Hyperglycaemia-induced chemoresistance of prostate cancer cells due to IGFBP2

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Abstract

Clinically relevant prostate cancer (PCa) is more frequent in Westernised societies and increasingly men have co-morbidities associated with a Western lifestyle, primarily diabetes, characterised by hyperinsulinaemia and hyperglycaemia. IGFs and their binding proteins (IGFBPs) are important mediators of the effects of nutrition on growth and play a key role in the development of PCa. We used DU145, PC3 and LNCaP PCa cell lines to examine how hyperglycaemia altered their response to docetaxel. Trypan Blue dye-exclusion assay was used to determine the percentage of cell death. Protein abundance was determined using western immunoblotting. Levels of IGFBP2 were measured using an ELISA. IGFBP2 gene silencing was achieved using siRNA technology. DNA methylation was assessed using combined bisulphide restriction analysis. Acetylation status of histones H3 and H4 associated with IGFBP2 gene was assessed using chromatin immunoprecipitation assay. Hyperglycaemia reduced docetaxel-induced apoptosis by 40% for DU145 cells and by 88% for LNCaP cells. This reduced cell death was mediated by a glucose-induced up-regulation of IGFBP2, as silencing IGFBP2 negated the survival effect of high glucose. Glucose increased IGFBP2 via increasing the acetylation of histones associated with the IGFBP2 gene promoter. This finding could have important implications in relation to therapeutic strategies as epigenetic modulation could be reversible.

Key Words

- prostate
- carcinoma
- chemotherapy
- IGF
- obesity

Introduction

Prostate cancer (PCa) is the most common non-cutaneous cancer diagnosed in men, with a one in six lifetime risk of developing the disease. Although mortality rates appear to be improving, this largely reflects the increased detection of mainly localised cancers due to PSA testing. Organ-confined disease, detected early is associated with good prognosis; the development of androgen independence followed by chemoresistance is however the critical markers of progression to an end-stage lethal disease. Although PCa is the most prevalent cancer in males in Westernised societies, clinical evidence of the disease is relatively rare in Eastern societies. Observations that PCa rates in migrants rapidly converge (within one generation) to that of their new locale excludes simple genetic explanations for these large geographical variations and implicates lifestyle/environmental exposures, particularly

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nutrition and metabolic status (Maskarinec & Noh 2004). In contrast, autopsy studies indicate that small, latent, occult prostate neoplasias are prevalent in the general population irrespective of geographic location (Zhou et al. 1991). If the effect of geographic location was due to environmental exposures initiating carcinogenesis, then the rates of latent cancers would increase in migrants who move to the regions of the world with higher incidence: this is not the case, despite the large increase in clinical cancers, indicating that the main effect of environmental exposures is on disease progression rather than on initiation. Increasingly, men who develop PCa also have one of the co-morbidities associated with a Western lifestyle (obesity, metabolic syndrome or diabetes), and these co-morbidities are associated with poor prognosis and more aggressive cancers (Hammarsten & Hogstedt 2004, 2005). Furthermore, aggressive tumours comprise more active cancer cells associated with increased metabolism and increased glucose uptake (Chen et al. 2007). The mainstay of treatment for advanced PCa is androgen deprivation therapy (ADT; Sharifi et al. 2005). Although ADT is a successful treatment in improving overall quality of life in patients with advanced PCa, there are side effects including a significant increase in the risk of metabolic disorders and diabetes (Saylor & Smith 2009, Taylor et al. 2009). Evidence therefore suggests that metabolic disturbances, either consequent to existing lifestyle or to ADT, have a negative impact on both outcomes of treatment and PCa mortality. The insulin-like growth factor (IGF) axis has been conserved throughout evolution as a means of mediating the effects of nutrition on cell growth and metabolism. Metabolic disturbances also affect this axis, which is itself strongly associated with PCa (Monti et al. 2007). Recent prospective epidemiology has consistently shown strong associations between circulating IGF1 levels and the subsequent risk of developing PCa (Roddam et al. 2008). The actions of the IGFs are modulated by a family of high-affinity IGF-binding proteins (IGFBPs 1–6) that can either inhibit or enhance the actions of the IGFs. Actions of IGFBPs that are independent of IGFs have also been recognised, indicating that IGFBPs can intrinsically modulate aspects of cell growth and survival (Bach et al. 2005). In particular IGFBP2 is considered a key player in PCa progression (Degraff et al. 2009), being an established mitogen for PCa cells (Chatterjee et al. 2004, Uzoh et al. 2011). IGFBP2 is also metabolically controlled (Martin et al. 2006) and is up-regulated in response to glucose in murine mesangial cells (Davis et al. 2008).

In this study, we assessed the effects of elevated glucose levels, as may result from long-term androgen deprivation or due to existing lifestyle, on the response of PCa cells to chemotherapy and determined any involvement of IGFBP2.

Materials and methods

Reagents

All chemicals, unless otherwise stated, were purchased from Sigma. Trichostatin A (TSA, 1406) was purchased from Tocris (Avonmouth, UK) and sirtinol (566320) from Merck. All siRNAs were purchased from Qiagen and the transfection reagent (SR-1003-04) from Synvolux Therapeutics (Groningen, The Netherlands). Foetal bovine serum (FBS) was purchased from Invitrogen, DMEM–25 mM glucose (cat no. BF-709) and DMEM–5 mM glucose (cat no. BF-708), RPMI 1640, penicillin–streptomycin solution and l-glutamine were bought from Lonza (Basel, Switzerland). ELISA Kit for IGFBP2 was purchased from Diagnostic System Laboratories (San Francisco, CA, USA). Human, recombinant non-glycosylated IGFBP2 was obtained from Sandoz (Camberley, Surrey, UK). Human, recombinant IGF1 peptide was from Gropep (Adelaide, SA, Australia).

Cell culture

Human PCa cell lines: androgen-independent DU145, PC3 and androgen-dependent LNCaP were purchased from ATCC (Teddington, Middlesex, UK). DU145 and PC3 cells were cultured in DMEM (cat no. BF-709) growth media (GM) supplemented with 10% FBS, penicillin–streptomycin (50 IU/ml) and 1% l-glutamine solution (2 mM) and LNCaP cells were cultured in RPMI 1640 GM supplemented with FBS and antibiotics. Cells were grown in a humidified 5% carbon dioxide atmosphere at 37 °C. For glucose experiments, all cell lines were seeded in 5 mM glucose GM for 24 h and transferred to serum-free media (SFM) with either 5 or 25 mM glucose, supplemented with sodium bicarbonate (1 mg/ml), BSA (0.2 mg/ml) and transferrin (0.01 mg/ml). After a further 24 h, the cells were dosed with docetaxel (0–60 nM), 5-aza-2'-deoxycytidine AZA (0–1 µM), TSA (0.75 µM), sirtinol (30 µM), AG1024 (1 µM) and RGD (10 µg/ml) according to the respective figure legends. Dead cells were counted using Trypan Blue staining as described previously (Thomas et al. 2010).

Silencing IGFBP2 using siRNA technology

Cells were seeded in 24-well plates (0.025 × 10^6 cells/well) in 5 mM glucose GM in the presence or absence of IGFBP2
siRNA (target sequence CCCGGAGCAGGTGTCGACAA) or with a random sequence negative control siRNA (NSsiRNA) (25 or 50 nM for DU145 and LNCaP cells respectively) before being switched to SFM for another 24 h before dosing with docetaxel (30 or 60 nM for DU145 and LNCaP cells respectively) for another 24 h. Cell death was assessed as described in Thomas et al.'s (2010) study and IGFBP2 abundance was monitored using western immunoblotting.

Western immunoblotting

Equal volumes of concentrated cell conditioned media were separated on a 12% SDS–PAGE gel and transferred to Hybond N+ nitrocellulose membranes (Amersham). Non-specific binding sites on the membranes were blocked for a minimum of 2 h with 5% milk in Tris-buffered saline/2% Tween (TBS/T) before overnight (4°C) probing with antiserum against IGFBP2 (1:1000, Santa Cruz, sc-6001) or tubulin (1: 5000, Thermo Fisher Scientific, Loughborough, UK; MZ05829). Secondary anti-goat antibody was used at 1:2000 for IGFBP2 or anti-mouse antibody (1:5000) for tubulin. Peroxidase binding was visualised by ECL and detected using the Chemi-Doc-It Imaging System (UVP; Watford, UK). Anti-RNA polymerase II and mouse IgG controls respectively. After release from the strip wells, the cross-linked DNA was reversed, purified and analysed with PCR. IGFBP2 primers for PCR were designed to target −413 to −350 in the promoter region with the following sequences: forward, 5′-GAC TGA AAT CTA CTT GAA GGT and reverse, 5′-GCT CTG GGG GTT CCC TGC TCA AA-3′ and reverse, 5′-GCT CGG GTT CCC TGC TCT TCC C-3′ (purchased from Thermo Fisher Scientific). PCR was carried out with HotStar Taq Plus PCR Kit (Qiagen). Primers for GAPDH, which were included in the kit, were used as a positive control. A total volume of 10 μl PCR mix contained 1 × PCR buffer, 0.1 mM dNTPs mix (Qiagen), 2.0 mM MgCl₂, 0.25 μM of each primer, 5% of DMSO and 0.25 U/10 μl Taq DNA polymerase. Annealing temperature for both primers was 60°C. PCR was performed with different numbers of cycles or DNA volumes to determine the linear range of the amplification. All results shown fall within this range. After 38 cycles, PCR products were run on 2% agarose gel stained with 0.5 μg/ml ethidium bromide and analysed by u.v. transilluminator (Bio-Rad, GEL DOC 1000).

Methylated PCR (combined bisulphide restriction analysis)

Genomic DNA was extracted from the cells, bisulphide converted and cleaned up with EZ DNA Methylation-Direct Kit (Zymo Research, Irvine, CA, USA) according to manufacturer’s instructions. Combined bisulphide restriction analysis (COBRA; modified from Xiong & Laird 1997) incubated, shaking at 500–700 r.p.m. on an orbital microplate shaker for 10 min at room temperature after which 100 μl of stop solution (0.2 M sulphuric acid) were added to each well. The absorbance of the solution in the wells was read within 30 min, using a microplate reader set to 450 nm wavelength, and the mean absorbance for each standard was plotted using the Genesis Software (Himayathnagar, Hyderabad, India), concentrations in samples were then calculated from these graphs.

ELISA for IGFBP2

Antibody–enzyme conjugate concentrate was diluted with assay buffer to prepare an antibody–enzyme conjugate solution: 50 μl of the standards, controls and samples of cell conditioned media were added into the appropriate wells following which 50 μl of the antibody–enzyme conjugate solution were added. Wells were then incubated, shaking at 500–700 per minute on an orbital microplate shaker for 3 h at room temperature, aspirated and washed five times with the wash solution using a multichannel pipette, blotted and dried by inverting the plate on absorbent material. Hundred microlitres of the TMB chromogen solution (tetramethylbenzidine in citrate buffer with hydrogen peroxide) was added to each well, avoiding exposure to direct sunlight and wells were
was used to quantitatively measure the percentage of methylated DNA. The primer pairs did not contain CpG dinucleotides so that the amplification did not discriminate methylation status in DNA templates. The primer pair regions in the promoter of IGFBP2 gene were designed using MethPrimer tool sequences of primers; IGFBP2 forward, 5′-GATTGAAAT-TTATTTGAAGGTTAAAA-3′ and reverse, 5′-ACTCTAAAAATT-CCCTACTCTTCC-3′ and purchased from Thermo Fisher Scientific. In the PCR products, restriction enzyme cutting sites were either retained (CpG methylated) or lost (CpG unmethylated) after bisulphide conversion. Approximately, 50 ng of bisulphide-modified DNA were denatured and hot-started at 95 °C for 10 min and then amplified for 38 cycles (94 °C 30 s, at annealing temperature of 57 °C 45 s, 72 °C 60 s) by PCR in a volume of 12 µl, using HotStarTaq Plus (Qiagen) DNA polymerase according to the manufacturer’s instructions. Ten microlitres PCR products were then digested with 20 units of restriction enzyme BstUI (10 000 U/ml, BioLabs, Hitchin, Hertfordshire, UK; R0518L) at 60 °C for 4 h. Ethidium bromide–stained agarose gels of digested COBRA PCR products were visualised by u.v. light (u.v. transilluminator, Bio-Rad, GEL DOC 1000). Band intensities were quantified using Image J for Windows Software. Linear response of the COBRA assays was validated by mixing known ratios of 0 and 100% methylated DNA and correlating the percentage of input fully methylated DNA vs the COBRA-measured percentage methylation.

**Statistical analysis**

Data were analysed with SPSS 12.0.1 for Windows using one-way ANOVA followed by least significant difference post-hoc test. A statistically significant difference was considered to be present at \( P<0.05 \).

**Results**

**Effects of hyperglycaemia on chemoresistance of PCa cells**

With DU145, LNCaP and PC3 PCa cells, we examined the effects of either euglycaemic (5 mM) or hyperglycaemic (25 mM) conditions on the efficacy of docetaxel (0–60 nM) in inducing cell death (Figs 1A, 2A and B respectively). With DU145 cells, we found that docetaxel caused a dose-dependent increase in cell death under euglycaemic conditions with a maximum increase at 45 nM. We have previously confirmed that cell death induced by docetaxel was due to apoptosis as indicated by an induction of PARP cleavage (Thomas et al. 2010) and in the current experiments the cell death was clearly regulated. Under hyperglycaemic conditions, we discovered that the cells were less sensitive to docetaxel with all doses. For example, with 45 nM docetaxel cell death was only increased by 17%, compared with a 28% increase under euglycaemic conditions (Fig. 1A). A similar protective effect of high glucose was observed in LNCaP cells with an 18% increase in cell death in 5 mM glucose vs 1.6% increase in 25 mM in cells treated with 50 nM docetaxel (Fig. 2A). In contrast to the increased survival observed in response to docetaxel under hyperglycaemic conditions with DU145 and LNCaP cells, we found that the response of PC3 cells to docetaxel under the same conditions was unaffected (Fig. 2B). Using DU145 cells we then examined a range of glucose concentrations from 5–25 mM (Fig. 1B), treated with one dose of docetaxel (30 nM), and showed that at glucose
concentrations of 5.625 and 6.25 mM, there was no significant changes in cell death compared with that at 5 mM glucose \( (P=0.4) \); however, at 7.5, 10, 15 and 25 mM; cell death was significantly reduced \( (P=0.04, 0.01, 0.01 \) and 0.001 respectively). We excluded that the differential effects of glucose concentrations were not simply due to changes in osmolality by using L-glucose (that cannot be metabolised by the cells). We seeded DU145 cells in DMEM with 4.5 g/l D-glucose (25 mM) and DMEM with 1 g/l \( \alpha \)-glucose made up to a concentration of 25 mM with \( \alpha \)-glucose as normal glucose and DMEM with 1 g/l \( \alpha \)-glucose made up to a concentration of 25 mM with \( \alpha \)-glucose as high glucose. For all figures dead cells were counted using Trypan Blue cell staining. Graph shows % cell death – mean of three experiments each repeated in triplicate.

**Figure 2**
Changes in % cell death in response to different concentrations of docetaxel in prostate cancer cell lines. (A) LNCaP cells were dosed with docetaxel (40–60 nM) for 24 h. (B) PC3 cells were dosed with docetaxel (20–30 nM) for 24 h. (C) DU145 cells seeded in DMEM with 4.5 g/l \( \alpha \)-glucose (25 mM) and DMEM with 1 g/l \( \alpha \)-glucose made up to a concentration of 25 mM with \( \alpha \)-glucose as normal glucose and DMEM with 1 g/l \( \alpha \)-glucose made up to a concentration of 25 mM with \( \alpha \)-glucose as high glucose. (D) DU145 cells seeded in DMEM with 1 g/l (5 mM) \( \alpha \)-glucose as normal glucose and DMEM with 1 g/l \( \alpha \)-glucose made up to a concentration of 25 mM with \( \alpha \)-glucose as high glucose. For all figures dead cells were counted using Trypan Blue cell staining. Graph shows % cell death – mean of three experiments each repeated in triplicate.

**Effects of hyperglycaemia on abundance of IGFBP2**

Using both ELISA \( (P<0.001) \) and western immunoblotting (Fig. 3A) techniques to measure IGFBP2, we found that under hyperglycaemic conditions there was a significant increase in the abundance of IGFBP2 secreted by DU145 cells. A significant increase in IGFBP2 in response to high glucose was also confirmed with LNCaP cells (Fig. 3B), no such effect on IGFBP2 was observed with PC3 cells (data not shown) consistent with the lack of effect on cell survival (Fig. 2B). To determine the significance of the hyperglycaemia-induced increase in IGFBP2, we then examined the response of the DU145 cells to docetaxel in eu- and hyper-glycaemic conditions following silencing of IGFBP2 using siRNA. We found that with IGFBP2 being silenced, hyperglycaemia could no longer confer survival and in fact cell death was actually greater than under euglycaemic concentrations \( (P<0.01; \) Fig. 3C). With LNCaP cells, similarly silencing IGFBP2 also negated
Figure 3

Different levels of IGFBP2 according to different glucose concentrations. (A, i) Graph shows changes in the levels of IGFBP2 secreted into the media from DU145 prostate cancer cell lines exposed to 5 and 25 mM glucose for 48 h. Supernatants were subjected to western blotting and densitometry was used to quantify changes (n = 3). (A, ii) Western immunoblot shows changes in abundance of IGFBP2 from DU145 cell supernatants exposed for 48 h to 5 or 25 mM glucose. (A, iii) Concentration of IGFBP2 in cell supernatants after 48 h exposure to different glucose conditions was measured using an ELISA (n = 3). (B, i) Changes in the levels of IGFBP2 secreted into the media from LNCaP prostate cancer cell lines. Cells were exposed to glucose as in (A, i) and subjected to western blotting and densitometry was used to quantify those changes (n = 3). (B, ii) Western immunoblot show changes in the abundance of IGFBP2 from LNCaP cells supernatants exposed for 48 h to 5 or 25 mM glucose conditions. (C) DU145 cells were transfected with siRNA (50 nM final concentration) for IGFBP2 with a random sequence negative control siRNA (NSsiRNA) for 24 h. After another 24 h, serum starved cells were dosed with or without docetaxel (30 nM) for a further 48 h. Western immunoblot insert for IGFBP2 abundance in conditioned media indicates successful silencing of IGFBP2. Graph show % cell death – mean of three experiments each repeated in triplicate. (D) LNCaP cells were set up as described in (C). Cells were dosed with 60 nM of docetaxel. Cell death was assessed using cell counting with Trypan Blue. Graphs show % cell death – mean of three experiments each repeated in triplicate. (E) LNCaP cells were predosed for 1 h with either 1 µM of IGF-IR tyrosine kinase inhibitor (AG1024) or 10 µg/ml of integrin receptor inhibitor (RGD). Cells were then spiked with IGFBP2 (250 ng/ml) and/or with docetaxel (60 nM) for a further 48 h. Dead cell number was assessed using Trypan Blue cell counting. Graph shows % cell death – mean of three experiments each repeated in triplicate.
the survival effect against docetaxel under hyperglycaemic conditions (Fig. 3D). Effective knock-down of IGFBP2 is illustrated by western immunoblotting for IGFBP2 in both cell lines (Fig. 3C and D, inserts). These data indicate that hyperglycaemia confers chemoresistance in DU145 and LNCaP cells via an up-regulation of IGFBP2 but not in PC3 cells because IGFBP2 was unaffected by glucose in these cells. We showed previously in DU145 cells that the hyperglycaemia-induced survival effect mediated by IGFBP2 was independent of the IGF-IR but required interaction with the beta 1 integrin receptor (Uzoh et al. 2011). With LNCaP cells, we found that IGFBP2 could similarly act as a survival factor against docetaxel-induced cell death (Fig. 3E). This was unaffected in the presence of an IGF-IR tyrosine kinase inhibitor (AG1024), but negated when integrin receptors were blocked using a short disintegrin, RGD-containing peptide. We also examined the effects of hyperglycaemia on the proliferative response to IGF1, insulin and an IGF1 analogue that does not bind to IGFBPs (LongR3-IGF1) and found that with these cells the responses to IGF1 were unaffected by the change in glucose levels, whereas the response to insulin was reduced, consistent with a hyperglycaemia-induced insulin-resistance (data not shown). In addition we found that level of endogenous IGF1 was less than that of IGF2 in the DU145 (~5 vs 34 ng/ml respectively), PC3 (~6 vs 30 ng/ml respectively) and LNCaP cells (~8 vs 24 ng/ml respectively) and that these levels were unaffected by the different glucose concentrations. In vivo hyperglycaemia would be associated with changes in insulin levels, but the survival effect associated with the hyperglycaemia-induced increase in IGFBP2 that we observed was unlikely to be due to changes in insulin responses as there was no insulin present in most of our experiments and IGFBP2 does not bind to insulin.

### Regulation of IGFBP2: epigenetic?

Having identified that hyperglycaemia-induced chemoresistance was dependent upon the up-regulation of IGFBP2, we investigated whether IGFBP2 was under epigenetic control. We initially assessed the methylation status of the IGFBP2 gene in DU145 cells using COBRA. Figure 4A shows that the IGFBP2 promoter is highly methylated and therefore as a positive control we used a chemical demethylating agent (AZA; 0.25–1 μM), which we effectively demonstrated that it could induce demethylation of the IGFBP2 gene. In contrast, in either eu- or hyperglycaemic conditions, the IGFBP2 gene remained methylated and there were no differences between the different levels of glucose.

As glucose has been shown previously to induce acetylation of histones H3 and H4 (Friis et al. 2009), we examined the level of acetylation of these histones associated with the IGFBP2 gene using ChIP assay and found that with DU145 cells both were increased under hyperglycaemic (H3, P=0.05; H4, P<0.05) compared with euglycaemic conditions (Fig. 4B). With LNCaP cells, we found that only the acetylation of H3 associated with IGFBP2 gene was increased in hyperglycaemic conditions (Fig. 4C) with no change in the acetylation of H4. We then examined PC3 cells as a negative control and found that there was no significant change in the association of acetylated histones H3 and H4 with the IGFBP2 gene (H3 and H4, P=NS; Fig. 4D).

In order to confirm that histone acetylation enhanced expression of IGFBP2, we treated DU145 cells with inhibitors of histone deacetylases (HDACs): trichostatin A (an HDAC inhibitor class I and II), or sirtinol and inhibitor of Sirt1. Sirt1 is an HDAC inhibitor whose activity is determined by the NAD:NADH ratio within the cell (which reflects energy status). Exposure to both TSA and sirtinol increased IGFBP2 secretion (Fig. 4E) and in combination there was an additive effect consistent with histone acetylation promoting IGFBP2 expression.

### Discussion

This study evaluated the effects of elevated glucose levels on the ability of PCa cell lines to respond to chemotherapy. Hyperglycaemia is often a consequence of a Western lifestyle that is associated with metabolic syndrome and type 2 diabetes or as a result of long-term ADT. The rationale for addressing this question was based on a number of epidemiology studies that suggested a relationship between diabetes and PCa. A meta-analysis on the association between diabetes and PCa showed a reduced risk of developing PCa in diabetic patients (Kasper & Giovannucci 2006). However, despite the risk of developing PCa being reduced by diabetes, patients with diabetes who develop PCa have lower survival rates than those without (Liu et al. 2012) consistent with metabolic exposures affecting progression and not initiation.

We first examined the effect of hyperglycaemia on chemotherapy in DU145 and LNCaP PCa cell lines and found that docetaxel treatment was less effective on cells exposed to elevated compared with normal levels of glucose. We had previously observed a similar protective effect of hyperglycaemia against chemotherapy-induced...
Figure 4

(A) Methylation status of IGFBP2 upon 48 h incubation in 5 or 25 mM glucose conditions. Results from COBRA showed that the treatment with AZA for (72 h) induced demethylation of the promoter of IGFBP2 DNA but no change was observed with different glucose concentrations.

(B) Changes in acetylation status of histones linked with IGFBP2 under 48 h exposure to 5 and 25 mM glucose were assessed using ChIP Kit. Inserted graph shows a fold change of acetylated histone H3 associated with IGFBP2 DNA of DU145 cells (n = 3).

(C) Changes in acetylation status of histone H3 linked with IGFBP2 gene of LNCaP cells. Inserted graph shows a fold change of acetylated histone H3 and H4 associated with IGFBP2 DNA of LNCaP cells (n = 3).

(D) Changes in acetylation status of histones H3 and H4 linked with IGFBP2 gene of PC3 cells. Inserted graph shows fold change of acetylated histones associated with the IGFBP2 gene (n = 3).

(E) Changes in levels of IGFBP2 according to different treatments. Concentration of IGFBP2 in cell supernatants was measured using an ELISA. DU145 cells were dosed with dose regimen of Trichostatin A (TSA), sirtinol and combined treatment for additional 48 h and media samples were collected (n = 3). M, methylated DNA; DM, demethylated DNA.
death of breast cancer cells (Zeng et al. 2010). These in vitro findings would be consistent with the epidemiology data indicating that patients with diabetes that do develop PCa respond less well to treatment. It should be noted that in most of our experiments we used 25 mM levels of glucose in order to accentuate any effect, but recognising that this would be experienced only in extremely poorly controlled patients with diabetes; we did however confirm that with a range of glucose concentrations from 5 to 25 mM, docetaxel-induced cell death of DU145 cells was affected by glucose concentrations above 7.5 mM (see Fig. 1B). These cells became less sensitive to docetaxel after chronic exposure to glucose levels above 7.5 mM, which are comparable with blood glucose levels commonly observed in patients with metabolic disturbance and diabetes. This is consistent with the literature that indicates metabolic disturbances, such as hyperglycaemia can compromise the response to chemotherapy in a range of different cancers, such as breast (Jiralerspong et al. 2009), colorectal (Gribovskaja-Rupp et al. 2011) and lung (Nakazawa et al. 2013).

In contrast to the increased survival observed in response to docetaxel under hyperglycaemic conditions with DU145 and LNCaP cells, we found that the response of PC3 cells to docetaxel under the same conditions was unaffected. One mechanism of resistance to docetaxel is caused by reduced drug accumulation within cells due to raised levels of proteins which promote efflux of the drug, such as the ATP-binding cassette transporter P-glycoprotein (P-gp; O’Neill et al. 2011). It will be interesting to investigate whether IGFBP2 alters the sensitivity to docetaxel by affecting the levels or activity of P-gp. To understand what mediated the survival effect of hyperglycaemia, we initially examined whether this was mediated via the activity of fatty acid synthase (FASN) as we had previously observed in breast cancer cells (Zeng et al. 2010). When we silenced FASN in the PCa cells, however, we found no reduction in the protective effect conferred by hyperglycaemia (in contrast to the amelioration of the effect that we observed with the breast cancer cells; data not shown). The IGF axis is strongly associated with PCa, and IGFBP2 is an established mitogen and survival factor for PCa (Chatterjee et al. 2004, Uzoh et al. 2011) and has been reported to be associated with metabolic status (Heald et al. 2006). IGFBP2 can act in both: an IGF-IR-dependent manner in PC3 cells and intrinsically (independent of IGF interaction) in DU145 cells (Uzoh et al. 2011). Recent studies have also reported that IGFBP2 is highly expressed in prostatic adenocarcinomas (Ambrosini-Spaltro et al. 2011). We observed a significant increase in the abundance of IGFBP2 secreted by DU145 and LNCaP cells in hyperglycaemic conditions, with no such effect observed in PC3 cells consistent with the survival effects observed in the former but not the latter cells. To further address the involvement of IGFBP2 in relation to chemoresistance, we silenced IGFBP2 with siRNA and found that hyperglycaemia could no longer confer survival against docetaxel in either DU145 or LNCaP cells, suggesting that IGFBP2 was responsible for the hyperglycaemia-induced chemoresistance in these cells. As we showed previously with DU145 cells, we also confirmed that IGFBP2 conferred survival in an IGF-IR-independent but integrin-dependent manner in LNCaP cells. As hyperglycaemia did not induce an increase in IGFBP2 in PC3 cells, this could explain why hyperglycaemia did not alter the sensitivity of PC3 cells to docetaxel-induced cell death.

There has been considerable recent interest in the epigenetic alterations contributing to PCa (Jeronimo et al. 2011). Environmental exposures including altered metabolic conditions can induce epigenetic alterations and these may affect disease progression and response to therapy. When screening tumours for aberrant DNA methylation, IGFBPs have consistently been among the most commonly identified genes (Davies et al. 1991, Wiley et al. 2006). Epigenetic modifications have been reported to be important during the progression to the advanced stage of PCa. Schayek et al. (2010) showed that the androgen receptor (AR) promoter undergoes methylation in advanced stages of the disease but not in early stages. Therefore, we investigated the methylation of IGFBP2 gene as a potential epigenetic regulation to alter gene expression; however, we found no effect of hyperglycaemic conditions. Knowing that glucose did not alter gene methylation, we then assessed the acetylation status of lysine residues in the conserved amino-terminal tails of histones associated with the IGFBP2 promoter in DU145, LNCaP and PC3 cells using a ChIP assay. Histone acetylation is mediated by lysine acetylases (HATs) and reversed by HDAC inhibitors and therefore we hypothesised that hyperglycaemia may be able to modulate such enzymes particularly as glucose has been shown previously to induce acetylation of histones H3 and H4 (Friis et al. 2009). We examined the level of acetylation of these histones associated with the IGFBP2 gene and found that both were increased under hyperglycaemic, compared with euglycaemic, conditions in DU145 cells, and in LNCaP only H3 acetylation was affected. As a negative control, we used PC3 cells as we had found that the response to docetaxel under eu-
Hyperglycaemic conditions in these cells was identical. In addition, that hyperglycaemia did not induce an increase of IGFBP2 secreted into the media. Therefore, as anticipated in the PC3 cells there was no significant change in association of acetylated histones associating with the IGFBP2.

Indeed, we confirmed that secretion of IGFBP2 was enhanced by histone acetylation by exposing the cells to HDAC inhibitors and found that both TSA (an HDAC inhibitor class I and II) and sirtinol (an inhibitor of sirtuins: SIRT1 and SIRT2 (silent mating type information regulation 2 homologue) 1 and 2 (Saccharomyces cerevisiae) resulted in elevated secretion of IGFBP2. This data would be consistent with an old report that a general HDAC inhibitor induced IGFBP2 production from intestinal epithelial cells (Nishimura et al. 1998). It has been shown previously that SIRT1 can modulate the promoter activity of another structurally similar IGFBP, IGFBP1 through FoxO-dependent and -independent mechanisms, and that activation of MAPK contributes to the effects of SIRT1 on IGFBP1 gene expression (Gan et al. 2005). In light of the effect of glucose on histone acetylation and IGFBP2 expression in DU145 and LNCaP, it will be of interest to delineate the specific mechanisms controlling the promoter activity in these cells.

As we observed changes in gene methylation and were aware that the AR has been reported to be methylated in DU145 cells (Hiraoka et al. 2012), we therefore investigated whether there were any changes in AR abundance in the DU145 cells following exposure to different glucose levels. We could not detect AR in the DU145 cells regardless of the level of glucose in the media.

**Conclusion**

Increased glucose concentrations appear to inhibit the efficacy of docetaxel at inducing apoptosis. This was associated with increased IGFBP2 production as a result of glucose-induced acetylation of histones bound to the IGFBP2 promoter. IGFBP2 is a secreted protein and one mechanism by which it may cause chemoresistance is via inactivation of PTEN as we described previously with DU145 cells (Uzoh et al. 2009). Here we observed that IGFBP2 mediated a similar effect with LNCaP cells that do not have a functional PTEN gene and hence IGFBP2 must also be able to affect survival via alternative mechanisms independent of PTEN, which are the subject for further investigations. This study shows that the metabolic environment can cause epigenetic modifications to genes that affect treatment efficacy; as such epigenetic modifications are reversible this indicates that improvements in metabolic status could enhance the response to chemotherapy. This may have implications for the management of androgen-independent advanced PCa, many such men will also have disturbed metabolism and improved metabolic control may ensure optimal response to docetaxel.

**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.

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