Research Article

Title: Insulin increases de novo steroidogenesis in prostate cancer cells

Running title: Insulin increases steroidogenesis in prostate cancer cells

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Abstract

Androgen-dependent pathways regulate maintenance and growth of normal and malignant prostate tissue. 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). While ADT generates a therapeutic response, it is also associated with a pattern of metabolic alterations consistent with metabolic syndrome including elevated circulating insulin. Since 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 10nM insulin increased mRNA and protein expression of steroidogenesis enzymes and upregulated the insulin receptor substrate IRS2. 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 DHT, insulin treatment resulted in increased mRNA expression of prostate specific antigen (PSA). CRPC progression also correlated with increased expression of IRS2 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.
Introduction

Organ confined prostate cancer can be treated with radical prostatectomy or radiation therapy; however, 25-40% of patients will experience biochemical recurrence with a rise 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 LHRH agonists or antagonists which disrupt the feedback loop within the hypothalamic gonadal axis to suppress testosterone production from the testes. Initially, inhibition of testicular androgen tumor regression. However, this remission is temporary and typically within 24 months patients recur with a rising PSA 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 appears to be further up-regulated by androgens indicating a feed forward loop of steroidogenesis. In the physiological 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.
While ADT provides control of prostate cancer growth, the systemic non-cancer effects include the induction of the metabolic syndrome with a key consistent feature of hyperinsulinaemia (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 demonstrate that insulin treatment on prostate cancer cells increases the expression at both the mRNA and protein levels of several key enzymes involved in de novo steroidogenesis including cytochrome p450 family members (CYP)11A1 and CYP17A1, RDH5 and hydroxysteroid dehydrogenase (HSD)3B2 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 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 substrate 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 (passage 36-48; American Type Culture Collection) and 22RV1 cells were cultured in phenol red-free RPMI 1640 (Invitrogen, Melbourne, Australia) and 5% fetal calf serum (FBS; Hyclone, Sigma, Australia). VCaP cells were
cultured in 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 24hrs, followed by 24hr in serum-free media. Cells were treated with 10nM insulin (Sigma) for various times (5, 10, 16, 24, and 48 hours). Insulin and DHT was refreshed if treatment exceeded 24hrs. Cells were incubated in the presence of 25μM bicalutamide or vehicle control for 2hr prior to addition of insulin or DHT.

**QRT-PCR:** RNA was extracted from prostate cancer cells using TriReagent (Applied Biosystems, Melbourne, Australia) before reverse transcription with superscript III reverse transcriptase (Invitrogen) according to the manufacturer’s protocol. Subsequent quantitative PCR was performed on 7900HT Fast Real Time PCR System (Applied Biosystems) with SYBR Green detection. Primers (Sigma Proligo) were designed by Primer 3 software from coding segments of genes, obtained from the NCBI data bank. The primers used were:

- **SREBP** (f) 5’-cgcctctccatacctagaca-3’, (r) 5’-tcgagaagcaatgtagtg-3’;
- **StAR** (f) 5’-gcccctggagctctatg-3’, (r) 5’-tccactccccccatgct-3’;
- **CYP11A1** (f) 5’-agtcttcggctctctgtctctgtc-3’;  CYP17A1 (f) 5’-gggcgccttcttgaga-3’, (r) 5’-cagcgaagggaagggctcttatg-3’;
- **HSD3B2** (f) 5’-ttgctgagctattctgactgttta-3’, (r) 5’-cagcgaagggaagggctcttatg-3’;
- **SRD5A1** 5’-acgggcatctcttacaaagagttctc-3’, (r) 5’-cagcgaagggaagggctcttatg-3’;
- **RDH5** (f) 5’-gcgccgagacacagtcag-3’, (r) 5’-cgccgagacctgcagtt-3’;
- **PSA** (f) 5’-agtcggctctctcttgtagttc-3’, (r) 5’-ccagcaagatgagctttttttg-3’, and **IRS-2** validated primers from Applied Biosystems. Gene expression was normalized to the housekeeping gene **RPL32** (f) 5’-ccctttgtgagaagccca-3’, (r) 5’-gactgtgctccgcaact-3’ then expressed relative
to the vehicle control at the same time point. Data was analyzed with SDS 2.3 software by means of the $2^{-\Delta\Delta C_T}$ method. Experiments were repeated a minimum of 5 times.

**Western Blotting:** Cells were lysed in radioimmunoprecipitation assay buffer (25mM Tris.HCl pH 7.6, 150mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS), and triplicate wells were pooled. SDS-PAGE was used to separate proteins (15ug/ lane), using standard protocols. Blots were visualized using secondary antibodies compatible with Li-Cor Odyssey Imager (Li-Cor Biosciences, Lincoln, USA). Antibodies used were: rabbit SREBP1, 1:200 (Santa Cruz Biotechnology, Santa Cruz, USA); rabbit StAR and CYP17A1, 1:1000 (kind gifts from Dr. DB Hales); rabbit CYP11A1, 1:1000 (Corgen, Taipei, Taiwan); goat HSD3B2 1:200 (Santa Cruz); goat SRD5A1 1:500 (Novus, Cambridge, UK), and RDH5 1:300 (Abnova, Taipei, Taiwan); GAPDH antibody (Santa Cruz) as loading control. Experiments were repeated minimum of three 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 LC/MS/MS:** Steroid analysis was performed as previously described (3). Briefly, cells were grown in 15cm plates and treated with 10nM insulin as described above. Two plates of cells were washed with PBS and pooled to give one sample. Media was collected and likewise combined for extraction. Steroids were extracted from the pellet with an MTBE/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, then sonicated and spun to remove any particulates. Samples were derivatized in 0.2M hydroxylamine HCL. Samples were run on the Waters Acquity Liquid Chromatography system and the Waters Quattro Premier LC/MS/MS, identified using standards of known retention times (Figure 1) and analyzed using BioLynx Software (Waters Corp, USA). 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, 6µCi/mL $^{14}$C-acetate (PerkinElmer) was added to each plate for co-incubation. Steroids from 2mL of media were extracted with 75/25 hexane/ethanoloacetate, dried down and resuspended into 75ul methanol (50%). Samples were analyzed on the Waters Alliance 2695 HPLC System and Packard Radiometric Detector 150TR Flow Scintillation Analyzers. Peaks were identified by comparison of retention times to Mix 10 steroid standard (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 dihydrotestosterone (DHT) were evaluated using ClinPro PSA kit as per kit instructions (ClinPro International, USA).
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 (preCx), 8 days after castration corresponding to a PSA nadir (N) and 28 days after castration corresponding to CRPC (CR). Tumors were removed and homogenized in Trizol (Invitrogen) and RNA was isolated for cDNA synthesis as described above.

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 dihydrotestosterone from intracellular cholesterol synthesis (Figure 1). The direct effect of insulin on steroidogenesis within prostate cancer cells was initially determined by QRT-PCR analysis to examine changes in expression of key genes within this pathway following chronic exposure of insulin. LNCaP cells treated with 10nM insulin for 5, 10 and 16hrs showed enzymes required for synthesis of androgens were upregulated at the mRNA level in the presence of insulin after 10hrs (Figure 2a). A parallel and
significant 3.5-fold increase in IRS-2 (p<0.05) suggests increased signaling via the insulin receptor (10). SREBP transcription factors are responsible for coordinately regulating the enzymes required for synthesis of cholesterol, its importation into the mitochondria, and steroidogenesis (11-13). At this time point, Sterol regulatory element binding protein (SREBP1) mRNA was also increased (p<0.05). StAR, which chaperones cholesterol into the mitochondria, increased 2.25-fold, CYP11A1 (C11A1), the rate limiting enzyme which commits cholesterol to steroid synthesis, is upregulated 2-fold. Many of the steroidogenic enzymes catalyze more than one step in the pathway to DHT synthesis. These include monooxygenase CYP17A1 (C17A1), oxidoreductase / dehydrogenases HSD3B2, HSD17B3 and 5a-reductase (SRD5A1). CYP17A1 was significantly increased 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), while aldo-ketoreductase (AKR)1C3 remained unchanged (data not shown). The enzyme responsible for conversion of testosterone to DHT, SRD5A1, showed a significant 30% increase from base level. In the ‘backdoor’ pathway of steroidogenesis (14), 11-cis retinol dehydrogenase (RDH5) converts androstenediol directly to DHT and was two-fold upregulated (p<0.05) with 10nM insulin.

These studies were extended into other prostate cancer cell lines with functional androgen receptors, 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 (Figure 2b), was observed for CYP11A1 (3-fold), CYP17A1 (1.5-fold), HSD17B3 (2.5-fold), and RDH5 (1.5-fold) (p<0.05). SREBP and
StAR were expressed but were not significantly changed with insulin (Figure 2b). In 22RV1 cells, similar increases in mRNA were demonstrated (Figure 2c). SREBP and StAR were upregulated 2-fold (p<0.05), while CYP11A1 was increased ~3-fold. CYP17A1 was upregulated 5-fold, while HSD3B2 and HSD17B3 increased 2.3 and 2-fold, respectively (p<0.05). RDH5 increased 2.6 fold (p<0.05) (Figure 2c). There was no significant change to IRS2 expression in VCaP or 22RV1 cells by insulin, at 48 hours.

The three 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). While the magnitude and temporal upregulation of steroidogenesis enzyme mRNA in these cells may differ, posttranslational modifications and subcellular localization also influences activity. Importantly, all enzymes necessary for steroidogenesis are expressed in various CaP cell lines.

We have previously shown that prostate cancer cells can use alternative steroidogenic pathways in a compensatory manner to synthesize DHT through either the classical pathway, ‘backdoor’ pathway or a combination (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 (Figure 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, while modestly increased following 10 hour insulin treatment at the mRNA level, increases greater than 3-fold at the protein level (p<0.05), and expression remains elevated (1.5 and 2 fold at 10 and 16hrs, respectively - data not shown). RDH5 protein was only upregulated by insulin at the 10hr time point (p<0.05) and HSD17B3 was not significantly increased until 16hrs insulin treatment (p<0.05), with steady expression at 5 and 10hrs (not shown). AKR1C3 protein levels were unchanged, however AKR1C3 is the predominant aldo-ketoreductase in prostate epithelial cells and it is possible that the activity of the enzyme is regulated post-translationally, 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 (Figure 2e) with significant increases in SREBP, CYP11A1, CYP17A1 and HSD17B3 (p<0.05).

While our data suggests that insulin increases StAR expression, biologically the more important effect is altered subcellular localization of StAR. Our data demonstrates a 1.7-fold increase in StAR translocation into the mitochondria following 16hr treatment with insulin in LNCaP cells (p<0.05; Figure 2f).

**Insulin increases intracellular steroids in prostate cancer cells.** To determine whether there is a parallel increase in steroid synthesis, we used HPLC-MS to measure total steroid content of LNCaP cells following 16hrs insulin treatment. Insulin dramatically increased intracellular LNCaP steroid levels (Figure 3a) with 2.5 fold increased levels of the first steroid converted from cholesterol in the pathway, pregnanolone, (Figure 3a; p<0.05).
observed a 15-fold increase in total intracellular 17-OH-progesterone levels (p<0.05), which is converted from progesterone by CYP17A1, an enzyme shown in Figure 2 to be significantly increased with insulin. CYP17A1 also catalyses the final reaction in the synthesis of DHEA, which was substantially increased 18-fold by insulin (Figure 3a; p<0.05). Notably, testosterone levels were increased ~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 0.65ng/g cells following 10nM insulin for 16hrs (Supplementary Figure 1a, c). These levels are consistent with the testosterone levels of our previous findings (3). Gregory et al. have demonstrated 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 et al report 1.25pmol/g tissue (0.498ng/g) of DHT in recurrent prostate cancer tissue specimens (20). Mostaghel found DHT levels in castrate patients are 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 10nM insulin for 16 hours. As expected, it was steroids in the latter part of the steroidogenic pathway that appeared in the media (Figure 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 0.037nM (Supplementary Figure 1b, c), with baseline levels consistent with our 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 72hrs before radiometric analysis of cell culture medium. Increased steroids in samples treated with insulin compared to vehicle control were consistent with steady-state data from LNCaP cells. In LNCaPs (Figure 3c), we demonstrated significant increases in testosterone (2-fold, p<0.05), androstenedione (1.5-fold, p<0.05), and 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 which did not correspond to Mix 10 standards (Supplementary Figure 2a, b) including a 34 min 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 Figure 2a, b). Furthermore, steroids beyond androstenedione and androsterone in the pathways were not detected in VCaP cells (Supplementary Figure 2a, b) suggesting rates of steroid synthesis differ between the cell lines. In 22RV1 cells, DHT secretion into the media was significantly increased from 0.23nM to 0.36nM (Figure 3d), following 48-hour treatment
with 10nM insulin. This is comparable to DHT secreted by LNCaP cells (Supplementary Figure 1) and sufficient to activate the AR (19-20).

**PSA expression and secretion are increased by insulin.** Serum PSA is the biomarker for CaP 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 10nM insulin or 10nM DHT for 16, 24, or 48 hours. Insulin treatment (24 hours) induced a 10-fold increase in PSA mRNA (Figure 4a), compared to a 20-fold increase by DHT, whereas mRNA levels decreased by 48hrs, likely due to the metabolism of these hormones. In contrast, the non-metabolizable androgen R1881 continued to increase PSA mRNA levels over this time course (Figure 4b). Levels of response were compared between 10nM insulin and increasing concentrations of DHT. At 24 hours PSA induction by 10nM insulin was equivalent to the level of induction seen with approximately 0.16(±0.29)nM DHT (Figure 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 due to insulin treatment after 16hrs (Figure 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 48hrs 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 48hrs (Supplementary Figure 3a, b). Insulin increased PSA
expression in VCaP cells 40% from baseline (p<0.05) following 24-hours (Figure 4e) and 2-fold 22RV1 cells at 48hr (Figure 4f). Treatment with the AR antagonist, bicalutamide attenuated the insulin induced increase in PSA expression in LNCaP and 22RV1 cells (Figure 4g-h) directly implicating insulin activation is mediated by the AR.

In LNCaP xenografts, mice which showed an increase in both PSA and RDH5 expression at 28 days post castration also displayed an increase in IR-A and IRS2 mRNA. CRPC progression can be modelled in vivo using LNCaP tumours injected subcutaneously into immunocompromised male mice; tumour growth is followed by monitoring tumor-derived PSA levels in the serum. Typically, after a 6-week period of growth the mice are castrated, PSA levels fall to a Nadir (N) within 7 days. In most mice PSA levels will begin to increase again by day 28 post-castration and this is referred to as castrate resistance (CR) 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, tumours were grown for 28 days post castration, and then analysed for the expression of markers relevant to steroidogenesis. From the isolated LNCaP tumors, QRT-PCR was performed for PSA, insulin receptor isoform A (IR-A), insulin receptor substrate 2 (IRS2), and RDH5. RDH5 is a key enzyme of the backdoor pathway to DHT synthesis, and tumours expressing this enzyme may be more steroidogenic (3, 14). In mice that exhibited increased serum PSA 28 days following castration, tumours showed significantly higher gene expression levels of PSA, RDH5 and IRS2 (Figure 5a; p<0.05) and IR- A showed a trend towards increased expression (Figure 5a). In contrast these genes were unchanged in mice bearing LNCaP tumors that did not show a serum PSA increase by 28
days (non-progressed; Figure 5b). Therefore, increased steroidogenesis correlates to increased androgen activation (PSA production) in vivo. Changes in key insulin signalling molecules suggest that insulin may act via IR-A and IRS2 in this model.

Discussion

Reactivation of the androgen receptor following ADT, defined by rising serum PSA 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 activation of AR (20). We have demonstrated that LNCaP, VCaP and 22RV1 prostate cancer cell lines expresses the enzymes required for de novo androgen synthesis (3). Following ADT, androgen levels continue to be substantial in prostate tissue, compared to 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 steroidogenesis during prostate cancer progression have not been largely explored.

Androgen deprivation therapy is associated with development of metabolic syndrome including an increase in fat mass and fasting plasma insulin (hyperinsulinaemia) (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 CaP and worse patient prognosis (6-7, 24, 26-27). Major findings from recent studies of men receiving ADT demonstrated 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 appear to have a decreased risk of CaP development (32-33). Increased insulin receptor (INSR) expression has recently been shown in neoplastic prostate specimens (7) suggesting increased insulin signaling in these cells.

Although there is mounting epidemiological evidence linking hyperinsulinaemia and CRPC, the direct action of insulin on prostate cancer cells has not been investigated. Insulin is able to promote steroidogenesis through upregulation of steroidogenic enzymes (34-36) in conditions such as polycystic ovarian syndrome (PCOS), and increased expression of insulin receptors have been reported on prostate cancer cell lines and prostate tumor tissue (7). The ability for prostate cell lines to produce steroids has been demonstrated (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 cells, leading to increased cell survival and exacerbating
CRPC progression.

We demonstrated 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 signalling 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 showed an increase in expression of mRNA and protein for SREBP, the transcription factor which is responsible for coordinating the initiation of cholesterol synthesis in LNCaP cells, following 10hr insulin treatment and 48hr 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 pregnanolone conversion and commitment to steroidogenesis. The enzymes which catalyse more than one step in the steroidogenesis pathway including CYP17A1, HSD3B2, HSD17B3 and SRD5A1 (Figure 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 suggests both pathways of de novo androgen synthesis are upregulated in CaP 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 the enzymes are functionally
active. The release of steroids by prostate cancer cells may provide paracrine activity of steroids within the microenvironment. Rising PSA following ADT is considered the sentinel for CRPC progression most likely driven by AR reactivation. We observed increased PSA mRNA expression in all three cell lines by insulin which demonstrates 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 where AR has acquired mutations leading to promiscuous activation by steroids and compounds other than testosterone and DHT (15) for example the mutation of the LNCaP AR ligand binding domain (T877A) can be activated by non-androgenic 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 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 signalling in prostate cancer cells through increased INSR. There are multiple studies correlating high insulin levels and CaP progression (8, 23, 27, 32, 43). The significance of cholesterol synthesis (steroid precursor) and steroidogenesis in CaP progression suggests treatments which target these pathways are pertinent for the treatment of patients with CRPC, particularly in the context of hyperinsulinaemia 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 castrate resistant prostate cancer.

Results of our study suggest that metabolic dysfunction of prostate cancer patients should be addressed. There are a number of pharmacological agents currently available for treatment of insulin resistance which can improve (reduce) circulating insulin levels including metformin and recent studies suggest targeting insulin resistance can have positive effects on cancer patient outcomes including prostate cancer (46). Upstream inhibitors of cholesterol synthesis such as the thiazolidinediones (TZDs) have been shown to be effective insulin sensitzizers and, in cancer, decrease androgen production in H295 cells by down regulation of CYP17A1 and HSD3B2 (47) and reduce cancer cell proliferation (48). Patients who are undergoing cholesterol lowering treatment with the statin class of HMG CoA inhibitors, show markedly lower PSA and tumor volumes (49). Currently, ADT-induced hyperinsulinaemia is not addressed in prostate cancer patients, despite a significantly increased risk of cardio-vascular 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.

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Figure Legends

Figure 1: Enzymes involved in both classical (left/centre) and backdoor (far right, bold font) steroidogenesis pathways. Many of the steroidogenic enzymes catalyze more than one step in the pathway. Standards available for underlined steroids.

Figure 2: Insulin regulates expression of key steroidogenic enzymes at the mRNA and protein level. RNA was processed from CaP cells for QRT-PCR analysis. Results were analyzed by \( \Delta \Delta \text{Ct} \) method normalized to RPL32, then normalized to vehicle-treated at equivalent time point. Insulin (10nM) 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 10nM insulin treatment in (d) LNCaP cells following 10nM insulin treatment (5 hours), RDH5 at 10hrs (white*), or HSD17B3 at 16hrs (double white*) and (e) 22RV1 cells following 48 hour treatment. (f) 16 hours insulin treatment (10nM) increased translocation of StAR to the mitochondria compared to vehicle-treated cells. Western blots quantitated using Odyssey software, version 1.2 with GAPDH loading control. Error bars +SE (*p<0.05).

Figure 3: Insulin increases steroid production in prostate cancer cells LNCaP cells were treated with 10nM insulin for 16 hours and steroids extracted. Intracellular steroids were identified by LC-MS-MS and quantitated (a) showing a significant increase in pregnanolone, 17-OH progesterone (17-OH P), DHEA and testosterone (T). (b) Extracellular steroids extracted from media were analyzed. Significantly increased levels of
androstendione (Andr), 17-OH progesterone (17-OH P), DHT and testosterone (T) were shown. Pregnanolone (Preg) was increased, but did not reach significance. Steroid levels were adjusted to cell pellet weight and recovery of deuterated testosterone used to calculate extraction efficiency. Results were compared to vehicle control. De novo steroid synthesis was measured by incubating cells with 6µCi/mL radiolabelled acetate (c). Increased ¹⁴C-labeled steroids steroid peaks were measured in insulin-treated versus control media samples after 72hr with significant increases in testosterone, androstenedione, androsterone and pregnan-3,20-dione. VCaP cells were also measured (Supplementary figure 2a, b). (d) DHT was measured by ELISA in media collected from 22RV1 cells after 48hr incubation with insulin and compared to vehicle control. Statistically significantly increased DHT levels were demonstrated. Error bars +SE (*p<0.05).

**Figure 4: Insulin treatment increases expression of PSA.** (a) Insulin-induced changes in PSA mRNA expression from LNCaP cells were compared to 10nM DHT at 16, 24 and 48hrs by QRT-PCR. Insulin-induced changes in PSA expression are detected at 24hrs. The effects of DHT/insulin are reduced after 48hrs culture. In contrast (b), increased expression of PSA in LNCaP cells is maintained at 48 hours by non-metabolizable AR agonist R1881. (c) PSA response of insulin and DHT was compared. LNCaP cells treated with DHT concentrations from 10nM to 0.1pM for 24hrs and the values compared to 10nM insulin. (d) Media was collected from LNCaP cells treated with insulin (10nM) for 5, 16 and 48 hours and 48hr control and PSA measured. Control = 48hrs. Increased expression of PSA
mRNA was shown following insulin (10nM) compared to vehicle following (e) 24hr treatment of VCaP cells and (f) 48hrs treatment of 22RV1 cells. Treatment with the AR-inhibitor bicalutamide attenuated the insulin induced increase in PSA expression in (g) LNCaP cells and (h) 22RV1 cells by ~50% (†p<0.05). Error bars +SE (*p<0.05).

Figure 5: In vivo tumor xenograft model. LNCaP tumor xenografts were collected from athymic nude mice at castration (pre-Cx), at PSA nadir (8 days post castration – N) and at castrate-resistant stage (28 days post castration – CR). QRT-PCR analyses of PSA, IRS2, 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).
**Figure 1**

- **Key:**
  1. Underlined steroids have reference standards available for LC-MS analysis.
  2. Backdoor pathway highlighted in **bold** font.

Figure 3

A

Fold change in intracellular steroids (LNCaP) (16hrs)

B

Fold change in steroids in Media (LNCaP) (16hrs)

C

Fold Change in $^{14}$C-Stereoids in LNCaP Media (72hr)

D

22RV1 Media DHT (nM)
Figure 4

A. LNCaP mRNA fold change from control (48hr)

B. LNCaP PSA mRNA fold change from control

C. LNCaP mRNA fold change from control (24hrs)

D. Concentration of PSA secreted into LNCaP media (ng/ml)

E. VCaP mRNA fold change from control (24hrs)

F. 22RV1 mRNA Fold Change from Control (48hr)

G. LNCaP mRNA Fold Change from Control (24hr)

H. 22RV1 mRNA Fold Change from Control (48hr)
Figure 5

A

Fold Change from pre Cx

PSA
IR-A
IRS 2
RDH5

pre Cx
N
CR

B

Fold Change from pre Cx

PSA
IR-A
IRS 2
RDH5

pre Cx
N
CR
Insulin increases *de novo* steroidogenesis in prostate cancer cells

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