Androgen responsiveness of the pituitary gonadotrope cell line LβT2

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Abstract

Androgens have a profound effect on the hypothalamic–pituitary axis by reducing the synthesis and release of the pituitary gonadotropin LH. The effect on LH is partly a consequence of a direct, steroid-dependent action on pituitary function. Although androgen action has been well studied *in vivo*, *in vitro* cell models of androgen action on pituitary gonadotropes have been scarce. Recently, an LH-expressing cell line, LβT2, was generated by tumorigenesis targeted to the LH-producing cells of the mouse pituitary. The purpose of these studies was to determine the presence of androgen receptor (AR) and establish its function in this cell line. RT-PCR analysis indicated that the LβT2 cell line expresses AR mRNA. Transient transfection assays, using the mouse mammary tumor virus

(MMTV) promoter, showed that a functional AR is also present. Testosterone (TEST), dihydrotestosterone (DHT), 7α-methyl-19-nortestosterone (MENT), and fluoxymesterone (FLUOXY) increased reporter gene activity in the rank order of potencies MENT>DHT>TEST>FLUOXY. Additionally, activation of MMTV promoter activity by DHT in LβT2 cells was diminished by the AR antagonists casodex and 2-hydroxy-flutamide, indicating that the effects of DHT are mediated through AR. In summary, these studies showed that the LβT2 cell line is a useful model for the evaluation and molecular characterization of androgen action in pituitary gonadotropes.

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Introduction

Androgen action is mediated by the androgen receptor (AR) which modulates activation of specific genes by homodimeric AR bound to DNA, or by interactions with other transcription factors. Both positive and negative regulation of target genes is a characteristic mode of action for steroid receptors (Beato *et al.* 1995). Androgens are clearly involved in reproductive tissue development, muscle and bone homeostasis, sex behavior, metabolism, etc. (see Mooradian *et al.* 1987 for a review). In the hypothalamic–pituitary axis, androgens negatively regulate gonadotropin secretion (Kalra & Kalra 1983, Gharib *et al.* 1990) and, consequently, reproductive function.

Existing models for the *in vitro* analysis of steroid action on reproductive endocrine tissues are either laborious or of limited availability. Recently, a number of pituitary cell lines have been developed using targeted tumorigenesis of pituitary gonadotropes in transgenic mice (Alarid *et al.* 1996, 1998). One of these cell lines, LβT2, expresses luteinizing hormone (LH) (Turgeon *et al.* 1996) and follicle-stimulating hormone (FSH) (Graham *et al.* 1999) under specific culture conditions. It has been reported that this cell line is responsive to gonadotropin-releasing

hormone, the secretagogue of the gonadotropins, as well as to estrogen (Turgeon *et al.* 1996, Schreihöfer *et al.* 2000). In these studies, we have investigated the potential use of this cell line as a model to evaluate androgen action at the molecular level in pituitary gonadotropes. We have identified the presence of endogenous AR in the LβT2 cell line. We have also defined the response to various androgens and assessed receptor specificity using AR antagonists. These studies have demonstrated the presence of a functional AR in the LβT2 cell line and establish these cells as a model for the evaluation of the molecular mechanisms of androgen action in pituitary gonadotropes.

Materials and Methods

Cell culture

LβT2 cells were licensed from the University of California, San Diego (La Jolla, CA, USA). Cells were cultured in high glucose, HEPES-buffered Dulbecco's modified Eagle's medium (DMEM; Biowhittaker, Walkersville, MD, USA) supplemented with 2 mM L-glutamine and 10% fetal bovine serum (Hyclone, Logan, UT, USA), penicillin and streptomycin (Gibco BRL,

Grand Island, NY, USA). Cells were grown at 37 °C in a humidified incubator in an atmosphere of 5% CO₂:95% air. Subculture of LβT2 cells was accomplished by gentle trypsinization when they approached 80% confluence. Subcultivation ratios of 1:5 to 1:20 were routinely used.

Total RNA extraction and RT-PCR

LβT2 cells were cultured to confluency and, after washing the monolayer with phosphate-buffered saline, total RNA was extracted by lysis in TRIzol reagent according to the manufacturer's instructions. Total RNA from mouse prostate was also isolated and served as a positive control for AR mRNA. After alcohol precipitation, total RNA pellets were dissolved in water and absorbance was measured at 260 and 280 nm. One microgram of total RNA from LβT2 cells and mouse prostate was DNase treated and reverse transcribed using the superscript II kit (Gibco BRL) and priming with oligo-dT, following the manufacturer's recommendations. cDNAs (equivalent to 1 µg reverse transcribed total RNA) were PCR amplified using a hot-start PCR protocol and AmpliTaq Gold (PE Applied Biosystems, Foster City, CA, USA). The reactions were carried out in 100 µl volume of 1 × AmpliGold buffer II containing 1 mM MgCl₂ and 400 nM of each primer. Samples were heated at 94 °C for 14 min followed by successive heating steps at 94 °C for 30 s, 52.5 °C for 30 s and 72 °C for 45 s. Thirty-five cycles were used for amplification followed by a final extension period at 72 °C for 10 min. Primers for mouse AR (accession number M37890) (Gaspar *et al.* 1990, 1991, He *et al.* 1990, Faber *et al.* 1991) used in the RT-PCR paradigm were as follows: forward primer: 5'-AACAACA GCAGCAGCACC-3' (bases 530 to 547), reverse primer 5'-GGATTGGAAGGTGAGAGAGCTT-3' (bases 953 to 934).

Southern blotting

Amplification products (1/10th of the PCR reaction) were resolved by electrophoresis through a 1% agarose gel and transferred to a nylon membrane (Hybond-N⁺; Amersham, Arlington Heights, IL, USA) using a trans-Vac TE80 vacuum blotter (Hoefer Pharmacia Biotech Inc., San Francisco, CA, USA). The identity of the amplification products was confirmed by hybridization using an oligonucleotide probe specific to the mouse AR (probe: 5'-AGCATTGGAACATCTGAGTCCAGGGG AAACA-3'). The oligonucleotide was labeled with ³²P using terminal transferase and the resulting probe was diluted in Rapid-Hyb solution (Amersham). The blot was hybridized to the diluted probe for 2 h at 42 °C and washed once at room temperature with 50 ml 5 × SSC, 0.1% SDS. Two additional stringency washes were performed at 42 °C utilizing 1 × SSC, 0.1% SDS and the blots were exposed to film.

Transfection assay

LβT2 cells were plated on Falcon primary plates (Becton Dickinson, Franklin Lakes, NJ, USA) at a density of 50 000 cells/cm² in phenol red-free, high glucose DMEM supplemented with 2 mM L-glutamine and 10% charcoal/dextran-stripped fetal bovine serum (Hyclone), penicillin and streptomycin (GIBCO/BRL). After growth overnight, the cell medium was replaced with fresh medium containing either vehicle or test compounds. Cells were immediately transfected with the reporter plasmid pGL3-MMTV (mouse mammary tumor virus; 10 µg/ml), consisting of the firefly luciferase reporter gene from the pGL3-basic reporter plasmid (Promega, Madison, WI, USA) under the transcriptional control of the androgen-sensitive MMTV LTR promoter element. As an internal control, cells were co-transfected with the pCMV-β-galactosidase reporter plasmid (1 µg/ml) (CLONTECH Laboratories, Inc., Palo Alto, CA, USA). The FuGENE 6 reagent (60 µl/ml; Roche Molecular Biochemicals, Indianapolis, IN, USA) was used for the transfection experiments following the manufacturer's recommendations. Cells were treated with vehicle or the various treatments concurrent with the transfection mix (5 µl/well) and were incubated for 24 h. Thereafter, cells were lysed with phosphate-buffered saline containing 0.1% Triton X-100. Both luciferase and β-galactosidase activity were measured in the same aliquot using the dual light assay system (Tropix, Bedford, MA, USA) in a LB96V luminometer (EG&G Berthold, Bad Wildbad, Germany) controlled by the WinGlow software (EG&G Berthold). Luciferase reporter gene activity normalized to the activity of the internal control β-galactosidase is reported.

Data analysis and statistics

Data are expressed as the means ± S.E.M. of six individual samples per group. The results were analyzed for statistically significant differences by a one-way analysis of variance and Dunnett's *post-hoc* test on data either untransformed or optimally transformed by the method of Box-Cox (Box & Cox 1964) using the JMP statistical analysis software (SAS Institute, Cary, NC, USA). Specific data transformations are mentioned in the figure legends. Estimation of EC₅₀ and IC₅₀ values was conducted using the four-parameter logistic equation on either untransformed or Box-Cox-transformed data by a previously described method (Ghosh *et al.* 1998). A *P* < 0.05 was designated as the minimum criterion for declaring statistically significant differences.

Results

To determine whether the pituitary cell line LβT2 expresses the AR gene, RT-PCR was conducted on total

Figure 1 LβT2 cells express AR mRNA. Primers specific for mouse AR were used to amplify reverse transcribed mouse AR cDNA from LβT2 cells or mouse prostate total RNA. Amplification products were resolved by electrophoresis through a 1% agarose gel and Southern blotted with an oligonucleotide probe corresponding to a sequence internal to the amplification primer sites. The + and – symbols indicate the inclusion or omission of RT in the reverse transcription step prior to amplification. Size indication is derived from a 100 bp ladder included in the agarose gel.

RNA isolated from LβT2 cells. Amplification products of predicted mobility were identified in RT-PCR reactions that included reverse transcriptase in LβT2 and prostate total RNA samples (Fig. 1; left panel). Reactions not containing reverse transcriptase did not yield any amplification product (Fig. 1; left panel). These data indicate that the source for the target sequence is mRNA rather than contaminating genomic DNA. As an additional verification of RT-PCR fidelity, PCR products were blotted and probed with a radiolabeled oligonucleotide derived from AR sequence internal to the primer sequences used in the PCR reaction. A correctly migrating band containing appropriate AR-derived sequences was observed (Fig. 1; right panel). Thus, the LβT2 cell line clearly expresses detectable levels of the AR mRNA.

The presence of functional AR in LβT2 cells was evaluated in transient transfection assays using an androgen-sensitive reporter. The pGL3-MMTV reporter plasmid used in these studies containing the MMTV long terminal repeat (LTR) promoter driving luciferase gene expression was chosen because of the well-documented sensitivity of the promoter to direct AR action at the level of transcription (Beato *et al.* 1996). Dihydrotestosterone (DHT), fluoxymesterone (FLUOXY), 7α-methyl-19-nortestosterone (MENT), or testosterone (TEST) caused a dose-dependent increase in pGL3-MMTV reporter gene activity in the LβT2 cells (Fig. 2). The rank potencies for the compounds tested were MENT>DHT>TEST> FLUOXY and are summarized in Table 1. Induction of reporter activity at maximal doses ranged approximately two- to fourfold. The dose-dependent increase in pGL3-MMTV reporter gene expression in androgen-treated cells indicated that LβT2 cells express a functional AR.

Although direct activation of AR leads to transcriptional responses dependent on the interaction of ligand-bound AR with a competent promoter element, other mechanisms for AR-dependent activation of transcription have been reported that are resistant to inhibition by the androgen receptor antagonists 2-hydroxy-flutamide and casodex (Peterziel *et al.* 1999). Therefore, to establish that the androgen responsiveness of pGL3-MMTV is dependent on the transcription activation properties of ligand-bound AR, we tested the ability of the AR antagonists 2-hydroxy-flutamide and casodex to block the DHT-evoked response of the reporter gene in LβT2 cells. Cells were treated with vehicle, 2 nM DHT (~EC₇₅) or 2 nM DHT and increasing concentrations of 2-hydroxy-flutamide or casodex concurrently with transfection. Both 2-hydroxy-flutamide and casodex blocked DHT-induced response of the reporter gene, indicating AR-dependent activity. In addition, both compounds demonstrated full efficacy, since they suppressed DHT-mediated reporter gene response to levels equivalent to the untreated vehicle controls (Fig. 3). Although both AR antagonists were fully efficacious, they exhibited a marked difference in potency (~56 nM for 2-hydroxy-flutamide versus ~1.2 μM for casodex; Table 1).

Discussion

The results of these studies indicate that the gonadotrope cell line LβT2 represents a valuable model for the analysis of androgen action in the pituitary that would facilitate the dissection of the molecular mechanisms involved in the regulation of gonadotropin production and secretion. Our data demonstrate that LβT2 cells express AR mRNA and functional protein as determined by activation of the androgen-sensitive MMTV promoter in a transient transfection paradigm. DHT, FLUOXY, MENT and TEST elicit robust, two- to fourfold increases in reporter gene activity with the following rank of potencies: MENT>DHT>TEST>FLUOXY. This response is AR dependent as evidenced by the blockade of DHT-evoked activity of the reporter gene by the well-characterized AR antagonists 2-hydroxy-flutamide and casodex. Our observations reinforce the notion that the gonadotrope itself represents a potential site for androgen action in regulating gonadotropin synthesis and secretion.

Androgens are the predominant peripheral signal regulating LH secretion by acting at both the hypothalamus (Damassa *et al.* 1976, Clayton *et al.* 1982) and pituitary (Kingsley & Bogdanove 1973, Drouin & Labrie 1976, Giguere *et al.* 1981, Kotsuji *et al.* 1988) sites, although their mechanism of action in either tissue is not well defined. Some evidence has been presented to suggest that the hypothalamus alone is the site of androgen feedback (Dubey *et al.* 1987, Tilbrook *et al.* 1991). With respect to the pituitary site of action, AR is expressed in the anterior

Figure 2 DHT, FLUOXY, MENT and TEST activate the pGL3-MMTV reporter transfected into LβT2 cells. Luciferase values are reported relative to β-galactosidase activity as an internal control. The open bar represents the vehicle-treated group for each treatment. Results from one of three independent experiments are presented. Data for each group correspond to the means ± S.E.M. of six wells. Asterisks denote statistically significant differences versus the respective vehicle-treated groups. For statistical analysis, data were converted to the natural logarithm.

pituitary (Naess *et al.* 1975, Sar & Stumpf 1977, Thieulant & Pelletier 1979, Schanbacher *et al.* 1984, Bonsall *et al.* 1985, Sar *et al.* 1990, Kimura *et al.* 1993, Abdelgadir *et al.*

1999, Pelletier *et al.* 2000). In particular, AR-like immunoreactivity has been identified in secretory cells of the anterior pituitary (Sar *et al.* 1990, Pelletier *et al.* 2000)

Table 1 Summary of potencies for androgens and antiandrogens when tested in multiple experiments

Compound	Potency estimate (nM)	95% Confidence limits (nM)	Interassay coefficients of variation (%)
Dihydrotestosterone	0.97 ± 0.12 (3)	0.75–1.25	12.81
Fluoxymesterone	13.41 ± 2.71 (3)	9.02–19.94	20.24
7α-Methyl-19-nortestosterone	0.28 ± 0.04 (3)	0.22–0.37	13.58
Testosterone	2.41 ± 0.32 (3)	1.85–3.12	13.32
2-Hydroxy-flutamide	56.30 ± 39.33 (2)	14.31–221.42	69.87
Casodex	1197.84 ± 333.59 (2)	693.96–2067.56	27.85

Data denote the results of (n) independent experiments using six replicates per group. The means are calculated as the average potency estimate for each experiment weighted by the variance of each experiment. The first four compounds were tested alone, the last two were tested in the presence of an EC₇₅ of DHT (2 nM). Weighted mean potency is reported ± weighted S.E.M.

Figure 3 AR antagonists 2-hydroxy-flutamide and casodex block DHT-induced reporter gene expression in L β T2 cells. Cells were co-transfected with the pGL3-MMTV and pCMV- β -galactosidase reporter genes. Transfected cells were concurrently treated with vehicle (open bars), an EC₇₅ dose of DHT (2 nM; solid bars) or DHT plus increasing concentrations of 2-hydroxy-flutamide (2HO-FLUTAMIDE) or casodex (symbols and curves). Results from one of two independent experiments are presented. Data for each group are represented as the mean $\pm$ S.E.M. of six wells. Asterisks denote statistically significant differences versus the DHT-treated groups. For statistical evaluation, data were elevated to -0.8 (flutamide experiment) or -0.6 (casodex experiment).

and AR-positive staining has been described in human pituitary FSH- and LH-producing cells (Kimura *et al.* 1993). Similarly, morphological/biochemical studies have shown abundant androgen-binding sites in rat pituitary gonadotropes (Sar & Stumpf 1977). Our data demonstrate the presence of AR mRNA and protein in immortalized LH-secreting cells. The L β T2 cell line therefore appears to have retained the ability to express AR as observed in the pituitary *in vivo*. The presence of functional AR in an immortalized, yet highly differentiated, cell line such as L β T2 cells provides a model system for addressing issues of direct androgen action at the level of the pituitary. This model offers a simpler system where the confounding influence of other endocrine effectors normally found *in vivo* or in primary cultures of gonadotropes is absent.

Functional studies suggest profound effects of androgens in the modulation of LH secretion in primary cultures of pituitary cells. Both TEST and DHT have been shown to readily suppress LH-releasing hormone (LHRH)-induced LH release from anterior pituitary cells in either static or dynamic culture conditions (Drouin & Labrie 1976, Kotsuji *et al.* 1988). DHT is approximately three times more potent than TEST in inhibiting LHRH-induced LH secretion with estimated ED₅₀ values of 0.16 and 0.5 nM

respectively (Drouin & Labrie 1976). In L β T2 cells, DHT was also approximately 2.5 times more potent than TEST in inducing activation of the reporter gene, indicating that in these immortalized cells both steroids behave as observed in primary cultures of pituitary cells. In addition, it has been shown that MENT is 12–25 times more potent than TEST in reducing orchidectomy-induced elevation of LH secretion (Kumar *et al.* 1992). This difference in potency is also observed in immortalized gonadotropes, since MENT has an approximately tenfold lower ED₅₀ than TEST in L β T2 cells. Overall, these observations support the notion that the L β T2 cell line represents a relevant model to assess androgen action in pituitary gonadotropes.

The establishment of an *in vitro* cell model of gonadotrope function such as the L β T2 cell line that is androgen responsive represents an important addition to the models used to evaluate tissue-selective actions of androgens. Synthetic androgens and antiandrogens exhibit tissue selectivity that results in different physiological consequences when administered *in vivo*. For example, both 2-hydroxy-flutamide and casodex cause the regression of seminal vesicles and ventral prostate in intact mature rats treated for 14 days; however, casodex does not cause significant

changes in circulating levels of LH or TEST, whereas 2-hydroxy-flutamide does (Furr *et al.* 1987). In a pituitary cell background, casodex appears 20-fold less potent than flutamide in blocking DHT-induced MMTV promoter activity in L β T2 cells. However, when tested in a co-transfection assay in CV1 cells, Hamann *et al.* (1998) report 15 and 157 nM IC₅₀ for 2-hydroxy-flutamide and casodex respectively. This difference in activity may be dependent on the cell background, suggesting that casodex may be less effective in pituitary gonadotropes than flutamide in blocking androgen action. Our observations suggest that the interaction of AR with intracellular molecular targets may differ between the two antiandrogens tested, leading to a decreased potency of the compound. Precedence for such an effect is found in the analysis of co-factor interaction of estrogen receptor with estrogen receptor modulators (Wijayarathne *et al.* 1999). In the case of the estrogen receptor, altered activity of the ligand-bound estrogen receptor can be correlated to altered conformation of the receptor. Furthermore, casodex, unlike 2-hydroxy-flutamide, increases the degradation of AR, thereby exerting antiandrogen activity by reducing receptor content (Veldscholte *et al.* 1992, Kempainen & Wilson 1996, Waller *et al.* 2000). More studies evaluating non-androgen response element (ARE)-dependent AR activity on the LH β promoter are required to further substantiate this idea.

Androgen-dependent reduction of LH secretion via direct action at the pituitary, rather than at the hypothalamus, could be explained by modulation of LHRH-receptor function, LH production, or both. Evidence for an androgen-dependent reduction in pituitary LHRH receptors has been demonstrated by Giguere *et al.* (1981). In agreement with this, pituitary cells from castrated rats show increased responsiveness to an LHRH challenge *in vitro* (O'Connor *et al.* 1980). Since, in functional studies using primary pituitary cell cultures, androgens do not alter basal LH secretion (Drouin & Labrie 1976, Kotsuji *et al.* 1988), it has been suggested that an important mechanism of action for androgens in the control of LH secretion in gonadotropes is the reduction of LHRH receptors rather than direct modulation of LH production. More recent studies have suggested that AR can suppress transcription of both α and β subunits of LH in a ligand-dependent fashion using multiple mechanisms. Binding of AR to an ARE in the α subunit promoter has been described (Clay *et al.* 1993). Mutational analysis of the ARE has indicated that this sequence is not required for liganded AR-dependent repression of the promoter (Heckert *et al.* 1997). In addition, two elements, the α basal- and tandem cAMP-regulatory elements, have been defined in the α subunit promoter as responsible for AR-mediated suppression of transcription (Heckert *et al.* 1997). Therefore, the AR-dependent inhibitory effect of α subunit and LH β promoter activity may occur via protein-protein interactions between AR and other

transcription factors binding to their own regulatory sequences in the promoters. Further examination of the co-factor interaction of AR with the α subunit and LH β promoters is necessary to determine the mechanism by which androgens inhibit transcriptional activity.

In summary, we have demonstrated that the L β T2 cell line is a useful model for the study of androgen action in the anterior pituitary. The availability of a highly differentiated cell line with a consistent gonadotrope phenotype facilitates the study of the mechanisms of androgen action in the gonadotrope. The L β T2 cells provide a consistent source of material for detailed examination of the transcriptional mechanisms of androgen action. Further study of AR action on endogenously expressed genes, as well as study of antiandrogen action via ARE-dependent and non-ARE, protein-protein interaction-dependent mechanisms will lead to a better mechanistic understanding of AR as a modulator of reproductive functions.

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