IGF2 increases *de novo* steroidogenesis in prostate cancer cells

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Abstract

IGF2 is a mitogenic foetal growth factor commonly over-expressed in cancers, including prostate cancer (PC). We recently demonstrated that insulin can activate *de novo* steroidogenesis in PC cells, a major pathway for reactivation of androgen pathways and PC progression. IGF2 can activate the IGF1 receptor (IGF1R) or insulin receptor (INSR) or hybrids of these two receptors. We therefore hypothesized that IGF2 may contribute to PC progression via *de novo* steroidogenesis. *IGF2* mRNA but not IGF2 receptor mRNA expression was increased in patient samples during progression to castrate-resistant PC as was immunoreactivity to INSR and IGF1R antibodies. Treatment of androgen receptor (AR)-positive PC cell lines LNCaP and 22RV1 with IGF2 for 48 h resulted in increased expression of steroidogenic enzyme mRNA and protein, including steroid acute regulatory protein (StAR), cytochrome p450 family member (CYP)17A1, aldo-keto reductase family member (AKR)1C3 and hydroxysteroid dehydrogenase (HSD)17B3. IGF2 treatment resulted in increased steady state steroid levels and increased *de novo* steroidogenesis resulting in AR activation as demonstrated by *PSA* mRNA induction. Inhibition of the IGF1R/INSR signalling axis attenuated the effects of IGF2 on steroid hormone synthesis. We present a potential mechanism for prostatic IGF2 contributing to PC progression by inducing steroidogenesis and that IGF2 signalling and related pathways present attractive targets for PC therapy.

Key Words

- Castrate-resistant prostate cancer
- IGF2
- steroidogenesis
- androgen receptor

Endocrine-Related Cancer
(2013) 20, 173–186

Introduction

Insulin-like growth factors (IGFs) play an integral role in the progression of many cancers (Pollak 2008a,b), regulating proliferation and transformation, as well as inhibiting apoptosis (Pollak 2008a). Both the related growth factors insulin and IGF1 have been implicated in prostate cancer (PC) progression (Pollak 2008a, Lubik et al. 2011); however, the role of IGF2 is less well explored. *IGF2*

expression is elevated in ovarian, colorectal and breast cancer associated with poor prognosis (Cardillo et al. 2003, Sayer et al. 2005, Kalla Singh et al. 2007, Pollak 2008a, Huang et al. 2010) and increased cell motility (Diaz et al. 2007). Dysregulated expression of *IGF2* in PC tumours and in surrounding prostate and stromal tissue occurs partially through loss of imprinting (Van Roozendaal et al. 1998,

Cui *et al.* 2003, Poirier *et al.* 2003, Bhusari *et al.* 2011, Wang *et al.* 2011) and correlates with tumour vs benign hyperplasia (Paradowska *et al.* 2009); however, tumour IGF2 expression levels are not reflected in serum (Rowlands *et al.* 2009, 2012). IGF2 can signal through the IGF1 receptor (IGF1R) or via the insulin receptor (INSR) to elicit insulin-like signalling (Cardillo *et al.* 2003, Pandini *et al.* 2004). In parallel to increased IGF2, increasing expression of these receptors has been demonstrated in PC progression (Cox *et al.* 2009).

Androgen deprivation therapy (ADT), which removes testicular androgens necessary for survival of prostate cells, is the standard treatment for advanced PC (So *et al.* 2005). Local tumour production and/or retention of steroids has been shown to differ significantly to levels in the circulation and contribute to PC progression (So *et al.* 2005, Stanbrough *et al.* 2006), so despite castrate levels of androgens in men undergoing ADT, it has been shown by our group and others that the local prostatic production of steroids (and conversion of adrenal precursors) may be instrumental in driving PC progression towards the terminal castrate-resistant PC (CRPC) (Locke *et al.* 2008). The clinical success of the CYP17A1 inhibitor, abiraterone (Attard *et al.* 2008, 2011), highlights the importance of this pathway in PC progression. At this stage of disease, circulating androgen levels are low, while the prostate tissue levels are high enough to reactivate the androgen receptor (AR), which may itself harbour mutations that give rise to constitutively active truncated receptors or increase AR sensitivity to low androgen and drive progression (Stanbrough *et al.* 2006, Locke *et al.* 2008). We recently reported that insulin, which rises in response to ADT, can drive expression of steroidogenesis enzymes in PC cells, increase intracellular androgens and increase expression of PSA (Lubik *et al.* 2011). Given the similarities in signalling between insulin and IGF2 (Pandini *et al.* 2004), we hypothesized that the increased IGF2 in the tumour microenvironment may accelerate prostatic *de novo* steroidogenesis. IGF2 has been shown to initiate steroidogenesis in thecal (Spicer & Aad 2007) and adrenocortical cells (Fottner *et al.* 1998). In this study, we observed increased IGF2 transcript in clinical tumour samples, which correlated with progression to castrate resistance in addition to increased expression of the receptors that facilitate IGF2 signalling: IGF1R and INSR. Our results demonstrate that IGF2 may accelerate PC progression through up-regulation of steroidogenesis enzymes and enhanced steroid production in PC cell lines. Levels of steroidogenic enzyme mRNA and protein in LNCaP and 22RV1 cells are increased after IGF2

treatment and result in increased DHT secretion. Increased *de novo* steroidogenesis in LNCaP and VCaP cells was measured using radiolabelled substrate, which resulted in androgen-mediated reactivation of AR and up-regulated expression of PSA.

Materials and methods

Laser capture microdissection and microarray analysis

Microarray analysis of mRNA was performed using samples of patient tissue after radical prostatectomy grouped into the following categories: 14 primary PCs from patients undergoing surgery with no therapy before surgery, 12 primary prostatectomy samples from patients receiving 1–3 months of neoadjuvant hormone therapy (NHT) before surgery, five primary PCs after 5–6 months of NHT before surgery, four primary PCs after 8–9 months of NHT before surgery and three hormone refractory PCs before surgery. Patients were further grouped according to the following risk factors: high (PSA >20, Gleason >7, clinical stage T3–T4), intermediate (PSA 10–20, Gleason 7, clinical stage T2) and low (PSA <10, Gleason <7, clinical stage 1).

The array preparation was performed as described previously (Chi *et al.* 2005). Tissues were flash frozen in OCT Compound (Tissue-Tek, VWR, Batavia, IL, USA) and frozen sections (8 µm) were cut and mounted on laser capture microdissection (LCM) slides (P.A.L.M. Microlaser Technologies, Bernried, Germany), sections were briefly thawed and fixed with 95% ethanol at –25 °C. Haematoxylin staining was followed by washes in diethyl pyrocarbonate (DEPC) water and then dehydration in 100% ethanol and LCM was performed on cancer cells using the photoactivation localisation microscopy (PALM). Microlaser system. Total RNA was isolated (PicoPure RNA Isolation Kit, ARCTURUS, Carlsbad, CA, USA) and amplified using the RiboAmp HS RNA Amplification kit (ARCTURUS), labelled with Cy5 using the AminoAllyl Message Amp IIa RNA Amplification Kit (Ambion, Streetsville, ON, Canada) and fragmented with RNA Fragmentation Reagents (Ambion) before hybridization. Custom-designed microarrays of 34 580 (70-mer) human oligos representing 24 650 genes and 37 123 gene transcripts (Human Operon V3.0, Operon Technologies, Huntsville, AL, USA) were supplied by the Microarray Facility of the Vancouver Prostate Centre. Scanned arrays were visualized using ImaGene 8.0 Software (BioDiscovery, San Diego, CA, USA). Feature data were subjected to background correction, print-tip-lowess within-array normalization and G quantile between-array normalization

(Limma, R/Bioconductor Software, Seattle, WA, USA). Significant differences between treatment groups were assessed using linear regression and Benjamini–Hochberg multiple test correction to estimate the false discovery rate.

Immunohistochemistry

Immunohistochemical (IHC) staining was conducted on sequential sections of the Gleason-graded tissue microarray (TMA) on patients from a similar cohort, also receiving NHT therapy before radical prostatectomy. Samples were probed using INSR receptor, β -subunit, rabbit immunoaffinity-purified IgG (Upstate Cell Signaling Solutions, Lake Placid, NY, USA) and IGF1R β rabbit polyclonal antibody (Santa Cruz Biotechnology, Inc.) with enzyme-labelled biotin streptavidin system and solvent-resistant DAB Map kit. Nonspecific reactivity was assessed by omission of the primary antibody. The specificity of staining for INSR was confirmed using placenta as a positive control. The slide was scanned with BLISS system (Bacus Lab, North Lombard, IL, USA) and IHC score staining intensity was evaluated by an independent pathologist. The biomarkers were scored using a four-point scale scoring system by a pathologist. Descriptively, 0 represents no staining by any tumour cells, 1 represents a faint or focal, 2 represents a stain of moderate intensity in a convincing number of cells and 3 represents intense staining by a sufficient number of cells expressing this antigen.

Cells

LNCaP and 22RV1 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and routine array analyses performed in our laboratory confirm expected gene profiles. Cells were maintained in phenol red-free RPMI 1640 (Invitrogen), 5% (v/v) foetal bovine serum (FBS; Invitrogen). VCaP cells were maintained in DMEM media (Hyclone, Hudson, NH, USA), 5% FBS. Cells were plated overnight in respective media containing 5% FBS before media were changed to media with 5% charcoal-stripped serum (CSS; Hyclone) for 24 h before 24-h starvation in serum-free media. Following optimization of concentration and duration, cells were treated with 85 ng/ml IGF2 (Novozymes, Thebarton, SA, Australia) in 0.2% BSA in SFM or 0.2% BSA (w/v) alone for 48–72 h. IGF2 was refreshed at 24-h intervals as necessary. Serum levels of IGF2 range from 200 to 600 ng/ml in men ~65 years of age (with and without PC; Harman et al. 2000).

Receptor inhibitor treatment

LNCaP and 22RV1 cells were grown in six-well plates for 24 h in FBS-supplemented media before incubation in 5% CSS media for 24 h and further 24-h incubation in serum-free media. For AR inhibition, cells were incubated for 2 h with 25 μ M bicalutamide and then for 24 h with 85 ng/ml IGF2 or 10 nM dihydrotestosterone (DHT). For inhibition of receptor activation by IGF2, cells were incubated with either 5 μ M BMS-754807 (Bristol-Myers Squibb, Montreal, Quebec, Canada) pan-INSR/IGF1R inhibitor or 12.5 μ g/ml of specific IGF1R inhibitor, CP-751,871 (Pfizer) for 2 h before addition of IGF2. To determine the difference between IGF2 and 10 nM insulin-induced steroid synthesis, 100 nM INSR-specific inhibitor, S661 (Novo Nordisk), was employed. Ketoconazole, 10 μ M (Sigma), demonstrated IGF2, or insulin-induced steroidogenesis could be suppressed with this steroidogenic inhibitor. All treatments were normalized to vehicle control. LNCaP cells were then treated as earlier with 14 C-acetate and IGF2 before *de novo* steroidogenesis analysis. 22RV1 cells, not treated with 14 C-acetate, were analysed using DHT ELISA. All inhibitors were used with kind permission.

Quantitative real-time PCR

Quantitative real-time PCR (QRT-PCR) was carried out using standard methods. Briefly, RNA was extracted from cell lines using Tri-Reagent (Applied Biosystems), before RT with Superscript III RT (Invitrogen). QRT-PCR was performed using SYBR Green detection on 7900HT Fast Real Time PCR System (Applied Biosystems). Primers used are listed in Table 1. Gene expression was normalized to the housekeeping gene and then expressed relative to vehicle control at the same time point. Data were analysed using SDS 2.3 Software (Life Technologies Australia Pty Ltd, Mulgrave, Victoria 3170, Australia) by means of the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen 2001). Experiments were repeated a minimum of five times.

Western blotting

Protein extraction and western blotting were carried out as described previously (Lubik et al. 2011). Briefly, cells were lysed in radioimmunoprecipitation assay buffer, proteins separated by SDS–PAGE and transferred to PVDF-FL membrane (Millipore, North Ryde, NSW, Australia). Antibodies were added in a 1:1 solution of Li-Cor blocking buffer (Li-Cor Biosciences, Lincoln, NE, USA) and 0.1% Tween 20–PBS and incubated overnight at 4 °C, before

Table 1 Primers used for QRT-PCR.

Gene	Forward primer (5'–3')	Reverse primer (5'–3')
StAR	gcccatggagaggctctatg	ttccactccccattgctt
CYP11A1	agttctcgggacttcgtcagt	ggagcccgccttctga
CYP17A1	ggcggcctcaaattgg	cagcgaaggcgaaggcga-taccctta
HSD3B2	cgggccaaactcctacaag	ttttcagaggctcttctcgt
AKR1C3	tgggaggccatggagaag	tttgacacccaatggacttg
HSD17B3	tgggacaftgggcagtga	cgagtacgctttccaattcc
SRD5A1	acgggcatcggtgcttaat	ccaacagtggcataggcttcc
RDH5	gcccgcagcaatgc	cgcccaaagcctgagtca
PSA	agtgcgagaagcattccaac	ccagcaagatcacgctttgtt
RPL32 ^a	ccccttgtaagccaaga	gactggtgccggaatgaact

^aHouse-keeping gene.

application of secondary antibody. Blots were visualized using the Li-Cor Odyssey Imager. Antibodies used were as described previously (Lubik et al. 2011). Experiments were repeated a minimum of three times.

Steroid analysis

LNCaP cells were grown in 15 cm plates and treated with either 85 ng/ml IGF2 in 0.5% BSA or 0.5% BSA in SFM for 48 h. Two plates of treated cells were washed with PBS and combined to give a single sample. Steroids were extracted from the pellet with methyl-tert-butyl ether (MTBE)/methanol/water extraction, which was dried down and resuspended in acetonitrile, sonicated, dried down and resuspended in 50% methanol and then sonicated and spun to remove any particulates. Samples were derivatized in 0.2 M hydroxylamine HCl. Water equilibrated ethylacetate was used instead of MTBE/methanol/water for extraction of secreted steroids from media samples. All samples were run on the Waters Acquity Liquid Chromatography system and the Waters Quattro Premier LC/MS/MS and analysed using BioLynx Software. Readings were normalized to cell pellet weight. Before extraction, d3T-deuterated testosterone standard (0.015 ng/ml final concentration) was added.

Radiolabelled analysis of *de novo* steroidogenesis in LNCaP and VCaP cells

Cells were grown in six-well plates and treated as earlier. At the time of IGF2 treatment, 6 µCi/ml ¹⁴C-acetate (PerkinElmer, Woodbridge, ON, Canada) was added to each plate for 72 h after which time medium was analysed for steroid content. Equal volumes of medium and hexane:ethylacetate (75:25) were incubated at room temperature for 1 h and the organic phase was extracted

twice. Samples were then dried and resuspended in 75 µl 50% methanol. These samples were analysed on the Waters Alliance 2695 HPLC. Before extraction, d3T-deuterated testosterone standard (0.015 ng/ml final concentration) was added.

Steroid analysis using DHT ELISA

DHT secreted into the media by 22RV1 cells was evaluated using a DHT ELISA (BioCore Pty Ltd.) kit according to the manufacturer's instructions. Limits of detection of this kit were 6 pg/ml, which is similar to that of our LC/MS/MS procedure.

Statistical analysis

Statistical analyses were performed using ANOVA on GraphPad Prism Software.

Results

IGF2 mRNA expression in prostate tissue from men undergoing NHT

We examined the levels of IGF2 mRNA expression in clinical tumour samples over the time of progression to castrate resistance (CRPC) in men undergoing NHT before radical prostatectomy. Prostatic tissue from patients undergoing radical prostatectomy after no NHT, 1- to 3-month NHT, 5- to 6-month NHT, 8- to 9-month NHT or having hormone refractory PCs, was examined by microarray analysis. Increased expression of IGF2 first occurred by 5–6 months. This reached statistical significance after 8–9 months and was maintained at CRPC (Fig. 1a). IGF2 receptor (IGF2R) mRNA levels remained constant (Fig. 1a), which suggests an altered ratio of free and bound IGF2, which may result in increased bioavailability of IGF2. IHC staining of NHT samples showed an increase in IGF1R and INSR protein immunoreactivity, both of which may be subject to activation by IGF2 (Fig. 1b and c). INSR demonstrated homogenous cytoplasmic staining within tumour cells and discontinuous staining in the basal cell layer of benign glands. IGF1R protein expression was apparent in basal and luminal cells of the benign glands as well as cancer cells. Immunoreactivity of IGF1R was localized predominantly to the cell membrane, with approximately double the staining in CRPC samples; no cytoplasmic staining was observed in early series samples (0–5 months) compared with occasional cytoplasmic staining in CRPC samples.

Figure 1

IGF2 and receptor expression in prostate cancer. Prostatic tissues included primary tumours from patients undergoing radical prostatectomy with no therapy before surgery (NHT 0), following 1–3 months of neoadjuvant hormone therapy (NHT), 5–6 months of NHT, 8–9 months of NHT and three castrate-resistant prostate cancers (CRPC). (a) *IGF2* (red) and *IGF2R* (black)

mRNA levels from patient samples were compared between groups (mean ± s.e.m.; * $P < 0.05$). IHC staining of (b) *IGF1R* and (c) *INSRb* from NHT-naïve tumour (left) and CRPC tumour (right) show increased staining at CRPC. Staining intensity was scored from 0 to 3 and graphed (mean ± s.e.m.; * $P < 0.05$).

IGF2 increases expression of steroidogenesis enzymes

Because IGF2 has been shown, in other tissues, to induce expression of enzymes in the steroidogenesis pathways, we investigated its potential to induce steroidogenesis in AR-positive prostate tumour cell lines, LNCaP and 22RV1. Working systematically through the steroidogenesis pathway (Fig. 2), we observed a statistically significant approximately twofold increase in mRNA levels of steroid acute regulatory protein (*StAR*), *CYP17A1*, *SRD5A1* and retinol 11-*cis* dehydrogenase *RDH5* ($P < 0.05$) in LNCaP

cells after 48 h, as well as a 2- and 1.5-fold increase in aldo-keto reductase family member (*AKR1C3*) ($P = 0.064$) and *HSD17B3* respectively (Fig. 3a).

Parallel increases in protein expression of steroidogenesis enzymes were observed in LNCaP cells following IGF2 treatment (Fig. 3b). Expression of the cholesterol chaperone protein *StAR* was significantly increased approximately threefold, along with a twofold increase in levels of the rate-limiting enzyme, *CYP17A1* as well as *HSD17B3* (Fig. 3b). Levels of *AKR1C3* and *SRD5A* proteins

Figure 2

Steroidogenesis pathways indicating enzymes involved in both classical (left) and backdoor (right) pathways of DHT production.

increased, although did not reach statistical significance. In contrast to increased mRNA levels, protein levels of RDH5 were unchanged at 48 h.

In comparison to LNCaPs, 22RV1 cells showed a similar pattern of mRNA induction for StAR (1.8-fold, $P > 0.05$), CYP17A1 (2.9-fold), AKR1C3 (2.6-fold, $P > 0.05$) and RDH5 (Fig. 3c). In contrast to LNCaPs, IGF2 induced an increase in *CYP11A1* mRNA (1.5-fold), and *HSD3B2* (twofold, $P < 0.05$), but not in *HSD17B3* mRNA. Despite having similar magnitude changes in gene expression, much greater absolute levels of most steroidogenic enzymes were detected in 22RV1 cells.

Parallel changes in protein levels were observed in 22RV1 cells, as StAR increased fivefold, CYP11A1 and CYP17A1 increased approximately twofold each and HSD3B2 and AKR1C3 increased approximately twofold each ($P < 0.05$; Fig. 3d). A small increase in HSD17B3 was also observed.

IGF2 increases intracellular and secreted steroids

HPLC on LNCaP cell pellets extracted with MTBE was used to investigate the differences in steroid content between

IGF2 and vehicle-treated cells. IGF2 treatment clearly increased intracellular steroid levels in LNCaP cells (Fig. 4a); a twofold increase in intracellular DHEA and 17OH-progesterone ($P < 0.05$) and a tenfold increase in androsterone ($P < 0.05$), an intermediate in the backdoor steroid biogenesis pathway, were also observed. Pregnenolone and progesterone increased five- and threefold respectively ($P < 0.05$) and intracellular testosterone increased fourfold ($P < 0.05$) from ~ 0.0131 – 0.053 ng/g cells with IGF2 treatment, which is consistent with our previous findings (Locke *et al.* 2008, Lubik *et al.* 2011). These concentrations would be sufficient to activate the AR, as it has been shown that androgen concentrations of $\sim 2.92 \times 10^{-6}$ ng/g may activate AR in PC cell lines (Gregory *et al.* 2001). Interestingly, no change was observed in intracellular DHT.

By contrast, dramatic increases (approximately tenfold, $P < 0.05$) in secreted steroids were observed for testosterone, DHT and androsterone (Fig. 4b). Small increases in DHEA and 17OH-progesterone were detected, as well as five- and nine-fold increases in pregnenolone and progesterone respectively. Notably, higher levels of steroids at the beginning of the steroidogenesis pathway

Figure 3

IGF2 up-regulates expression of steroidogenic enzyme mRNA and protein. (a) RNA from LNCaP cells treated with 85 ng/ml IGF2 for 48 h increased *StAR*, *CYP17A1*, *SRD5A1* and *RDH5* expression. Results analysed by $\Delta\Delta CT$ method normalized to RPL32 and then to vehicle-treated controls for the equivalent time point ($n=5$). (b) Quantitated densitometry of western blot from LNCaP lysates treated for 48 h, 85 ng/ml IGF2 showed increased *StAR*, *CYP17A1*, *HSD17B3*, *AKR1C3* and *SRD5A1* ($n=3$). (c) Identical treatment in 22RV1 cells showed significant increase in *StAR*, *CYP17A1*, *HSD3B2* and *AKR1C3* mRNA ($n=6$) and (d) increased 22RV1 proteins *CYP11A1*, *CYP17A1*, *HSD3B2*, *AKR1C3* ($n=3$). Western blots were quantitated using GAPDH loading control (mean $\pm$ S.E.M.; * $P < 0.05$).

are increased intracellularly, while the levels of more potent steroids and androgens increased further down in the pathway were measured in the media. Testosterone and DHT concentrations increased to 44.9 pg/ml (1.56×10^{-10} mol/l) and 19.5 pg/ml (6.74×10^{-11} mol/l) following IGF2 treatment, once again levels sufficient to activate the AR (Gregory *et al.* 2001, Titus *et al.* 2005, Locke *et al.* 2008). Furthermore, IGF2 treatment was demonstrated to increase DHT secretion in 22RV1 cell medium (Fig. 4c) from 100 to 135 pg/ml ($P > 0.05$), well within the range necessary for AR activation. It is possible that IGF2 may activate the AR through mechanisms other than direct activation via steroidogenesis. To address this, our experiments were performed in serum-free medium, assuming that all androgens in the medium capable of inducing AR activation are newly formed. Therefore, it is unlikely that IGF2 activates the receptor in conjunction with the steroids present; our observations are that IGF2 does not enhance steroid activation of PSA.

IGF2 increases induction of AR-regulated genes

We demonstrated that IGF2-mediated androgen biosynthesis could potentiate AR-mediated gene transcription, using PSA mRNA expression as a surrogate for AR activation in the presence and absence of the AR antagonist bicalutamide. We compared the potency of IGF2 to 10 nM DHT (Fig. 5a). PSA mRNA expression was doubled in LNCaP cells after IGF2 treatment ($P < 0.05$) and we demonstrated that this PSA response was mediated through AR; the addition of bicalutamide abrogated the IGF2-induced increase in PSA expression. Following this, we titrated PSA mRNA expression following IGF2 treatment with increasing concentrations of DHT (Fig. 5b) and found that the concentration of IGF2 used in our studies is approximately as potent as 1.6×10^{-12} M DHT in PSA induction, which is consistent with our measurement of 6.74×10^{-11} M in media.

IGF2 increases *de novo* steroidogenesis in LNCaP and VCaP medium

We measured *de novo* steroidogenesis by treating LNCaP or VCaP cells for 72 h with 14 C-labelled acetate and subsequent HPLC and radiometric detection was used to measure *de novo* steroidogenesis. In VCaP cells, a fivefold increase in androstenedione and a threefold increase in androsterone levels were detected after IGF2 treatment (Fig. 6a). It is notable that a threefold increase in a steroid peak with a retention time close to that of progesterone

Figure 4

IGF2 treatment increased steroid production in LNCaP cells. LNCaP cells were treated with 85 ng/ml IGF2 for 48 h. (a) LC–MS used to identify and quantitate intracellular steroids showing a significant increase in 17-OH progesterone (17-OH Prog), testosterone (Test), androsterone (Andro), pregnenolone (Preg) and progesterone (Prog). (b) Extracellular steroids identified and quantitated by LC–MS in media detected significantly increased levels of testosterone (Test), androsterone (Andro) and pregnenolone (Preg) were detected. Steroid levels normalized to cell pellet weight and deuterated testosterone for extraction efficiency and compared to the vehicle time point control ($n=6$). (c) 22RV1 cells were treated with 85 ng/ml IGF2 for 48 h, secreted significantly more DHT compared with vehicle treated ($n=6$; mean $\pm$ S.E.M.; $*P<0.05$).

was also detected. We have demonstrated that cold progesterone is present in LC/MS/MS of LNCaP cells in a similar concentration to that of testosterone and androsterone, and this peak proximal to progesterone is also similar in magnitude to those steroids. It is possible that progesterone was compromised in our derivatization procedure. Furthermore, IGF2 treatment in VCaP cells increased pregnan-3,20-dione, 2.6-fold ($P<0.05$; Fig. 6a), and *de novo* cholesterol production, the building block of steroid synthesis (Leon et al. 2010). As the method of extraction used in this experiment is more specific to steroids, the assessment of cholesterol levels should be considered more qualitative than quantitative.

IGF2-treated LNCaP cells significantly increased testosterone, 4.5-fold ($P<0.05$), as well as androstenedione, androsterone and pregnan-3,17-diol-20-one (approximately fourfold, $P<0.05$; Fig. 6b). In a similar manner to that observed in VCaP cells, there was an increase in the steroid peak resembling progesterone (approximately threefold, $P<0.05$). IGF2 also induced a 2.4-fold increase in pregnan-3,20-dione (Fig. 6b). *De novo* DHT synthesis was not detected in these experiments, as our current methods for steroid extraction of radiolabelled steroids are not effective for extracting DHT.

Receptor blockade of IGF2

Our IHC analysis of patient TMAs demonstrated increased expression of INSR and IGF1R, which correlated with PC progression; IGF2 can signal through both these receptors or through hybrid INSR:IGF1R. In order to address the relative contribution of each receptor to IGF2-induced steroidogenesis, inhibitors of the insulin signalling axis, including the BMS-754807 tyrosine kinase inhibitor (Carboni et al. 2009, Huang et al. 2010) and the highly specific IGF1R neutralizing antibody, CP-751,871 (Cohen et al. 2005), were used and their effects on IGF2-induced steroidogenesis were assessed. BMS-754807 inhibits IGF1R and INSR activity with equal affinity (Carboni et al. 2009), whereas CP-751,871 is highly specific at inhibiting IGF1R activation, including hybrid receptors, with no effect on the INSR (Cohen et al. 2005). Optimal concentrations of inhibitors were determined empirically to assess effectiveness in each cell line but were not optimized for comparison of efficacy.

At the concentrations used, IGF2-induced testosterone synthesis was completely abolished with CP-751,871 compared to 50% reduction with BMS-754807 (Fig. 7a). An ~50% decrease in androstenedione, androsterone and pregnan-3,17-diol-20-one was

Figure 5

IGF2 increases expression of androgen receptor-regulated genes (a) Increased PSA mRNA following IGF2 treatment was compared to PSA induction by 10 nM DHT. Both IGF2-induced and DHT-induced expression was blocked by AR antagonist bicalutamide. (b) PSA mRNA expression following IGF2 treatment was compared to with increasing concentrations of DHT (0.01 pM–1 nM). DHT induction of PSA mRNA was plotted as a function of concentration. The equation of the line was used to compare IGF2 induction. 85 ng/ml IGF2 is equipotent to 1.6×10^{-12} M DHT for PSA induction. ($n=3$; mean $\pm$ s.e.m.; * $P<0.05$).

demonstrated with CP-751,871 treatment, with minimal change by BMS-754807 (Fig. 7a). There was little impact by the inhibitors on pregnan-3,20-dione or the steroid peak close to progesterone. QRT-PCR analysis of steroidogenesis enzyme mRNA in LNCaP cells showed that CP-751,871 treatment blocked CYP17A1 expression, which is upstream of androstenedione and may account for the decrease in that key steroid with the IGF1R inhibitor, whereas BMS did not appear to decrease CYP17A1 in LNCaP cells. Both inhibitors decreased levels of StAR, AKR1C3, and HSD17B3 (Fig. 7b). These data suggest that inhibition of the IGF/insulin signalling axis, particularly IGF1R in LNCaPs, reduces IGF2-mediated steroidogenesis.

The same concentrations of inhibitor used in 22RV1 cells completely blocked IGF2-induced DHT secretion with both BMS-754807 and CP-751,871 (Fig. 7c). IGF2-induced increases in StAR, CYP17A1 and AKR1C3 protein were equally blocked by both inhibitors (Fig. 7d). Differences in endogenous, basal levels of enzymes, cholesterol and steroid metabolism between the cell lines may explain some of the differences in the relative sensitivity of LNCaPs, VCaPs and 22RV1s to inhibitor treatment (Locke et al. 2009).

We have recently demonstrated that insulin induces steroidogenesis in PC cells (Lubik et al. 2011). We compared the relative potency of 10 nM insulin and 85 ng/ml IGF2 on PSA mRNA induction in the presence and absence of multiple receptor inhibitors (Fig. 8). Co-treatment with CP-751,871 reduced IGF2-induced PSA expression, as did BMS-754807 (Fig. 8a). Specific inhibition of INSR (and hybrid receptors) with S-661 (Schäffer et al. 2008) had very little effect of induction of PSA by IGF2, suggesting that it signals predominantly through IGF1R over hybrid receptors in this cell line. Ketoconazole, a pan-CYP enzyme inhibitor (Locke et al. 2009), completely abolished PSA induction. By contrast, induction of PSA by insulin was not inhibited by CP-751,871 (Fig. 8b). It was, however, blocked by BMS-754804 treatment, as well as S-661, and ketoconazole. In summary, both IGF2 and insulin, albeit with different preferential use of different receptors, induced PSA expression, which was inhibited with the steroidogenesis inhibitor, ketoconazole.

Discussion

ADT effectively reduces systemic androgens; however, elevated androgen levels persist within PC tumours (So et al. 2005). Previous studies from our group and others indicate that one mechanism driving progression to castrate resistance is the ability of PC cells to initiate a program of *de novo* steroidogenesis from cholesterol or other precursors, providing androgens to the tumour microenvironment at concentrations sufficient to activate the AR and promote PC growth (Gregory et al. 2001, Locke et al. 2008, 2009, Leon et al. 2010), a process that has rationalized the recent addition of the CYP17A1 inhibitor, abiraterone, to advanced PC therapies. Understanding the mechanisms that regulate intratumoural steroidogenesis in PC is part of ongoing studies in our laboratory aimed at providing new therapies for this stage of disease.

Increased prostatic IGF2 mRNA and protein concentrations have previously been shown to increase during

Figure 6

IGF2 increases *de novo* steroidogenesis in prostate cancer cells. Steroids were extracted from media from VCaP and LNCaP cells after 72-h incubation with 85 ng/ml IGF2 and 6 μ Ci/ml 14 C-acetate, quantitated by HPLC and radiometric detection. (a) VCaPs responded to IGF2 with increased steroid levels for pregnane3,20dione and a peak close to progesterone in retention time, though trend of increase is evident for androstenedione, androsterone and cholesterol. (b) LNCaPs increased levels of every detected steroid in response to IGF2 treatment (mean $\pm$ S.E.M. of $n=3$; * $P<0.05$).

progression from normal to PIN to PC (Cardillo *et al.* 2003, Trojan *et al.* 2006) and correlated with high Gleason scores (Cardillo *et al.* 2003, Pollak 2008a). In models of androgen deprivation, e.g. LNCaP xenografts in castrated mice, IGF2, IGF1R and INSR mRNA increases significantly from pre-castrate to PSA recurrence (Nickerson *et al.* 2001, Lubik *et al.* 2011). Our microarray analysis of clinical samples revealed a steady rise in IGF2 with duration of

NHT, which persisted in CRPC and contrasted with the static expression of IGF2R. Furthermore, studies have shown a correlation between elevated levels of prostatic IGF2 and DHT (Monti *et al.* 1998). Given the similar signalling pathways between insulin and IGF2, we hypothesized that increased local IGF2 coupled with increased receptors may accelerate local steroid synthesis.

Here, we demonstrate for the first time that IGF2 can promote steroidogenesis in PC cells, LNCaP and 22RV1, via up-regulation of obligatory enzymes (mRNA and protein), coupled with increased total content and *de novo* synthesis of steroids to concentrations sufficient to activate the AR (Gregory *et al.* 2001, Locke *et al.* 2008). IGF2 increased mRNA expression of StAR, CYP17A1, AKR1C3, SRD5A1 and RDH5. The importance of increased StAR is a reflection of its key role ferrying cholesterol into the mitochondria for initiation of steroidogenesis. CYP17A1 catalyses several reactions in the pathway including conversion of pregnenolone; both AKR1C3 and HSD17B3 can convert androstenedione to testosterone; and HSD17B3 also contributes to 'backdoor' steroidogenesis through conversion of androsterone to androstanediol (Locke *et al.* 2008). The 'backdoor' pathway differs from the classical pathway in that it bypasses testosterone in the formation of DHT. SRD5A1 and RDH5 catalyse the final reactions in the synthesis of DHT in the classical and 'backdoor' pathways respectively (Auchus 2004). Once made, DHT is the most potent activator of AR and its pathways and vital to PC survival (So *et al.* 2005).

The observed differences between LNCaP and 22RV1 cells in IGF2-induced enzyme expression may be explained by their differing origins, LNCaP from lymph node metastasis and 22RV1s from a subclavicular metastasis (Horoszewicz *et al.* 1980, Sramkoski *et al.* 1999). The main difference between the two cell lines at 48 h was the absence of CYP11A1 and HSD3B2 induction in LNCaPs (data not shown) in contrast to significant induction in 22RV1 cells. It is possible that these early enzymes in the steroidogenesis pathway are up-regulated rapidly in LNCaPs in response to IGF2, with levels restored by 48 h. Alternatively, these enzymes may be more active/abundant in LNCaP cells. Studies have demonstrated that steroidogenic PC cells will bypass inhibition to achieve steroidogenesis depending on cell needs and enzyme availability (Locke *et al.* 2009).

IGF1 can activate many pathways in PC cells, aside from steroidogenesis, which would contribute to AR-mediated progression. However, in contrast to IGF1, we have observed that IGF2 does not enhance steroid

Figure 7

IGF2 increased *de novo* steroidogenesis in prostate cancer cells, and is blocked by inhibition of IGF1R and hybrid receptors. (a) Medium was collected from LNCaP cells after 72-h incubation with 85 ng/ml IGF2 and 6 μ Ci/ml 14 C acetate and in the presence or absence of BMS-754807 pan-INSR and IGF1R inhibitor (5 μ M) or anti-IGF1R antibody (12.5 μ g/ml) CP-751,871. Steroids were quantitated via HPLC/radiometric detection. Steroidogenesis was blocked by CP-751,871 and partially blocked by BMS-754807. (b) Both inhibitors potently inhibited mRNA expression of

StAR, CYP17A1, AKR1C3 and HSD17B3 in LNCaP cells. (c) Medium from 22RV1 cells following 48-h incubation with IGF2 in the presence or absence of CP-751,871 or BMS-754807 showed inhibition of IGF2-induced steroidogenesis with both inhibitors. (d) Western blot showed that IGF2 increased steroidogenesis enzymes in 22RV1s following IGF2 treatment was blocked by CP-751,8871 or BMS-754807 (mean $\pm$ s.e.m. of $n=3$; * $P<0.05$). * $P<0.05$ compared to control. # $P<0.05$ compared to IGF2 treatment.

activation of PSA, as IGF1 has been reported to do (Kim & Coetzee 2004). Furthermore, we performed our experiments in serum-free medium, assuming that all androgens in the medium capable of inducing AR activation, as measured by PSA expression, were newly formed by IGF2 stimulation and PSA induction was amplified by IGF2 in concert with pre-existing steroids. Furthermore, our preliminary studies with IGF1 suggested that IGF2 was a more potent enhancer of steroidogenesis than IGF1 (data not shown). This is similar to findings reported in human

adrenocortical cells (l'Allemand *et al.* 1996); however, because IGF2 clearly functions at least in part through IGF1R, it is plausible that IGF1 may also enhance steroidogenesis in PCa cells.

The modulation of both intracellular and extracellular steroid profiles in LNCaP cells by IGF2 compared to our earlier studies with insulin showed considerable differences between the growth factors. In our previous study, 10 nM insulin increased intracellular testosterone levels 60-fold in LNCaPs; here, IGF2 increased testosterone

Figure 8

IGF2- and insulin-induced AR activation was blocked by ketoconazole. PSA mRNA expression in LNCaP cells was measured in response to (a) 85 ng/ml IGF2 treatment and (b) 10 nM insulin in the presence or absence of or 12.5 µg/ml CP-751,871, 5 µM BMS-754807 or 10 µM ketoconazole. (a) IGF2 induction of PSA mRNA was blocked in the presence of inhibitors to IGF1R and hybrid receptors, whereas (b) insulin-induced PSA was reduced in the presence of INSR inhibitors (100 nM S661 and BMS-754807. Mean ± s.e.m. of $n=3$; * $P<0.05$.

levels fourfold but IGF2 increased secreted DHT and testosterone approximately tenfold, where insulin saw a smaller, twofold increase. In 22RV1 cells, DHT production induced by the two growth factors was similar; insulin increased DHT by 1.7-fold where IGF2 increased DHT 1.4-fold. BMS-754807, an equipotent inhibitor of INSR and IGF1R, prevented IGF2-induced steroidogenesis in 22RV1s but was less effective in LNCaP cells. One reason for this may be the relative abundance of IGF1R, INSR and hybrid receptors.

The varying amounts of steroidogenesis occurring in our cell lines, in absolute levels, as well as induction, may partially reflect their relative AR mutation status. LNCaP, VCaP and 22RV1 are androgen responsive; however, each cell line expresses different forms of AR; LNCaP AR harbours the T877A mutation, which renders it

promiscuous (Veldscholte *et al.* 1990), and 22RV1 cells express the less promiscuous AR mutation, H874Y, along with a truncated, constitutively active AR (Tepper *et al.* 2002), whereas VCaPs express wild-type AR (Wu *et al.* 2011). Interestingly, 22RV1s, which have the most cumulative AR mutations, appear to be most steroidogenic in regard to absolute levels of DHT and testosterone synthesis; furthermore, LNCaP cells produce more *de novo* testosterone and DHT than VCaP cells, which are documented to have the least AR mutations. This supports the concept that steroidogenesis and AR mutations work synergistically to promote PCa (Knudsen & Penning 2010).

IGF2 signalling can be targeted by pharmacological agents currently in clinical trials (Huang *et al.* 2010). Inhibition of IGF1R leads to decreased concentrations of steroids downstream of CYP17A1 activity in LNCaP cells, which may indicate that CYP17A1 up-regulation by IGF2 is crucial to steroidogenesis. Further, a decrease in IGF2-induced SRD5A1 would account for the decrease in backdoor pathway steroids, as SRD5A1 may facilitate synthesis through the backdoor pathway, and decreased HSD17B3 and AKR1C3 would result in reduced testosterone (LNCaP) or DHT (22RV1) synthesis in the presence of treatments that target the insulin/IGF receptor signalling axis. Given the temporal changes in IGF2 expression with NHT, IGF2-induced steroidogenesis could also be targeted using CYP17A1 inhibitors, such as abiraterone, which have shown tremendous promise in the clinical setting (Attard *et al.* 2008, 2011). This is supported by our finding that the pan-CYP inhibitor, ketoconazole, blocked IGF2 activation of AR-mediated PSA expression.

In breast cancer cell lines, it has been shown that blocking either the INSR or the IGF1R can result in increased expression of the other and therefore may not hinder IGF2-related tumour progression (Ulanet *et al.* 2010). Inhibition of both receptors, or of common downstream effectors, may have therapeutic potential for PC (Sayer *et al.* 2005). Both CP-751,871 and BMS-754807, which are in clinical trials (Gualberto & Pollak 2009), demonstrate efficacy in reducing IGF2-induced steroidogenesis and AR activation. IGF2 activates numerous intracellular pathways leading to cancer cell survival, thus combining receptor inhibitors and steroidogenesis inhibitors, such as abiraterone, may also help improve patient outcomes. In summary, IGF2 has been identified as a candidate PC gene target and we provide evidence that IGF2 activates *de novo* steroidogenesis in PC cell lines.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

M P consulted for Pfizer and for Bristol-Myers and received research support from Pfizer. This work was funded by grants from the Queensland Smart Futures Premier's Fellowship, Prostate Cancer Foundation of Australia (PCFA PG25), Terry Fox Program Project (National Cancer Institute of Canada grants 012003 and 017007).

Acknowledgements

The authors would like to thank Marie-Jose Blouin for excellent technical support.

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Received in final form 8 December 2012

Accepted 21 December 2012

Made available online as an Accepted Preprint

14 January 2013