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Androgen-Induced PSA Expression Requires Not Only Activation of AR But Also Endogenous IGF-I or IGF-I/PI3K/Akt Signaling in Human Prostate Cancer Epithelial Cells

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Abstract

BACKGROUND—Prostate cancer (PrCa) risk is positively associated with levels of insulin-like growth factor I (IGF-I) and prostate specific antigen (PSA), both androgen receptor (AR) signaling target genes in PrCa cells. Although activated AR is required for androgen-induction of expression of both genes, effects of the IGF-I signaling pathways on the androgen-induction of PSA have not been studied.

METHODS—Human prostate stromal and epithelial cancer cells were treated alone or in coculture with steroid hormone and/or inhibitors. Gene or protein expression was analyzed by real time RT-PCR or Western blotting of lysates, nuclear extracts, or immunoprecipitated products.

RESULTS—In PrCa epithelial cells, endogenous IGF-I, significantly induced by R1881, was required for R1881-induction of PSA. Increased IGF-I correlated with accumulation of cytoplasmic dephospho β -catenin (CPDP β -catenin), a co-activator of AR signaling. Exogenous IGF-I enhanced R1881-induced PSA and accumulation of CPDP β -catenin in LAPC-4 cells. Functional depletion of IGF-I or IGF-I receptor diminished PSA induction. Induction of IGF-I reached a plateau while PSA consecutively increased. Inhibiting PI3K abolished R1881-induced Akt phosphorylation, CPDP and nuclear β -catenin and nuclear association of AR/ β -catenin, consequently abrogating R1881-induced expression of IGF-I and/or PSA.

CONCLUSIONS—By integrating androgen, IGF-I and β -catenin signaling pathways, these data reveal that androgen-induced PSA expression requires activation of AR and endogenous IGF-I or IGF-I/PI3K/Akt signaling, suggesting a positive feedback cycle for increased production of PSA associated with PrCa.

Keywords

Akt; AR; β -catenin; IGF-I; prostate cancer; PSA; R1881

INTRODUCTION

Androgens and their cognate receptor, androgen receptor (AR) play an essential role in mediating male sexual differentiation in utero, sperm production at puberty, prostate development in the adult, and also promoting prostate cancer (PrCa) growth in PrCa patients [1]. In PrCa cells, the androgenic response cross-talks with multifunctional growth factor

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signaling pathways (such as EGF, FGF, IGF, TGF- β , and VEGF) [2]. Among these growth factors, IGF-I may have the most inseparable connection with PrCa. IGF-I is found in both stromal and epithelial prostate tissue compartments but changes with cancer progression. In normal prostate, cells IGF-I expression is higher in stroma than in epithelium and is primarily a paracrine signal. But in PrCa tissue, it is more strongly expressed in epithelial malignant cells becoming an autocrine signal to the epithelial compartment [3].

Previous studies in this laboratory addressed steroid hormonal-induced prostate stromal paracrine effects on epithelial cells and demonstrated that androgen-induced PSA levels in LAPC-4 cells were consistently increased when they grown in the presence of 6S primary prostate stromal cells [4,5]. This suggested stromal cell paracrine factors may amplify androgenic signals. One factor likely to be involved in this amplification is IGF-I. Although activated AR is required for androgen-induction of expression of both IGF-I and PSA, the role of IGF-I in androgen-induction of PSA has not been elucidated. It is understood that androgen interacts with the AR and can induce PSA transcription via androgen response elements (AREs) in the PSA promoter [6]. Androgens (testosterone and dihydrotestosterone) induced IGF-I in LNCaP cells [7]. Two AREs have been identified in the IGF-I upstream promoter, leading to androgen activation of IGF-I expression in PrCa cells [8]. Androgens also stimulate expression of IGF-I in human primary PrCa-associated stromal cells (6S cells) but not in normal human prostate stromal cells [9]. The evidence of androgen induction of IGF-I and the existence of ARE in the IGF-I promoter in PrCa cells suggest that genetic mutations enhancing androgen induction of IGF-I expression may contribute to promotion of PrCa, as IGF-I is a pro-cancer factor. IGF-I activates both phosphoinositide 3-kinase (PI3K)/Akt and Raf/mitogen-associated/extracellular regulated kinase (MEK)/extracellular regulated kinases (ERK) pathways via IGF-I receptor (IGF-IR), resulting in promotion of growth and reduction of apoptosis in cells, increasing the possibility of cancer promotion [10]. Androgens increase IGF-IR expression in HepG2 and LNCaP cells, providing more receptors for IGF signaling in the PrCa cells [8]. Additionally, elevated levels of IGF-I in blood have been associated with increased risk of PrCa [11–14]. IGF-I increases AR transactivation in PrCa cells, under very low/absent androgen levels [15,16] and PrCa cell growth [17]. Overexpression of FOXO-1 suppresses androgen-induced AR transactivation, by binding to ligand-AR to the AR promoters and disrupting of androgen-induced AR nuclear localization; this suppression is attenuated by IGF-I administration via activation of PI3K/Akt pathway, leading to phosphorylating and inactivating FOXO-1 [18].

We previously reported that IGF-I rescued camptothecin-induced apoptotic death in normal prostate epithelial (NPE) cells when co-cultured with prostate stromal (6S) cells after treatment with androgen, and that IGF-I also promoted NPE cell proliferation by activating PI3K/Akt then inactivating glycogen synthesis kinase-3 (GSK3) [19]. Akt reduction of GSK3 activity consequently leads to stabilization of β -catenin [20], a co-activator of AR signaling to enhance transcription of IGF-I and prostate specific antigen (PSA) [19,21,22].

IGF-I has been shown to induce cellular levels of β -catenin in LNCaP prostate cancer cells which have a mutation in the AR ligand binding domain [23]. The present study contributes understanding of additional pathways for regulation of PSA expression using the co-culture of LAPC-4, containing the wild-type AR, and primary prostate stromal (6S) cells or mono-culture of these cells. It demonstrates that while IGF-I alone up to 50 ng/ml cannot induce PSA in LAPC-4 cells, endogenous production of IGF-I by 6S stromal or PrCa epithelial cells or IGF-I/PI3K/Akt signaling is required for R1881-stimulated expression of IGF-I and PSA. PSA values from LAPC-4 cells cocultured with stromal cells were consistently increased, indicating contributions of IGF-I from stromal cells. This study recreates autocrine/paracrine positive feedback loop via genomic and non-genomic pathways of endogenous IGF-I production in both prostate stromal and epithelial cells, upregulation of

cytoplasmic β -catenin, stabilization of the AR, increased PSA expression, and additional IGF-I production.

MATERIALS AND METHODS

Cell Culture

The sources of primary prostate stromal 6S cells and LAPC-4 cells and the co-culture and mono-culture conditions have been described [4,24]. LNCaP cells were purchased from ATCC (Manassas, VA). LNCaP cells were cultured as LAPC-4 and 6S cells. Cocultures were prepared in Treatment Media consisting of Medium 199 (phenol red-free)/F12 phenol red-reduced media (Invitrogen, Carlsbad, CA) (1:1) supplemented with penicillin (100 U/ml), streptomycin (100 mg/ml), glutamine (100 mg/ml; Invitrogen), and 2% charcoal dextran-treated FBS (CDS, Hyclone Labs). LAPC-4 cells were seeded at a density of 10,000 cells per well onto Millipore PICM 12 mm inserts (Billerica, MA) coated with a 1:10 dilution in H₂O of Matrigel™ (BD Biosciences, Bedford, MA). Parallel cultures of stromal cells (6S) cells were seeded at 10,000 cells per well in 24-well plates. LAPC-4 and 6S cells were grown separately for 2 or 3 days. Cocultures were initiated by placing inserts containing epithelial cells into wells containing stromal cells and adding appropriate hormone or reagent treatments.

Antibodies and Reagents

Antibodies against IGF-I, Insulin like growth factor-I receptor (IGF-IR) and normal IgG without sodium azide have been described [19]. Anti-(α -)AR, α -pAkt308T, α - β -catenin, α -IGF-IR β , α -Lamin A/C, control siRNA and IGF-IR $\alpha\beta$ siRNA were purchased from Santa Cruz (Santa Cruz, CA); α -Akt, α -pERK, α -ERK were from Cell Signaling Technology (Danvers, MA), and α -GAPDH from Advanced ImmunoChemical, Inc. (Long Beach, CA). α -Cytoplasmic dephospho-(CPDP) β -catenin was obtained from EMD Chemicals (Gibbstown, NJ) and α -PSA from Abcam (Cambridge, MA). R1881 was bought from PerkinElmer (Wellesley, MA) dissolved in ethanol and 10 nM was used in cell media (final ethanol concentrations <0.02%). IGF-I, LY-294002 (LY-1 μ g/ml applied to cells), cell permeable PI3K inhibitor, and PD 98059 (PD-1 μ g/ml applied to cells), inhibitor of the activation of mitogen-activated protein kinase kinase (MAPKK) were obtained from Sigma-Aldrich (St. Louis, MO).

RNA Extraction and Real-Time Quantitative RT-PCR

Cells were left untreated, or treated with R1881 (10 nM) and/or LY (1 μ g/ml) for 2 days. RNA was extracted and evaluation of gene expression was performed as reported [5,9]. For real-time PCR of gene expression in LNCaP cells, LNCaP cells were plated with 1,500 cells/well in a 96-well plate and transfected with control siRNA or IGF-IR $\alpha\beta$ siRNA for 2 days. Transfection was performed according to siRNA manufacturer's protocol. Then, the cells were treated without or with R1881 for 2 days. Real-time PCR of IGF-I, PSA, and RPLP0 was performed using Power SYBR Green Cell to CT Kit (Applied Biosystems, Foster City, CA) and RPLP0 values were used for normalization. Each value of real-time PCR fold change was averaged from 3 to 6 replicates and each point shown in real-time PCR figures was averaged from the values derived from three separate experiments.

Immunoprecipitation and Western Blot Analysis

Cells were treated without or with R1881 and/or other reagents (normal IgG, Anti IGF-I and IGF-IR, IGF-I, LY, or PD) for 3 days and lysed using lysis buffer and protocols from Cell Signaling Technology. Protein concentrations were assessed in the cell lysates using the

bicinchoninic acid protein (BCA) assay kit (Thermo Scientific, Rockford, IL). Western blotting procedures have been described [25].

Nuclear Fractionation and Immunoprecipitation/Western Blot Analysis to Detect Localization and/or Association of AR and β -Catenin

The nuclear localization and/or association of AR and β -catenin were studied by immunoprecipitation/Western blot according to previously reported methods [25] with minor modifications. Treated LAPC-4 or 6S cells were suspended with Trypsin EDTA (Invitrogen) and pelleted. The pellets were lysed using lysis buffer containing 20 mM Tris-HCl (pH 7.8), 0.5% Nonidet P-40, 137 mM NaCl, 50 μ M EDTA, 1 mM NaF, 1 mM NaVO₃, and protease inhibitors (Roche Diagnostics, Mannheim, Germany) for 10 min on ice. Cell lysates were spun at 2,000 rpm for 5 min at 4°C. The pellets were resuspended in the lysis buffer, passed through a 30.5-gauge needle to disrupt nuclei and then cleared at 3,000 rpm for 5 min. Protein extracts were directly loaded onto NuPAGE 4–12% Bis-Tris Gels for Western blot to measure AR or β -catenin concentrations, or incubated with α -AR overnight at 4°C, followed by 1 hr incubation with protein A agarose (Sigma-Aldrich). Control immunoprecipitation samples were performed with normal rabbit IgG (Santa Cruz). After three washes with 1 ml of the lysis buffer, the pellets were suspended in SDS sample buffer, heated at 95°C for 5 min, and loaded onto NuPAGE for Western blot.

Confocal Microscopy Analysis

LAPC-4 cells were plated onto sterile cover slips. Cells were left untreated, or treated with R1881 and/or LY for 2 days. The fixing, blocking and staining procedures have been described [26]. Samples were stained with primary antibodies, α -AR (rabbit IgG, 1:100) and α - β -catenin (goat IgG, 1:50), and secondary antibodies, donkey anti-rabbit conjugated rhodamine 1:200 and donkey anti-goat conjugated FITC 1:100 (Santa Cruz). VECTA-SHIELD HardSet Mounted Medium with DAPI (Vector Labs, Burlingame, CA) was employed for mounting cover slips. Cells were viewed with a confocal laser microscope (Lenses: 63W; LSM510; Carl Zeiss MicroImaging, Inc.) in the Lab of Cellular and Developmental Biology, NIDDK, NIH.

Statistical Analysis

All shown data represent two or more separate experiments. Quantitative data are presented as mean values $\pm$ SEM, averaged from three or more separate experiments. The statistical analysis was performed using the JMP (SAS Institute, Cary, NC) statistical package based on *t*-test. An adjusted *P*-value of ≤ 0.05 was considered significant. Densitometric values for protein bands were reported in arbitrary units.

RESULTS

Effects of R1881 and Primary Stromal Cells on LAPC-4 Expression of PSA, AR, and β -Catenin

Previously, we determined R1881-induced LAPC-4 PSA gene and protein expression by ~2-fold when in co-culture with 6S cells compared to that from LAPC-4 cells in mono-culture using ELISA and real-time PCR [4,5]. In the current study, we used Western blot to measure PSA production in LAPC-4 cell lysates in co-culture versus monoculture treated without or with R1881. There was no detectable PSA from untreated LAPC-4 cells grown in co-culture or mono-culture whereas R1881 induced PSA protein expression in both co-cultured and mono-cultured LAPC-4 cells (Fig. 1A). The PSA production from LAPC-4 cells in co-culture was ~2-fold (*P* < 0.001) over that from cells in monoculture (Fig. 1B), which is similar to the increase as previously determined using ELISA [4,5]. AR was expressed in

LAPC-4 cells and R1881 treatment greatly stabilized the protein, but no differences of AR expression were found between cells grown in mono- and co-culture (Fig. 1A). Because β -catenin is a co-activator of AR signaling for androgen-induced PSA [21,22], R1881 might also induce β -catenin stabilization. Indeed, R1881 increased cytoplasmic dephospho- (CPDP) β -catenin but not whole β -catenin (Pan β -catenin) in LAPC-4 cells in co-culture or mono-culture (Fig. 1A). Additionally R1881 induced CPDP β -catenin expression >2.5-fold in LAPC-4 cells in co-culture over that in cells in mono-culture (Fig. 1A, C, $P < 0.01$). The increased levels of CPDP β -catenin in co-cultured LAPC4 cells may contribute to the increased PSA as induced by R1881.

R1881-Induced IGF-I Stabilizes β -Catenin and Is a Prerequisite for R1881 Induction of PSA

The link between increased levels of CPDP β -catenin and PSA was investigated by analyzing how β -catenin is stabilized by R1881. Androgens induce IGF-I expression in 6S cells [9] and exogenous IGF-I can stabilize β -catenin by activating PI3K/Akt followed by inactivation of GSK3, resulting in accumulation of cytosolic β -catenin [27], which may be a resource of CPDP β -catenin. This pathway was evaluated using both stromal and epithelial PrCa cells grown in monoculture or co-culture. IGF-I mRNA expression was measured in LAPC-4 cells and 6S cells grown in mono- or co-culture using real-time PCR. R1881 induced IGF-I mRNA expression four- to fivefold (Fig. 2A, $P < 0.001$) in LAPC-4 cells compared to controls in both monoculture and co-culture. These results indicate that androgen also induces IGF-I expression in PrCa epithelial LAPC-4 cells expressing normal AR. R1881 also induced IGF-I mRNA expression in 6S cells in mono-culture by four- to fivefold (Fig. 2B, $P < 0.05$), consistent with the previous results in 6S cells induced by androgens [9,19] and in co-culture by two- to threefold ($P < 0.05$) (Fig. 2A, B). Therefore, R1881 significantly induced more expression of IGF-I in both cell types in both culture conditions.

Previously IGF-I was shown to induce and enhance androgen-induced PSA in the AR-mutant LNCaP cells [15], but its effect on a normal AR such as in LAPC-4 cells was unknown. LAPC-4 cells were left untreated or treated with R1881 and/or IGF-I (1 ng, 10 ng, or 50 ng/ml) for 3 days. Figure 2C shows that IGF-I alone, at concentrations up to 50 ng/ml, could not induce PSA; however, increasing amounts of IGF-I progressively increased R1881-induced PSA. Cells treated with R1881 plus exogenous IGF-I evidently produced more PSA than that done by cells treated with R1881 alone. The inability of IGF-I alone to induce PSA in LAPC-4 cells may be due to inability of IGF-I to activate the normal AR in LAPC-4 cells as IGF-I alone could not induce AR stability (Fig. 2C), as had been found in the mutant AR LNCaP cells [15]. Finally, CPDP β -catenin but not AR stability was positively associated with IGF-I concentrations. This demonstrates that increased IGF-I (either R1881-induced or exogenous), stabilizes β -catenin which, together with the R1881-activated AR, promotes PSA expression.

To determine the importance of the IGF pathway in PSA production, endogenous IGF-I was blocked by treating LAPC-4 cells in mono-culture or co-culture with antibodies against both IGF-I and IGF-IR. PSA and CPDP β -catenin were used as read-outs of endogenous IGF-I as shown in similar studies using exogenous IGF-I [12,27]. PSA was induced by 10 nM R1881 in LAPC-4 cells in mono-culture or co-culture for 3 days (Fig. 2D). Normal IgG (3.5 μ g/ml), used as a control antibody, slightly reduced R1881-induced PSA induction and CPDP β -catenin expression whereas the R1881-induced PSA and CPDP β -catenin were strongly inhibited by the combination of anti-IGF-I (2.5 μ g/ml) and anti-IGF-IR (1.0 μ g/ml), compared to R1881-induced PSA and CPDP β -catenin in cells treated without antibody (Fig. 2D). However, R1881-induced AR stability was not affected by antibody treatments, suggesting that IGF-I induction by R1881 plays a critical role in accumulation of CPDP β -catenin, and consequently the PSA induction. In another words, β -catenin stabilization is

required for R1881-induced PSA induction. Because IGF-IR mediates biological activities of both IGF-I and IGF-II [28], anti-IGF-IR antibody may also block IGF-II activity. However, IGF-II was not increased by androgens in either cell type (data not shown).

To elucidate kinetics of androgen induction of IGF-I and PSA gene expression, LAPC-4 cells were treated with R1881 for 0, 1, 2, 4, 8, 24, 48, and 72 hr. At the early time points of 1, 2, 4, and 8 hr, R1881-induced mRNA levels of IGF-I and PSA were minimal and similar to those at time 0 (data not shown). However, R1881-induced mRNAs of IGF-I and PSA were significant from Days 1 to 3 (Fig. 2E). R1881-induced IGF-I mRNA reached a plateau on Day 2 ($P < 0.05$) whereas induction of PSA consecutively increased with significant increases with each day ($P < 0.05$ or 0.001). IGF-I mRNA expression therefore transiently precedes and may be involved in androgen induction of PSA mRNA.

R1881 Induces PSA Via IGF-I/PI3K/Akt Pathway

The requirement of IGF-I for R1881-induced PSA and accumulation of CPDP β -catenin was demonstrated (Fig. 2). It has been previously suggested that IGF-I induces the accumulation of cytosolic β -catenin via PI3K/Akt; additionally, the IGF-I/Raf/MEK/ERK pathway is required for β -catenin/TCF signaling (β -CTS) [27]. The following studies validate that the IGF-I/PI3K/Akt pathway is involved in R1881-induced PSA and in accumulation of CPDP β -catenin in LAPC-4 cells and 6S cells, and investigate whether Raf/MEK/ERK pathway was also involved in β -catenin/AR signaling.

LAPC-4 cells (mono-culture or co-culture; Fig. 3A–D) and 6S cells (mono-culture Fig. 3E–G) were left untreated or treated with R1881 or R1881 plus 1 μ g/ml LY (inhibitor of PI3K) or 1 μ g/ml PD (inhibitor of MAPKK) for 3 days. In LAPC-4 cells, R1881-induced expression of PSA, CPDP β -catenin and pAkt308T (mono-culture) was abolished by LY but not PD (Fig. 3A, B), whereas R1881-induced pERK aggregate (AGG) (mono-culture) increased ~40% of the control ($P < 0.01$) and was down-regulated by PD to ~70% of the control ($P < 0.01$) (Fig. 3C, D). Interestingly, R1881-induced pERK aggregate increased >3-fold of the control ($P < 0.01$) by LY treatment. In the stromal 6S cells (Fig. 3E–G), R1881-induced CPDP β -catenin was inhibited >40% ($P < 0.001$) by LY but not PD (Fig. 3E, F). Also, in 6S cells, R1881-induced pAkt was abrogated by LY but not PD, whereas R1881-induced pERK was reduced to ~40% by PD ($P < 0.05$) (Fig. 3E, G). In both cell types, the R1881-induced AR stability was not affected by either LY or PD (Fig. 3A, B, E). Androgen-induced AR stability might contribute to β -catenin stabilization in 6S cells [19] so that LY abrogation of pAkt did not completely inhibit R1881-induced accumulation of CPDP β -catenin (Fig. 3E, F). R1881-induced pERK was also dramatically increased by LY treatment in 6S cells. The increased pERK in cells treated with R1881 plus LY might be androgen-dependent, as pERK did not increase in LAPC-4 cells treated with LY alone compared with that in control cells (data not shown). In both cell types (6S and LAPC-4 cells) with long term (3-day) treatment, increased expression of pERK1/2 (Thr202/Tyr204 and Thr185/Tyr187) required R1881-induced IGF-I activation of upstream kinases of ERK and those kinases were perhaps also activated by GSK3, as LY inhibition of PI3K/Akt activities might result in unchecked GSK3 activities.

These experiments confirmed that both R1881-induced PSA and accumulation of CPDP β -catenin use PI3K/Akt pathway but not MAPK pathway in these prostate cells. Also, IGF-I activated Raf/MEK/ERK pathway is required for β -CTS [27] but not β -catenin/AR signaling.

PI3K Pathway Is Involved in R1881 Induction of IGF-I and PSA Gene Expression, β -Catenin Nuclear Localization, and/or Association of AR/ β -Catenin

The ability of LY to abolish R1881-induced PSA protein expression is likely partly due to genomic effects, because LY also abrogated R1881-induced accumulation of CPDP β -catenin, a co-activator for AR transactivation in the nuclei of the LAPC-4 cells. To confirm LY inhibition of R1881-induced target gene expression, IGF-I and PSA mRNA levels were measured in LAPC-4 cells either left untreated, or treated with R1881 and/or LY for 2 days. Figure 4A shows that R1881 induced >400-fold increase in expression of PSA mRNA, whereas LY down-regulated R1881-induced PSA mRNA ~10-fold, compared to those of the control cells and cells treated with LY alone ($P < 0.001$). Since androgen-induced IGF-I mRNA expression also requires nuclear accumulation of β -catenin [19], LY abolished R1881-induced accumulation of CPDP β -catenin (as seen in Fig. 3B), which may be a resource of nuclear β -catenin, and is now shown to inhibit R1881-induced IGF-I mRNA expression in LAPC-4 cells ($P < 0.01$) (Fig. 4B).

In 6S stromal cells, androgen induction of nuclear co-localization of AR and β -catenin are essential for androgen-induced IGF-I expression [19]. This phenomenon may also be true in LAPC-4 cells. As noted above, CPDP β -catenin may be a resource of nuclear β -catenin as β -catenin is translated in cytosol. Induced by R1881 in 6S and LAPC-4 cells (Fig. 2A, B, E), endogenous IGF-I increased pAkt (Fig. 3A, E) and might inhibit GSK3 phosphorylation of β -catenin as exogenous IGF-I [27], so that newly translated β -catenin may evade GSK3 phosphorylation and accumulate in the cytosol. The accumulation of β -catenin can be detected by α - β -catenin in cytosolic fraction [27] or α -CPDP β -catenin in lysates (Figs. 1–3), and may be translocated into nuclei with androgen-activated AR [19,29,30]. To quantify concentrations of AR and β -catenin and study association of the two proteins in nuclei, nuclear extracts of LAPC-4 cells were prepared according to the Materials and Methods Section. In control or LY treated cells, AR and β -catenin were minimally expressed whereas R1881 increased expression of AR and β -catenin. The R1881-induced expression of β -catenin but not AR was inhibited by LY. Similar expression of Lamin A/C was detected in nuclear extracts of cells with any of the four treatments. Similarly, α -AR pulled-down more AR from nuclear extracts of LAPC-4 cells with R1881 or R1881 plus LY treatment than from those of cells treated without R1881, or more β -catenin from nuclear extracts of cells with R1881 treatment than from those of cells with any other treatment (Fig. 4C). Substantial β -catenin was pulled-down by AR from nuclear extracts of cells treated with R1881 because the highest concentration of β -catenin was detected in the nuclear extracts, compared with that in nuclear extracts of cells with any other treatment. The same concentration of normal rabbit IgG as that of α -AR did not pulled-down detectable AR or β -catenin from a combination of nuclear extracts of cells with the four different treatments, possessing the same protein concentration as those nuclear extracts incubated with α -AR (data not shown).

To visualize the dynamics of AR and β -catenin, LAPC-4 cells were treated without or with R1881 and/or LY for 2 days. Localization of AR and β -catenin was analyzed using confocal microscopy. In control cells and cells treated with LY alone, staining of AR (red) and β -catenin (green) represent their basal expression (Fig. 4D) where AR was localized in the cytoplasm and β -catenin was concentrated in plasma membrane. AR and β -catenin occasionally localized in nuclei but they were separate, as purple staining (AR) and cyanine staining (β -catenin) are observed in the merged images. R1881 considerably induced expression of AR and β -catenin, as well as their nuclear co-localization, indicated by increased white color visualized in the merged image. LY treatment diminished R1881-induced white color by strongly reducing expression of β -catenin but not AR, leaving R1881-induced AR in nuclei (blue DAPI) seen as purplish red color in the merged image. Although AR was still strongly associated with nuclei, which is suggested by purplish red

color (Fig. 4D), LY strongly inhibited R1881-induced PSA and IGF-I (Fig. 4A, B), because of lacking β -catenin, a co-activator of AR signaling, in nuclei (Fig. 4C, D). These data support that androgen induction of β -catenin but not AR, and induction of androgen signaling for PSA and IGF-I transcription require R1881-induced IGF-I/PI3K/Akt signaling axes in LAPC-4 cells.

LY also abrogated R1881-induced IGF-I mRNA expression in 6S cells (Fig. 4E); however, LY did not block R1881-induced AR expression but reduced R1881-induced CPDP β -catenin expression by >40% and the amount of CPDP β -catenin was still considerable, in 6S cell lysates (Fig. 3E, F). Western blot of nuclear extracts of 6S cells shows that LY significantly inhibited R1881-induced nuclear expression of AR and β -catenin; again, lamin A/C was used as a loading control (Fig. 4F). These data suggest that LY inhibits R1881-induced AR/ β -catenin translocation into nuclei in 6S cells (Fig. 6).

Depletion of IGF-IR Diminishes R1881-Induced Gene Expression of IGF-I and PSA

The requirement of IGF-I signaling for androgen-induction of IGF-I and PSA gene expression was additionally demonstrated using siRNA to deplete IGF-IR. Because transfection efficiency of LAPC-4 cells is very low, we used LNCaP cells, another PrCa epithelial cell line that induces IGF-I and PSA after treated with androgen [7,15]. LNCaP cells were transfected with control siRNA or IGF-IR $\alpha\beta$ siRNA for 2 days, and then treated without or with R1881 for 24 hr. The cells were lysed for Western blots. The α -IGF-IR β antibody stains two bands. One is IGF-IR precursor, and the other is IGF-IR β . IGF-IR was depleted at least greater than 50% by IGF-IR $\alpha\beta$ siRNA, independent of treatment with R1881 (Fig. 5A). In cells transfected with control siRNA or IGF-IR $\alpha\beta$ siRNA, R1881 increased IGF-IR expression (IGF-IR precursor and IGF-IR β) substantially greater than that of cognate controls (Fig. 5A). This observation is consistent with a previous report of androgen-induced increase of IGF-IR expression in LNCaP cells [8]. R1881 also increased AR stability greater than 40% regardless of either siRNA administration, whereas IGF-IR $\alpha\beta$ siRNA alone slightly decreased AR expression, compared to that of cells treated with control siRNA alone (Fig. 5A). R1881 induction of CPDP β -catenin, pAkt308T, and pERK1/2 expression was substantially reduced by IGF-IR $\alpha\beta$ siRNA. Therefore, IGF-IR $\alpha\beta$ siRNA decreased R1881-induced IGF signaling but androgen-induced AR expression remained as the cells expressed >90% AR level compared to that of cells treated with control siRNA and R1881 (Fig. 5A). In LNCaP cells, R1881 induced greater than eight- or sixfold of mRNA expression of IGF-I or PSA ($P < 0.01$) whereas IGF-IR $\alpha\beta$ siRNA significantly inhibited >50% of the induction (Fig. 5B). The reduction can be attributed to IGF-IR $\alpha\beta$ siRNA inhibition of R1881-induced IGF signaling (Fig. 5A).

The summary figure (Fig. 6) displays the interaction of these pathways in the 6S (stromal) and LAPC-4 (cancer-epithelial) cells. Both cell types produce endogenous IGF-I with androgen stimulation. Downstream of the IGF-IR, PI3K-induced pAkt inactivates GSK3, resulting in stabilization of β -catenin and an increase of AR/ β -catenin association. This complex is translocated to the nucleus where genomic transcription of PSA in epithelial cells and IGF-I in both cell types is further promoted.

DISCUSSION

The study presented here provides a molecular dissection of IGF-I in cell-cell paracrine and/or autocrine interactions within prostate stromal and epithelial cells in response to androgens. It is well established that stromal-epithelial interactions are important for prostate development and maintenance [31] and that these interactions may become disordered in prostate pathology and cancer progression [32]. The data compared here

analyze the paracrine secondary effects of IGF-I and androgen in stromal cells and the accumulative effects to the epithelial cells, compared to epithelial autocrine effects.

The IGF-I pathway is an important mediator of growth and prostate pathology [32,33]. It is linked with the PSA pathway as PSA is an IGF-I binding protein 3 (IGFBP3) protease, allowing release of IGF-I into microenvironment [33]. PSA is the player and IGF-I is the responder in this non-genomic pathway. Our study demonstrates the interconnecting relationship of these two proteins in non-genomic and genomic pathways. A diagram representing the highlights of the results is shown in Figure 6 which reviews the pathways of AR, β -catenin and IGF-I interactions in both 6S and LAPC4 cells. IGF-I can induce accumulation of CPDP β -catenin, leading to enhancing R1881-induced PSA expression. R1881 binds to AR in both PrCa epithelial and 6S cells and induces the stabilization (ST) of the receptor. AR stabilization also stabilizes β -catenin [34–36] which together localize to the nuclei of 6S cells and LAPC-4 cells and activate IGF-I and PSA (epithelial only) gene expression. The induced IGF-I is secreted from 6S cells and LAPC-4 cells and can then bind to IGF-IR in 6S cells and LAPC-4 cells, thus activating PI3K/Akt pathway and further stabilizing β -catenin, resulting in more of the AR co-activator and more expression of IGF-I and/or PSA. The androgen-induced expression of IGF-I and PSA is associated with accumulation of cytoplasmic and nuclear β -catenin, since functional depletion of IGF-I or blocking of PI3K/Akt signaling by LY or IGF-IR $\alpha\beta$ siRNA down-regulated R1881-induced accumulation of CPDP and/or nuclear β -catenin, and abrogated R1881-induced expression of IGF-I and/or PSA. Increasing exogenous IGF-I enhanced ligand-AR signaling via increasing β -catenin, a co-activator of AR signaling, resulting in the increased PSA expression.

In normal prostate tissues, IGF-I mRNA expression is predominantly paracrine expressed in the stromal compartment which has nine fold IGF-I expression over epithelial cells [37]. A common feature of prostate cancer is the shift from paracrine (stromal) to autocrine (epithelial) expression of growth factors and the shift to androgen independence [38]. In prostate cancer tissues, the IGF-I expression levels are increased in both stromal and epithelial components [3]. Increased IGF-I expression was also reported in reactive stromal cells compared to normal prostate stromal cells [9]. IGF-I also protects cells from apoptosis [39] especially in the reactive stromal microenvironment [19]. This study adds to the increasing literature in cell–cell interactions and by elucidating mechanisms of paracrine/steroid hormone-induced changes in cellular differentiation, proliferation, and gene expression [3–5,8,40,41].

CONCLUSION

The analysis of 6S prostate stromal cell and LAPC-4 IGF production represents the microenvironment of prostate cancer with locally increased intraprostatic IGF levels. The epithelial autocrine production augments the stromal paracrine production to amplify the IGF-PSA pathway expression. This study combines several signaling pathways and also demonstrates that the ERK pathway is not involved signaling pathways from androgen to PSA in LAPC-4 cells. It is the first to demonstrate that androgen induced-PSA requires androgen-induced IGF-I in the PrCa cells, suggesting a cellular and molecular basis of increased production of human prostate PSA associated with PrCa.

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Fig. 1.

PSA protein production in LAPC-4 cells grown in co-culture with 6S cells or in monoculture. **A:** LAPC-4 cells were treated and cultured as indicated. Western blots of the LAPC-4 lysates were probed by indicated antibodies. **B:** Relative intensity of R1881-induced PSA. The intensity of R1881-induced PSA protein in monocultured LAPC-4 cells was set as one for comparison. **C:** Relative intensity of R1881-induced CPDP β-catenin. Shown intensities were subtracted from their basal levels. The intensity of R1881-induced CPDP β-catenin protein in mono-cultured LAPC-4 cells was set as one. **B, C:** Data are averaged from three blots including the shown one in **A** and presented as mean values $\pm$ SEM. *** versus the other: $P < 0.001$ and **versus the other: $P < 0.01$.

Fig. 2.

IGF-I is required for and enhances R1881 induction of PSA. **A, B:** R1881 increased IGF-I mRNA expression in LAPC-4 and 6S cells in mono-culture or co-culture. The expressed fold changes are relative to the basal levels of IGF-I mRNA expression in monoculture which were set as one. Data are averaged from three separate experiments and presented as mean values $\pm$ SEM. **C:** IGF-I enhanced R1881 induction of PSA. LAPC-4 cells were treated as indicated (**D**). IGF-I is required for R1881-induced PSA and CPDP β -catenin. LAPC-4 cells were cultured and treated as indicated. **C, D:** Western blots of the lysates were probed by the indicated antibodies. Shown results represent three separate experiments. **E:** Kinetics of R1881-induced mRNA expression of IGF-I and PSA. The highest fold changes in each group were set as one. Data are averaged from four separate experiments and presented as mean values $\pm$ SEM. * and *** versus control treatments: $P < 0.05$ or 0.001; and # or ### versus the others in the same group: $P < 0.05$ or 0.001.

Fig. 3.

R1881 induction of PSA via IGF-I/PI3K/Akt pathway. Western blots of the lysates of LAPC-4 or 6S cells were probed by indicated antibodies. **A, B:** LAPC-4 cells were treated as indicated and grown in mono-culture (A) or in co-culture for 3 days (B) LY but not PD treatment reduced R1881-induced PSA, CPDP β -catenin and pAkt. **C:** LY increased but PD decreased R1881-induced pERK AGG in LAPC-4 cells in monoculture. **D:** Relative intensity of pERK AGG with intensity of the control set as one. **E:** In stromal 6S cells LY reduced CPDP β -catenin and pAkt whereas PD decreased pERK. 6S cells were treated as indicated. **F:** Relative intensity of CPDP β -catenin in 6S cells. **G:** Relative intensity of pERK in 6S cells. F, G: The intensity of the proteins induced by R1881 is arbitrarily set as one. A, C, E: Shown results represent three or more separate experiments. D, G, F: Data are averaged from three or more blots including shown ones in (C, E), and presented as mean

values $\pm$ SEM. ** versus any other treatment or the other: $P < 0.01$ and * versus the other: $P < 0.05$.

Fig. 4.

LY inhibited R1881 induction of expression of PSA and IGF-I mRNAs via diminishing R1881-induced nuclear β -catenin. **A:** LY strongly reduced R1881-induced expression of PSA mRNA in LAPC-4 cells. **B:** LY inhibited R1881-induced expression of IGF-I mRNA in LAPC-4 cells. **C:** LY decreased R1881-induced nuclear β -catenin and association of AR/ β -catenin in LAPC-4 cells. **D:** LY reduced R1881-induced nuclear β -catenin observed using confocal microscopy. LAPC-4 cells were treated as indicated. Cells were fixed and blocked as described in the Materials and Methods Section, and stained by the indicated antibodies and DAPI [AR (red), β -catenin (green) and DAPI (blue)]. Data represent three separate experiments. **E:** LY inhibited R1881-induced IGF-I mRNA expression in 6S cells. **A, B, E:** The control levels of mRNA expression are set as one. Data are averaged from three separate experiments and presented as mean values $\pm$ SEM. ***versus any other treatment: $P < 0.001$ and ** versus any other treatment: $P < 0.01$. LY inhibited R1881-induced nuclear expression of AR and β -catenin in 6S cells. **C, F:** Preparation of nuclear extracts of LAPC-4 and 6S cells for Westernblots and immunoprecipitation/Westernblots is described in the Materials and Methods Section. Westernblots were stained by the indicated antibodies. Data represent three separate experiments.

Fig. 5. Depletion of IGF-IR diminishes R1881-induced gene expression of IGF-I and PSA in LNCaP cells. **A:** IGF-IR $\alpha\beta$ siRNA inhibited R1881-induced CPDP β -catenin, pAkt, and pERK1/2. The treatments and antibodies are indicated. Data represent two separate experiments. **B:** IGF-IR $\alpha\beta$ siRNA reduced R1881-induced mRNAs of IGF-I and PSA. Data are averaged from three separate experiments and presented as mean values $\pm$ SEM. **versus any other treatment in the cognate group: $P < 0.01$.

Fig. 6. Feedback cycle of R1881-induced cross-talk between AR signaling and IGF-signaling in prostate stromal 6S cells and LAPC-4 cancer epithelial cells. Androgens induce IGF-I expression in 6S and LAPC-4 cells and stabilize β -catenin by activating PI3K/Akt resulting in accumulation of cytosolic β -catenin. The AR- β -catenin complex is translocated to nucleus for additional production of PSA (LAPC-4) and IGF-I (6S and LAPC-4). Activities inside nuclei suggest genomic effects whereas activities outside nuclei including paracrine or autocrine or intracytosolic communications suggest non-genomic effects. ST, stabilization; GT, genetic transcription/translation. Several critical protein components of both pathways (IGF-I/PI3K/Akt and IGF-I/Raf/MEK/ERK), such as PI3K, GSK3, Raf, etc. are omitted for simplification.