblotting of ER β and AR in LNCaP cells transfected with ER β antisense. Protein expression was evaluated on the day of the cell count. (C) Proliferation of LNCaP was assayed as above, except that cells were transfected with the empty vector or ER β antisense before treatments. Each data point represents the mean \pm s.d. of three independent experiments performed in triplicate. * $P < 0.05$ compared with ethanol alone (-).

note, ICI 182,780 failed to reverse the E2-induced transactivation of AR, but it blocked the proliferative stimulus exerted by E2. In addition, the results obtained using an antisense approach corroborated

our conclusion from these pharmacological experiments. Thus ER β and AR are involved in hormone-stimulated proliferation of LNCaP cells, because each of the two antagonists, casodex and

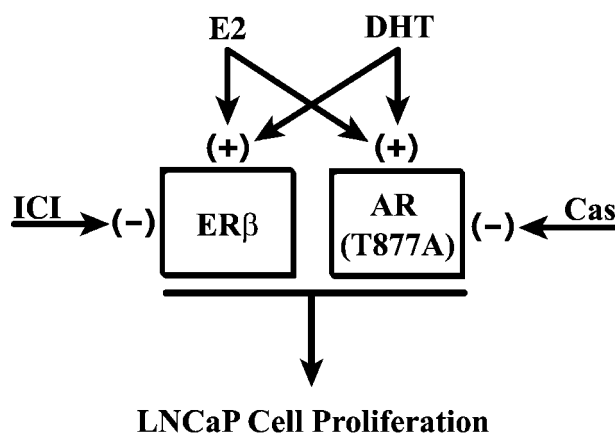


Figure 8 Schematic representation of hormone/antihormone action in AR and ERβ-mediated transcription that may lead to LNCaP cell proliferation.

ICI 182,780, repressed the activation of AR and ERβ in an exclusive manner, but both compounds inhibited the proliferation of LNCaP cells (Fig. 8).

Prostate cancer is a leading cause of mortality and morbidity among men in Western countries (Parker *et al.* 1997, Wingo *et al.* 2001). After an initial stage characterized by androgen dependence and responsiveness to androgen deprivation, the disease exhibits no sensitivity to further hormonal manipulation in the ultimate progression (Garnick 1993, Catalona 1994, Klocker *et al.* 1994). The AR mutation T877A, characteristic of LNCaP cells, has also been found in prostatic tissue from cancer patients (Suzuki *et al.* 1993, Kuil & Brinkman 1996). This and other mutations that alter the AR ligand-binding specificity and hence confer responsiveness to different compounds including oestrogens (Veldscholte *et al.* 1990, Culig *et al.* 1993, Elo *et al.* 1995, Tan *et al.* 1997, Maggiolini *et al.* 2002) may provide a potential mechanism by which prostate cancer cells escape the effects of antiandrogen treatments. Nevertheless, the role played by oestrogens in prostate cancer remains controversial. In the stromal compartment of human and rodent prostates, their actions are believed to be mediated via ERα (Kirschenbaum *et al.* 1994, Ehara *et al.* 1995, Hiramatsu *et al.* 1996, Prins *et al.* 1998, Bonkhoff *et al.* 1999). The discovery of ERβ (Kuiper *et al.* 1997), its localization in the epithelial compartment of rodent prostates (Couse *et al.* 1997, Lau *et al.* 1997) and its expression in human malignant cells (Lau *et al.* 2000) have raised the possibility that oestrogens and antioestrogens could

exert important biological effects through an ERβ signalling pathway. In line with these observations, our findings extend to androgen-responsive LNCaP cells results previously obtained with DU145 prostate cancer cells, which express only ERβ and no ERα. Although DU145 cells do not depend on added steroids for proliferation, ICI 182,780 severely inhibits their proliferation (Lau *et al.* 2000), suggesting that these cells require at least a basal ERβ activity. Of interest, recent studies have also demonstrated that the selective ER modulator, raloxifene, induces apoptosis of androgen-sensitive or -insensitive prostate cancer cells through an androgen-independent pathway (Kim *et al.* 2002a, b).

How do both oestrogens and androgens stimulate the proliferation of LNCaP cells? As stated above, we propose that either type of hormone activates both ERβ and AR, and that both steroid receptors are required for the proliferative stimulus. Our experiments do not address the question of whether these nuclear receptors function as ligand-activated transcription factors or act in combination with rapid non-genomic signalling, although the Src inhibitor PP2 was not able to block the response to hormones. In a previous study (Migliaccio *et al.* 2003), cross-talk between steroid receptors was reported to regulate the non-genomic proliferative pathways of different steroids. In LNCaP cells, either E2 or the synthetic androgen R1881 induced the assembly of a ternary complex of AR, ERβ and Src, stimulated the Raf-1/Erk signal transduction cascade, and triggered entry into S-phase (Migliaccio *et al.* 2000). ICI 182,780, casodex and an inhibitor of Src prevented assembly of this complex and non-genomic signalling elicited by either agonist. These findings are compatible with a role for non-genomic signalling in driving the proliferation of LNCaP cells. However, sustained hormone-stimulated proliferation is likely to be more than entry into the first S-phase, as stimulated by non-genomic signalling. A large number of studies support a contribution of transcriptional effects (Beato 1989, Beato *et al.* 1996, Yeh & Chang 1996). New genetic and pharmacological tools will have to be developed if the contributions of genomic and non-genomic pathways mediated by endogenous steroid receptors are to be elucidated.

Progressive loss of ERβ expression has been found in prostatic hyperplasia and, to a greater

extent, in invasive cancer (Horvath *et al.* 2001). A similar pattern was observed after the development of breast and colon tumours (Enmark *et al.* 1997, Foley *et al.* 2000). Interestingly, prostate tumours that retain ER β appear to be associated with a greater rate of relapse (Horvath *et al.* 2001). These data imply that the role of ER β in regulating the growth of different tumours is complex and that further studies are needed to clarify the link between ER β and cancer. As it concerns prostate carcinoma, the presence of functional ER β may represent a potential target for treatment with antioestrogens.

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