Published OnlineFirst July 11, 2011; DOI: 10.1158/0008-5472.CAN-10-2470

Insulin Increases *De Novo* Steroidogenesis in Prostate Cancer Cells

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Abstract

Androgen-dependent pathways regulate maintenance and growth of normal and malignant prostate tissues. Androgen deprivation therapy (ADT) exploits this dependence and is used to treat metastatic prostate cancer; however, regression initially seen with ADT gives way to development of incurable castration-resistant prostate cancer (CRPC). Although ADT generates a therapeutic response, it is also associated with a pattern of metabolic alterations consistent with metabolic syndrome including elevated circulating insulin. Because CRPC cells are capable of synthesizing androgens *de novo*, we hypothesized that insulin may also influence steroidogenesis in CRPC. In this study, we examined this hypothesis by evaluating the effect of insulin on steroid synthesis in prostate cancer cell lines. Treatment with 10 nmol/L insulin increased mRNA and protein expression of steroidogenesis enzymes and upregulated the insulin receptor substrate insulin receptor substrate 2 (IRS-2). Similarly, insulin treatment upregulated intracellular testosterone levels and secreted androgens, with the concentrations of steroids observed similar to the levels reported in prostate cancer patients. With similar potency to dihydrotestosterone, insulin treatment resulted in increased mRNA expression of prostate-specific antigen. CRPC progression also correlated with increased expression of IRS-2 and insulin receptor *in vivo*. Taken together, our findings support the hypothesis that the elevated insulin levels associated with therapeutic castration may exacerbate progression of prostate cancer to incurable CRPC in part by enhancing steroidogenesis. *Cancer Res; 71(17); 1–11. ©2011 AACR.*

Introduction

Organ-confined prostate cancer can be treated with radical prostatectomy or radiation therapy; however, 25% to 40% of patients will experience biochemical recurrence with an increase in serum levels of prostate-specific antigen (PSA), used as a marker of tumor growth, and continue to progress with metastatic disease (1). The most common therapy for progression is androgen deprivation therapy (ADT) usually administered by luteinizing hormone–releasing hormone agonists or antagonists that disrupt the feedback loop within the hypothalamic gonadal axis to suppress testosterone production from the testes. Initially, inhibition of testicular androgen causes tumor regression; however, this remission is temporary, and typically within 24 months patients recur with an increasing PSA level despite castrate androgen levels in the serum. This is termed castrate-resistant prostate cancer (CRPC) and is currently viewed as incurable (1, 2). The underlying mechanisms for progression to CRPC are complex; however, we have recently shown that one major mechanism is that in the face of ADT, prostate tumors are capable of synthesizing androgens *de novo*, reactivating androgen driven processes, and thereby promoting tumor growth (3). This *de novo* biosynthesis seems to be further upregulated by androgens, indicating a feed-forward loop of steroidogenesis. In the physiologic setting, the systemic circulating weak androgens derived from the adrenal gland can also serve as substrates for further conversion to more active androgens within the prostate using the steroidogenic enzymes shown to be present (2, 4). The key question remains as to what initiates and augments steroidogenesis in prostate cancer following castration.

Although ADT provides control of prostate cancer growth, the systemic noncancer effects include the induction of the metabolic syndrome with a key consistent feature of hyperinsulinemia (5, 6). We have hypothesized (7, 8) that these high circulating levels of serum insulin may act directly on prostate cancer cells and enhance steroidogenic pathways. Herein, we show that insulin treatment on prostate cancer cells increases the expression at both the mRNA and protein levels of several

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Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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doi: 10.1158/0008-5472.CAN-10-2470

Published OnlineFirst July 11, 2011; DOI: 10.1158/0008-5472.CAN-10-2470

key enzymes involved in *de novo* steroidogenesis including cytochrome p450 family members CYP11A1 and CYP17A1, RDH5, and hydroxysteroid dehydrogenase HSD3B2 in LNCaP, VCaP, and 22RV1 prostate cancer cell lines. Furthermore, in LNCaP cells, insulin promotes the translocation of steroidogenic acute regulatory protein (StAR) to the mitochondria, a rate-limiting step in steroidogenesis. We show that synthesis of steroid hormones, including testosterone and dihydrotestosterone (DHT), are increased in prostate cancer cells with insulin treatment to levels sufficient enough to activate PSA production. *In vivo* studies of LNCaP xenografts show increased expression of insulin receptor, and insulin receptor substrates are associated with progression to castrate resistance. We conclude from this study that insulin acts directly on prostate cancer cells to increase *de novo* androgen synthesis in CRPC.

Materials and Methods

In vitro model

LNCaP cells (passages 36–48; American Type Culture Collection) and 22RV1 cells were cultured in phenol red–free RPMI 1640 (Invitrogen) and 5% fetal bovine serum (FBS; HyClone; Sigma). VCaP cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) containing 10% FBS. For modeling androgen deprivation, cells were plated in FBS and changed to 5% charcoal-stripped serum (CSS; HyClone) media for 24 hours, followed by 24 hours in serum-free media. Cells were treated with 10 nmol/L insulin (Sigma) for various times (5, 10, 16, 24, and 48 hours). Insulin and DHT were refreshed if treatment exceeded 24 hours. Cells were incubated in the presence of 25 μmol/L bicalutamide or vehicle control for 2 hours prior to the addition of insulin or DHT.

Quantitative real time-PCR

RNA was extracted from prostate cancer cells using TRI Reagent (Applied Biosystems) before reverse transcription with SuperScript III Reverse Transcriptase (Invitrogen) according to the manufacturer's protocol. Subsequent quantitative PCR was carried out on 7900HT Fast Real Time PCR System (Applied Biosystems) with SYBR Green detection. Primers (Sigma Proligo) were designed by Primer3 Software from coding segments of genes obtained from the NCBI data bank. The primers used were as follows: SREBP [forward (f)] 5′-cgctcctccatcaatgacaa-3′, [reverse (r)] 5′-tcgagaaagcgaatgtagtcgat-3′; StAR (f) 5′-gcccatggagaggctctatg-3′, (r) 5′-ttccactcccccattgctt-3′; CYP11A1 (f) 5′-agttctcgggacttcgtcagt-3′, (r) 5′-ggagcccgccttcttga-3′; CYP17A1 (f) 5′-gggcggcctcaaatgg-3′, (r) 5′-cagcgaaggcgaaggcgatacccctta-3′; HSD3B2 (f) 5′-cgggcccaactcctacaag-3′, (r) 5′-ttttccagaggctcttcttcgt-3′; SRD5A1 (f) 5′-acgggcatcggtgcttaat-3′, (r) 5′-ccaacagtggcataggctttc-3′; RDH5 (f) 5′-gcccgccagcaatgc-3′, (r) 5′-cgcccaaagcctgagtca-3′; PSA (f) 5′-agtgcgagaagcattcccaac-3′, (r) 5′-ccagcaagatcacgcttttgtt-3′, and insulin receptor substrate 2 (IRS-2) validated primers from Applied Biosystems. Gene expression was normalized to the housekeeping gene *RPL32* (f) 5′-ccccttgtgaagcccaaga-3′ and (r) 5′-gactggtgccggatgaactt-3′ and then expressed relative to the vehicle control at the same time point. Data were analyzed with SDS 2.3 software by means of the $2^{-\Delta\Delta Ct}$ method. Experiments were repeated a minimum of 5 times.

Western blotting

Cells were lysed in radioimmunoprecipitation assay buffer (25 mmol/L Tris-HCl, pH 7.6, 150 mmol/L NaCl, 1% NP-40, 1% sodium deoxycholate, and 0.1% SDS), and triplicate wells were pooled. SDS-PAGE was used to separate proteins (15 μg/lane), using standard protocols. Blots were visualized using secondary antibodies compatible with LI-COR Odyssey Imager (LI-COR Biosciences). Antibodies used were as follows: rabbit SREBP1, 1:200 (Santa Cruz Biotechnology); rabbit StAR and CYP17A1, 1:1,000 (kind gifts from Dr. D.B. Hales); rabbit CYP11A1, 1:1,000 (Corgen); goat HSD3B2, 1:200 (Santa Cruz Biotechnology); goat SRD5A1, 1:500 (Novus); and RDH5, 1:300 (Abnova); glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (Santa Cruz Biotechnology) was used as a loading control. Experiments were repeated a minimum of 3 times.

Mitochondrial fractionation assay

MitoSciences cell fractionation kit was used for mitochondrial fractionation of LNCaP cells. GAPDH was used as a control for mitochondrial isolation.

Steroid quantitation (total steroids) by liquid chromatography/tandem mass spectrometry

Steroid analysis was carried out, as previously described (3). Briefly, cells were grown in 15-cm plates and treated with 10 nmol/L insulin, as described previously. Two plates of cells were washed with PBS and pooled to give one sample. Media were collected and likewise combined for extraction. Steroids were extracted from the pellet with an methyl *tert*-butyl ether/methanol/water extraction for cell lysates of water-equilibrated ethyl acetate for media, which was dried down and resuspended in acetonitrile, sonicated, dried down, and resuspended in 50% methanol, and then further sonicated and spun to remove any particulates. Samples were derivatized in 0.2 mol/L hydroxylamine HCl. Samples were run on a Waters Acquity Liquid Chromatography system and Waters Quattro Premier liquid chromatography/tandem mass spectrometry (LC/MS-MS) system, identified using standards of known retention times (Fig. 1), and analyzed using BioLynx Software (Waters Corporation). Readings were adjusted using cell pellet weight and normalized to vehicle-treated samples.

De novo steroid analysis using radiometric detection

LNCaP and VCaP cells were grown in 6-well plates and treated as described previously (9). At the time of insulin treatment, 6 μCi/mL ^{14}C-acetate (PerkinElmer) was added to each plate for coincubation. Steroids from 2 mL of media were extracted with 75:25 hexane/ethanol acetate, dried down, and resuspended into 75 μL methanol (50%). Samples were analyzed on the Waters Alliance 2695 HPLC System and Packard Radiometric Detector 150TR Flow

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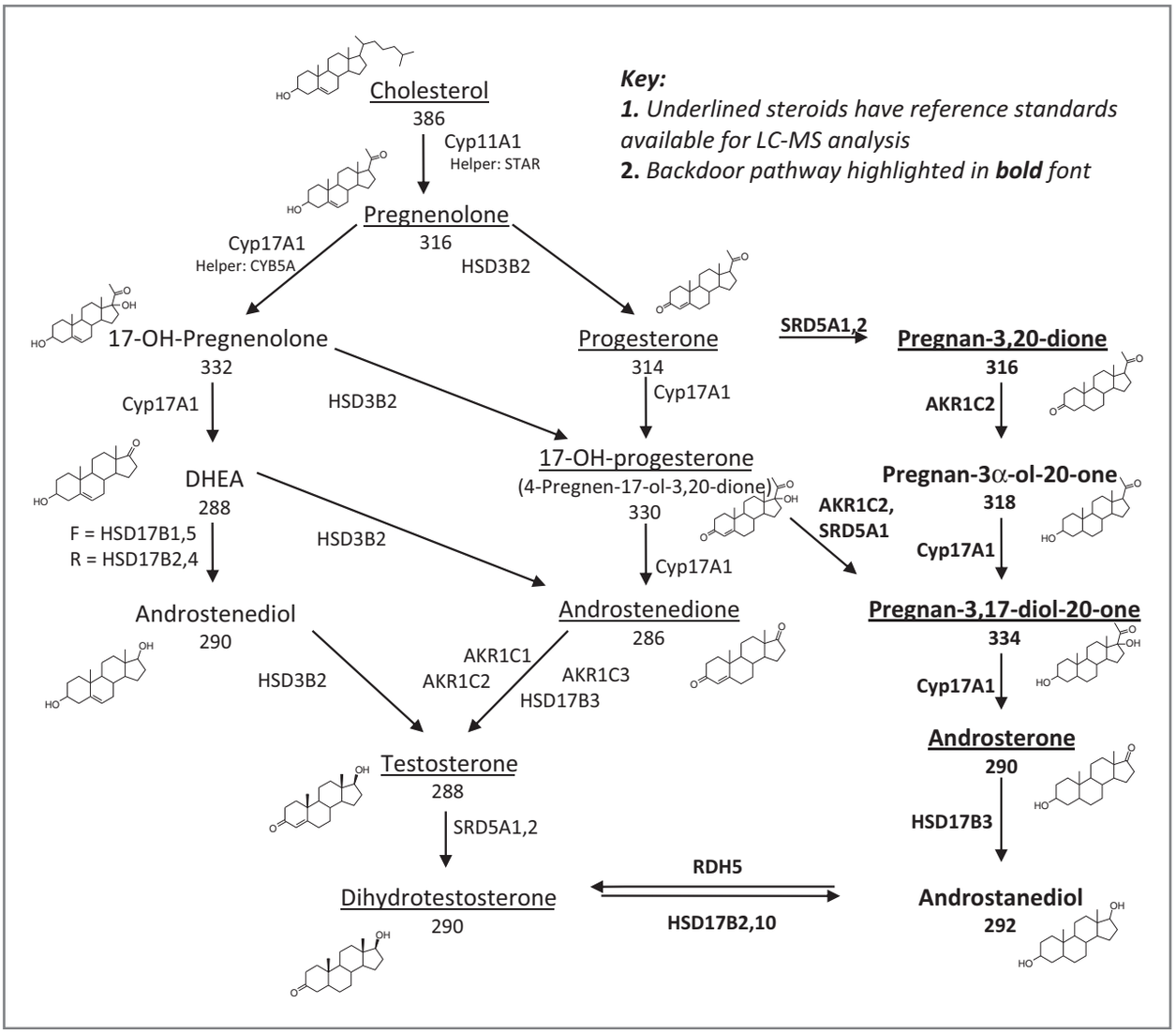

Figure 1. Enzymes involved in both classical (left/middle) and backdoor (far right, bold font) steroidogenesis pathways. Many of the steroidogenic enzymes catalyze more than 1 step in the pathway. Standards available for underlined steroids. Adapted from Auchus and colleagues (14) and Locke and colleagues (3).

Scintillation Analyzers. Peaks were identified by comparison of retention times to Mix 10 steroid standards (Sigma).

Steroid analysis using DHT ELISA

DHT secreted into the media by 22RV1 cells was evaluated using a DHT ELISA (BioCore Pty Limited) kit according to the manufacturer's instructions.

PSA analysis of LNCaP media

PSA levels of LNCaP cells treated with insulin or DHT were evaluated using ClinPro PSA kit as per kit instructions (ClinPro International).

In vivo model: LNCaP tumor progression to castrate resistance

All animal experimentation was conducted in accordance with accepted standards of the University of British Columbia Committee on Animal Care, as described previously (3). Briefly, LNCaP xenograft tumors were grown in athymic nude mice at 4 sites. PSA levels were measured weekly from tail vein serum samples. Six weeks following inoculation of tumors, mice were castrated. Tumors were harvested from the same mouse before castration, 8 days after castration corresponding to a PSA nadir, and 28 days after castration corresponding to CRPC. Tumors were removed and homogenized in TRIzol (Invitrogen), and RNA was isolated for cDNA synthesis as described previously.

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Statistics

Two-tailed Student's *t* test assuming unequal variance was used throughout.

Results

Insulin upregulates expression of enzymes necessary for steroidogenesis at the mRNA and protein levels

A number of interlinked pathways can lead to the production of testosterone and DHT from intracellular cholesterol synthesis (Fig. 1). The direct effect of insulin on steroidogenesis within prostate cancer cells was initially determined by quantitative real time-PCR (qRT-PCR) analysis to examine changes in expression of key genes within this pathway following chronic exposure of insulin. LNCaP cells treated with 10 nmol/L insulin for 5, 10, and 16 hours showed that enzymes required for synthesis of androgens were upregulated at the mRNA level in the presence of insulin after 10 hours (Fig. 2A). A parallel and significant 3.5-fold increase in IRS-2 ($P < 0.05$) suggests increased signaling via the insulin receptor (10). Sterol regulatory element–binding protein (SREBP) transcription factors are responsible for coordinately regulating the enzymes required for synthesis of cholesterol, importation of cholesterol into the mitochondria, and steroidogenesis (11–13). At this time point, SREBP1 mRNA was also increased ($P < 0.05$). StAR, which chaperones cholesterol into the mitochondria, increased 2.25-fold, and CYP11A1 (C11A1), the rate-limiting enzyme that commits cholesterol to steroid synthesis, is upregulated 2-fold. Many of the steroidogenic enzymes catalyze more than 1 step in the pathway to DHT synthesis. These include monooxygenase CYP17A1 (C17A1), oxidoreductase/dehydrogenases HSD3B2, HSD17B3, and 5-α-reductase (SRD5A1). CYP17A1 was significantly increased by 3.7-fold ($P < 0.05$), HSD3B2 showed a significant 1.5-fold increase with insulin ($P < 0.05$), and HSD17B3 was upregulated approximately 7.5-fold ($P = 0.11$), whereas aldo-keto reductase (AKR)1C3 remained unchanged (data not shown). SRD5A1, the enzyme responsible for conversion of testosterone to DHT, showed a significant 30% increase from base level. In the "backdoor" pathway of steroidogenesis (14), RDH5 converts androstenediol directly to DHT and was 2-fold upregulated ($P < 0.05$) with 10 nmol/L insulin.

These studies were extended into other prostate cancer cell lines with functional androgen receptors (AR), VCaP and 22RV1s. The single time point 48 hours was used to allow simultaneous assessment of accumulated steroids synthesized *de novo*. Similarly to LNCaP cells, increased mRNA in VCaP cells ($P < 0.05$; Fig. 2B) was observed for CYP11A1 (3-fold), CYP17A1 (1.5-fold), HSD17B3 (2.5-fold), and RDH5 (1.5-fold). SREBP and StAR were expressed but were not significantly changed with insulin (Fig. 2B). In 22RV1 cells, similar increases in mRNA were shown (Fig. 2C). SREBP and StAR were upregulated 2-fold ($P < 0.05$), whereas CYP11A1 was increased approximately 3-fold. CYP17A1 was upregulated 5-fold, whereas HSD3B2 and HSD17B3 increased 2.3- and 2-fold, respectively ($P < 0.05$). RDH5 increased 2.6-fold ($P < 0.05$; Fig. 2C). There was no significant change in IRS-2 expression in VCaP or 22RV1 cells by insulin at 48 hours.

The 3 cell lines tested are androgen responsive and derived from prostate cancer patient metastases. However, their response to differing stimuli is variable and likely underpinned by differences in genomics and other molecular characteristics of the lesions (15, 16). Although the magnitude and temporal upregulation of steroidogenesis enzyme mRNA in these cells may differ, posttranslational modifications and subcellular localization also influence their activity. Importantly, all enzymes necessary for steroidogenesis are expressed in various prostate cancer cell lines.

We have previously shown that prostate cancer cells can use alternative steroidogenic pathways in a compensatory manner to synthesize DHT through the classical pathway, "backdoor" pathway, or a combination of both (17). Furthermore, many of the enzymes in the steroidogenesis pathway can function bidirectionally, permitting alternative pathways of steroid synthesis (18).

Protein levels of steroidogenic enzymes were increased in LNCaP cells following insulin treatment (Fig. 2D). Specifically, the protein levels of CYP11A1 and CYP17A1 were significantly increased 2.5- and 2-fold, respectively ($P < 0.05$). Levels of HSD3B2 protein increased 3.6-fold but did not reach statistical significance. SRD5A, although modestly increased following 10-hour insulin treatment at the mRNA level, it increased greater than 3-fold at the protein level ($P < 0.05$) and showed elevated expression (1.5- and 2-fold at 10 and 16 hours, respectively; data not shown). RDH5 protein was only upregulated by insulin at the 10-hour time point ($P < 0.05$), and HSD17B3 was not significantly increased until 16-hour insulin treatment ($P < 0.05$), with steady expression at 5 and 10 hours (not shown). AKRIC3 protein levels were unchanged; however, AKR1C3 is the predominant AKR in prostate epithelial cells, and it is possible that the activity of the enzyme is regulated posttranslationally, without exceptional change to protein levels. It is important to note that AKR1C3 was expressed in all cell lines investigated. In 22RV1 cells, the profile of upregulated enzymes was similar to LNCaPs (Fig. 2E), with significant increases in SREBP, CYP11A1, CYP17A1, and HSD17B3 ($P < 0.05$).

Although our data suggest that insulin increases StAR expression, biologically the more important effect is altered at subcellular localization of StAR. Our data show a 1.7-fold increase in StAR translocation into the mitochondria following 16-hour treatment with insulin in LNCaP cells ($P < 0.05$; Fig. 2F).

Insulin increases intracellular steroids in prostate cancer cells

To determine whether there is a parallel increase in steroid synthesis, we used high-performance liquid chromatography–mass spectrometry to measure total steroid content of LNCaP cells following 16-hour insulin treatment. Insulin dramatically increased intracellular LNCaP steroid levels (Fig. 3A), with 2.5-fold increased levels of pregnenolone, the first steroid converted from cholesterol in the pathway ($P < 0.05$; Fig. 3A). We observed a 15-fold increase in total intracellular 17-hydroxyprogesterone (17-OH-progesterone) levels ($P < 0.05$), which is converted from progesterone by CYP17A1, an enzyme shown

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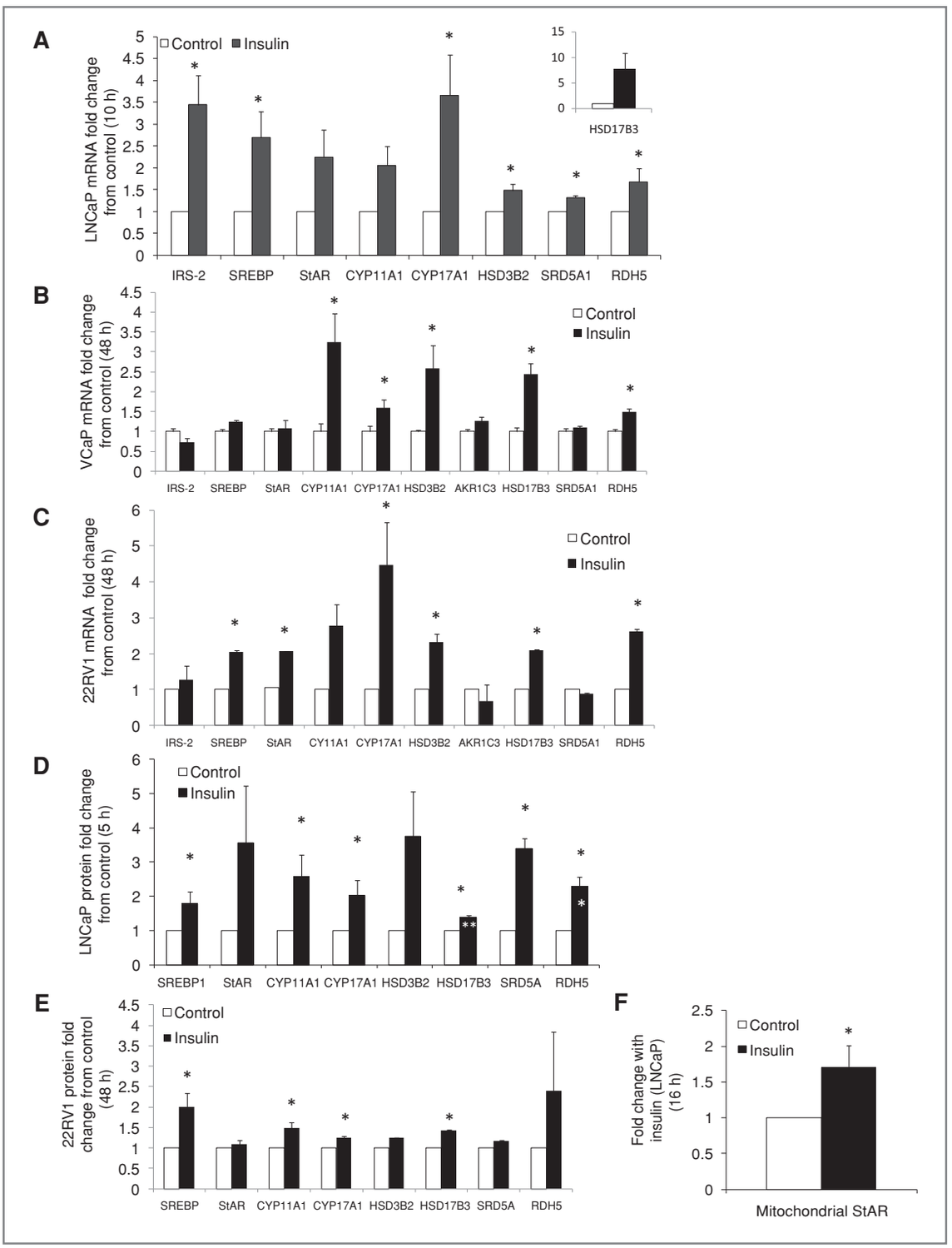

Figure 2. Insulin regulates expression of key steroidogenic enzymes at the mRNA and protein levels. RNA was processed from prostate cancer cells for qRT-PCR analysis. Results were analyzed by $\Delta\Delta C_t$ method normalized to RPL32 and then normalized to vehicle treated at equivalent time point. Insulin (10 nmol/L) increased mRNA expression in (A) LNCaP cells following 10-hour treatment or following 48-hour insulin treatment in (B) VCaP cells and (C) 22RV1 cells. Western blot protein analysis showed increased levels of steroidogenesis enzymes with 10 nmol/L insulin treatment in (D) LNCaP cells following 10 nmol/L insulin treatment (5 hours), RDH5 at 10 hours (white *), or HSD17B3 at 16 hours (double white *) and (E) 22RV1 cells following 48-hour treatment. F, 16-hour insulin treatment (10 nmol/L) increased translocation of StAR to the mitochondria compared with vehicle-treated cells. Western blots were quantitated using Odyssey software (version 1.2) with GAPDH loading control. Error bars, ±SE (*, $P < 0.05$).

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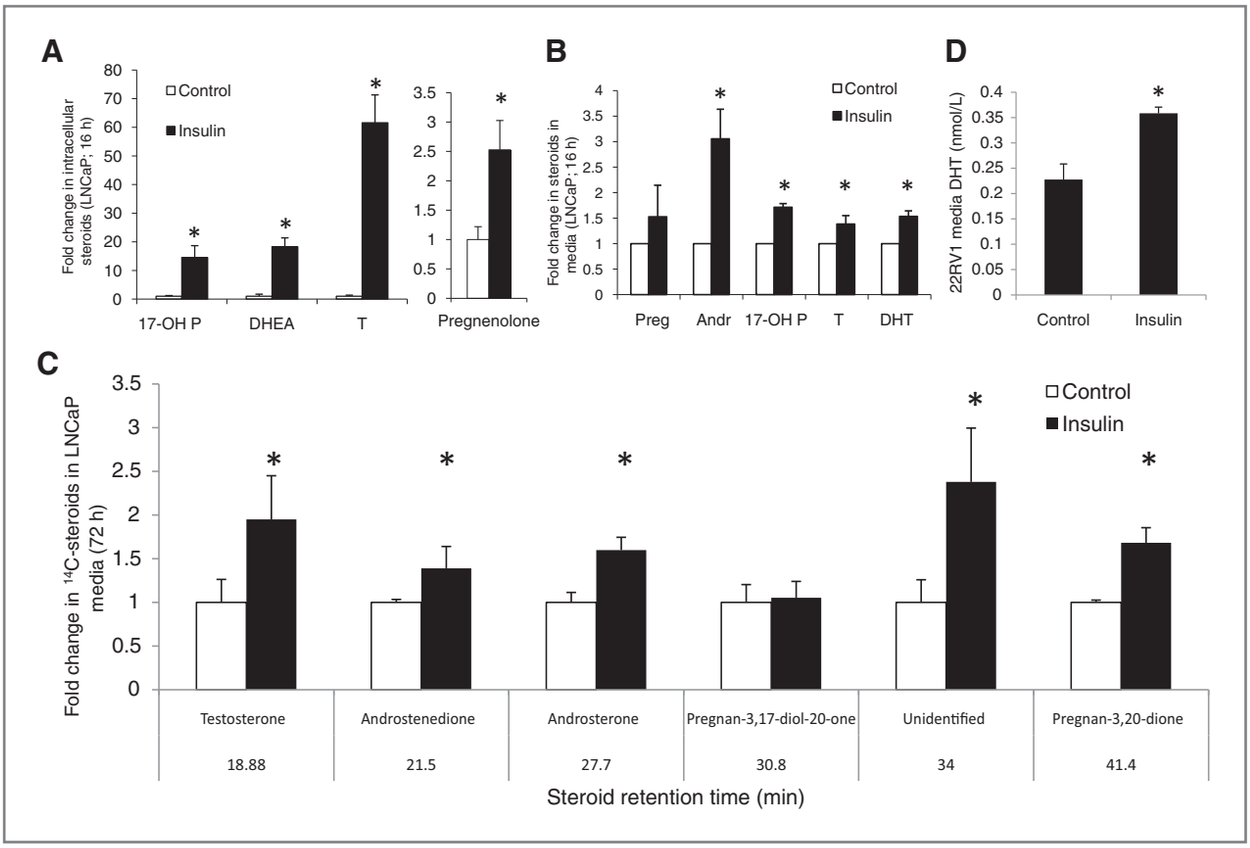

Figure 3. Insulin increases steroid production in prostate cancer cells. LNCaP cells were treated with 10 nmol/L insulin for 16 hours, and steroids were extracted. Intracellular steroids were identified by LC/MS-MS and quantitated (A), showing a significant increase in pregnenolone, 17-OH-progesterone (17-OH P), DHEA, and testosterone (T). B, extracellular steroids extracted from media were analyzed. Significantly increased levels of androstenedione (Andr), 17-OH-progesterone (17-OH P), DHT, and testosterone (T) were shown. Pregnenolone (Preg) was increased but did not reach significance. Steroid levels were adjusted to cell pellet weight, and recovery of denatured testosterone was used to calculate extraction efficiency. Results were compared with vehicle control. *De novo* steroid synthesis was measured by incubating cells with 6 μCi/mL radiolabeled acetate. C, increased ^{14}C-labeled steroid peaks were measured in insulin-treated versus control media samples after 72 hours with significant increases in testosterone, androstenedione, androsterone, and pregnan-3,20-dione. VCaP cells were also measured (Supplementary Fig. S2A and B). D, DHT was measured by ELISA in media collected from 22RV1 cells after 48-hour incubation with insulin and compared with vehicle control. Statistically significantly increased DHT levels were shown. Error bars, ±SE (*, $P < 0.05$).

in Figure 2 to be significantly increased with insulin. CYP17A1 also catalyzes the final reaction in the synthesis of dehydroepiandrosterone (DHEA), which was substantially increased 18-fold by insulin ($P < 0.05$; Fig. 3A). Notably, testosterone levels were increased approximately 60-fold in LNCaP cells following treatment with insulin ($P < 0.05$). Intracellular levels of testosterone were calculated to increase from approximately 0.011 to 0.65 ng/g cells following 10 nmol/L insulin for 16 hours (Supplementary Fig. S1A and C). These levels are consistent with the testosterone levels of our previous findings (3). Gregory and colleagues have shown that DHT concentrations as low as 1×10^{-14} mol/L (2.92×10^{-6} ng/g) to transactivate AR in prostate cancer cell lines (19), and Titus and colleagues report 1.25 pmol/g tissue (0.498 ng/g) of DHT in recurrent prostate cancer tissue specimens (20). Mostaghel found DHT levels in castrate patients to be 0.2 to 1.78 ng/g (4); therefore, the androgen concentrations detected in our study were consistent with levels needed for AR activation.

Insulin increases secretion of steroids from prostate cancer cells

As steroidogenic cells differentially secrete specific steroids, we measured steroid levels in the media of prostate cancer cells cultured in serum-free media versus media supplemented with 10 nmol/L insulin for 16 hours. As expected, it was steroids in the latter part of the steroidogenic pathway that appeared in the media (Fig. 3B). Of greatest significance, 17-OH-progesterone was 2-fold increased ($P < 0.05$), testosterone 1.3-fold and DHT 1.5-fold increased ($P < 0.05$), and androstenedione was increased 3-fold ($P < 0.05$).

Importantly, these data indicate that insulin-stimulated intracellular steroidogenesis by prostate cancer cells could provide steroids, including androgens, to the tumor microenvironment. Concentrations of DHT and testosterone secreted into the media by LNCaP cells after 16 hours of insulin treatment in our studies were calculated to be approximately 0.0249 and 0.037 nmol/L (Supplementary Fig. S1B and C), with baseline levels consistent with our

Published OnlineFirst July 11, 2011; DOI: 10.1158/0008-5472.CAN-10-2470

previous studies (3) and within the range necessary to activate AR (19, 20).

To investigate *de novo* steroidogenesis, LNCaP and VCaP cells were treated with ^{14}C-labeled acetate in the presence or absence of insulin for 72 hours before radiometric analysis of cell culture medium. Increased steroids in samples treated with insulin compared with vehicle control were consistent with steady-state data from LNCaP cells. In LNCaPs (Fig. 3C), we showed significant increases in testosterone (2-fold, $P < 0.05$), androstenedione (1.5-fold, $P < 0.05$), androsterone (2-fold, $P < 0.05$), and pregnan-3,20-dione (1.7-fold, $P < 0.05$), as well as several other peaks within the steroid region that did not correspond to Mix 10 standards (Supplementary Fig. S2A and B) including a 34-minute peak with a retention time between progesterone and pregnan-3,20-dione, which was significantly increased (2.4-fold, $P < 0.05$). Steroid peaks were significantly increased in VCaP extracts following insulin treatment as well as a 4-fold increase in cholesterol (Supplementary Fig. S2A and B). Furthermore, steroids beyond androstenedione and androsterone in the pathways were not detected in VCaP cells (Supplementary Fig. S2A and B), suggesting that rates of steroid synthesis differ between the cell lines. In 22RV1 cells, DHT secretion into the media was significantly increased from 0.23 to 0.36 nmol/L (Fig. 3D) following 48-hour treatment with 10 nmol/L insulin. This is comparable with DHT secreted by LNCaP cells (Supplementary Fig. S1) and sufficient to activate the AR (19, 20).

PSA expression and secretion are increased by insulin

Serum PSA is the biomarker for prostate cancer recurrence and for progression to CRPC following ADT. We and others have shown that a key mechanism underlying CRPC progression is the activation of androgen-driven pathways through the AR (21, 22); therefore, we have used PSA as a functional surrogate of AR reactivation via increased androgen production. A direct comparison of insulin stimulation of PSA with DHT was made with LNCaP cells exposed to 10 nmol/L insulin or 10 nmol/L DHT for 16, 24, or 48 hours. Insulin treatment (24 hours) induced a 10-fold increase in PSA mRNA (Fig. 4A), compared with a 20-fold increase by DHT, whereas mRNA levels decreased by 48 hours, likely due to the metabolism of these hormones. In contrast, the nonmetabolizable androgen R1881 continued to increase PSA mRNA levels over this time course (Fig. 4B). Levels of response were compared between 10 nmol/L insulin and increasing concentrations of DHT. At 24 hours, PSA induction by 10 nmol/L insulin was equivalent to the level of induction seen with approximately 0.16 ($\pm$0.29) nmol/L DHT (Fig. 4C), calculated by linear regression. Mean concentration of PSA with vehicle did not change over time (data not shown). PSA secreted into the media was increased because of insulin treatment after 16 hours (Fig. 4C), which suggests a lag in PSA response consistent with requisite steroid production. These data clearly show that PSA was increased at 16 hours and significantly accumulated in the media by 48 hours to 1.8-fold of baseline following insulin exposure ($P < 0.05$). This is supported by data showing sustained intracellular androgen levels by insulin at 48 hours (Supplementary Fig. S3A and B). Insulin increased PSA expression in VCaP cells by 40% from baseline ($P < 0.05$) after 24 hours (Fig. 4E) and 2-fold 22RV1 cells at 48 hours (Fig. 4F). Treatment with the AR antagonist bicalutamide attenuated the insulin induced increase in PSA expression in LNCaP and 22RV1 cells (Fig. 4G and H), directly implicating that insulin activation is mediated by the AR.

In LNCaP xenografts, mice that showed an increase in both PSA and RDH5 expression at 28 days postcastration also displayed an increase in insulin receptor isoform A and IRS-2 mRNA

CRPC progression can be modeled *in vivo* using LNCaP tumors injected subcutaneously into immunocompromised male mice; tumor growth is followed by monitoring tumor-derived PSA levels in the serum. Typically, after a 6-week period of growth, the mice are castrated and PSA levels fall to a nadir within 7 days. In most mice, PSA levels begin to increase again by day 28 postcastration and this is referred to as castrate resistance in this model. However, in some mice there is a greater lag of PSA production not arising until after 35 days. In a blinded study for PSA level following castration, tumors were grown for 28 days postcastration and then analyzed for the expression of markers relevant to steroidogenesis. From the isolated LNCaP tumors, qRT-PCR was carried out for PSA, insulin receptor isoform A (IR-A), IRS-2, and RDH5. RDH5 is a key enzyme of the backdoor pathway to DHT synthesis, and tumors expressing this enzyme may be more steroidogenic (3, 14). In mice that exhibited increased serum PSA levels 28 days following castration, tumors showed significantly higher gene expression levels of PSA, RDH5, and IRS-2 ($P < 0.05$; Fig. 5A), and IR-A showed a trend toward increased expression (Fig. 5A). In contrast, these genes were unchanged in mice bearing LNCaP tumors that did not show an increase in serum PSA levels by 28 days (nonprogressed; Fig. 5B). Therefore, increased steroidogenesis correlates to increased androgen activation (PSA production) *in vivo*. Changes in key insulin signaling molecules suggest that insulin may act via IR-A and IRS-2 in this model.

Discussion

Reactivation of the AR following ADT, defined by rising serum PSA levels is a hallmark of CRPC progression. Several mechanisms including hypersensitivity of the receptor to low steroid concentrations and ligand promiscuity arising from mutations in the receptor ligand–binding domain can play a role in AR reactivation (19, 22). We and others have previously identified that intratumoral androgen production is also associated with the activation of AR (20). We have shown that LNCaP, VCaP, and 22RV1 prostate cancer cell lines express the enzymes required for *de novo* androgen synthesis (3). Following ADT, androgen levels continue to be substantial in prostate tissue compared with the dramatic decrease of androgens in sera. These low levels are sufficient to activate AR (2, 3). These studies indicate that androgen synthesis plays an important role in CRPC progression; however, the biological factors inducing and regulating

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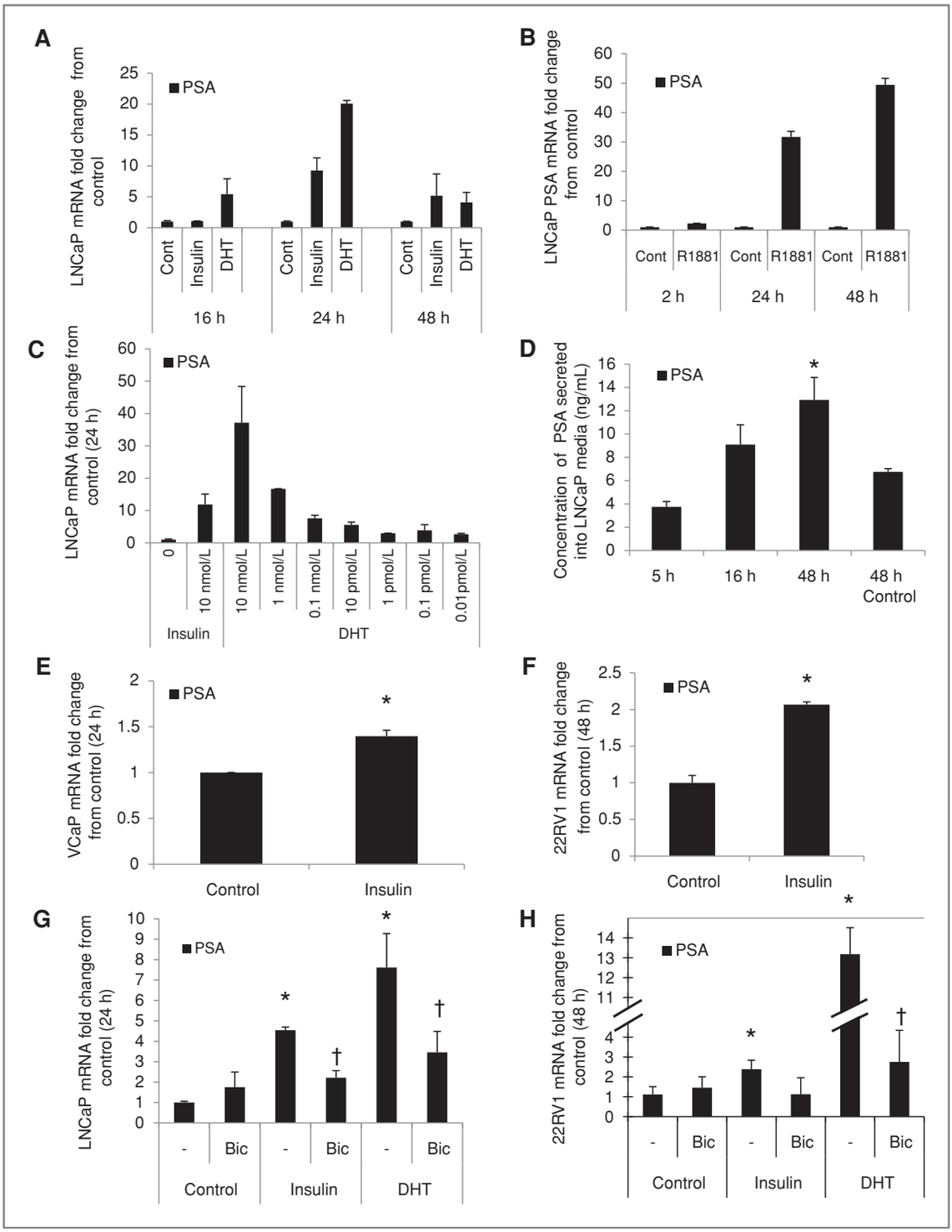

Figure 4. Insulin treatment increases expression of PSA. A, insulin-induced changes in PSA mRNA expression from LNCaP cells were compared with 10 nmol/L DHT at 16, 24, and 48 hours by qRT-PCR. Insulin-induced changes in PSA expression are detected at 24 hours. The effects of DHT/insulin are reduced after 48 hours of culture. Cont, control. B, increased expression of PSA in LNCaP cells is maintained at 48 hours by the nonmetabolizable AR agonist R1881. Cont, control. C, PSA response of insulin and DHT was compared. LNCaP cells were treated with DHT concentrations from 10 nmol/L to 0.1 pmol/L for 24 hours, and the values were compared with 10 nmol/L insulin. D, media were collected from LNCaP cells treated with insulin (10 nmol/L) for 5, 16, and 48 hours, and control at 48 hours and PSA were measured. Control = 48 hours. Increased expression of PSA mRNA was shown following insulin treatment (10 nmol/L) compared with vehicle following (E) 24-hour treatment of VCaP cells and (F) 48-hour treatment of 22RV1 cells. Treatment with the AR inhibitor bicalutamide (Bic) attenuated the insulin-induced increase in PSA expression in (G) LNCaP cells and (H) 22RV1 cells by approximately 50% (†, $P < 0.05$). Error bars, ±SE (*, $P < 0.05$).

Published OnlineFirst July 11, 2011; DOI: 10.1158/0008-5472.CAN-10-2470

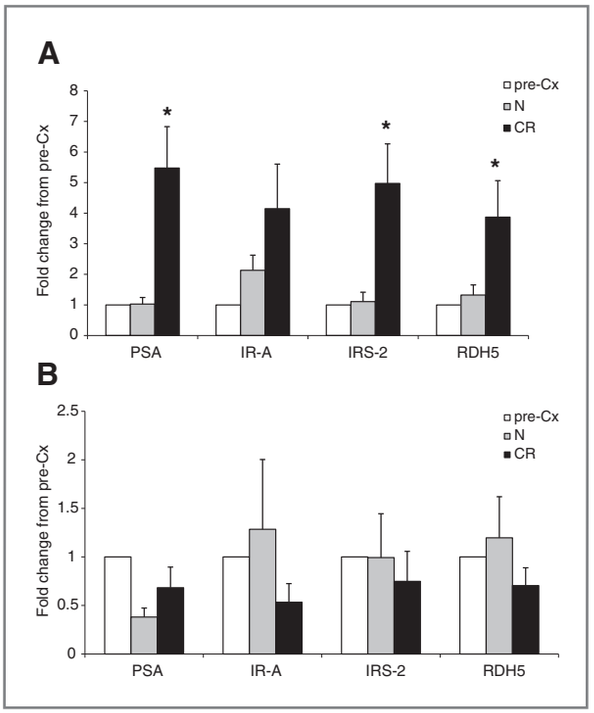

Figure 5. *In vivo* tumor xenograft model. LNCaP tumor xenografts were collected from athymic nude mice at castration (pre-Cx), at PSA nadir (N; 8 days postcastration), and at castrate-resistant stage (CR; 28 days postcastration). qRT-PCR analyses of PSA, IRS-2, IR-A, and RDH5 in tumors show statistically significant increases in expression in mice that experienced PSA recurrence (A). In contrast, expression of these genes was not changed in mice that did not progress to castrate resistance (B). Error bars, ±SE (*, $P < 0.05$).

steroidogenesis during prostate cancer progression have not been largely explored.

ADT is associated with the development of metabolic syndrome including an increase in fat mass and fasting plasma insulin levels (hyperinsulinemia refs. 6, 23). Emerging evidence suggests that both body mass index and high serum insulin levels are independently predictive of poorer patient outcomes including increased disease aggression and increased cancer mortality (24); obese men are more likely to have higher grade cancers, high recurrence rates, and high prostate cancer–specific mortality (25). Recent studies have specifically identified a correlation between elevated C-peptide levels, a surrogate measure of insulin levels, with high-grade prostate cancer and worse patient prognosis (6, 7, 24, 26, 27). Major findings from recent studies of men receiving ADT showed a relationship between elevated C-peptide levels and more rapid progression to castrate resistance (28, 29), suggesting a role for insulin in disease progression. Diet-induced hyperinsulinemia leads to more aggressive tumor growth in mice (8), and insulin is known to stimulate proliferation in breast and prostate cancer cells (30, 31). In contrast, men with low insulin levels due to diabetes seem to have a decreased risk of prostate cancer development (32, 33). Increased insulin receptor expression has recently been shown in neoplastic prostate specimens (7), suggesting increased insulin signaling in these cells.

Although there is mounting epidemiologic evidence linking hyperinsulinemia and CRPC, the direct action of insulin on prostate cancer cells has not been investigated. Insulin is can promote steroidogenesis through upregulation of steroidogenic enzymes (34–36) in conditions such as polycystic ovarian syndrome, and increased expression of insulin receptors have been reported on prostate cancer cell lines and prostate tumor tissue (7). The ability of prostate cell lines to produce steroids has been shown (3, 35, 37–39). Therefore, we investigated whether insulin plays a role in prostate cancer progression through the promotion of *de novo* steroidogenesis. We show for the first time that insulin upregulates steroidogenesis in AR-responsive prostate cancer cell lines, LNCaP, VCaP, and 22RV1, leading to increased cell survival and exacerbating CRPC progression.

We have shown that RNA and protein for many enzymes required for steroidogenesis, via both the classical and backdoor pathways, are upregulated following insulin treatment. Expression of the insulin signaling molecule IRS-2 is significantly increased at the RNA level in LNCaPs; increased expression of IRS-2 has been associated with increased steroidogenesis in both ovarian thecal and breast cancer cells (40, 41). Importantly, we have shown an increase in expression of mRNA and protein for SREBP, the transcription factor that is responsible for coordinating the initiation of cholesterol synthesis in LNCaP cells following 10-hour insulin treatment and 48-hour treatment of 22RV1 cells. CYP11A1 was increased in all cell types and StAR in LNCaPs and 22RV1s; these enzymes are responsible for importation of cholesterol into the mitochondria for pregnenolone conversion and commitment to steroidogenesis. The enzymes that catalyze more than 1 step in the steroidogenesis pathway including CYP17A1, HSD3B2, HSD17B3, and SRD5A1 (Fig. 1) were all significantly upregulated by insulin treatment. Significantly increased expression of SRD5A1 was seen in LNCaP cells only but RDH5 expression was increased in all cell lines; these enzymes convert testosterone and androstenediol into DHT, respectively. Taken together, our data suggest that both pathways of *de novo* androgen synthesis are upregulated in prostate cancer cells following insulin treatment, allowing for versatile means of androgen synthesis as seen in our previous studies (17).

Insulin consistently stimulated an increase in intracellular steroids and steroids released into the media including androgens in all cell lines, indicating that the enzymes are functionally active. The release of steroids by prostate cancer cells may provide paracrine activity of steroids within the microenvironment. Rising PSA levels following ADT is considered the sentinel for CRPC progression most likely driven by AR reactivation. We observed increased PSA mRNA expression in all 3 cell lines by insulin, which shows that there is adequate AR activation to stimulate PSA expression (3, 22, 42) and this can be inhibited by bicalutamide treatment. An increase in structurally related steroids may still be relevant in cancer progression, in the cases wherein AR has acquired mutations, leading to promiscuous activation by steroids and compounds other than testosterone and DHT (15), for example, the

Published OnlineFirst July 11, 2011; DOI: 10.1158/0008-5472.CAN-10-2470

mutation of the LNCaP AR ligand–binding domain (T877A) can be activated by nonandrogenic steroids (15).

In summary, our research has shown that insulin increases steroidogenesis in AR-positive prostate cancer cell lines by increasing the mRNA and protein levels of steroidogenic enzymes and increases steroid production, including androgens. Subsequent increased PSA secretion suggests that insulin can affect prostate cancer cell survival and CRPC progression. Increased expression of the insulin receptor in the LNCaP xenograft model during progression to castrate resistance provides further evidence that insulin may be increasing signaling in prostate cancer cells through increased insulin receptor. There are multiple studies correlating high insulin levels and prostate cancer progression (8, 23, 27, 32, 43). The significance of cholesterol synthesis (steroid precursor) and steroidogenesis in prostate cancer progression suggests that treatments that target these pathways are pertinent for the treatment of CRPC patients, particularly in the context of hyperinsulinemia and the metabolic syndrome (3, 4, 9, 22, 37–39, 42, 44). Abiraterone, an inhibitor of CYP17A1, has shown promising results in clinical trials with men who are no longer responsive to ADT (45). This is one pathway by which insulin may contribute to cancer progression; however, insulin is expected to activate multiple pathways in cancer cells (25). Further understanding of the direct action of insulin on prostate cancer cells may provide important insight into new therapeutic strategies to prevent progression of CRPC.

Results of our study suggest that metabolic dysfunction of prostate cancer patients should be addressed. There are a number of pharmacologic agents currently available for the treatment of insulin resistance that can improve (reduce) circulating insulin levels including metformin, and recent studies suggest that targeting insulin resistance can have positive effects on cancer patient outcomes including prostate cancer (46). Upstream inhibitors of cholesterol synthesis such as the thiazolidinediones have been shown to be effective insulin sensitizers and, in cancer, decrease androgen production in H295 cells by downregulation of CYP17A1 and HSD3B2 (47) and reduce cancer cell proliferation (48). Patients who are undergoing cholesterol-lowering treatment with the statin class of 3-hydroxy-3-methyl-glutaryl CoA inhibitors show markedly lower PSA levels and tumor volumes (49). Currently, ADT-induced hyperinsulinemia is not addressed in prostate cancer patients, despite a significantly increased risk of cardiovascular mortality in these patients (50); however, we provide further evidence that management of the metabolic consequences of ADT may be as important as treatment of the cancer itself.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

The authors thank Steven Pham for skilled technical assistance (Vancouver Prostate Centre, University of British Columbia, Vancouver, British Columbia, Canada) and acknowledge the support of Australian-Canadian Prostate Cancer Research Alliance and the Queensland Government.

Grant Support

This work was funded by grant from the Queensland Smart Futures Premier's Fellowship—CCN, Prostate Cancer Foundation of Australia (PCFA PG25), and Terry Fox Program Project (National Cancer Institute of Canada grants 012003 and 017007).

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Received July 7, 2010; revised June 12, 2011; accepted June 28, 2011; published OnlineFirst July 11, 2011.

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Published OnlineFirst July 11, 2011; DOI: 10.1158/0008-5472.CAN-10-2470

Cancer Research
The Journal of Cancer Research (1916–1930) | The American Journal of Cancer (1931–1940)

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Insulin Increases *De Novo* Steroidogenesis in Prostate Cancer Cells

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Cancer Res Published OnlineFirst July 11, 2011.