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Androgen increases androgen receptor protein while decreasing receptor mRNA in LNCaP cells

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Summary

We have examined the effect of androgen treatment on androgen receptor mRNA and protein expression in the LNCaP human prostate carcinoma cell line. Incubation with androgen caused a decrease in cellular androgen receptor mRNA content that was concentration and time dependent. Maximal suppression to approximately 35% of control level was observed after 49 h of exposure to androgen. By contrast, incubation of LNCaP cells with androgen resulted in a 2-fold increase in the cellular content of androgen receptor protein at 24 h. At 49 h androgen receptor protein increased 30% as assayed by immunoblots and 79% as assayed by ligand binding. These results suggest that ligand-induced changes in androgen receptor stability and/or the translational efficiency of androgen receptor mRNA account for the phenomenon of androgen receptor upregulation observed in cultured LNCaP cells. Furthermore, the suppression of androgen mRNA and protein that is caused by prolonged incubation with androgen is incomplete and is reversible upon removal of ligand.

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Introduction

Proteins of the steroid receptor family are ligand-responsive transcription factors that control a wide range of biological processes (Evans, 1988). The expression of many members of this gene family is under complex developmental and physiological control (Ylikomi et al., 1984; Clarke et al., 1990; Hodin et al., 1990; Ishikawa et al., 1990). One such influence is the ligand that binds to the receptor (Kalinyak et al., 1987; Hodin et al., 1990; Takane et al., 1990). Investigation of steroid receptor gene expression has produced examples

of ligand-specific positive and negative feedback regulation (McDonnell et al., 1987; Rosewicz et al., 1988; Alexander et al., 1989; Saceda et al., 1989).

When conventional ligand-binding assays are employed, diverse responses to the presence or absence of androgen are observed in different tissues. For example, studies by Van Doorn et al. (1976) and Blondeau et al. (1982) suggested that androgen induces upregulation of its receptor in rat ventral prostate and epididymis. Likewise, in human genital skin fibroblasts the androgen receptor is upregulated in response to 5α-dihydrotestosterone or the synthetic androgen methyltrienolone (Kaufman et al., 1983). In contrast, age-related downregulation of the androgen receptor has been described in the rat penis (Rajfer et al., 1980; Takane et al., 1990). Other studies have suggested a biphasic response as nuclear androgen receptor levels in the lateral lobe of the rat prostate initially decrease and then increase beginning at 3 days following castration (Prins, 1989). This result contrasts sharply with the response in ventral and dorsal lobes of the rat prostate where androgen receptor levels are reduced and remain low following castration. Thus, modulation of androgen receptor expression at the binding level is not uniform and may be age- and tissue-specific.

The molecular process by which androgens influence the expression of the androgen receptor gene is poorly understood. Quarmby et al. (1990) reported upregulation of androgen receptor mRNA in response to androgen withdrawal in the ventral prostate, coagulating gland, epididymis, kidney and brain of the rat and in the human prostate carcinoma cell line, LNCaP. Conversely, treatment with androgen decreases androgen receptor mRNA levels in these tissues and in LNCaP cells. To define the mechanisms controlling androgen receptor expression, we have studied the effects of androgens on the levels of both androgen receptor mRNA and protein in four human cell lines, including LNCaP.

Materials and methods

Tissue culture

Stock cultures of the LNCaP prostate carcinoma cell line were maintained in RPMI-1640 (Gibco, Grand Island, NY, U.S.A.) medium containing 10% (v/v) fetal calf serum (FCS) (Hazleton, Kansas City, KS, U.S.A.). Monolayer cultures of T47D breast cancer cells were propagated in RPMI-1640 medium containing 10% FCS and 0.20 units/ml of regular pork insulin. SA-OS osteosarcoma cells were grown in McCoy's medium containing 10% FCS and normal human genital skin fibroblasts were cultured in minimum essential medium (MEM) containing 10% FCS.

For experiments, cells were trypsinized and plated at a density of 1×10^6 cells/10 cm dish on day 1. On day 5 the medium was removed and replaced with phenol red-free RPMI-1640 containing 5% dextran-coated charcoal-stripped FCS (DCSS). Androgen content of charcoal-treated stripped serum was analyzed by chromatography on celite (Johns-Manville Products Corp., Lompoc, CA, U.S.A.) (Johnson et al., 1986). Concentrations of testosterone were < 17 fM, and concentrations of 5α-dihydrotestosterone (DHT) were approximately 100 fM. Hormones were added in ethanol, with a final ethanol concentration of 0.02% (v/v) while control dishes were treated with ethanol alone.

In each experiment parallel dishes were grown in duplicate to determine cell number. Medium was aspirated and the cells were rinsed twice with cold phosphate-buffered saline and then incubated in 1 ml of 0.25% trypsin/1 mM EDTA (Gibco, Grand Island, NY, U.S.A.) for approximately 5 min. The reaction was stopped by the addition of cold modified Eagle's medium (Gibco, Grand Island, NY, U.S.A.). Dilutions of cell suspensions were counted on a hemacytometer. Cell density was calculated from the mean of 6–8 hemacytometer fields. Over the incubation periods examined, no consistent change in cell numbers was observed following treatment with mibolerone or DHT compared with control (for example, see Table 1).

Materials

[^{3}H]Mibolerone (87 Ci/mmol) and mibolerone were obtained from New England Nuclear-Dupont (Boston, MA, U.S.A.). 5α-Dihydrotestosterone, dexamethasone, 17β-estradiol, and progesterone were obtained from Steraloids (Wilton, NH, U.S.A.).

Preparation of radiolabelled hybridization probes

A 275 bp fragment of the human androgen receptor cDNA (nucleotides 890–1165) (Tilley et al., 1989) was prepared by amplification using the polymerase chain reaction in the DNA Thermal Cycler (Perkin Elmer Cetus, Hartford, CT, U.S.A.). The oligonucleotides employed (No. 211: 5′-CATGGGCCTGGGTGTGGAGGCGTTG-3′ and No. 213: 5′-CGCTGCAGCAGGGAGCTC-CGGGACAC-3′) contained artificial *Eco*RI restriction endonuclease cleavage sites at their 5′ termini which permitted the insertion of the amplified fragment into the *Eco*RI restriction site of the plasmid, BSKSM13 + (Stratagene Cloning Systems, La Jolla, CA, U.S.A.). Orientation of the subcloned fragment in the plasmid (designated pBShAREX1) was established by restriction endonuclease mapping. Uniformly labelled RNA probes were then synthesized according to the manufacturer's specifications. To synthesize RNA probes of polarity that is complementary to that of native androgen receptor mRNA, i.e. an antisense probe, a sample of the plasmid pBShAREX1 was linearized with the restriction endonuclease *Hin*dIII and the T7 RNA polymerase was employed for in vitro transcription. Final [^{32}P]UTP concentration was 1.3 μM (specific activity of approximately 800 Ci/mmol).

Construction of the standard curve

To prepare synthetic RNA transcripts of the same polarity as native androgen receptor mRNA, a sample of the plasmid pBShAREX1 was linearized by digestion with the restriction enzyme *Bam*HI. Following template purification, RNA was transcribed in vitro using the T3 RNA polymerase and 0.2 μM [^{32}P]UTP (specific activity approximately 800 Ci/mmol). The integrity of this standard was determined by denaturing gel electrophoresis and sizing compared to ^{32}P-labelled molecular weight markers. From the nucleotide sequence of the fragment and the specific activity of the [^{32}P]UTP, the molar quantity of sense probe was calculated. Using this method 1350 pmol of synthetic androgen receptor mRNA standard were synthesized and stored as aliquots at −80 °C. The solution was diluted as necessary in diethylpyrocarbonate-treated water for use in individual experiments. Notably, dilutions of up to 10,000-fold, stored for up to 10 months at −80 °C, gave consistent data without evidence of deterioration of the androgen receptor mRNA standard.

Measurement of cellular androgen receptor mRNA content

Total cellular RNA was extracted by the hot phenol method as previously described (Maniatis et al., 1982). Recovery of RNA using this method was consistently 60% as assayed by recovery of an exogenous labelled synthetic RNA standard. Samples of the synthetic androgen receptor mRNA standard and samples of total cellular RNA were hybridized to the uniformly labelled 'antisense' probe in hybridization buffer (0.4 M NaCl, 0.04 M Pipes (pH 6.5), 1 mM EDTA, and 60% deionized formamide) at 48 °C for 12 h. Following hybridization, the samples were digested with 2000 units S_1 nuclease (Boehringer-Mannheim, Indianapolis, IN, U.S.A.) (3 U/μl) for 2 h at 42 °C in S_1 nuclease buffer (0.25 M NaCl, 1 mM $ZnSO_4$, 5% glycerol, and 30 mM NaAc, pH 4.5). The digestion products were extracted with phenol/chloroform (1 : 1), purified by two precipitations from 2 M ammonium acetate, and run on 6% polyacrylamide/8 M urea gels. Standard curves were included in each experiment. The specifically protected bands were visualized by autoradiography, cut out of the gel, and quantified by scintillation counting. Background activity typically represented less than 5% of the counts. Linear regression analysis of scintillation counts generated by standard probes yielded correlation coefficients of > 0.98 and were linear from 0.45 to 18 fmol of synthetic androgen receptor mRNA standard. Values are presented as molecules mRNA per cell. Each experimental result represents the mean of two replicates.

Measurement of [^{3}H]mibolerone binding

The effect of stimulation with androgen on androgen receptor levels was assayed by ligand binding and immunoblots at 24 and 49 h. For these experiments, cultures of LNCaP cells were plated into 3 cm dishes (for binding experiments) or into 10 cm dishes (for Western analysis). Four days after plating, the cultures were changed to RPMI medium containing 5% DCSS containing no added steroid (group I), 2 nM [^{3}H]mibolerone

(group II), 2 nM [^{3}H]mibolerone and 500 nM unlabelled mibolerone (group III), or 2 nM unlabelled mibolerone (group IV).

To compare the effect of 24 h incubation with mibolerone to the effect of a 1 h incubation with mibolerone, the medium from four dishes in group I was replaced with RPMI containing 5% DCSS and 2 nM [^{3}H]mibolerone (two dishes) or 2 nM [^{3}H]mibolerone and 500 nM unlabelled mibolerone (two dishes) after 23 h. After incubation at 37°C for an additional hour, the medium was removed from these four dishes, from two dishes in group II, and from two dishes in group III, and the level of specific androgen binding assayed (Griffin and Wilson, 1977).

The effect of incubation with mibolerone for 49 h or 1 h was assessed in a similar fashion. Medium was removed and replaced with fresh medium for four dishes in group I, two dishes in group II, and two dishes in group III after 24 h. At 48 h, the medium was removed from four dishes from group I and replaced with RPMI containing 5% DCSS and 2 nM [^{3}H]mibolerone (two dishes) or 2 nM [^{3}H]mibolerone and 500 nM unlabelled mibolerone (two dishes). After incubation for an additional hour at 37°C, the medium was removed from these four dishes, the two dishes from group II and the two dishes from group III and the level of specific [^{3}H]mibolerone bound was determined (Griffin and Wilson, 1977). On each day, the level of immunoreactive androgen receptor was assayed in parallel cultures incubated in medium containing unlabelled 2 nM mibolerone (24 h or 49 h).

Western analysis

Immunoblots were prepared as described by Husmann et al. (1990). In brief, washed cell pellets from individual culture dishes were solubilized in sample buffer (100 mM dithiothreitol, 70 mM sodium dodecyl sulfate (SDS), 10% glycerol, 0.004% bromophenol blue, 80 mM Tris, pH 6.9) by sonication and heating in a boiling water bath and diluted to the appropriate concentration with sample buffer. Protein concentration was determined by the method of Lowry et al. (1951) after precipitation with 10% (v/v) trichloroacetic acid in the presence of 0.8 mM sodium deoxycholate. 50 or 100 μl samples containing 52–155 μg protein were applied to 7.5% acrylamide gels containing 3.5 mM SDS, separated by electrophoresis, transferred to nitrocellulose filters and incubated sequentially with affinity-purified antibodies that recognize the amino- (N-) terminal region of the androgen receptor protein and with ^{125}I-labelled anti-rabbit IgG. Immunoreactive bands were visualized by autoradiography. Anti-N-terminal antibodies were raised in rabbit U402 in response to a synthetic peptide containing the N-terminal 21 amino acids of the androgen receptor protein and purified on a peptide affinity column as described (Husmann et al., 1990). M_r values were estimated by comparison with ^{14}C-methylated protein molecular weight markers (Rainbow Markers, Amersham Corp., Arlington Heights, IL, U.S.A.) run in the same gel.

Autoradiograms of the immunoblots were scanned in a 300A Computing Densitometer (Molecular Dynamics, Sunnyvale, CA, U.S.A.). All data were processed using Imagequant Software V1.21. Data is expressed as arbitrary densitometer units per mg protein. Each value represents the average obtained with samples from two replicate dishes.

Results

Culture medium affects cellular androgen receptor mRNA

To establish growth conditions for cell culture that would maximize hAR transcripts, a comparison was made of androgen receptor mRNA expression in LNCaP monolayer cultures using several different types of medium. Stock cultures were maintained in 10% FCS as described (Materials and Methods), seeded into 10 cm dishes (initial density 1×10^6 cells/plate), and grown for 5 days. 24 h prior to harvesting, the medium was changed to phenol-red-free RPMI medium containing one of the following additional components: (1) 10% FCS, (2) 5% dextran-coated charcoal-stripped serum (DCSS), (3) 5 mM bovine serum albumin (BSA) (Sigma, St. Louis, MO, U.S.A.), or (4) 1% ITS$^+$ serum substitution (Collaborative Research, Bedford, MA, U.S.A.). Total cellular RNA was isolated by solubilization of the cell pellets with guanidinium thiocyanate and purification over a 5.7 M cesium chloride cushion.

10 μg samples of total RNA were hybridized to the radiolabelled hybridization probe and quantified as described. The quantity of androgen receptor mRNA per μg of total RNA was determined in each sample. The relative levels of androgen receptor mRNA expressed in these samples were as follows: 10% FCS, 1.0; 5% DCSS, 1.4; 5 mM BSA, 1.0; and 1% ITS$^+$, 1.3. All subsequent experiments were performed using medium supplemented with 5% DCSS.

Androgens decrease the levels of androgen receptor mRNA in the LNCaP prostate carcinoma cell line

A preliminary experiment was performed to examine the effect of DHT (20 nM) and the synthetic androgen mibolerone (10 nM) on the cellular androgen receptor mRNA content of LNCaP cells. Following a 40 h incubation, androgen receptor mRNA levels were determined for the control cells and those treated with the various agents. When compared to control cells, the treated cells showed a reduction of androgen receptor mRNA concentration from 950 androgen receptor mRNA molecules per cell to 659 (69%) and 401 (42%) for the DHT- and mibolerone-treated cells, respectively (see Table 1).

An additional experiment was performed utilizing a 49 h incubation, with medium changes every 12 h, to define optimal treatment conditions for the androgen treatment. As indicated in Table 1, both DHT and mibolerone at 10 nM concentrations were effective at lowering mRNA levels, although 1 nM DHT appeared somewhat less effective. Based on the results of these two experiments, mibolerone was chosen for use in more detailed studies on the effect of androgens on androgen receptor expression in cultured human cells.

Decrease of androgen receptor mRNA by mibolerone is concentration and time dependent

As our initial experiments employed supraphysiological concentrations of androgens, we examined androgen receptor mRNA expression following 49 h incubations of LNCaP cultures with varying concentrations of mibolerone (Fig. 1). In this figure the level of androgen receptor mRNA is expressed as a percentage of the mean control value of 1271 ± 233 molecules per cell. Maximal reduction in androgen receptor mRNA (66%) was achieved with concentrations of mibolerone equal to or greater than 1 nM.

We next examined the time course of this decline in androgen receptor mRNA after incubation with 2 nM mibolerone. A graphic summary of the results of three separate experiments is shown in Fig. 2. These data indicate that little change in cellular androgen receptor mRNA content occurs within the first 24 h of androgen treatment. However, by 49 h of androgen stimulation, androgen

TABLE 1

DIHYDROTESTOSTERONE AND MIBOLERONE DECREASE THE LEVEL OF CELLULAR ANDROGEN RECEPTOR mRNA

Monolayer LNCaP cultures were grown in 10 cm dishes at 37°C in 5% CO_2 with initial density 1×10^6 cells per dish. On day 5, the growth medium was replaced with RPMI-1640 supplemented with 5% charcoal-stripped fetal calf serum, and containing either no additives or the compounds indicated above. Following incubation, cellular RNA was extracted using the hot phenol method, hybridized to radioactive probe complementary to exon 1 of the hAR cDNA, and digested with S_1 nuclease. The digestion products were purified by ammonium acetate precipitation, separated by electrophoresis on 6% denaturing polyacrylamide gels and autoradiographed. Individual bands were cut out of the gel and quantified by scintillation counting of the samples and standard curves included in every experiment. Numbers represent means of two experimental replicates as a percentage of two control replicates.

Additions	Number of cells per dish $\times 10^6$	Androgen receptor mRNA per cell (% of control)
Experiment I [a]		
None	13.7	100 [b]
5α-Dihydrotestosterone, 20 nM	14.3	69
Mibolerone, 10 nM	18.6	42
Experiment II [c]		
None	10.2	100 [d]
5α-Dihydrotestosterone, 1 nM	10.2	59
5α-Dihydrotestosterone, 10 nM	10.2	33
Mibolerone, 1 nM	9.6	39
Mibolerone, 10 nM	10.0	35

[a] Experiment I: Cells were incubated with or without additions for 40 h. Medium was changed after 24 h.
[b] 950 molecules of androgen receptor mRNA per control cell.
[c] Experiment II: Cells were incubated with or without additions for 49 h. Medium was changed every 12 h.
[d] 2090 molecules of androgen receptor mRNA per control cell.

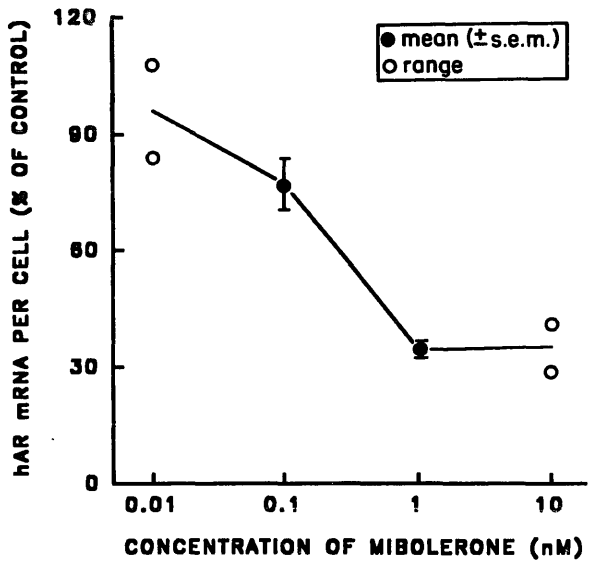


Fig. 1. Downregulation of human androgen receptor (hAR) mRNA by mibolerone is dose dependent. Monolayer cultures of LNCaP were grown as described in the text. On day 5, stock medium was replaced with experimental medium containing no added steroid or a variable concentration of mibolerone. Medium was changed every 24 h. Following 49 h incubations, RNA was extracted and the level of AR mRNA quantified. Four separate experiments were done, examining the effect of 0.1 nM and 1 nM mibolerone 3 times, and 0.01 and 10 nM mibolerone twice. Each experiment was performed on a different day. Each data point represents mean androgen receptor mRNA level in two experimental replicate (i.e., handled identically) cultures expressed as a percentage of androgen receptor mRNA detected in two untreated control dishes.

receptor mRNA levels had declined to 45% of control cells. Cellular androgen receptor mRNA levels at 80 h were similar to the levels determined at 49 h.

Downregulation of androgen receptor mRNA by ligand does not occur in all types of cultured human cells

The downregulation of androgen receptor mRNA in the LNCaP cell line suggested that this response might provide a useful marker of androgen action. For this reason, we examined other cell types to determine whether the downregulation of androgen receptor mRNA was a universal phenomenon. Androgen receptor mRNA levels were assayed in non-transformed genital skin fibroblasts, the osteosarcoma cell line SA-OS, and the breast cancer cell line T47D, in the presence or absence of DTH or mibolerone for 49 h using a protocol identical to that employed for the LNCaP cell line. The results of these experiments are shown in Table 2. The T47D cell line showed a decline in androgen receptor mRNA levels of a magnitude similar to that detected for the LNCaP cell line. By contrast, neither the SA-OS nor genital skin fibroblasts showed an appreciable decline in androgen receptor mRNA levels in response to androgen.

Ligand transiently increases levels of specific mibolerone binding and immunoreactive androgen receptor protein in the LNCaP cell line

The observation that androgen receptor mRNA decreased following incubation of LNCaP or T47D cells with mibolerone was in contrast to reports of increased specific androgen binding in response to androgen in other cell culture systems (Kaufman

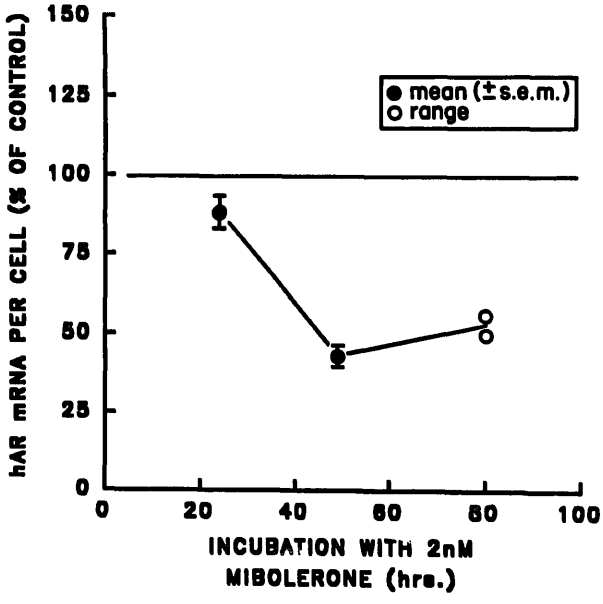


Fig. 2. The decrease in cellular hAR mRNA content in response to mibolerone is time dependent. LNCaP cells were cultured as described in the text. Stock medium was removed and replaced with experimental medium containing 2 nM mibolerone on day 5, and the medium was changed every 24 h thereafter. Cells were harvested after 24, 49, or 80 h. Samples of RNA were prepared as described, and analyzed by the S_1 nuclease protection assay. Data points from two (80 h) or three (24 and 49 h) separate experiments are summarized in this graph. Each individual data point represents the mean of two experimental replicates as a percentage of two control replicates.

TABLE 2

COMPARATIVE EXPRESSION AND MODULATION OF ANDROGEN RECEPTOR mRNA IN DIFFERENT HUMAN CELL LINES

Monolayer cultures of LNCaP prostate cancer cells, T47D breast cancer cells, non-transformed foreskin fibroblasts (strain 704), and SA-OS osteogenic sarcoma cells were propagated as described in the Materials and Methods section. On day 5, growth medium was replaced with appropriate medium supplemented with 5% charcoal-stripped fetal calf serum, and either no additives or the compounds listed below for 49 h. Following incubation, cellular RNA was extracted using the hot phenol method, hybridized to radioactive probe complementary to exon 1 of the hAR cDNA, and digested with S_1 nuclease. The digestion products were purified by ammonium acetate precipitation, separated by electrophoresis on 6% denaturing polyacrylamide gels and autoradiographed. Individual bands were cut out of the gel and quantified by scintillation counting of the samples. Standard curves were included in every experiment. Data represent means of two experimental replicates as a percentage of two control replicates. Numbers in the first column represent mean value of hAR mRNA content in all control cells in the given row ±SEM. Numbers in parentheses represent number of independent experiments performed under the given conditions.

	Androgen receptor mRNA					
	Molecules per untreated control cell	Percent of control value				
		Steroid treatment				
		5α-Dihydrotestosterone		Mibolerone		
		1 nM [a]	10 nM [a]	1 nM [a]	10 nM [a]	10 nM [b]
LNCaP	1576±202	53 (2)	58 (2)	39 (1)	36 (1)	35 (2)
T47D	293± 22	43 (2)	60 (1)	–	–	31 (1)
Foreskin fibroblasts	248± 23	–	–	–	–	102 (3)
SA-OS	90	–	–	–	–	92 (1)

[a] Medium changed every 12 h.
[b] Medium changed every 24 h.

et al., 1983). To examine this phenomenon, we incubated monolayer cultures of LNCaP cells with mibolerone for 24 or 49 h and monitored the effect on both androgen receptor ligand-binding activity and immunoreactivity. As shown in Table 3, there was a 2.5-fold increase in specific [^{3}H]-mibolerone binding after 24 h and a 1.8-fold increase after 49 h in androgen-treated cells compared to untreated control cells. Furthermore, this increase in specific ligand binding was accompanied by an increase in immunoreactive receptor protein in androgen-treated cells.

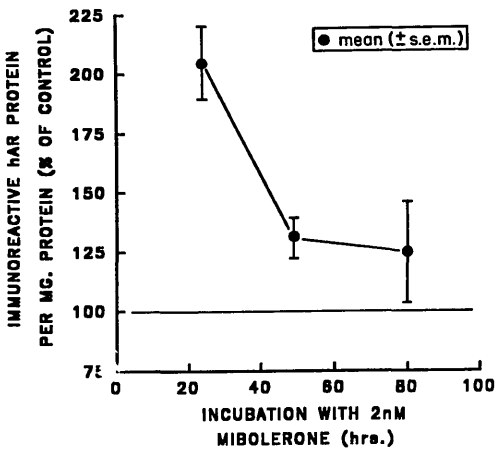
A graphic summary of data from four experiments designed to determine the time course of induction of immunoreactive androgen receptor protein is presented in Fig. 3. The average level of immunoreactive protein in LNCaP cells after 24 h of incubation with mibolerone increased 2-fold compared to untreated control cells. A smaller increase also was seen after 49 and 80 h of exposure to the androgen. These results are in contrast to the effect of mibolerone treatment on the

TABLE 3

LIGAND INCREASES THE CELLULAR CONTENT OF SPECIFIC ANDROGEN BINDING AND THE IMMUNOREACTIVE ANDROGEN RECEPTOR PROTEIN

Measurement of [^{3}H]mibolerone binding was performed as described in the text. Specific binding is the total [^{3}H]mibolerone bound minus that bound in the presence of a 200-fold excess of unlabelled mibolerone. Each binding value is the average of replicate assays. The percent change from control is the difference in specific binding detected in cells following a 24 or 49 h incubation with [^{3}H]mibolerone compared to cultures incubated with [^{3}H]mibolerone for 1 h. Immunoreactive androgen receptor was assayed as described in the text in cultures incubated with 2 nM mibolerone for 24 or 49 h.

Preincubation time (h)	Specific [^{3}H]mibolerone binding		Immunoreactive androgen receptor
	fmol/mg protein	Percent of control value	Percent of control value
0	193	100	100
24	491	254	177
0	203	100	100
49	364	179	130



Incubation Time	24 Hours		49 Hours		80 Hours	
Steroid Treatment	None	2nM Mb	None	2nM Mb	None	2nM Mb
Sample Size (µg)	132 116	120 100	108 106	120 108	96 96	92 104

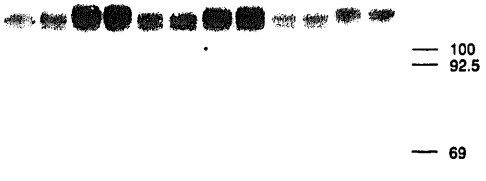


Fig. 3. 'Upregulation' of specific androgen binding is paralleled by increases in the cellular content of immunoreactive androgen receptor. LNCaP cells were cultured in parallel with the cells analyzed in the experiment depicted in Fig. 2. The cells were scraped into phosphate-buffered saline, pelleted, suspended in loading buffer and homogenized by sonication and boiling. Suspensions were brought to similar protein concentrations, and aliquots were fractionated by SDS-polyacrylamide gel electrophoresis on 7.5% gels. These were transferred onto a nitrocellulose filter and probed sequentially with polyclonal rabbit antibodies that detect the amino terminus of the human androgen receptor followed by ^{125}I-goat anti-rabbit IgG. The immunoreactive band was visualized by autoradiography and quantified by densitometry. Upper panel: Graphic summation of data points from four separate experiments. Each individual data point represents the mean of two experimental replicates as a percentage of two control replicates. Lower panel: Autoradiogram of a representative experiment. The position and size of standard molecular weight markers are shown in the right margin.

cellular content of androgen receptor mRNA in LNCaP cells (Fig. 2).

Ligand induces a change in the mobility of the androgen receptor protein

The immunoblot presented in Fig. 3 shows that androgen receptor obtained from the LNCaP cell line migrates as a doublet of bands of approximately 110 kDa. In the absence of hormone, substantial quantities of both the slower and faster migrating species can be detected. After 24 h of incubation with 2 nM mibolerone, however, the slower migrating species increases and becomes the predominant form of the receptor. Similar mobility shifts are also detected in the T47D cell line androgen receptor in response to mibolerone (data not shown).

Long-term incubation of LNCaP cells with ligand does not effect a complete disappearance of androgen receptor mRNA or the receptor protein

The changes in androgen receptor mRNA and protein described above resulted from incubations with hormone for 80 h or less. To examine the effects of long-term stimulation by androgen, two flasks of LNCaP were incubated with experimental medium, with or without 2 nM mibolerone. On day 8, cells were harvested, reseeded in 10 cm dishes (initial density approximately 1×10^6 cells per dish) and propagated with daily medium changes for another 9 days. Androgen receptor mRNA was readily detectable in both groups, with the mibolerone-treated LNCaP cells showing 36% androgen receptor mRNA per cell in comparison to the control cells, which contained 1990 androgen receptor mRNA molecules per cell. Little or no change in immunoreactive androgen receptor protein was observed in the mibolerone-treated cells compared to control cells (data not shown).

Because the LNCaP cells do not grow well in charcoal-treated serum for extended periods, this same issue was approached by the long-term incubation of LNCaP cell cultures in stock medium containing mibolerone. Stock cultures of LNCaP were grown for 13 days in RPMI-1640 supplemented with 10% FCS and 2 nM mibolerone. On day 14, cell cultures were changed to RPMI medium containing 5% DCSS with or without 2 nM mibolerone. The group cultured in medium without androgen on the last 2 days showed an increase of 30% in androgen receptor mRNA per cell compared to cells grown continuously in medium containing mibolerone which contained 2552 androgen mRNA molecules per cell. This

increase was paralleled by a 23% increase in immunoreactive androgen receptor protein.

Discussion

The regulation of androgen receptor expression has been investigated in a number of tissues prior to the availability of nucleic acid and antibody probes specific for the androgen receptor (Van Doorn et al., 1976; Rajfer et al., 1980; Blondeau et al., 1982; Kaufman et al., 1983; Prins, 1989). Results of these studies indicated that androgen receptor expression is complex and likely to be influenced by changes in tissue composition and hormonal milieu.

In the current study, we examined the changes in the cellular content of androgen receptor mRNA and protein in continuous human cell lines. These experiments demonstrate that androgen receptor mRNA decreases in the LNCaP cell line in response to androgens. The decrease is concentration and time dependent, with a small decrease after 24 h and maximal effect at 49 h. Downregulation of androgen mRNA by treatment with androgen has also been observed by Quarmby et al. (1990) in castrate rats. Decreased levels of androgen receptor mRNA in rat tissues were detected by Northern analysis within 8 h of androgen administration. In contrast, we found that more than 24 h of exposure to androgen are required to produce an appreciable change in the level of androgen receptor mRNA in LNCaP and T47D cells. The reasons for this difference in the kinetics of change in androgen receptor mRNA levels are not clear.

This study also provides several additional insights into the regulation of androgen receptor expression. First, the level of androgen receptor mRNA expression cannot be completely suppressed by treatment with androgens. In our experiments, androgen receptor mRNA was never decreased below 30% of the levels detected in parallel control cultures, even following prolonged incubation with androgen. Furthermore, this suppression was reversible following the removal of androgens from the culture medium. This pattern of androgen receptor mRNA regulation is in contrast to several experimental models of androgen receptor expression in which the developmental regulation of the androgen receptor results in the irreversible disappearance of androgen receptor from specific compartments of the rat penis (Rajfer et al., 1980; Takane et al., 1990), rat gubernaculum (Husmann, 1990), and human penis (Roehrborn et al., 1987). This inability of ligand to suppress androgen receptor expression completely in the prostatic carcinoma cell line LNCaP may indicate a fundamental difference of androgen receptor regulation between the human prostate and human penis and may account for the continued androgen-mediated growth of the human prostate throughout adult life.

To assess the generality of these findings, we also investigated the regulation of androgen receptor mRNA in other cell types in response to androgens. The levels of androgen receptor mRNA decreased to 31% of control values in T47D breast cancer cells, a change that is similar to that observed in the LNCaP prostate cancer cell line. However, we could not demonstrate changes in androgen receptor mRNA levels in genital skin fibroblasts (strain 704) or in the osteosarcoma cell line SA-OS in response to androgens. These results suggest that the mechanisms controlling androgen receptor mRNA expression may differ among different cell types, even those that employ the same promoter within the androgen receptor gene (Tilley et al., 1990).

Previous studies have demonstrated an increase in specific androgen binding in some cell strains in response to incubation with ligand (Kaufman et al., 1983). Our studies demonstrate that this increase also occurs in LNCaP cells, and is accompanied by an increase in the cellular content of immunoreactive androgen receptor. The increased level of androgen receptor protein observed after 24 h incubation with mibolerone appears to be a posttranscriptional effect since experiments conducted with parallel cell cultures revealed little or no change in the level of androgen receptor mRNA under the same conditions. These results suggest that androgen receptor 'upregulation' in LNCaP cells occurs principally because of increased translational efficiency and/or stabilization of the receptor protein and not due to changes in the level of androgen receptor mRNA.

It is intriguing to note that changes in receptor level in response to mibolerone were accompanied

by altered mobility of the immunoreactive androgen receptor protein, from a faster to a slower migrating form. These two forms of immunoreactive androgen receptor have been observed previously in LNCaP cells by Van Laar et al. (1990), but they found no change in isoform ratio in response to treatment with the synthetic androgen methyltrienolone in short-term experiments. Similar ligand-induced mobility shifts in progesterone (Sheridan et al., 1988) and vitamin D (Pike and Sleator, 1985) receptors have been associated with alterations in phosphorylation of receptor proteins.

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