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Transforming growth factor β1 increase of hydroxysteroid dehydrogenase proteins is partly suppressed by red clover isoflavones in human primary prostate cancer-derived stromal cells

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Transforming growth factor β1 (TGF-β1) increases dehydroepiandrosterone (DHEA) metabolism to androgens and prostate-specific antigen (PSA) in a prostate tissue model where stromal (6S) cells and epithelial (LAPC-4) cells are cocultured. Red clover (RC) isoflavones inhibits transforming growth factor (TGF)-β-induced androgenicity. Mechanisms controlling those activities were explored. Three hydroxysteroid dehydrogenases (HSDs), 3β-HSD, HSD-17β1 and HSD-17β5 involved in metabolizing DHEA to testosterone (TESTO) were investigated. Individual depletion of HSDs in 6S cells significantly reduced TGF-β1/DHEA-induced PSA in LAPC-4 cells in cocultures. Monomer amounts of 3β-HSD were similar without or with TGF-β1 in both cell types but aggregates of 3β-HSD in 6S cells were much higher than those in LAPC-4 cells and were upregulated by TGFβ in 6S cells. Basal and TGF-β1-treated levels of HSD-17β1 and HSD-17β5 in LAPC-4 cells were significantly lower than in 6S cells, whereas levels of HSD-17β1 but not HSD-17β5 were TGFβ inducible. 6S cell HSD genes expression induced by TGFβ or androgen signaling was insignificant to contribute TGF-β1/DHEA-upregulated protein levels of HSDs. RC decreased TGF-β1- upregulation of aggregates of 3β-HSD but not HSD-17β1. Depletion of TGFβ receptors (TGFβ Rs) reduced TGF-β1/DHEA-upregulated HSDs and TESTO. Immunoprecipitation studies demonstrated that TGF-β1 disrupted associations of TGFβ Rs/HSDs aggregates, whereas RC suppressed the dissociations of aggregates of 3β-HSD but not HSD-17β1 from the receptors. Given that TGFβ Rs are recycled with or without ligand, TGF-β1-induced disassociation of the HSDs from TGFβ Rs may increase stability and activity of the HSDs. These data suggest a pathway connecting overproduction of TGFβ with increased PSA in prostate cancer.

Introduction

Transforming growth factor (TGF)-β has paradoxical and multiple roles in the tumor microenvironment. On one hand, TGFβ receptor knockout studies show that loss of TGFβ signaling induces tumor growth and immune cell infiltration; yet in advanced cancers, TGFβ becomes a tumor progression factor (1,2). In the prostate, TGFβ can induce a reactive phenotype in the stromal cells (3,4) and also plays a pivotal role in wound healing (5) supporting the notion that cancer is similar to a 'wound that does not heal' (6). Reactive stroma is present as an early lesion in prostate cancer progression and is seen as a shift from a smooth muscle cell phenotype (expressing smooth muscle actin and calponin) to a myofibroblast phenotype, expressing smooth muscle actin and vimentin (7). These reactive fibroblasts have unique gene signatures, characterized by gene expression profiling (8).

TGF-β1 may also be an important contributor to altered steroid metabolism in the altered microenvironment of the prostate (9). Dehydroepiandrosterone (DHEA) is an adrenal androgen circulating in humans. DHEA levels are 10 and 1000 times those of androgens and estrogens, respectively (10). Normally, large amounts of circulating DHEA or DHEA localized in the tissues may not contribute to altered functions (11). We hypothesize that in the context of reactive stroma, as induced by TGFβ, the local inflammatory response increases DHEA metabolism to androgenic metabolites and that TGFβ-treated prostate stromal cells are stimulated to metabolize adrenal androgens (12). This provides new insights into potential of increased androgen metabolism associated with early cancer reactive stromal phenotype that may contribute to progression of the epithelial cancer. Previously, we reported that prostate cancer LAPC-4 cells, expressing normal androgen receptor (AR), were responsive to DHEA treatment only in the presence of stromal cells (13) as measured by increased testosterone (TESTO) and prostate-specific antigen (PSA) levels. Upon treatment with the cytokine, TGF-β1, the induction of TESTO and PSA were greatly increased over DHEA alone, whereas red clover (RC) isoflavones inhibited the TGFβ induction (9).

The objective of this study was to determine mechanisms involved in TGF-β1-induced increases in androgenic effects in DHEA-treated prostate cocultures. We evaluated the effects of TGF-β1 on hydroxysteroid dehydrogenase (HSD) enzymes involved in DHEA metabolism, especially the isoforms 3β-HSD, HSD-17β1 and HSD-17β5. Protein expression levels of these HSDs were compared between the prostate stromal and epithelial cells. We show that these enzymes in the prostate stromal cells are contributors to epithelial PSA production in cocultures. In 6S stromal cells, of the three HSDs, protein expression of two HSDs were TGF-β1 inducible, whereas RC inhibited one of the HSDs upregulated by TGF-β1. Using immunoprecipitation, we have detected associations of the two HSDs with TGFβ Rs and the associations were modulated by TGF-β1 and/or RC. We propose a non-genomic mechanism involved in TGF-β1/DHEA-upregulation of TESTO and PSA and elaborate on how RC antagonized these processes in 6S cells and/or 6S/LAPC-4 cocultures. It is the first time TGFβ and RC regulation of cell culture androgenicity by altering protein expression of the HSDs has been observed.

Abbreviations: AR, androgen receptor; DHEA, dehydroepiandrosterone; 4-DIONE, androstenedione; HSD, hydroxysteroid dehydrogenase; mRNA, messenger RNA; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PCR, polymerase chain reaction; PSA, prostate-specific antigen; RC, red clover; TGF, transforming growth factor; TESTO, testosterone.

Materials and methods

Cell culture

Prostate cancer epithelial LAPC-4 cells, were generously provided by Dr Charles Sawyers (University of California at Los Angeles, Los Angeles, CA). Primary human prostate cancer-derived stromal cells were isolated from radical prostatectomy specimens (6S) and have previously been described (14). Both cell types were grown in Dulbecco's modified Eagle's medium/F12 (1:1) medium (Invitrogen, Gaithersburg, MD) L-glutamine (292 μg/ml; Invitrogen) and 5% fetal bovine serum (HyClone Laboratories, South Logan, UT) at 37°C in 5% CO_2 and propagated at 1:5 dilutions. Cells were kept as frozen stocks and used within seven passages after thawing.

Antibodies and reagents

Anti-(α-) 3β-HSD, recognizing 3β-HSD1 and 3β-HSD2, α-AR, α-β-catenin, α-HSD-17β1 and α-TGFβ RI, II or III, were purchased from Santa Cruz (Santa Cruz Biotechnology, CA); α-GAPDH from Advanced ImmunoChemical (Long Beach, CA); α-HSD-17β5 was obtained from (Sigma–Aldrich, St Louis, MO) and α-PSA from Abcam (Cambridge, MA). Control siRNA, 3β-HSD1 siRNA, HSD-17β1 siRNA and TGFβ RI, II or III siRNA were purchased from

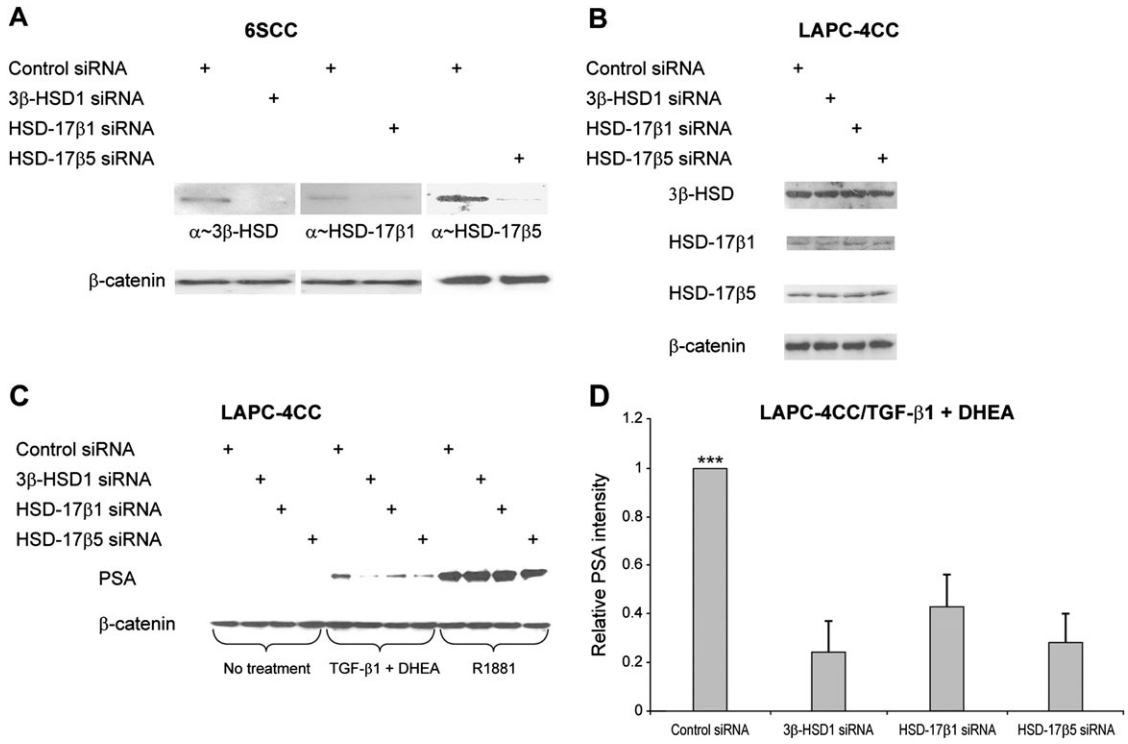

Fig. 1. DHEA metabolic enzymes in prostate stromal (6S) cells are required for TGF-β1/DHEA to induce PSA from LAPC-4 cells cocultured with 6S cells. (**A**) 3β-HSD AGG, HSD-17β1 or HSD-17β5 was depleted by its cognate siRNA in 6S cells in coculture (6SCC). (**B**) siRNA treatment had little effect on the protein expression of any of the DHEA metabolic enzymes in LAPC-4 cells in coculture (LAPC-4CC). (**C**) Each of the HSD siRNAs significantly decreased TGF-β1/DHEA-induced PSA from LAPC-4CC. (A–C) Treatment of siRNAs and reagents in the coculture of LAPC-4 and 6S cells and time for collection of cell lysates for immunoblots have been described in the Materials and Methods. The blots were probed with the indicated antibodies. (**D**) Relative PSA intensity. The intensity value of PSA expression from TGF-β1/DHEA-treated cells (Figure 1C), pretreatment with control siRNA is set as one. Data are averaged from three blots including the shown one in (C) and represented as mean ± SD. ***versus any other treatment: $P < 0.001$.

Santa Cruz. HSD-17β5 (Accession #: NM_003739) siRNA was designed by Qiagen online software and synthesized by Qiagen (Valencia, CA) and the target sequence is AACCGGAGTAAATTGCTAGAT. Androstenedione (4-DIONE or A in Figure 2C) and DHEA were bought from Sigma–Aldrich and R1881 from PerkinElmer (Wellesley, MA). The preparation of RC isoflavones has been described (9) and consists of biochanin A (CID 5280373), daidzein (CID 5281708), formononetin (CID 5280378) and genistein (CID 5280961) and their molecular structures can be found in NCBI PubChem Compound. For all experiments performed, steroid hormones were dissolved in 100% ethanol and used at the following concentrations: 100 nM DHEA, 10 nM 4-DIONE and 10 nM R1881 (final ethanol concentrations $< 0.02\%$). Concentration of RC applied to cells was 100 nM. TGF-β1 concentration applied to cells was 40 pM (1 ng/ml), unless specifically indicated.

PSA and TESTO assays

Protocols for the coculture assays have been previously reported (9) and are summarized here. LAPC-4 cells were seeded in serum-reduced Treatment Medium onto Millipore PICM 12 mm inserts coated with a 1:10 dilution of Matrigel/H_2O, at a density of 5×10^5 cells per insert, so that LAPC-4 cells were seeded in high confluence. 6S cells were seeded in treatment media at 40–50% confluence in 24-well plates. LAPC-4 and 6S cells were plated separately and combined after the cells attached. 6S cells in coculture with LAPC-4 cells were pretreated with control siRNA, 3β-HSD siRNA, HSD-17β1 siRNA or HSD-17β5 siRNA for 2 days according to Santa Cruz siRNA transfection protocol. Then the cocultures were left untreated or treated with TGF-β1/DHEA or R1881 for an additional 3 days after which lysates of LAPC-4 cells from the inserts were collected for immunoblot of PSA. Additional cocultures were treated as above with the same siRNAs for a total of 3 days (no additional steroid treatments) and the 6S and LAPC-4 cell lysates were separately collected for HSDs protein level assessment as measured by immunoblots. In other separate experiments, 6S cells were seeded in treatment media at 40–50% confluence in 24-well plates and treated with control siRNA and TGFβ RI, II or III siRNA for 2 days. Then, the cells were left untreated or treated with DHEA or DHEA plus TGF-β1 for 2 days. Media were collected for TESTO assay using a kit from ALPCO (Salem, NH). To measure TESTO metabolized from 4-DIONE, 6S cells were seeded in treatment media in 24-well plates and grown to confluence. Cells were left untreated or treated with 4-DIONE. Each TESTO value was determined in triplicate, averaged and normalized to mean cell number of 6S cells using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay value of 6S cells in three separate experiments. Modified MTT assay (Promega, Madison, WI) has been described previously (15).

Gene expression real-time polymerase chain reaction

6S cells were plated in 96-well plates and left untreated or treated as indicated in Figure 3C for 24 h. Quantitative real-time polymerase chain reaction (RT PCR) was performed using the Power SYBR Green Cell to CT kit from Applied Biosystems (Foster City, CA). Primers to amplify 3β-HSD1, 3β-HSD2 or HSD-17β1 or RPLP0 have been described (16,17). Each value of messenger RNAs (mRNAs) was averaged from means, determined by three to six replicates and normalized by RPLP0 mRNA, in three separate experiments. Gene expression in untreated cells was used as control to calculate change following any treatment.

Immunoprecipitation/immunoblot

Treated cells were lysed using lysis buffer and a protocol from Cell Signaling Technology (Danvers, MA). Protein concentrations of cell lysates were assessed in the cell lysates using the bicinchoninic acid protein (BCA) assay kit (Thermo Scientific, Rockford, IL). Lysates were loaded onto NuPAGE 4–12% Bis–Tris (Invitrogen) for immunoblot or incubated with α-TGFβ RI, RII or RIII (2 μg/0.5 ml) and protein A agarose in an immunoprecipitation buffer (18) for 3.5 h. Washed and precipitated complexes were eluted in sodium dodecyl sulfate-loading buffer. Heated at 95°C for 5 min, the eluted samples were cleared and loaded onto NuPAGE for immunoblots. Densitometry of protein bands was performed using Kodak 1D 3.6 software.

Statistical analysis

All shown data represent three or more separate experiments. Quantitative data are presented as mean values ± SD, 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

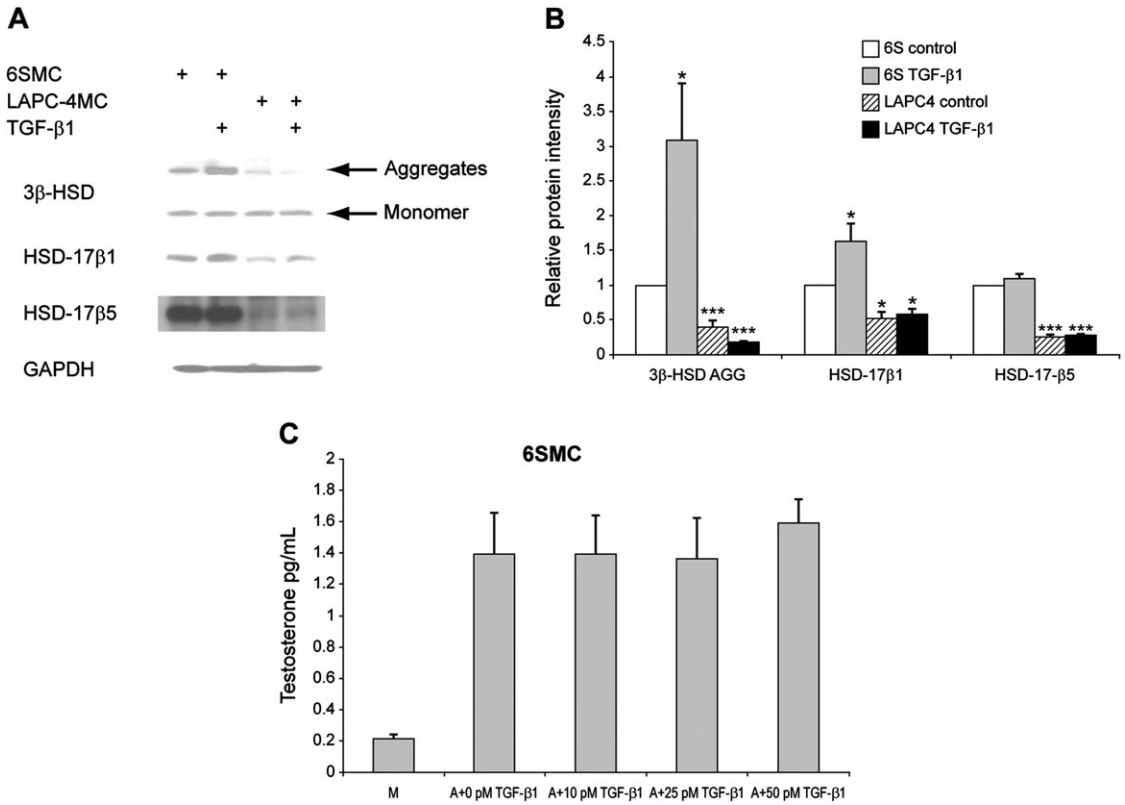

Fig. 2. TGF-β1 effects on expression and activity of DHEA metabolic enzymes in 6S and LAPC-4 cells. (**A**) Comparison of expression DHEA metabolic enzymes, 3β-HSD, HSD-17β1 or HSD-17β5, in 6S and LAPC-4 cells in monoculture (MC). 6S or LAPC-4 cells were treated without or with TGFβ-1 for 24 h and lysates were collected for immunoblots, probed with indicated antibodies. GAPDH expression was used as a loading control. (**B**) Relative intensity of bands (from 2A) of DHEA metabolic enzymes in 6S or LAPC-4 cells in MC. The intensity value of 3β-HSD AGG, HSD-17β1 or HSD-17β5 from 6S control cells (6S control) was set as one. Data were averaged from three blots including the shown ones in (A) and represented as mean ± SD. *versus the cognate 6S control: $P < 0.05$; ***versus any other treatment in the cognate group: $P < 0.001$. (**C**) TESTO production in 4-DIONE + TGF-β1-treated 6S cells. 6S cells were left untreated (M) or treated with 10 nM 4-DIONE (A) plus TGF-β1 at 0, 10, 25 or 50 pM) for 48 h. Measurement of TESTO in media was performed using ELISA. Data are averaged from three separate experiments and represented as mean ± SD.

0.05 was considered significant. Densitometric values for protein bands were reported in arbitrary units.

Results

DHEA steroid metabolizing enzymes in prostate stromal 6S cells are required for TGF-β1/DHEA induction of PSA in cocultured LAPC-4 cells

In the human prostate, DHEA can be metabolized to 4-DIONE by 3β-HSD or to androstenediol (5-DIOL) by HSD-17β1. 4-DIONE may be further metabolized by HSD-17β3 or 5 to TESTO, whereas the 5-DIOL can be metabolized by 3β-HSD to TESTO (10). To confirm stromal-driven effect on PSA (9) via these HSD metabolic enzymes in 6S cells, 6S cells were grown to 40–50% confluence and cocultured with LAPC-4 cells. The cultures were treated with siRNAs against 3β-HSD1, HSD-17β1 or HSD-17β5 for 3 days. Figure 1A and B shows that 3 days targeted siRNA treatment of 6S/LAPC-4 cocultures depleted 3β-HSD, HSD-17β1 or HSD-17β5 in the 6S cells. None of the HSD siRNAs inhibited the expression of these enzymes in the cocultured LAPC-4 cells (Figure 1B), most probably due to their very low transfection efficiency (19) and high confluence, described in the Materials and Methods. The 3β-HSD represented in Figure 1A was aggregates as described for Figure 2. The pretreatment of each of the HSDs siRNAs in 6S cells significantly reduced PSA protein expression from LAPC-4 cells in the cocultures treated with TGF-β1/DHEA for 3 days ($P < 0.001$, Figure 1C and D), whereas PSA protein expression in LAPC-4 cells without TGF-β1/DHEA treatment was not detectable. There was no attenuation of R1881-induced PSA expression other than in control siRNA-treated cocultures where LAPC-4 cells produced comparable amounts of PSA (Figure 1C). β-Catenin was used as a loading control for lysates of 6S or LAPC-4 cells in the experiments (Figure 1A–C). These data support the crucial role of HSD enzymes in the prostate stromal 6S cells for PSA expression from LAPC-4 cells in the coculture treated with TGF-β1/DHEA because PSA produced from the cocultured LAPC-4 cells was associated with expression of HSDs in 6S cells but not LAPC-4 cells.

TGF-β1 effects on expression and activity of DHEA metabolic enzymes in 6S and LAPC-4 cells

TGF-β1 treatment can increase metabolism of DHEA to TESTO in cocultures of 6S and LAPC-4 cells (9), suggesting that TGF-β1 alters the expression and/or activity of DHEA metabolic enzymes. Protein expression of DHEA metabolic enzymes, 3β-HSD, HSD-17β1 and HSD-17β5 was compared in monocultured 6S or LAPC-4 cells treated without or with TGF-β1 for 24 h as illustrated in Figure 2A and B. Although similar amounts of 3β-HSD monomer were detected in 6S and LAPC-4 cells treated without or with TGF-β1, basal expression of 3β-HSD aggregates (AGG), HSD-17β1 and HSD-17β5 was significantly higher in 6S cells compared with that in LAPC-4 cells ($P < 0.001$ or 0.05). Moreover, in 6S cells, TGF-β1-upregulated expression of 3β-HSD AGG by ∼3-fold ($P < 0.05$) and HSD-17β1 by ∼1.8-fold ($P < 0.05$) but did not significantly change protein expression of HSD-17β5.

HSD-17β5 metabolizes the conversion of 4-DIONE to TESTO (10); therefore, we used a TESTO ELISA to assay conditioned media from 6S stromal cells treated with 4-DIONE to determine TGFβ effects on HSD-17β5 activity. There was no significant change in TESTO levels in culture media from 6S cells treated with 4-DIONE

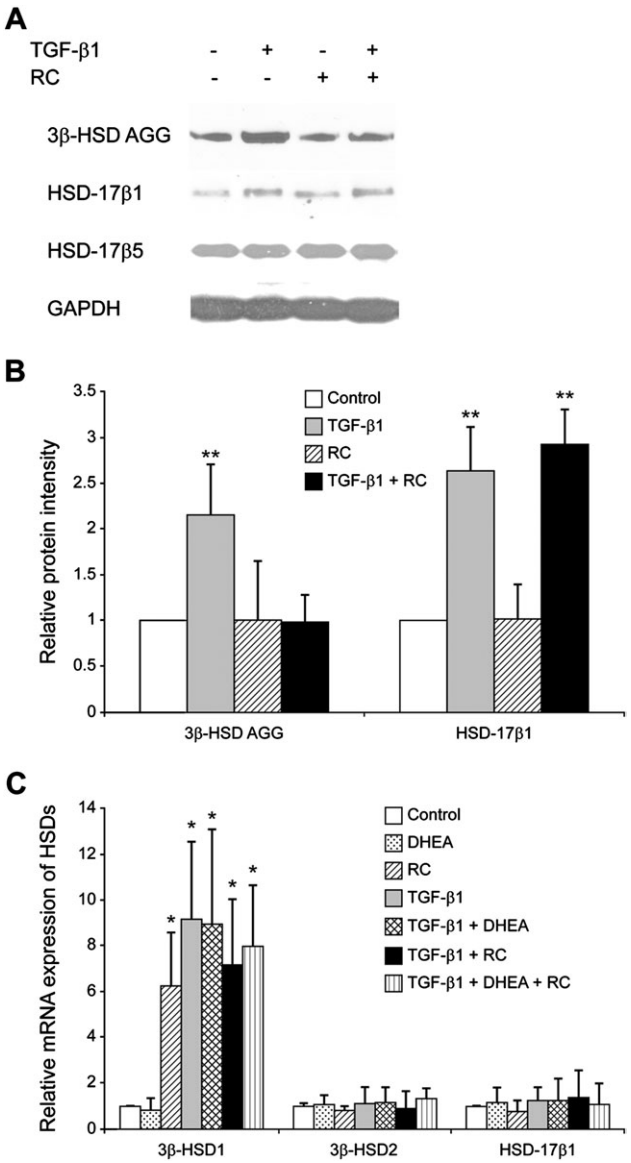

Fig. 3. Effects of TGF-β1 and RC on protein and gene expression of DHEA metabolic enzymes in prostate stromal (6S) cells. (**A**) TGF-β1 induced increase of 3β-HSD AGG and HSD-17β1 but not HSD-17β5, whereas RC inhibited TGF-β1-upregulated 3β-HSD. 6S cells were left untreated or treated with TGF-β1 and/or RC for 24 h. Cell lysates were collected for immunoblots, probed with antibodies to 3β-HSD, HSD-17β1 and HSD-17β5. (**B**) Relative intensity of indicated proteins. The intensity value of 3β-HSD AGG or HSD-17β1 from 6S control cells was set as one. Data were averaged from three or more blots including those shown in (A) and represented as mean ± SD. **versus any other treatment in the cognate group: $P < 0.01$. (**C**) HSDs mRNA expression. 6S cells were left untreated or treated with indicated reagents for 24 h. RT PCR of 3β-HSD1, 3β-HSD2 or HSD-17β1 and the control gene, RPLP0 was performed. Values of mRNAs from untreated cells were set as one. Data were averaged from three or more separate experiments and represented as mean ± SD. *versus any other treatment in the cognate group: $P < 0.05$.

(A) compared with A plus TGF-β1 up to 50 pM for 48 h, (Figure 2C; 'M' = untreated). These data suggest that TGF-β1 exerts little effect on protein expression and activity of HSD-17β5 in 6S cells.

Effects of TGF-β1 and RC isoflavones on protein and gene expression of DHEA metabolic enzymes in 6S stromal cells

The protein expression of the DHEA metabolic enzymes was significantly higher in the stromal 6S cells than in the cancer epithelial LAPC-4 cells and was upregulated by TGF-β1 in 6S cells (Figure 2A and B). These data suggest that the stromal cells play major roles in metabolism of DHEA to TESTO and are a main resource of androgens in the cocultured cells treated with TGF-β1/DHEA. The following studies were focused on these DHEA metabolic enzymes in 6S cells.

Previous studies identified RC isoflavones as possessing inhibitory effects on TESTO and PSA production in 6S/LAPC-4 cocultures treated with TGF-β1/DHEA (9). We have also repeated these results in normal prostate stromal cells, PrSC (see Supplementary Figure S1). The current studies address whether RC interferes with TGF-β1 upregulation of protein expression of the DHEA metabolic enzymes. For HSD protein analysis, 6S cells were left untreated or treated with TGF-β1 and/or RC for 24 h. TGF-β1-induced 3β-HSD AGG protein expression ($P < 0.01$) was abrogated by RC, whereas RC did not inhibit TGF-β1-induced HSD-17β1 protein expression ($P < 0.01$) (Figure 3A and B). As shown in Figure 2A and B, TGF-β1 did not change HSD-17β5 protein expression nor did RC (Figure 3A). The RC isoflavones have similar molecular structures to those of steroid hormones. They could potentially bind to DHEA metabolic enzymes and affect their stability and/or activities. However, there was no change in TESTO production after 6S cells were treated with 4-DIONE for 48 h in the absence or presence of RC (data not shown), suggesting that neither protein expression (Figure 3A) nor activity of HSD-17β5 was altered by RC. To determine TGFβ effects on HSD gene expression, real-time PCR was performed in 6S cells treated as follows: no treatment, DHEA, RC, TGF-β1, TGF-β1 + DHEA, TGF-β1 + RC or TGF-β1 + DHEA + RC, for 24 h and the total RNA samples from the untreated or treated cells were probed with primers to 3β-HSD1, 3β-HSD2 or HSD-17β1. As seen in Figure 3C, there was no significant difference in mRNA levels of 3β-HSD2 or HSD-17β1 in 6S cells, whereas mRNA levels of 3β-HSD1 were induced 6- to 9-fold ($P < 0.05$) by RC, TGF-β1, TGF-β1/DHEA, TGF-β1/RC or TGF-β1/DHEA/RC compared with control. However, the protein amounts of 3β-HSD AGG in RC-treated cells were similar to that in control cells (Figure 3A and B). In addition, TGF-β1 or RC or TGF-β1 + RC induced comparable amounts of 3β-HSD1 mRNA (Figure 3C) but cells with the treatment of TGF-β1 or RC or TGF-β1 + RC differentially expressed amounts of 3β-HSD AGG (Figure 3A and B). Therefore, gene expression induced by TGF-β1 for contribution of TGF-β1-upregulated protein levels of the HSDs was insignificant. We concluded that the TGF-β1 and/or RC regulation of protein expression of 3β-HSD AGG and/or HSD-17β1 is via non-genomic pathways. 3β-HSD metabolizes DHEA to 4-DIONE and 5-DIOL to TESTO, which are critical steps in the enzymatic pathways from DHEA to TESTO (10). Figure 3A and B explains why RC inhibits TGF-β1/DHEA-induced TESTO and PSA in the cocultures (9). As cells treated with TGF-β1 and/or RC expressed similar levels of HSD-17β5 protein (Figure 3A), we did not measure mRNA expression of HSD-17β5.

Effects of individual depletion of TGFβ receptors on TGF-β1/DHEA-upregulated 3β-HSD AGG, HSD-17β1 and TESTO

TGF-β1 is a ligand for TGFβ receptors (TGFβ Rs, including RI, RII and RIII). Cells can read TGFβ signals in a number of ways, one of which is that TGFβ ligand initiates signaling by assembling a membrane receptor complex (20) which requires that TGFβ interacts with TGFβ Rs. We tested which of the three TGFβ Rs were required for TGF-β1 or TGF-β1/DHEA-upregulated 3β-HSD AGG and HSD-17β1 protein expression or TESTO. In control siRNA-treated 6S cells, the levels of the respective TGFβ Rs were variable in the absence or presence of TGF-β1 for 24 h (Figure 4A), as TGFβ Rs are destined to be recycled without or with ligand (21) and recycle rates of different TGFβ Rs might be different in the absence or presence of TGF-β1. TGF-β1 treatment increased protein levels of 3β-HSD AGG and HSD-17β1 as seen in control siRNA-treated cells, which is consistent with the observations in Figures 2 and 3. TGFβ RI, RII or RIII was depleted by its cognate siRNA over the 3 days treatment. Depletion of each receptor consequently abolished TGF-β1-upregulated protein expression of 3β-HSD AGG and HSD-17β1 ($P < 0.001$, Figure 4A

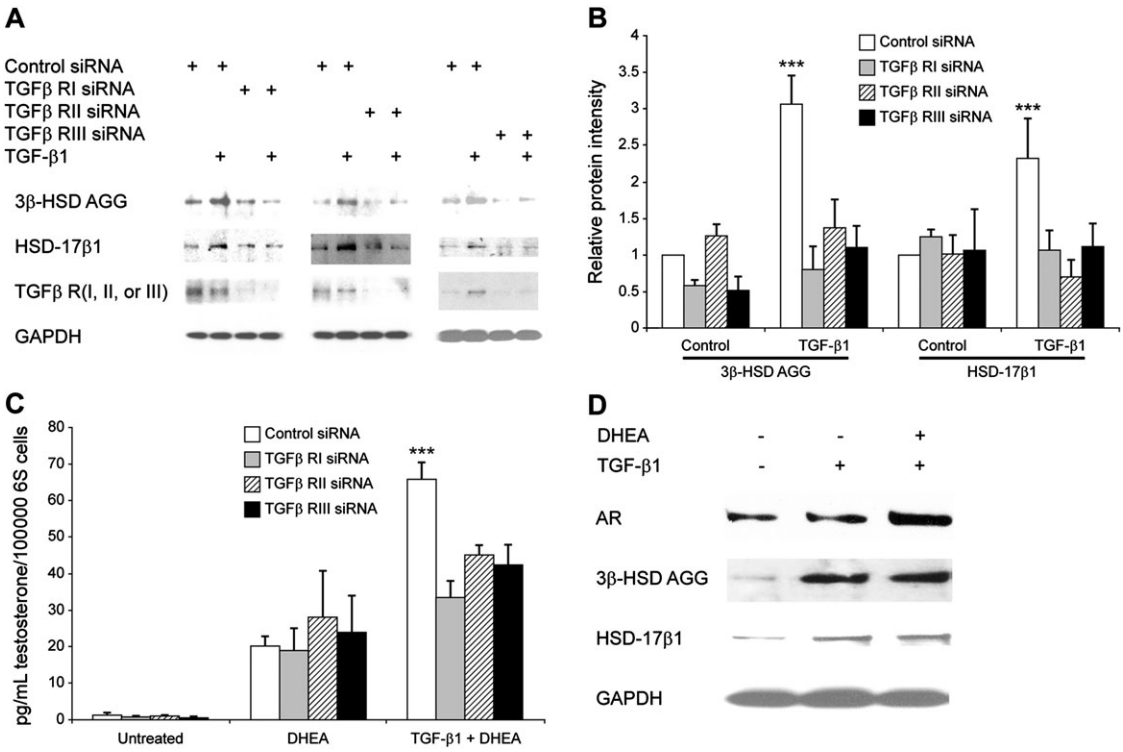

Fig. 4. Effects of individual depletion of TGFβ receptors on TGF-β1/DHEA-upregulated 3β-HSD AGG, HSD-17β1 and TESTO in prostate (6S) stromal cells. (**A**) TGFβ Rs were required for TGF-β1-increased 3β-HSD AGG and HSD-17β1 in 6S cells. 6S cells were treated with control siRNA or TGFβ RI, RII or RIII siRNA for 2 days. Then the cells were treated without or with TGF-β1 for 24 h. Lysates were collected for immunoblots, probed with indicated antibodies. (**B**)Relative intensity of indicated proteins. The intensity value of 3β-HSD AGG or HSD-17β1 from 6S control cells is set as one. Data are averaged from three blots including the shown ones in (A) and represented as mean ± SD. ***versus any other treatment in the cognate group: $P < 0.001$. (**C**) TGFβ Rs were required for TGF-β1-increased TESTO in 6S cells. 6S cells were treated with control siRNA or TGFβ RI, RII or RIII siRNA for 2 days. Then the cells were left untreated or treated with DHEA or TGF-β1/DHEA for 48 h. Media were collected for TESTO assay. Cell densities were determined by MTT assay. TESTO assay values were normalized against MTT assay values. Data are averaged from three separate experiments and represented as mean ± SD. ***versus any other treatment: $P < 0.001$. (**D**) TGF-β1 increased 3β-HSD AGG or HSD-17β1 was not via androgen signaling in 6S cells. 6S cells were left untreated or treated with TGF-β1 or TGF-β1/DHEA for 24 h. Lysates were collected for immunoblots, probed with indicated antibodies.

and B). Depletion of each receptor also resulted in significant decrease of TESTO production in 6S cells treated with TGF-β1/DHEA for 48 h ($P < 0.001$, Figure 4C). These data suggest that TGF-β1 or TGF-β1/DHEA-upregulation of protein expression of 3β-HSD AGG and HSD-17β1 or TESTO requires all the intact TGFβ Rs in 6S cells. TGF-β1-upregulated protein expression of 3β-HSD AGG and HSD-17β1 did not appear to be affected by androgen signaling, which stimulated >2-fold of AR protein expression in 6S cells treated with TGF-β1/DHEA (Figure 4D), as androgen levels (TESTO) are increased in the culture medium compared with those in the culture medium administrated with TGF-β1 alone (9); however, protein expression of 3β-HSD AGG and HSD-17β1 was similarly upregulated in 6S cells treated with TGF-β1 or TGF-β1/DHEA for 24 h (Figure 4D). These results suggest that DHEA does not play any role in upregulation of the HSDs.

TGF-β1 and RC effects on TGFβ Rs interaction with 3β-HSD AGG and HSD-17β1 AGG in 6S stromal cells

These above results suggest that TGF-β1 and RC regulation of 3β-HSD AGG and/or HSD-17β1 protein expression is via non-genomic pathways involving TGFβ Rs. A possible regulatory mechanism may be through protein–protein interactions between TGFβ Rs and 3β-HSD or HSD-17β1. To test this hypothesis, 6S cells were treated with TGF-β1 and/or RC for 1 h. Equal volumes of the lysates with the same protein concentrations were immunoprecipitated with α-TGFβ RI, RII or RIII. The immunoprecipitated complexes were eluted by loading buffer and then loaded onto NuPAGE to be immunoblotted with 3β-HSD, HSD-17β1 or TGFβ Rs antibodies. Interestingly, we found interactions between TGFβ Rs and 3β-HSD AGG or HSD-17β1 AGG in 6S cells at basal status or treated with RC alone, whereas TGF-β1 blocked the interactions between TGFβ Rs and 3β-HSD AGG or HSD-17β1 AGG (Figure 5A and B, $P < 0.01$). Moreover, RC treatment reversed the inhibitory effect of TGF-β1 on the associations of TGFβ Rs with 3β-HSD AGG but not with HSD-17β1 AGG (Figure 5A and B). The protein expression of 3β-HSD AGG and HSD-17β1 AGG was not changed following each treatment (Figure 5A). Associations of TGFβ Rs with 3β-HSD monomer or HSD-17β1 monomer were minimal or similar in cells with the treatments (data not shown).

Discussion

The role of steroid metabolism within prostate tissues is important in understanding prostate cancer pathogenesis. Although some work has elucidated steroid metabolizing enzymes in the prostate stroma (22,23), most studies have concentrated on prostate epithelial cells (10) including TGF effects on androgen metabolism in epithelial cells (24). Prostate cancer stromal cells and LNCaP cells can co-ordinate activation of the AR through synthesis of TESTO and dihydrotestosterone from DHEA (25). It is provocative in that the concentrations of adrenal steroid, DHEA in the prostate are much higher than testosterone and estrogens (10). Although DHEA itself appears to be undisruptive as a prohormone, locally increased DHEA metabolism to androgens or estrogens may be problematic in the prostate (12). Previously, we reported that DHEA-treated LAPC-4 prostate cancer cells containing a functionally normal AR did not induce PSA protein or mRNA except when cocultured with the stromal cells (13). This stromal-driven response to DHEA was amplified in the presence of TGF-β1, which substantially induces PSA and TESTO in 6S/LAPC-4

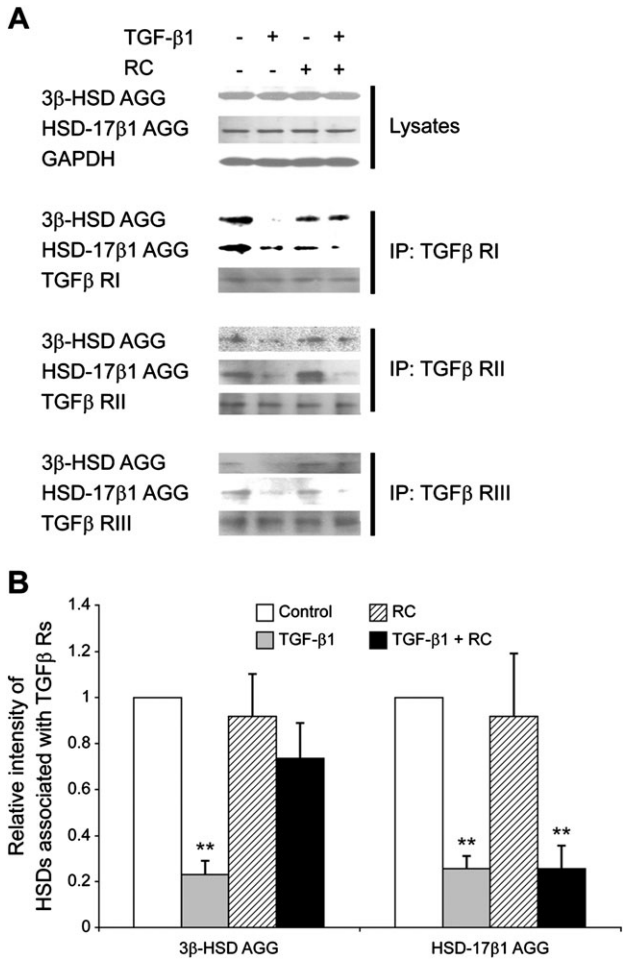

Fig. 5. TGF-β1 and RC effects on TGFβ Rs interaction with 3β-HSD AGG or HSD-17β1 AGG in prostate (6S) stromal cells. (**A**) 6S cells were left untreated or treated with TGF-β1 and/or RC for 1 h. Lysates were collected for immunoblots, probed with indicated antibodies or incubated with indicated antibodies for immunoprecipitation. The immunoprecipitated complexes were loaded onto NuPAGE for western blots, probed with indicated antibodies. (**B**) Relative intensity of indicated proteins associated with TGFβ Rs. The intensity values of either of HSD AGGs associated with any of TGFβ Rs from 6S control cells are set as one. Each value was compiled from nine blots, including the shown ones in (A), in three separate immunoprecipitation assays and represented as mean ± SD. **versus any other treatment in the cognate group: $P < 0.01$.

cocultures over treatment with DHEA alone or LAPC-4 cells in monoculture (9). These studies suggest that steroid metabolic enzymes in 6S cells are involved in DHEA to TESTO conversion.

In these studies, we propose a non-genomic mechanism how TGF-β1 and/or RC may regulate protein expression of 3β-HSD and HSD-17β1 in 6S cells based on the results of protein–protein interactions between TGFβ Rs and 3β-HSD or HSD-17β1 shown in Figure 5. Because TGFβ Rs are recycled in ligand-dependent or -independent ways (21), 3β-HSD AGG and HSD-17β1 AGG may be co-recycled with TGFβ Rs due to their associations in control cells. When cells are treated with TGF-β1, TGF-β1 binds to TGFβ Rs, resulting in altering conformation of TGFβ Rs, which dissociate 3β-HSD AGG and HSD-17β1 AGG. Dissociated from TGFβ Rs, 3β-HSD AGG and HSD-17β1 AGG may reduce their recycle rates and thus increase their stability. As stated in the above, RC has similar structures to those of steroid hormones and may bind to the 3β-HSD-1 or 17β HSD-1 altering their conformation,

RC inhibits TGF-β1-upregulated protein expression of 3β-HSD AGG but not HSD-17β1 (Figure 3A and B). RC may act by suppressing the ability of TGF-β1 to disrupt associations between TGFβ Rs and 3β-HSD AGG (Figure 5). The RC differential effects on 3β-HSD and HSD-17β1 protein expression and associations with TGFβ Rs may be explained by different primary structures of the HSD proteins. Moreover, associations of 3β-HSD AGG and HSD-17β1 AGG with TGFβ Rs were approximately the same in RC-alone-treated cells and control cells (Figure 5), so that the HSDs might have similar recycle rates in RC-alone-treated cells to those in control cells as the protein expression of the HSDs from RC-treated or control cells was approximately the same (Figure 3A and B).

The aggregates of the HSDs are probably dimers of the enzymes, as the apparent molecular weights of the AGGs appear approximately double of those of the monomers. Native forms of the enzymes may be dimers in cells, given that HSD-17β1 exists as a homodimer in cells (26). The 3β-HSD AGG in 6S cells is very resistant to dissociation even when heated in sodium dodecyl sulfate-loading buffer with dithiothreitol and is substantially identified by immunoblots. Therefore, the HSDs monomers associations with TGFβ Rs probably occurred postcell lysis and were meaningless for TGF-β1 upregulation of protein expression and activity of the HSDs in live 6S cells. This is why we did not pay much attention to associations of the HSDs monomers with TGFβ Rs in 6S cells.

The positive control (R1881) demonstrates levels of PSA produced from a direct effect of the androgen on LAPC-4 cells, which does not require additional androgen metabolized from stromal 6S cells. In these experiments, the R1881-induced PSA expression levels were much higher than that from TGF-β1/DHEA-treated LAPC-4 cells, pretreated with control siRNA, in the coculture (Figure 1C). This may be due to the decreased number of 6S cells in the cocultures, which had to be plated at 40–50% confluence for siRNA treatment. TGF-β1/DHEA-induced PSA from LAPC-4 cells in cocultures can be dependent on confluence of 6S cells since in other studies, we found that LAPC-4 cells cocultured with confluent 6S cells and treated with TGF-β1/DHEA can produce similar levels of PSA to the cocultures treated with R1881 (9).

The expression of metabolic enzymes for conversion of DHEA to TESTO is higher in 6S cells compared with LAPC-4 cells (Figure 2A and B). This explains why DHEA treatment alone can induce TESTO and PSA in 6S/LAPC-4 coculture but not in LAPC-4 monoculture (13). TGF-β1 induces upregulation of 3β-HSD AGG and HSD-17β1 in 6S cells, which explains why TGF-β1/DHEA treatment can greatly induce TESTO and PSA in 6S/LAPC-4 coculture (9).

We propose a novel pathway of TGFβ signaling in prostate stromal (6S) cells in mono- or coculture with LAPC-4 epithelial cells. TGF-β1 dissociates HSDs from TGFβ Rs, resulting in enhancing stability and thus activity of the HSDs, accelerating conversion of DHEA to TESTO and increasing androgen target gene expression of insulin-like growth factor-I (15,17,19) and PSA (9,13,19) associated with prostate cancer. RC suppresses TGF-β1-induced dissociation of 3β-HSD from TGFβ Rs and inhibits TGF-β1-upregulated stability of the enzyme in 6S cells. The study not only reveals a mechanism of TGF-β1/DHEA and RC regulation of androgenicity in the coculture but also suggests a pathway linking TGFβ to increased androgenicity and PSA in prostate cancer. The overproduction of TGFβ by cancer epithelial cells (27) or in the inflammatory microenvironment of reactive stromal cells may increase DHEA metabolism to TESTO via the proposed pathway in prostatic stromal cells and thus increase PSA expression in androgen-responsive prostatic epithelial cells.

Supplementary material

Supplementary Figure S1 can be found at http://carcin.oxfordjournals.org/

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