Androgens Up-regulate the Insulin-like Growth Factor-I Receptor in Prostate Cancer Cells

Giuseppe Pandini,1 Rossana Mineo,1 Francesco Frasca,1 Charles T. Roberts, Jr.,2 Marco Marcelli,3 Riccardo Vigneri,1 and Antonino Belfiore4

1Dipartimento di Medicina Interna e di Medicina Specialistica, Cattedra di Endocrinologia, University of Catania, Ospedale Garibaldi, Catania, Italy; 2Department of Pediatrics, Oregon Health and Science University, Portland, Oregon; 3Department of Medicine, Division of Endocrinology, Diabetes and Metabolism, Baylor College of Medicine and Veterans Affairs Medical Center, Houston, Texas; and 4Dipartimento di Medicina Sperimentale e Clinica, Cattedra di Endocrinologia, Policlinico Mater Domini, University of Catanzaro

Abstract

In this study, we show that androgens up-regulate insulin-like growth factor-I receptor (IGF-IR) expression and sensitize prostate cancer cells to the biological effects of IGF-I. Both dihydrotestosterone and the synthetic androgen R1881 induced an ∼6-fold increase in IGF-IR expression in androgen receptor (AR)–positive prostate cancer cells LNCaP. In accordance with IGF-IR up-regulation, treatment with the nonmetabolizable androgen R1881 sensitized LNCaP cells to the mitogenic and motogenic effects of IGF-I, whereas an IGF-IR blocking antibody effectively inhibited these effects. By contrast, these androgens did not affect IGF-IR expression in AR-negative prostate cancer cells PC-3. Reintroduction of AR into PC-3 cells by stable transfection restored the androgen effect on IGF-IR up-regulation. R1881-induced IGF-IR up-regulation was partially inhibited by the AR antagonist Casodex (bicalutamide). Two other AR antagonists, cyproterone acetate and OH-flutamide, were much less effective. Androgen-induced IGF-IR up-regulation was not dependent on AR genomic activity, because two AR mutants, AR-C619Y and AR-C574R, devoid of DNA binding activity and transcriptional activity were still able to elicit IGF-IR up-regulation in HEK293 kidney cells in response to androgens. Moreover, androgen-induced IGF-IR up-regulation involves the activation of the Src-extracellular signal-regulated kinase pathway, because it was inhibited by both the Src inhibitor PP2 and the MEK-1 inhibitor PD98059. The present observations strongly suggest that AR activation may stimulate prostate cancer progression through the altered IGF-IR expression and IGF action. Anti-androgen therapy may be only partially effective, or almost ineffective, in blocking important biological effects of androgens, such as activation of the IGF system. (Cancer Res 2005; 65(5): 1849-57)

Introduction

Prostate cancer is the most common malignancy in men. Androgen stimulation is essential for growth and resistance to apoptosis in ∼70% of prostate carcinomas. This is the basis of therapeutic approaches based on androgen deprivation. The palliative clinical benefits of androgen deprivation are temporary, however, because although initially responsive to anti-androgen treatments these carcinomas eventually progress to androgen-independent tumors, for which no efficacious treatment is currently available. The molecular basis of androgen stimulation of prostate cancer growth and the switch to androgen independence are incompletely understood. Recent evidence suggests that androgens may regulate prostate cancer proliferation by up-regulating autocrine loops involving peptide growth factors and their cognate receptors (1). The progression to androgen independence may be explained by the appearance of malignant cell clones that are responsive to growth factors other than androgens. These cells therefore are only partially responsive to androgen deprivation and can be successfully killed only by a combined approach targeting not only the androgen receptor (AR) but also other relevant growth regulators. Hopefully, such a combined approach could slow down the progression of androgen-independent prostate cancer.

The insulin-like growth factor (IGF) system plays a key role in regulating growth, resistance to apoptosis, and invasion in a variety of human malignancies (2–5). Various lines of evidence suggest a role for the IGF system in prostate cancer (6, 7). First, clinical and epidemiologic studies indicate that increased IGF-I serum levels are associated with an increased risk of prostate cancer (6, 8). Second, IGF-I may increase mitogenic and transcriptional activity in nonmetastatic AR-transfected prostate cancer cells, whereas antisense-mediated inhibition of IGF-I receptor (IGF-IR) expression suppresses in vivo tumor growth and prevents prostate cancer cell invasiveness (9). Third, in human prostate cancer cell xenografts, progression to androgen independence in some experimental models is associated with increased expression of both IGF-IR and IGF-I (10).

Our understanding of the mutual regulation of the androgen and IGF systems in human prostate cancer is limited, however. Different studies have established that IGF-I may influence AR signaling. Early reports have indicated that IGF-I may transactivate the AR in transfected DU-145 human prostate cancer cells (11). Other authors found that IGF-I enhanced androgen-mediated AR transcriptional activity in DU-145 cells but was unable to transactivate AR in the absence of androgens (12). Recently, Plymate et al. showed that the IGF-I effect on AR transcriptional activity is even more complex and depends on the cell context (13). They found that IGF-I enhanced dihydrotestosterone-stimulated, but not basal, AR transcriptional activity in nonmetastatic AR-transfected prostate cancer cells (PRI cells). However, IGF-I suppressed AR activity in response to dihydrotestosterone in PRI-derived metastatic cells.

Lin et al. have shown that IGF-I, through the activation of the phosphatidylinositol 3-kinase/Akt serine-threonine kinase pathway, phosphorylates the AR at Ser210 and Ser790. This AR phosphorylation may inhibit AR-mediated apoptosis possibly by inhibiting the interaction between AR and coregulators (14). In addition, activation...
of the Ras/mitogen-activated protein kinase pathway by IGF-I may sensitize the AR transcrip
tional complex to subphysiologic levels of androgen in LNCaP prostate cancer cells (15).

On the contrary, data regarding androgen regulation of the IGF system in prostate cancer are lacking. We studied whether in human AR-positive prostate cancer cells androgens influence the expression of receptors of the IGF system and found that androgens induce a selective and marked up-regulation of the IGF-IR. Cell proliferation and invasiveness in response to IGF-I was greatly increased by androgens. The effect of androgens on IGF-IR involved an increase in both mRNA and protein expression and occurred through the activation of a nongenomic AR signaling pathway.

Materials and Methods

Cell media and all chemicals, unless otherwise stated, were obtained from Sigma (St. Louis, MO). The following materials were also purchased: FCS and geneticin (G418) from Invitrogen Laboratories (Paisley, United Kingdom); IGF-I, L2949002, PD98059, and PP2 from Calbiochem (San Diego, CA); dihydrotestosterone from Fluka (Buchs, Switzerland); synthetic nonpolymorphic androgen R1881 from NEN Life Science Products (Boston, MA); FuGene transfection reagent from Roche Diagnostics (Mannheim, Germany); luciferase assay system from Promega Corp. (Madison, WI); monoclonal antibody anti-IGF-IR (a13R-3) from Oncogene Research (Cambridge, MA); polyclonal anti-IGF-IR antibody and monoclonal antibody anti-AR from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); monoclonal antibody anti-phosphotyrosine (4G10) from UBI (Lake Placid, NY); and polyclonal anti-phospho-extracellular signal-regulated kinase1/2 (ERK1/2) and anti-ERK1/2 antibodies from New England Biolabs (Beverly, MA). Casodex (bicalutamidine), an androgen antagonist, was kindly provided by AstraZeneca (Milan, Italy). The cDNA encoding the human AR cloned into the expression vector pSV0 was kindly provided by Dr. E. Baldi (Florence, Italy) and maintained in DMEM American Type Culture Collection (Manassas, VA) and maintained as described for certain MMtV-luc cDNAs encoding the LNCaP cells were grown to 60% confluence, serum starved, and further cultured in the presence or absence of 10 nmol/L R1881 for 24 hours. Cells were lysed and processed for the Dual Luciferase Assay (Promega). Luciferase activity was normalized for transfection efficiency (Ronull a activity, b-galactosidase activity, or GFP amount). b-galactosidase activity was measured according to the manufacturer’s instructions. In GFP-transfected cells, the activity of each sample (5 lL) was measured by a spectrophotometer (Wallac 1420 Victor 2, PerkinElmer, Boston, MA).

IR, IGF-IR, and Hybrid Receptor Measurements

Cell lysates were prepared as described previously and used for receptor measurement by both ELISA and Western blot analysis.

ELISA. The characteristics and specificity of receptor ELISAs have been described previously (20, 21). Receptors were captured by incubating cell lysates (10–60 mg protein per well) in Maxisorp immunoplates precoated with 2 mg/mL specific monoclonal antibody as described previously (21, 22). Immuno
captured receptors were incubated with the specific biotinylated monoclonal antibodies at 0.3 mg/mL in 50 mmol/L HEPS-buffered saline (pH 7.6) containing 0.05% Tween 20, 1% bovine serum albumin, 2 mmol/L sodium orthovanadate, 1 mg/mL bacitracin, and 1 mmol/L phenyl-
methylsulfonyl fluoride and then with peroxidase-conjugated streptavidin. Peroxidase activity was determined colorimetrically by adding 100 mL of 3,3’,5,5’-tetramethylbenzidine [0.4 mg/mL in 0.1 mol/L citrate-
phosphate buffer (pH 5.0) with 0.4 mL of 30% H2O2]. The reaction was stopped by the addition of 1.0 mol/L H3PO4 and the absorbance was measured at 450 nm (21).

Western Blot. To confirm data obtained by ELISA, IR, IGF-IR, and hybrid receptor (Hybrid-R) were also measured in the same cell lysates by Western Blot analysis using specific antibodies as described previously (22).

IGF-I Binding Studies

LNCaP cells were grown to ~60% confluence, serum starved, and further cultured in the presence or absence of 10 nmol/L R1881 for 24 hours. Cells (3 106) were then incubated with 125I-IGF-I (10 pmol/mL) for a further 16 hours at 4°C in the presence of increasing concentrations of cold IGF-I. Then, cell-associated radioactivity was measured in a gamma counter. Scatchard analysis was done using GraphPad Prism 4 software.

IGF-IR Autophosphorylation

Cell monolayers were serum starved in medium without phenol red 24 hours before stimulation with 10 nmol/L IGF-I for 10 minutes. Cells were lysed in cold radioimmunoprecipitation assay buffer containing 50 mmol/L Tris (pH7.4), 150 mmol/L NaCl, 1% Triton X-100, 0.25% sodium deoxycholate, 10 nmol/mL sodium pyrophosphate, 1 mmol/mL NaF, 1 mmol/L sodium orthovanadate, 2 mmol/L phenylmethylsulfonyl fluoride, 10 mg/mL aprotinin, 10 mg/mL pepstatin, and 10 mg/mL leupeptin, and the insoluble material was separated by centrifugation at 10,000 g for 10 minutes at 4°C. Cell lysates were incubated at 4°C under rotation for 2 hours with 4 mg anti-IGF-
IR c13R-3 antibody coated with protein G-Sepharose. Immunoprecipitates

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were subjected to SDS-PAGE. The resolved proteins were transferred to nitrocellulose membranes, immunoblotted with anti-phosphotyrosine 4G10 monoclonal antibody, and detected by enhanced chemiluminescence. The nitrocellulose membrane was then stripped with buffer Restore (Pierce, Rockford, IL) and subsequently reprobed with an anti-IGF-IR rabbit polyclonal antibody.

**ERK1/2 Phosphorylation in Response to Androgen**

Cells were stimulated with 10 nmol/L R1881 or dihydrotestosterone for 5 minutes. Cell lysates obtained by the addition of 4× sample buffer were heated at 95°C to 100°C for 5 minutes and subjected to reducing SDS-PAGE on 10% polyacrylamide gel. The resolved proteins were transferred to nitrocellulose membranes and immunoblotted with anti-phosphospecific ERK1/2 polyclonal antibody. The nitrocellulose membranes were then stripped with buffer Restore and subsequently reprobed with anti-ERK1/2 polyclonal antibody.

**Real-time PCR**

Total RNA (5 μg) was reverse transcribed by ThermoScript RT (Invitrogen) and oligo(dT) primers. Synthesized cDNA (0.15 μL) was then combined in a PCR reaction using primers 5′-GGGCATCACGGATTTGA-GAA-3′ (forward) and 5′-CACAGGCGGTGCTGGTCTGCA-3′ (reverse) specific for the IGF-IR (fragment size 330 bp), EGE-1 (housekeeping gene) amplification was done using the following primers: 5′-ATTGAA-GAAATTGGCGGCTC-3′ (forward) and 5′-TGGAGAAGGAGAAGCTGTATC-3′ (reverse; fragment size 280 bp). Quantitative real-time PCR was done on an ABI Prism 7700 (PE Applied Biosystems, Foster City, CA) using SYBR Green PCR Master Mix (PE Applied Biosystems) following the manufacturer’s instructions. Amplification reactions were checked for the presence of nonspecific products by agarose gel electrophoresis. Relative quantitative determination of target gene levels was done by comparing ΔCt as described previously (23).

**Incorporation of [3H]Thymidine**

[3H]Thymidine incorporation was carried out as described previously (24). Briefly, LNCaP cells, preincubated or not with androgens for 24 hours, were seeded in 24-well plates and allowed to attach for 24 hours. Complete medium was replaced with serum-free medium without phenol red and containing 0.1% bovine serum albumin for 48 hours and then treated for a further 18 hours with IGF-I in the presence or absence of anti-IGF-IR monoclonal antibody αIR-3. After incubation, 0.5 μCi/mL [3H]thymidine was added for 4 hours. The cells were washed with ice-cold PBS and incubated with 1 mL of 10% ice-cold trichloroacetic acid for 30 minutes. The acid-insoluble fraction was solubilized with 0.1 N NaOH and the incorporation of [3H]thymidine into DNA was determined by scintillation counting in a β counter.

**Migration Assays**

LNCaP cells were serum starved for 24 hours in medium without phenol red and stimulated with R1881 for a further 24 hours. Cells were then removed from plates with HBSS containing 5 mmol/L EDTA and 25 mmol/L HEPES (pH 7.2) and 0.01% trypsin and resuspended at 106/mL. Cells were added to the top of each migration chamber. Cell migration assays were done as described previously (25), with minor modifications, using modified Boyden chambers (6.5 mm diameter, 10 μm thickness, 8 μm pores, Transwell, Costar Corp., Cambridge, MA) containing polycarbonate membranes coated at the lower side with 250 μg/mL collagen VI. Cells were allowed to migrate to the underside of the top chamber for 18 hours in response to 10 nmol/L IGF-I added to the lower chamber and in the presence or absence of anti-IGF-IR antibody αIR-3. Cells that had migrated to the lower side of the filter were fixed and stained with 0.1% crystal violet in 20% ethanol for 20 minutes. After three washes with water and complete drying, the crystal violet was solubilized by in 10% acetic acid and its concentration was evaluated as absorbance at 590 nm.

**Figure 1.** Expression of IR, IGF-IR, and Hybrid-R and IGF-I binding in prostate cancer cells in response to androgen. IR, IGF-IR, and Hybrid-R expression: serum-starved AR-positive LNCaP (A and B) or AR-negative PC-3 cells (C and D) were incubated in the presence or absence of 10 nmol/L R1881 for 24 hours. Cells were then lysed and receptor subtypes were immunopurified by specific antibodies and measured by ELISA (A and C) or Western blot (B and D) as described in Materials and Methods. Columns, mean ELISA data of three independent experiments; bars, SD. Representative Western blot experiments. IGF-I binding: competition-inhibition curves of 125I-IGF-I binding were carried out in LNCaP cells (E) and PC-3 wt cells (F) preincubated in the presence (●) or absence (●) of 10 nmol/L R1881 for 24 hours. Inset, Scatchard plot analysis of binding data.
incubated with a tracer dose of 125I-IGF-I and with increasing presence or absence of 10 nmol/L R1881 for 24 hours were an increased IGF-I binding capacity, LNCaP cells cultured in the R1881 in both cell lines (data not shown).

The effects of testosterone or dihydrotestosterone (10 nmol/L) on IGF-IR and IR expression were almost identical to those of AR-negative PC-3 cells (Fig. 1). Hybrid-R expression in AR-negative PC-3 cells (Fig. 1). To evaluate whether the increased IGF-IR expression reflected an increased IGF-I binding capacity, LNCaP cells cultured in the presence or absence of 10 nmol/L R1881 for 24 hours were incubated with a tracer dose of 125I-IGF-I and with increasing concentrations of cold IGF-I. Scatchard plot analysis of binding data showed a 7-fold increase of specific IGF-IRs (5.6-36.0 pmol/L/10⁶ cells) in androgen-exposed LNCaP cells compared with unexposed cells. The dissociation constant (Kd), however, was very similar (0.32 versus 0.22 nmol/L in androgen-exposed and nonexposed cells, respectively), showing that androgen increases IGF-I binding sites but only minimally affects the receptor affinity for the ligand (Fig. 1E). As expected, no change in IGF-I binding was observed in AR-negative PC-3 cells exposed or unexposed to androgen (Fig. 1F).

Dose-response experiments showed that R1881 was able to increase IGF-IR protein expression in a dose-dependent manner. IGF-IR started to increase at a dose as low as 0.01 nmol/L and reached maximum levels at 1 to 10 nmol/L R1881 (Fig. 2A). Time course experiments with 10 nmol/L R1881 showed that an increase of IGF-IR protein expression was already evident at 12 hours and increased steadily up to 24 to 48 hours (Fig. 2B). A 24-hour incubation length was therefore used in subsequent studies.

**IGF-IIR Phosphorylation.** As shown in Fig. 2C, LNCaP cells preincubated with R1881 exhibited increased ligand-induced IGF-I tyrosine phosphorylation compared with untreated cells, indicating that the increase in IGF-IIR expression resulted in increased signal transduction.

**Androgens Up-regulate IGF-IIR in AR-Transfected PC-3 Cells.** To evaluate whether the effect of androgens on IGF-IIR was restricted to LNCaP cells, we carried out studies in PC-3 cells transfected with the wild-type AR cDNA. Two cell clones (PC-3-AR6 and PC-3-AR13) with different AR expression levels were used. As shown in Fig. 2D, the PC-3-AR6 clone had an AR expression level, as measured by Western blot, which was only slightly lower than

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**Results**

**Androgens Up-regulate the IGF-IIR, but not the IR, in AR-Positive Prostate Cancer Cells**

The expression of the IGF-IIR and IR was determined in serum-starved LNCaP and PC-3 cells exposed to the non-metabolizable androgen R1881 (10 nmol/L) for 24 hours. In AR-positive LNCaP cells, exposure to R1881 increased IGF-IIR expression by ~5-fold (0.8-4.0 ng IGF-IIR/100 µg protein as measured by ELISA; Fig. 1A and B). In contrast, R1881 did not affect LNCaP cell IR content, showing that the androgen effect on the IGF-IIR was specific.

We also studied IR/IGF-IIR Hybrid-Rs that are formed by random assembly of IR and IGF-IIR hemidimers and behave as functional IGF binding sites (21, 22, 26). Hybrid-Rs were also increased in LNCaP cells exposed to R1881 (Fig. 1A and B) presumably as a consequence of increased IGF-IIR expression. Both ELISA measurements and Western blot analysis gave very similar results. Exposure to R1881 did not affect IGF-IR or Hybrid-R expression in AR-negative PC-3 cells (Fig. 1C and D). The effects of testosterone or dihydrotestosterone (10 nmol/L) on IGF-IIR and IR expression were almost identical to those of R1881 in both cell lines (data not shown).

To evaluate whether the increased IGF-IIR expression reflected an increased IGF-I binding capacity, LNCaP cells cultured in the presence or absence of 10 nmol/L R1881 for 24 hours were incubated with a tracer dose of 125I-IGF-I and with increasing concentrations of cold IGF-I. Scatchard plot analysis of binding data showed a 7-fold increase of specific IGF-IIRs (5.6-36.0 pmol/L/10⁶ cells) in androgen-exposed LNCaP cells compared with unexposed cells. The dissociation constant (Kd), however, was very similar (0.32 versus 0.22 nmol/L in androgen-exposed and nonexposed cells, respectively), showing that androgen increases IGF-I binding sites but only minimally affects the receptor affinity for the ligand (Fig. 1E). As expected, no change in IGF-I binding was observed in AR-negative PC-3 cells exposed or unexposed to androgen (Fig. 1F).

Dose-response experiments showed that R1881 was able to increase IGF-IIR protein expression in a dose-dependent manner. IGF-IIR started to increase at a dose as low as 0.01 nmol/L and reached maximum levels at 1 to 10 nmol/L R1881 (Fig. 2A). Time course experiments with 10 nmol/L R1881 showed that an increase of IGF-IIR protein expression was already evident at 12 hours and increased steadily up to 24 to 48 hours (Fig. 2B). A 24-hour incubation length was therefore used in subsequent studies.

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IGF-I Receptor Up-regulation by Androgens

Androgens Induce IGF-IR mRNA Expression

The increase in IGF-IR expression induced by R1881 in LNCaP cells was completely inhibited by either actinomycin D or cycloheximide, suggesting that both de novo mRNA and protein synthesis are required for this effect (data not shown). IGF-IR mRNA expression was then studied in LNCaP cells by quantitative real-time PCR after cell exposure to 10 nmol/L R1881. Dose-response experiments carried out in cells exposed to R1881 for 24 hours showed that IGF-IR mRNA increased at a R1881 dose of 0.001 nmol/L and reached a plateau at 1 to 10 nmol/L (Fig. 3A). Time course experiments carried out with 10 nmol/L R1881 indicated that mRNA started to increase at 4 hours and reached levels ~15-fold higher after 16- to 24-hour exposure to R1881 (Fig. 3B). The increase in IGF-IR mRNA in response to androgen treatment was partially inhibited by preincubation with cycloheximide, suggesting that new protein synthesis was required for androgen stimulation of IGF-IR gene expression (Fig. 3C).

Androgen-Induced IGF-IR Up-regulation Is Partially Inhibited by the AR Antagonist Casodex and Is Sensitive to c-Src and MEK-1 Inhibition

We next evaluated whether AR antagonists could block IGF-IR up-regulation in response to androgens. LNCaP cells were incubated with 10 nmol/L R1881 (or dihydrotestosterone) in the presence of 50 nmol/L cyproterone acetate or 3 μmol/L OH-flutamide or 10 μmol/L Casodex. Cells were then lysed and IGF-IR expression was studied by Western blot. The results showed that R1881-induced IGF-IR up-regulation was partially inhibited by Casodex (~40 ± 6%), whereas cyproterone acetate and OH-flutamide were almost ineffective (about ~13% for both; Fig. 4A). Dose-response experiments with these anti-androgens showed that cyproterone acetate at concentrations >50 nmol/L was actually stimulatory and that OH-flutamide at concentrations >3 μmol/L was cytotoxic (data not shown).

These results suggested that IGF-IR up-regulation might not be mediated by classic AR transactivation pathway, which are sensitive to AR antagonists. An alternative AR signaling pathway has been described, which involves the activation of a Src/Raf-1/ERK pathway that in turn elicits important biological effects, such as cell proliferation (27).

To evaluate the involvement of the Src/Raf-1/ERK pathway in IGF-IR up-regulation, LNCaP cells were incubated with R1881 in the presence or absence of various kinase inhibitors, including PP2, a Src inhibitor, PD98059, a MEK-1 inhibitor, and LY294002, a phosphatidylinositol 3-kinase inhibitor. IGF-IR expression was then measured by Western blot analysis. The R1881 effect was blocked almost completely by both PP2 (10 μmol/L) and PD98059 (50 μmol/L). LY294002 (up to 20 μmol/L) was ineffective (Fig. 4B).

As expected, incubation with R1881 for 30 minutes induced a marked ERK1/2 activation, and both PP2 and PD98059, but not LY294002, at the doses used, completely blocked this effect (Fig. 4C). Taken together, these data strongly suggest that IGF-IR up-regulation by R1881 involves the activation of the Src/Raf-1/ERK pathway as reported previously for other nongenomic, androgen-mediated effects. This effect was, however, specific of androgens, as a potent stimulator of the ERK1/2 activity, such as epidermal growth factor (10 nmol/L), was ineffective in inducing IGF-IR up-regulation (data not shown).

Two Transcriptionally Inactive AR Mutants Are Able to Increase IGF-IR Promoter Activity and IGF-IR Protein Levels

To further evaluate whether IGF-IR up-regulation can occur independently of the transcriptional activity of the AR, we used two different AR mutants, AR-C619Y and AR-C574R, both reported to be unable to bind DNA and activate transcription (16–18). In particular, AR-C574R is additionally unable to translocate into the nucleus (17, 18). AR-negative HEK293 cells were then transfected with either the ARwt or the AR-C619Y or AR-C574R cDNAs, and IGF-IR protein expression was measured, after exposure to androgen, by Western blot analysis. Cells transfected with each of the two transcriptionally inactive AR

![Figure 3. IGF-IR mRNA up-regulation by androgen in LNCaP cells.](image-url)
mutants showed an up-regulation of IGF-IR protein expression at a level comparable with that observed in cells transfected with the ARwt, thus indicating that AR DNA binding and transcriptional activity were unnecessary for this effect (Fig. 5A).

To confirm the absence of transcriptional activity of the two AR mutants in our system, HEK293 cells were transiently transfected with either the ARwt or each of the two AR mutants along with the androgen-responsive MMTV-luc reporter. To avoid possible spurious Renilla luciferase activation by androgen described in certain systems (28), transfection efficiency was measured by using a GFP vector that is completely insensitive to androgens as described in Materials and Methods. As shown in Fig. 5B, R1881 was able to induce increased activity of the MMTV-luc reporter in the presence of the ARwt but not in the presence of AR mutants AR-C619Y or AR-C574R. R1881 also caused an increase of the MMTV-luc activity in LNCaP cells.

HEK293 cells were then transiently cotransfected with plasmids encoding either the ARwt or the AR-C619Y or AR-C574R and luciferase constructs containing each of the three IGF-IR promoter sequences (full-length, 5′ flanking fragment, and 5′ untranslated region fragment). Cotransfection with the GFP vector was used to normalize for transfection efficiency. The promoter activity of the full-length fragment (bp −476/+41) was increased mitogenic effect of IGF-I. Serum-starved cells obtained in AR-transfected PC-3 cells (data not shown).

**Figure 4.** Inhibition of androgen-induced IGF-IR expression by anti-androgens or kinase inhibitors. A, inhibition of androgen-induced IGF-IR expression by anti-androgen: serum-starved LNCaP cells were treated with 10 nmol/L R1881 for 24 hours in the presence or absence of either 50 nmol/L cyproterone acetate, 3 μmol/L OH-flutamide, or 10 μmol/L Casodex. Whole-cell lysates containing equal amounts of protein were separated by SDS-PAGE and immunoblotted with an antibody to the IGF-IR. Representative of four independent experiments (top). Mean Columns, mean densitometric values of IGF-IR/β-actin ratios from four independent experiments; bars, SD (bottom). B, inhibition of androgen-induced IGF-IR up-regulation by kinase inhibitors: LNCaP cells were incubated with 10 nmol/L R1881 for 24 hours and in the presence or absence of either 10 μmol/L PP2 (c-Src inhibitor), 50 μmol/L PD98059 (MEK-1 inhibitor), or 20 μmol/L LY294002 (phosphatidylinositol 3-kinase inhibitor). IGF-IR expression was then measured by Western blot analysis (top), as described in Materials and Methods, using an antibody to the IGF-IR. Membranes were reblotted with an anti-β-actin antibody to control for protein loading (bottom). Representative of three independent experiments (top). Columns, mean densitometric values of IGF-IR/β-actin ratios from three independent experiments; bars, SD (bottom). C, androgen activation of ERK1/2 and inhibition by both PD98059 and PP2: serum-starved LNCaP cells were incubated with 10 nmol/L R1881 for 5 minutes in the presence or absence of either 10 μmol/L PP2 (c-Src inhibitor), 50 μmol/L PD98059 (MEK-1 inhibitor), or 20 μmol/L LY294002 (phosphatidylinositol 3-kinase inhibitor). ERK1/2 activation was then evaluated by Western blot analysis using a phosphospecific antibody (top). Membranes were stripped and reblotted with an anti-EPK1/2 antibody (bottom).

**Biological Effects of Androgen-Induced IGF-IR Up-regulation**

**Cell Growth.** We evaluated whether androgen-induced IGF-IR up-regulation in LNCaP prostate cancer cells may result in increased mitogenic effect of IGF-I. Serum-starved cells preincubated with or without R1881 for 24 hours were exposed to IGF-I for 18 hours. IGF-IR levels were ~7-fold higher in cells incubated with R1881 (Fig. 6A). In these cells, IGF-I increased [3H]thymidine incorporation by 3-fold, whereas no effect was observed in control cells (Fig. 6B). Most of this effect was blocked by the anti-IGF-IR monoclonal
antibody αIR-3, thus demonstrating the specificity of IGF-I effect (Fig. 6B).

Cell Invasiveness. Chemoinvasion, measured as the cell ability to migrate in response to IGF-I in Boyden chambers, was studied in LNCaP cells preincubated with or without R1881. IGF-I effectively stimulated chemoinvasion in cells preincubated with R1881. This effect was inhibited by αIR-3 and was not observed in control cells (Fig. 6C).

Discussion

The main findings of the present work can be summarized as follows: (a) androgens are able to induce up-regulation of IGF-IR, Hybrid-Rs, and IGF-I binding capacity in AR-positive prostate cancer cells; (b) this effect cannot be blocked by the most frequently used AR antagonists, such as cyproterone acetate and OH-flutamide (almost ineffective) and Casodex (only partial inhibition); (c) IGF-IR up-regulation by androgens does not involve AR binding to DNA and classic transcriptional activity but rather occurs via the activation of a Src-ERK pathway; and (d) IGF-IR up-regulation by androgens sensitizes cells to the mitogenic and motogenic effects of IGF-I.

Recent work has clearly showed a role of the IGF system in prostate cancer (3, 6–8, 19, 29–34). The IGF-IR may be overexpressed in prostate cancer at initial stages (35). Disease progression may be associated with IGF-IR down-regulation (36), although recent evidence indicates that IGF-IR is overexpressed at metastatic sites (37). The molecular mechanisms underlying IGF-IR regulation at the different stages of prostate cancer are still unclear. Our data show that LNCaP cells are unresponsive to IGF-I, unless they are preincubated with androgens, which induce a marked up-regulation of the IGF-IR and sensitize cells to the mitogenic and motogenic effects of IGF-I. This effect is quite rapid, as it is already detectable 12 hours after exposure to androgens, and occurs at an androgen dose as low as 0.01 nmol/L R1881. This effect is not restricted to LNCaP cells and is also observed in transfected PC-3 cells. Scatchard plot analysis of binding data confirmed a marked increase in IGF-I binding sites after androgen exposure with a minimal increase in binding affinity. These data
and Methods. \[^{3}H\]Thymidine incorporation into nuclei was measured as described in Materials and Methods. Columns, means of three independent experiments; bars, SE. **, \(P = 0.003\), IGF-I versus basal; *, \(P = 0.007\), IGF-I + IR-3 versus IGF-I (two-tailed Student's \(t\) test for paired values). C, cell migration assay: R1881-pretreated cells were seeded at the top of Boyden chambers coated with 250 \(\mu\)g/mL collagen VI and stimulated with 10 nmol/L IGF-I in the presence or absence of an IGF-IR blocking antibody (αIR-3) for a further 18 hours. After stimulation, \[^{3}H\]Thymidine (0.5 \(\mu\)Ci/well) was added for 4 hours and thymidine incorporation into nuclei was measured as described in Materials and Methods. Columns, means of three independent experiments; bars, SE. **, \(P = 0.001\), IGF-I versus basal; *, \(P = 0.02\), IGF-I + αIR-3 versus IGF-I (two-tailed Student's \(t\) test for paired values).

are in partial agreement with the findings of Iwamura et al. (38), who showed previously that IGF-I is mitogenic in LNCaP cells only after pretreatment with dihydrotestosterone. These authors, however, could not explain the mechanism of this finding, as they were unable to show an increase in cell IGF-I binding sites after dihydrotestosterone exposure. The reason for the discrepancy between our study and the study of Iwamura et al. (38), as far as the increase of IGF-I binding is concerned, is unclear and may involve cell variability. Moreover, in our study, the direct measurement of IGF-IR protein or mRNA expression confirmed the increase of IGF-I binding sites after exposure to androgens. These measurements were not undertaken in the Iwamura et al. study (38).

The effect of androgens on IGF-IR is specific, as androgens did not affect the expression of the closely related IR (39–42). However, because these two receptors form hybrids (Hybrid-R) by random assembly of α/β-subunit hemireceptors (21, 22, 26, 43), androgen-induced IGF-IR up-regulation resulted in an increase of Hybrid-Rs. These Hybrid-Rs bind IGF-I with high affinity and could contribute therefore to the increased cell sensitivity to IGF-I.

In some cell models, prostate cancer progression to androgen independence is associated with increased expression of IGF-IR (10). Among the multiple mechanisms hypothesized for prostate cancer progression to androgen independence is the emergence of cell clones with activating AR mutations (44, 45). We hypothesize that these activating AR mutations may contribute to IGF-IR up-regulation in metastatic cancer. It is noteworthy that the most frequently used AR antagonists are either almost ineffective in blocking AR-induced IGF-IR up-regulation (cyproterone acetate and OH-flutamide) or only minimally effective (Casodex). As cyproterone acetate is concerned, 50 nmol/L was the most effective inhibitory dose and higher doses were stimulatory (data not shown) according to previous reports (46). These observations strongly suggest that in clinical practice most anti-androgen treatments targeting the AR are ineffective in blocking androgen-induced activation of the IGF system, which may play an important role in cancer progression to androgen independence. The mechanism(s) by which androgens up-regulate the IGF-IR do not involve binding and transactivation of androgen response elements in DNA, because two different AR mutants (AR-C619Y and AR-C574R), both devoid of DNA binding capacity and transcriptional activity, are still able to elicit this effect. AR-C619Y has been described recently as a naturally occurring mutated AR in prostate cancer that is unable to bind DNA in vivo and in vitro and to activate transcription (16). The AR-C574R mutant is characterized by a mutation of the DNA binding domain of the AR and is unable to translocate into the nucleus and to bind DNA (17, 18). Nevertheless, both mutants induced IGF-IR up-regulation with an activity similar to the ARwt.

The existence of AR signaling pathways other than the classic one involving DNA binding and transactivation has been the focus of numerous recent studies (27, 47–50). In particular, it has been shown that dihydrotestosterone leads to the activation of the ERK pathway, which is insensitive to androgen blockade (49). This effect was associated to an increased activity of the transcription factor Elk-1, which in turn activates c-fos expression and may elicit gene transcription independently of AR binding to DNA response elements (49). According to this model, androgens may induce/activate primary-response genes via pathways that do not include AR binding to DNA; these genes will then code for transcriptional factors that in turn influence the regulation of secondary-response genes.

Interestingly, both the AR-C619Y and the AR-C574R variants, although transcriptionally inactive, maintain the ability to activate the ERK pathway. These findings further corroborate our data, indicating that the ERK pathway is involved in the androgen-induced increase of IGF-IR in prostate cancer cells. Recently, Migliaccio et al. reported that ERK activation by androgens requires the activation of c-Src (27). In agreement with these findings, we observed that IGF-IR up-regulation by androgens was blocked by the MEK-1 inhibitor PD98059 and by the c-Src inhibitor PP2. Moreover, in cells containing either the ARwt or each of the two transcriptionally inactive AR mutants, the increase of luciferase activity driven by the IGF-IR promoter in response to androgen was completely inhibited by cotransfection with either a dominant-negative Src or a dominant-negative MEK-1.

Taken together, these data strongly suggest that the IGF-IR up-regulation by androgens involves the activation of a Src-ERK pathway, although it does not involve AR binding to specific DNA response elements. The present study therefore also suggests that naturally occurring AR mutants in prostate cancer unable to bind...
DNA, such as AR-C619Y, may still sensitize cells to IGF-I and play a role in cancer progression. Agents targeting these signaling pathways or the IGF-IR itself should therefore be considered as a complement of standard anti-androgen therapy to induce tumor regression and possibly reduce the chance or delay the time of tumor progression to androgen independence. Alternatively, new anti-androgen compounds able to completely block IGF-IR upregulation should be developed.

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Giuseppe Pandini, Rossana Mineo, Francesco Frasca, et al.


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