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Dihydrotestostenone increase the gene expression of androgen receptor coregulator FHL2 in human non-transformed epithelial prostatic cells

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The actions of androgens are mediated through an androgen receptor (AR), and AR activity is modulated by coregulators. The aim of this study was to assess the action of androgens in the expression of AR and the coregulators *FHL-2* and *SHP-1* in human non-transformed epithelial prostatic cells (HNTEP) treated with androgens. Prostate tissues were obtained from 12 patients between 60 and 77 years of age. HNTEP cells were grown in basal medium and treated with DHT in different conditions. HNTEP cells under treatment with DHT (10^{-13} M) induced an increase in *FHL-2* expression. In turn, high DHT concentrations (10^{-8} M) induced an increase in the expression *SHP-1*. The present data suggest that the *SHP-1* and *FHL-2* genes play a role in the control of responsiveness and androgen-dose-dependent cell proliferation in HNTEP cells. Further studies are needed to assess the influence of androgens in AR and its coregulators and the implications in the pathophysiology of prostate diseases.

Key words: Androgens, *FHL-2*, *AR,* prostate, proliferation, coregulators.

INTRODUCTION

Androgens are mediators of a wide range of developmental and physiological responses. The effects of androgens occur through the androgen receptor (AR). The androgen interactions with the AR result in transcriptional activation of target genes which are important in male sexual differentiation and puberal sexual maturation (Marker et al., 2003). AR is a ligand inducible transcriptional factor, member of the nuclear

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Abbreviations: AR, Androgen receptor; **HNTEP,** human non-transformed epithelial prostatic cells; **LBD,** ligand binding domain; **DBD,** DNA-binding domain; **NTD,** NH_2- terminal transactivation domain; **AF-1,** activation function domain 1; **BPH,** benign prostate hyperplasia; **PCa,** prostate carcinoma; **LUTS,** lower urinary tract symptoms; **ARE,** androgen responsive elements; **SDS,** sodium dodecyl sulfate; T_m, melting temperature; C_T, cycle threshold; **SEM,** standard error of mean; **SPSS,** statistical package for social sciences.

receptor superfamily that mediates the expression of target genes in response to specific stimulus (Aranda and Pascual, 2001). The human AR gene is localized in the chromosome X at the q11-q12 position. There are eight exons and the gene shares a characteristic structure with other nuclear receptors, four domains: the ligand binding domain (LBD) containing activation function domain 2 (AF-2), the zinc-finger-type DNA-binding domain (DBD), a hinge region and the variable NH_2- terminal transactivation domain (NTD) possessing activation function domain 1 (AF-1).

AF-1 acts as a ligand-independent manner and can be more active when in contact with basal transcription factors, while the activity of AF-2 requires the ligand binding. Also, the interaction between NTD and LDB could also be necessary for the activation of AR (Dehm and Tindall, 2007; He et al., 2000).

Androgens play an important role in controlling the growth of the normal prostate gland, and also in promoting benign prostate hyperplasia (BPH) and prostate carcinoma (PCa). Histologic evidence of BPH is found in 50% of men at the age of 50 and up to 90% of males at the age of 80 (Roehrborn et al., 2006). The pathogenesis of BPH is still poorly understood, but there are two accepted permissive factors: the presence of circulating androgens and advancing age (Untergasser et al., 2005). The proliferative effects of androgens in prostate are controversial; some studies demonstrated a biphasic effect of androgen action in prostatic cells proliferation where lower androgen concentrations have a maximum mitogen effect whereas higher concentrations do not (Joly-Pharaboz et al., 2000; Joly-Pharaboz et al., 1995; Lee et al., 1995; Shao et al., 2007; Sonnenschein et al., 1989).

In contrast, other studies were not able to show any effect of androgens, at different concentrations, on cell proliferation of normal, hyperplasic or tumoral prostatic cells (Berthon et al., 1997; Heisler et al., 1997; Krill et al., 1999). Androgen concentrations are determinant to prostate enlargement and dependent on AR activity (Li et al., 2007; Yuan et al., 2006). Prior to androgen binding, AR is held inactive through association with heat shock proteins. Androgen binding releases inhibitory proteins and the AR translocates to the nucleus, where it interacts with DNA sequences, called androgen responsive elements (ARE). After binding to ARE, AR is able to recruit all the compounds of the transcriptional machinery for target genes (Balk and Knudsen, 2008).

Activation of gene expression is one aspect of AR signaling. Repressed or activated androgen-responsive genes appear to play important roles in regulating cell growth and differentiation. The activation of AR by androgens is a complex process involving a large number of activating and repressing proteins called coregulators. Several studies have indicated that altered expression of these molecules may modify transcriptional activity of AR suggesting that these coregulators could also contribute to the progression of prostatic pathologies (Heemers and Tindall, 2007; Urbanucci et al., 2008, Muramatsu et al., 2013, Toropainen et al., 2015).

SHP-1 (short heterodimer partner) is an orphan nuclear receptor which interacts with a large variety of nuclear receptors and has been shown to be expressed in androgen target tissues (Johansson et al., 1999). Gobinet et al. (2001) demonstrated that *SHP-1* interacts both *in vitro* and *in vivo* with the full-length AR and inhibited both the AR ligand-binding domain and the N-terminal domain dependent transactivation. *SHP-1* could also inhibit AR activity by competing with AR coactivators.

FHL-2 is an LIM-only member of the LIM protein superfamily. It is a selective agonist-dependent coactivator of the AR, but not of other nuclear receptors. *FHL-2* increases the transcriptional activity of the AR in an agonist and AF-2 dependent manner. Also a study showed a FHL2 overexpression in prostatic cells and in the presence of DHT, endogenous FHL2 bind at the ARE to enhance AR-transcriptional activity (Kollara and Brown, 2010). It is expressed in myocardium and in the prostate gland (Muller et al., 2000). The action of androgens in AR is controversial and complex, because it has become clear that the transcriptional activity of AR is regulated by coregulators, including both coactivators and corepressors, by various mechanisms. The aim of the present study was to determine the effect of androgens at different concentrations on AR expression and the coregulators *SHP-1* and *FHL-2* in HNTEP cells.

MATERIALS AND METHODS

Cell culture

Samples of prostatic tissue were obtained from retropubic prostatectomy from 12 patients between 56 and 75 years of age, diagnosed with BPH. Patients with malignant tumors were excluded. The study protocol was approved by the local Ethics Committee (UFRGS, protocol 99001). Informed consent was obtained from all subjects. HNTEP cells were cultured as previously described (Brum et al., 2003). Briefly, after removal of blood clots, prostatic tissue was washed with Hank's balanced salt solution (HBSS, Gibco BRL Grand Island, N.Y., USA) plus kanamycin (0.5 mg/ml) (Sigma Chem Co., St Louis, MO,USA), and then finely minced into 2 to 3 mm pieces. Tissue fragments were treated with type IA collagenase (7.5 mg/g of tissue) (Sigma Chem Co., St Louis, MO, USA) in HBSS. Enzymatic digestion proceeded for 3 h at 37°C with gentle shaking. The enzymatic reaction was interrupted with the addition of warm 199 culture medium (Gibco BRL Grand Island, N.Y., USA) with kanamycin (0.5 mg/ml) and 10% fetal bovine serum (FBS) (Gibco BRL Grand Island, N.Y., USA). Epithelial cells were separated by differential filtration. Cell suspensions were distributed into 35 mm tissue culture dishes (Corning, Glassworks, NY, USA), 1×10^5 cells per dish, or into 24 well tissue culture plates (NUNC^{TM}, Denmark), 2×10^4 cells/ml per plate, and maintained at 37°C in a humidified atmosphere of 95% air/5% CO_2 (NuAire, Inc., Minnesota, USA). In addition, since it is hard to observe the stimulatory effect of androgen on cell growth *in vitro*, we used a culture medium that was free of growth factors other than those present in FBS. Basal medium consisted of 199 medium containing kanamycin (0.5 mg/ml) enriched with 5%

charcoal-stripped FBS (cFBS). Cultures were kept in the same medium for the first 2 days and then the medium was changed every two days.

Western blot

Protein was obtained from the extraction of RNA with Trizol® (Invitrogen, Carlsbad, CA, USA) reagent following the protocol of the manufacturer. The protein concentration was determined by the Bradford method. Sodium dodecyl sulfate (SDS) polyacrylamide gel eletrophoresis (8%) was carried out using a miniprotein system (Bio-Rad, Hercules, CA, USA) with broad-range molecular weight standards (Bio-Rad, Hercules, CA, USA). Protein (30 µg) was loaded in each lane with loading buffer containing [(0.375 M Tris 6.8 pH), 50% glycerol, 10% SDS, 0.5 M dithiothreitol, and 0.002% bromophenol blue]. Samples were heated at 100°C for 2 min prior to gel loading. After eletrophoresis, proteins were transferred to nitrocellulose membranes using an eletrophoretic transfer system at 110 V for 1 to 2 h. The membranes were then washed with TTBS (20 mM Tris-HCl, 7.5 pH; 150 mM NaCl; 0.05% Tween-20, 7.4 pH) and 8% nonfat dry milk for 90 min. The membranes were incubated overnight at 4°C with the primary antibody diluted in TTBS. A rabbit polyclonal antibody for AR (2 µg/ml) (Upstate Biotechnology) was used. After washing, the membranes were incubated for 2 h at room temperature with secondary antibody (1:20.000) (anti-rabbit IgG peroxidase conjugated; Upstate Biotechnology), washed with TBS (20 mM Tris-HCl; 150 mM NaCl, 7.5 pH), and developed with the chemiluminescence Western Blot system (Amersham Biosciences) followed by apposition of the membranes to autoradiographic films (Kodak X-Omat) exposure for 15 to 60 s. Ponceau S staining was used as protein loading control.

Extraction of RNA and synthesis of cDNA

Cells were grown in serum deprived basal medium for 4 h, and then treated with DHT or ethanol vehicle in different conditions. Cultured Prostatic cells were washed twice with PBS and homogenized in phenol-guanidine isothiocyanate (Trizol, Invitrogen, Carlsbad, CA, USA). Total RNA was extracted with chloroform and precipitated with isopropanol by 12.000 x*g* centrifugation at 4°C. The RNA pellet was washed twice with 75% ethanol, resuspended in diethylpyrocarbonate-treated water, and quantified by light absorbance at 260 nm. First strand cDNA was synthesized from 1 µg total RNA, using the SuperScript Preamplification System (Invitrogen, Carlsbad, CA, USA). After denaturing the template RNA and primers at 65°C for 5 min, 50 U of reverse transcriptase was added in the presence of 20 mM Tris-HCl (8.4 pH) plus 50 mM KCl, 2.5 mM $MgCl_2$, 0.5 mM dNTP mix and 10 mM dithiothreitol, and incubated at 42°C for 50 min. The mixture was heated at 70°C for interruption of the reaction and incubated with 2 U *Escherichia coli* RNase for 20 min at 37°C for destruction of untranscribed RNA.

Real-time PCR conditions

Amplification and detection were performed with the MiniOpticon Real Time PCR detection system (Bio- Rad Life Scicence Research, USA). Duplicate samples were used. The PCR mixture contained 1.25 µl of SYBR green, 2 ng of cDNA at 1:50 dilution, 3 mM of $MgCl_2$, 20 mM Tris-HCl (8.4 pH) plus 50 mM KCl ,0.2 mM dNTP mix, 1.25 U of taq polymerase and 0.4 µM of each primer in a 25 µl of final volume. The reaction conditions were 94°C for 2 min for initial denaturation and the cycling conditions were designed for each gene. The fluorescence emitted by SYBR green I was measured in every cycle at the end of elongation step. The reaction conditions were 94°C for 2 min for hot-start, and 35 cycles of 94°C for 30 s, 55°C for 40 s and 72°C for 40 s for the AR gene, 39 cycles of 94°C for 40 s, 58°C for 40 s and 72°C for 40 s for the *SHP-1* gene and 39 cycles of 94°C for 40 s, 59°C for 40 s and 72°C for 40 s for the *FHL*-2 gene. The sequences of primers employed were: AR gene sense 5' CATGGTGAGCAGAAGTGCCCTATC3' antisense 5' TCCCAGAGTCATCCCTGCTTCAT 3' (Taplin et al., 1995), *SHP-1* gene sense 5' CAGCTATGTGCACCTCATCG 3' antisense 5'AGCCACCTCAAAGGTCACAG3' (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and *FHL-2* gene sense 5' AAACTCACTGGTGGACAAGC 3' antisense 5'AGATGAAGCAGGTCTCATGC 3' (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). For normalization of the expression levels, the expression of *β2-microglobulin* (sense: 5' ATCCAGCGTACTCCAAAGATTCAG3', antisense: 5' AAATTGAAAGTTAACTTATGCACGC 3' (Taplin et al., 1995), was used as a housekeeping gene.

Standard curves and efficiency

All samples were automatically processed for melting curve analysis of amplified cDNA. The T_m (melting temperature) is specific to each amplicon. Standard curves were created by plotting the C_T (cycle threshold) values of the real-time PCR performed on dilution series of standard. The real-time PCR assay was analyzed in the linear phase, and a linear function was fitted of the log of relative fluorescence versus cycle number with a typical R^2 value greater than 0.8 (AR R^2=0.91, *SHP-1* R^2=0.881 and *FHL-2* R^2 = 0.89) (Figure 1).

Statistical analysis

Data are reported as means and standard error of mean (SEM). Analysis of the data revealed a normal distribution. Differences between groups were assessed by analysis of variance, followed by Duncan's test. All analysis was performed using the Statistical Package for Social Sciences (SPSS, Chicago, IL, USA). Data were considered to be significant at $P < 0.05$.

RESULTS

The action of DHT treatment on AR, *SHP-1* and *FHL-2* mRNA levels in HNTEP cells was estimated by quantitative analysis. To confirm the presence of the AR in HNTEP cells, we evaluated the protein levels of AR after 1 and 2 h of incubation (Figure 2). The AR mRNA levels were assessed after 4 h of incubation with DHT (10^{-13} and 10^{-8} M) alone or in association with hydroxyflutamide (OH-FLU) at 10^{-6} M and no effect on AR gene expression was observed (Figure 3) in these conditions. The *SHP-1* gene expression was evaluated after different concentrations of DHT after 4h of incubation and we observed an increase in *SHP-1* mRNA levels with the higher dose of DHT (10^{-8} m) in comparison with the control group (data not show). FHL-2 levels in HNTEP cells were analyzed after 4h of treatment with two concentrations of DHT (10^{-8}, 10^{-13} m) alone or in association with the anti-androgen agent hydroxyflutamide (Oh-Flu) at 10^{-6} m. The coactivator of AR, *FHL-2,* showed an increase in mRNA levels in the group treated with the lower dose of androgen (10^{-13} m DHT) in comparison with the other (Figure 4).

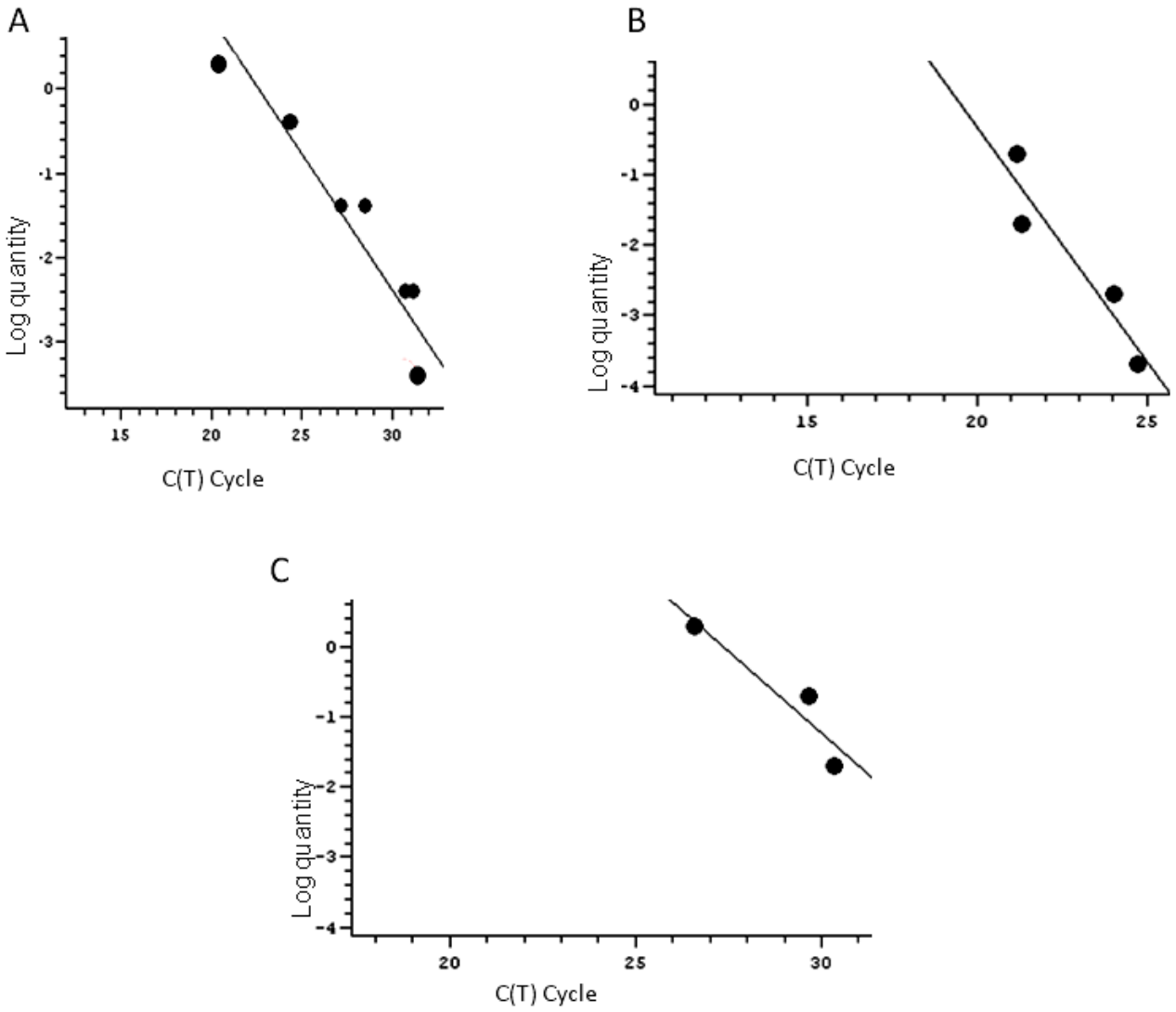

Figure 1. Standard curves of real-time PCR. Standard curves of real-time PCR for AR (A), FHL-2 (B) and SHP-1(C) genes performed on a dilution series of cDNA standard. Real-time PCR assay was analyzed in the linear phase and a fit linear function of the log of relative fluorescence vs. cycle number with a typical R^2 value > 0.8.

DISCUSSION

There is little information about the cell biology of the human prostate and about how hyperplasic and malignant lesions develop. The epithelial prostate cells (HNTEP) were established as an *in vitro* model to study the androgen dependence of human prostate and previously we showed that low concentrations of androgens exerted a positive effect on cell proliferation in HNTEP cells, and high concentrations maintained proliferation similar to that of the control (Pozzobon et al., 2012). In this study, the action of DHT treatment on AR, *SHP-1* and *FHL-2* mRNA levels in HNTEP cells was estimated by quantitative analysis.

HNTEP cells in primary culture expressed a functional AR. The AR mRNA levels were assessed after 4 h of incubation with DHT (10^{-13} and 10^{-8} M) alone or in association with hydroxyflutamide (OH-FLU) at 10^{-6} M and no effect on AR gene expression was observed (Figure 1) in these conditions.

The expression of AR mRNA was detected in several cell types like LNCaP and the androgen-independent cell lines (DU-145 and PC-3) when transfected with AR (Alimirah et al., 2006). In this case, AR gene expression did not change with different treatments of DHT, but the addition of the anti-androgen agent hidroxyflutamide abolished the proliferative effect in HNTEP cells, giving support to the notion that the mitogenic effect of the low dose of DHT in HNTEP cells is regulated by its own receptor, the AR. These results are consistent with those of Mestayer et al. (2003) who reported that AR expression is the same in normal or tumoral prostatic

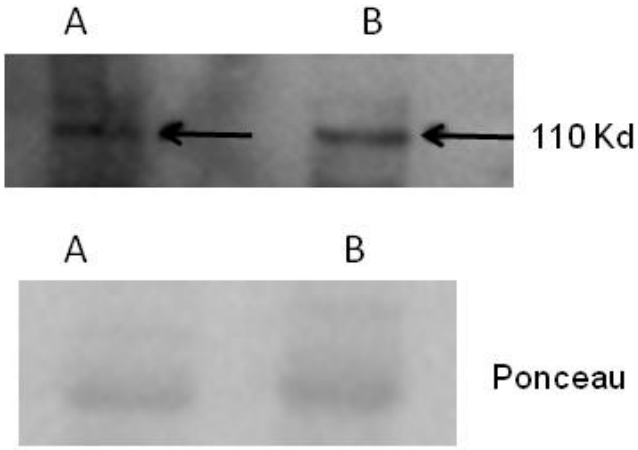

Figure 2. AR Protein. Autoradiogram of AR protein levels of HNTEP cells after 1 h (A) and 2 h (B) of incubation. Samples were separated in 8% SDS-PAGE, transferred to a nitrocellulose membrane and incubated with anti-AR antibody. Ponceau S staining was used as protein loading control.

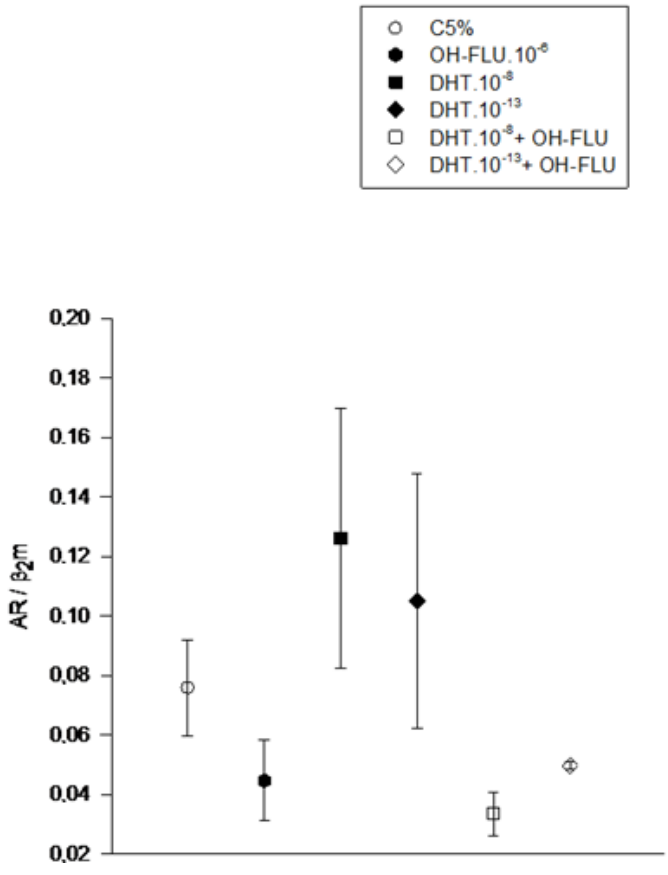

Figure 3. AR expression in HNTEP cells. Graphic display the relative expression of AR gene. Each bar represents the mean (± SEM) of product amplified (ng) at 4 h of treatment with DHT (10^{-8} and DHT.10^{-13} M) alone or in association with the antiandrogen hidroxyflutamide (OH-FLU.10^{-6} M). Comparisons between groups were analyzed by ANOVA followed by Duncan's test.

tissue. However in the immortalized cell lines PNT1A and DU-145, transfected with functional AR other author showed an increased activity in AR expression with DHT (10^{-9}m) treatment (Avances et al., 2001). These controversial results may be explained by the different experimental models employed. The modulation of androgen receptor is a complex phenomenon which involves several mechanisms. It is important to note that the AR may also mediate important cellular functions in the cytoplasm, independent of its role as a transcriptional factor. For example, AR has been shown to participate in rapid signaling cascades which involve the activation of the MAPK pathway and thereby potentially induce a mitogenic reponse (White et al., 2005). The mechanisms underpinning the capacity of AR to induce a mitogenic program may be diverse and dependent on cell context. A study demonstrated in LNCaP that protein levels of AR were unaffected during 5 days of treatment with 5.10^{-11} M R1881, a very similar dose to the one used in our study (Nesslinger et al., 2003).

The AR expression is influenced by several coregulator molecules, which facilitate domain interactions and consequently AR transativation. As a general definition, AR coregulators are proteins that are recruited by the AR and either enhance or reduce its transativation. More than 200 nuclear receptor coregulators have been identified since the isolation of the first nuclear receptor coactivator, SRC-1 in 1995 (Onate et al., 1995). The isolation of a multitude of proteins with AR coregulatory properties leads to speculation about the manner in which the formation of the AR transcriptional complex is orchestrated. The balance of corepressors and coactivetors in the AR complex determines AR transcriptional activity. In the present study, we observed an increase in the mRNA levels of *SHP-1* when HNTEP cells were treated with a high dose of androgens. *SHP-1* was described as an inhibitor of AR activity, and this result can indicate a negative modulation of AR with higher doses of androgens and consequently a lower proliferation of HNTEP cells. The repression of AR activity by androgen treatment with 10^{-9} M R1881 was demonstrated in a monkey kidney cell line CV1 and in CHO cells, co-transfected with a functional AR and *SHP-1* (Gobinet et al., 2001). It is possible that the mechanism of SHP-1 repression could be through competition with coactivators in AF-2 region, and this process depends on a higher concentration of *SHP-1* or greater affinity (Jouravel et al., 2007).

In this article, we examined the role of the AR coregulator *FHL-2.* This gene does not bind to DNA, but it has an intrinsic transactivation domain and interacts with AR in a ligand-dependent manner (Kahl et al., 2006). Moreover, *FHL-2* interacts with several AR-associated coactivators (Johannessen et al., 2006; Nair et al., 2007). However, the molecular mechanism by which *FHL-2* modulates AR transactivation remains unclear. *FHL-2* expression occurs in the cytoplasm of normal prostate

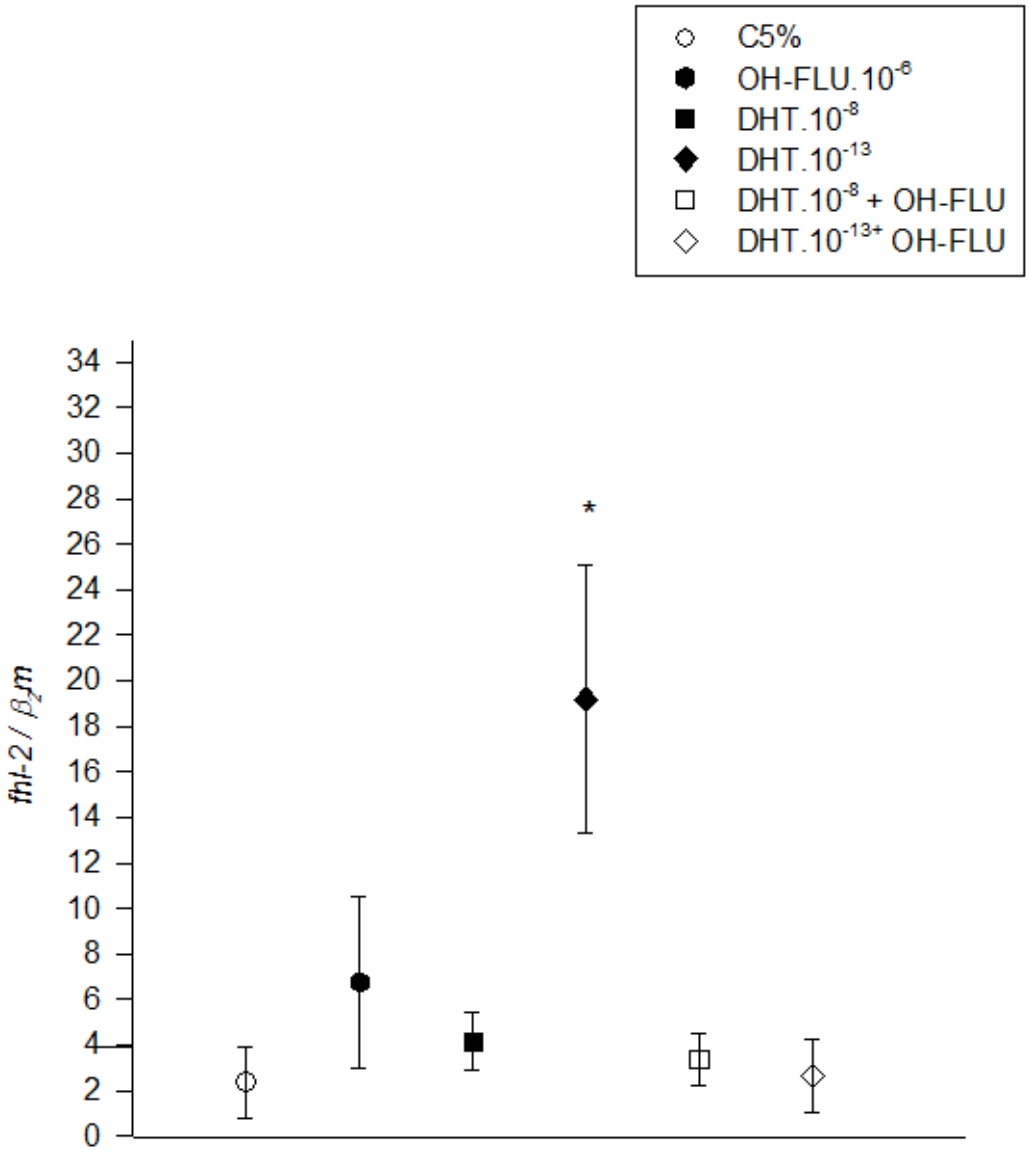

Figure 4. FHL2 expression in HNTEP cells. Graphic display the relative expression of *FHL2* gene. Each bar represents the mean (± SEM) of product amplified (ng) at 4 h of treatment with DHT (10^{-8} and DHT.10^{-13} M) alone or in association with the antiandrogen hidroxyflutamide (OH-FLU.10^{-6} M). Comparisons between groups were analyzed by ANOVA followed by Duncan's test. *p< 0.05 for all groups analyzed.

cells, and the degree of nuclear translocation increases in less-differentiated cancer cells (Muller et al., 2002). In our primary cell culture, we found an increase in mRNA levels of *FHL-2* after incubation with a lower concentration of androgens, the same concentration that stimulated cellular proliferation. This effect was abolished by the anti-androgen agent OH-flutamide suggesting a positive modulation of androgen levels by *FHL-2* in HNTEP cells. A study also described an increase in AR activity in CV1 cells cotransfected with AR and *FHL-2* and treated with androgens (Gobinet et al., 2001). Another study showed that a coregulator of multiple nuclear receptors, PELP1 interacts with *FHL-2* and synergistically enhances the transcriptional activity of *FHL-2* in PC-3 cells cotransfected with MMTV-luc, β-Gal reporter gene and AR when incubated with androgen R1881. The same study has shown that *FHL-2* also enhanced AR-mediated transativation of PSA promoter activity (Nair et al., 2007). Some authors did not show an *FHL-2* expression in LNCaP cells (Nessler-Menardi et al., 2000), but the androgen induction of *FHL-2* was demonstrated by Heemers (Heemers et al., 2007). The androgen exposure led to a marked increase in *FHL-2* expression, both at the mRNA and protein levels in LNCaP cells. They also demon-

strated a time dependent androgen stimulation of *FHL-2*, reaching a maximum at 48 h. Finally, they also demonstrated the influence of *FHL-2* in other androgen dependent genes containing androgen responsive elements (ARE) in their promoter regions.

There are few studies regarding *FHL-2* and *SHP-1* expression in the normal prostatic gland, and most of the data used immortalized cell lines. The dependence of AR coregulators to form a functional transcriptional complex suggests an important role in the development and maintenance of androgen-responsive tissues and can be involved in pathologies like benign prostatic hyperplasia and prostatic carcinoma.

This is the first article that shows a relation between *FHL-2* and *SHP-1* expressions and androgen levels in human non-transformed prostatic cells. In summary, we showed that the expression of the AR coactivator *FHL-2* is stimulated by lower androgen concentrations whereas the corepressor *SHP-1* is inhibited by this concentration and stimulated by higher androgen concentrations. We speculate that an increase in *FHL-2* may lead to enhanced AR signaling or sensitize HNTEP cells to low levels of androgens and thus proliferate. This finding provides insights into the regulation of AR in prostatic diseases and identifies a possible mechanism by which the AR is able to assure its aberrant activity in prostatic diseases.

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Conflict of interests

The authors did not declare any conflict of interest.

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