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

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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)
of the Ras/mitogen-activated protein kinase pathway by IGF-I may activate 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 genetin (G418) from Invitrogen Laboratories (Paisley, United Kingdom); IGF-I, LY294002, PD98059, and PP2 from Calbiochem (San Diego, CA); dihydrotestosterone from Fluka (Buchs, Switzerland); synthetic nonmetabolizable 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 (aIR-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-extraacellular signal-regulated kinase1/2 (ERK1/2) and anti-ERK1/2 antibodies from New England Biolabs (Beverly, MA). Casodex (bicalutamide), an androgen antagonist, was kindly provided by AstraZeneca (Milan, Italy). The cDNA encoding the human AR cloned into the expression vector pSV50 was kindly provided by Dr. A.O. Brinkmann (Rotterdam, the Netherlands). The cDNAs encoding the kinase-inactive MEK-1 (Ser259,Ala) and the kinase-inactive form of Src (Lys422,Met) were kindly provided by Dr. G. Castoria (Naples, Italy). The cDNAs encoding the MMTV-lac reporter gene and the 5′ untranslated region (+41/+640) ligated to the Renilla luciferase reporter gene (pGL3-V) were kindly provided by AstraZeneca (Milan, Italy). The monoclonal antibody anti-IGF-IR (clone 3A2) was kindly provided by AstraZeneca (Milan, Italy). The cDNA encoding the kinase-inactive MEK-1 (Ser259,Ala) and the kinase-inactive form of Src (Lys422,Met) were kindly provided by Dr. G. Castoria (Naples, Italy). The cDNAs encoding the MMTV-lac reporter gene was kindly provided by Dr. Farsetti (Rome, Italy). The expression vectors for the human transcrip-

tionally inactive AR mutants, AR-C619Y and AR-C574R, have been described previously (19). Briefly, 2.5 × 10⁶ cells were seeded in 12-well plates and grown for 24 hours in medium without phenol red. A transfection mixture containing 1 μg DNA and 4 μL Fugene 6 in 40 μL of medium without serum was added to each well. After 18 hours, the medium was changed to serum-containing medium for 30 hours. Cells were then serum starved overnight and incubated with 10 nmol/L R1881 for 24 hours. Cells were then lysed and processed for Dual Luciferase Assay (Promega). Luciferase activity was normalized for transfection efficiency (Renilla activity, β-galactosidase activity, or GFP amount). β-galactosidase activity was measured according to the manufacturer’s instructions. In GFP-transfected cells, the activity of each sample (5 μL) 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 μg protein per well) in Maxisorp immunoplates precoated with 2 μg/mL specific monoclonal antibody as described previously (21, 22). Immunocaptured receptors were incubated with the specific biotinylated monoclonal antibodies at 0.3 μg/mL in 50 mmol/L Hepes-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 streptav- din. Peroxidase activity was determined colorimetrically by adding 100 μL of 3,3′,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. 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 (pH 7.4), 150 mmol/L NaCl, 1% Triton X-100, 0.25% sodium deoxycholate, 10 mmol/L sodium pyrophosphate, 1 mmol/L NaF, 1 mmol/L sodium orthovanadate, 2 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL aproti-

nin, 10 μg/mL pepstatin, and 10 μg/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 μg anti-IGF-
IR eIR-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 4X 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′-GGCCCATACGATTGAGAAGAA-3′ (forward) and 5′-TGAAGGACTGGTGCACTAC-3′ (reverse) specific for the IGF-IR (fragment size 330 bp). ELL-1 (housekeeping gene) amplification was done using the following primers: 5′-ATTGGAAGAATTGCCAGGCTC-3′ (forward) and 5′-TGGAAGAAGAGGAGCTGTATCT-3′ (reverse; fragment size 280 bp). Quantitative real-time PCR was done on an ABI Prism 7700 (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 6iR-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 10^6/mL. 500 μL were subjected 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 determined by absorbance at 590 nm.

![](image1.png)

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

The expression of the IGF-IR 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-IR expression by ~5-fold (0.8-4.0 ng IGF-IR/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-IR was specific.

We also studied IR/IGF-IR Hybrid-Rs that are formed by random assembly of IR and IGF-IR 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-IR 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-IR and IR expression were almost identical to those of R1881 in both cell lines (data not shown).

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/10^6 cells) in androgen-exposed LNCaP cells compared with unexposed cells. The dissociation constant (K_d), 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-IR Phosphorylation. As shown in Fig. 2C, LNCaP cells preincubated with R1881 exhibited increased ligand-induced IGF-IR tyrosine phosphorylation compared with untreated cells, indicating that the increase in IGF-IR expression resulted in increased signal transduction.

Androgens Up-regulate IGF-IR in AR-Transfected PC-3 Cells.

To evaluate whether the effect of androgens on IGF-IR 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...
LNCaP cells, whereas the PC-3-AR13 clone expressed the AR at ∼6- to 8-fold lower levels than LNCaP cells.

R1881 markedly up regulated IGF-IR expression in AR-positive but not in AR-negative transfected cells, and the IGF-IR response was proportional to the AR expression level. In LNCaP and PC-3-AR6 cells that exhibited the highest level of AR expression, IGF-IR content increased 6- to 8-fold and 4- to 6-fold, respectively. Only a slight increase was observed in PC-3 AR13 cells (Fig. 2E), which had a lower AR content (Fig. 2D), and no effect of R1881 on IGF-IR expression was observed in control PC-3-NEO cells and PC-3wt cells not expressing AR (Fig. 2D and E).

**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 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

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**Figure 3. IGF-IR mRNA up-regulation by androgen in LNCaP cells.** Dose-response experiments (A) were carried out by incubating serum-starved LNCaP cells in the presence or absence of increasing concentrations of R1881 for 24 hours. For time course experiments (B), LNCaP cells were incubated with or without 10 nmol/L R1881 for the indicated times. Total RNA prepared from LNCaP cells was used as template for real-time reverse transcription-PCR as described in Materials and Methods. Relative mRNA amounts were normalized to the abundance of the ELE-1 mRNA. Actinomycin D (1 μg/mL) completely blocked IGF-IR mRNA up-regulation by R1881, whereas cycloheximide (10 μg/mL) was only partially effective (C). Columns, mean of three separate experiments; bars, SD.
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 IGF-IR promoter activity in LNCaP cells.

A similar increase in luciferase activity (+154% increase) was obtained in AR-transfected PC-3 cells (data not shown).

Results similar to those obtained in HEK293 cells were also obtained in AR-transfected PC-3 cells (data not shown).

**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.

**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 stripped and 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 PD98059 (MEK-1 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 phospohespecific antibody (top). Membranes were stripped and reblotted with an anti-EPK1/2 antibody (bottom).
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 analyses of binding data confirmed a marked increase in IGF-I binding sites after androgen exposure with a minimal increase in binding affinity. These data

Figure 5. IGF-IR expression and transcription is up-regulated by transcriptionally inactive AR mutants. A, IGF-I protein expression is up-regulated by each of the two different transcriptionally inactive AR mutants: HEK293 cells were stably transfected with expression plasmids coding for either the wild-type AR (ARwt) or a transcriptionally inactive AR mutant (AR-C619Y or AR-C574R). Control cells were transfected with an empty vector (EV). Transfected cells were then exposed to 10 nmol/L R1881 for 24 hours and IGF-I expression was measured by Western blot analysis (top) as described in Materials and Methods. Filters were reblotted with an anti-AR antibody (middle) and then with an anti-β-actin antibody (bottom). Representative of three independent experiments. B, androgen-induced activity of a MMTV-luc reporter: HEK293 cells were cotransfected with either the ARwt or the AR-C619Y or AR-C574R mutant and the androgen-responsive MMTV-luc reporter. R1881 induced a marked increase of MMTV activity in cells transfected with the ARwt but not in those transfected with the AR mutants. R1881 stimulation of the endogenous AR in LNCaP cells was also able to induce MMTV-luc reporter activity. C–E, androgen induces IGF-IR promoter activity in AR-transfected cells: HEK293 cells were transiently cotransfected with expression plasmids coding for either the wild-type AR (ARwt; C) or each of the two transcriptionally inactive AR variants (AR-C619Y or AR-C574R; D and E) together with the IGF-IR promoter/luciferase vector containing the full-length promoter fragment (bp −476/+641) of the rat IGF-IR gene. In androgen-stimulated cells, the IGF-IR promoter activity was then assayed in the presence or absence of plasmids coding for either a Src k−/k− dominant-negative or a MEK-1 k−/k− dominant-negative. Relative luciferase activity is expressed as fold activation with respect to unstimulated cells. Columns, mean of three independent experiments normalized for transfection efficiency with a GFP vector as described in Materials and Methods; bars, SD.
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 up-regulation should be developed.

Acknowledgments

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