Peroxiredoxin-3 is overexpressed in prostate cancer and promotes cancer cell survival by protecting cells from oxidative stress

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Objective: We have previously identified peroxiredoxin-3 (PRDX-3) as a cell-surface protein that is androgen regulated in the LNCaP prostate cancer (PCa) cell line. PRDX-3 is a member of the peroxiredoxin family that are responsible for neutralising reactive oxygen species.

Experimental design: PRDX-3 expression was examined in tissue from 32 patients using immunohistochemistry. Subcellular distribution was determined using confocal microscopy. PRDX-3 expression was determined in antiandrogen-resistant cell lines by western blotting and quantitative RT–PCR. The pathways of PRDX-3 overexpression and knockdown on apoptosis and response to oxidative stress were investigated using protein arrays.

Results: PRDX-3 is upregulated in a number of endocrine-regulated tumours; in particular in PCa and prostatic intraepithelial neoplasia. Although the majority of PRDX-3 is localised to the mitochondria, we have confirmed that PRDX-3 at the cell membrane is androgen regulated. In antiandrogen-resistant LNCaP cell lines, PRDX-3 is upregulated at the protein but not RNA level. Resistant cells also possess an upregulation of the tricarboxylic acid (TCA) pathway and resistance to H2O2-induced apoptosis through a failure to activate pro-apoptotic pathways. Knockdown of PRDX-3 restored H2O2 sensitivity.

Conclusion: Our results suggest that PRDX-3 has an essential role in regulating oxidation-induced apoptosis in antiandrogen-resistant cells. PRDX-3 may have potential as a therapeutic target in castrate-independent PCa.

The mammalian peroxiredoxins (PRDXs) are a highly conserved family of thiol-containing peroxidases that has six members (PRDX1–6) (Leyens et al, 2003; Hall et al, 2009). The PRDX proteins catalyse the reduction of molecules that cause oxidative stress such as reactive oxygen species (ROS), for example, peroxides that are essential metabolic intermediates and regulators of growth factor signalling but are often produced as a result of cellular stress (Wood et al, 2003; Giorgio et al, 2007; Cox et al, 2009; Lenaz, 2012; Miki and Funato, 2012; Ray et al, 2012). If left unchecked, ROS can damage DNA and induce tumourigenesis (Trachootham et al, 2008; Ruckenstuhl et al, 2009). Alternatively, the induction of ROS formation by chemotherapy and ionising radiation can be used therapeutically to cause DNA damage-induced cell death (Lee et al, 2011). Catalysis of ROS leads to oxidised SO2 and SO3 forms of the PRDX proteins which can be reduced, and therefore reactivated, via a thioreredoxin and

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Received 21 March 2013; revised 19 June 2013; accepted 24 June 2013; published online 23 July 2013

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www.bjcancer.com | DOI:10.1038/bjc.2013.396

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ATP-dependent mechanism (Fujii and Ikeda, 2002; Aran et al., 2009; Hanschmann et al., 2010). The PRDX protein family have previously been linked to cancer development (Shen and Nathan, 2002). All of the PRDX protein that have been linked to the development or progression of tumourigenesis as summarised in Table 1. PRDX-1, 2, 5 and 6 have also been linked to resistance to chemotherapy, in particular cisplatin, and ionising radiation, in a variety of tumours including ovarian, oesophageal and hepatocellular (Kikuta et al., 2010; Dittmann et al., 2011; Gao et al., 2011; Lee et al., 2011; Pak et al., 2011; Cho et al., 2012; Zhiyu et al., 2011). Increased PRDX-2 is thought to induce resistance by preventing DNA damage-induced apoptosis (Lee et al., 2011).

Prostate cancer (PCa) is the most prevalent male cancer in the Western world and it requires androgens such as testosterone or dihydrotestosterone (DHT) for continued growth and development (Friedlander and Ryan, 2012). Treatment for PCa targets this reliance on androgens by blocking androgen production or the androgen receptor (Kuil and Mulder, 1994). However, prostate cells eventually escape this androgen blockade and continue to grow, becoming resistant to therapy, despite continued antiandrogen treatment. The mechanisms involved in the progression to castrate-independent disease remains poorly understood, but may include androgen receptor mutation or amplification, receptor activation by alternative ligands such as oestrogen, changes in cofactor expression or alterations in growth factor signalling (Bevan, 2005). Second line chemotherapy with docetaxel is only effective in 30–50% of patients (Tannock et al., 2004). PRDX-1 is known to interact with the androgen receptor, enhancing ligand binding and regulating transactivation of androgen-regulated genes (Park et al., 2007; Chhipa et al., 2009; Shiota et al., 2011; Miki and Funato, 2012; Ray et al., 2012).

PRDX-3 is a known c-myc, miR-383 and miR-23b target gene required for mitochondrial homoeostasis and neoplastic transformation (Wosney et al., 2002; Vivas-Mejia et al., 2009; He et al., 2012; Li et al., 2012). A high level of expression is found in the breast, cervical, hepatocellular and prostate carcinomas (Noh et al., 2001; Choi et al., 2002; Wosney et al., 2002; Karihata et al., 2003; Lin et al., 2007; Kim et al., 2009; Basu et al., 2011). In PCa, the expression of PRDX-3 has been negatively correlated with TMPRSS–ERG fusion status and overexpression has been shown to increase cellular proliferation regardless of the hormone dependency of the cell line (Ummanni et al., 2012). The subcellular localisation of the different PRDX proteins is diverse (Fujii and Ikeda, 2002). The majority of PRDX-3 has been localised to the mitochondria with a small proportion also reported at the cell surface, similar to reports of membrane-associated PRDX-6 (Araki et al., 1999; Liu et al., 2005; Whicker et al., 2007; Ambrosio et al., 2012). PRDX-3 expression is induced by oxidants in the cardiovascular system (Araki et al., 1999; Kumar et al., 2009) and is particularly sensitive to oxidative stress following treatment with pro-apoptotic drugs, such as auranofin, isothiocyanates and arsenic trioxide (Brown et al., 2008; Cox et al., 2008a; Vivas-Mejia et al., 2009).

Using immunohistochemistry (IHC), we have shown upregulation of PRDX-3 in endocrine-regulated tumours and in particular prostastic intraepithelial neoplasia (PIN) and PCa. PRDX-3 protein, but not mRNA, is significantly upregulated in antiandrogen-resistant PCa cell lines, resulting in increased resistance to oxidative stress and failure to activate pro-apoptotic pathways. Conversely, knockdown of PRDX-3 leads to raised susceptibility to oxidative stress. These results suggest that PRDX-3 may be essential for the development of PCa and the development of resistance to treatment.

### MATERIALS AND METHODS

**Patient cohorts.** Prostate tissue from radical prostatectomies performed at Addenbrookes Hospital, Cambridge, UK between 2001 and 2005 was used to make tissue microarrays (TMAs) using duplicate 0.6-mm cores taken from paraffin-embedded tissue and a Beecher Manual TMA Arrayer. Details of this array have previously been published (Whitaker et al., 2010). In total, the tissue from 32 different patients was used to generate the TMA. Regions of benign or normal prostate (n = 4), PIN (n = 4) and malignancy (n = 2–6) were identified by a specialist uro-pathologist (AYW) for each patient (Whitaker et al., 2010). Malignant tissue was obtained from at least one and, where possible, up to three different tumour foci from each patient. Stage and Gleason grade was confirmed by a specialist uro-pathologist (AYW) before scoring any IHC staining. Full ethical approval was obtained before beginning this study.

**IHC and image analysis.** A TMA containing one matched normal and one tumour core from a variety of different organs (Stretton Scientific, Stretton, UK) or the prostate TMA outlined above was probed with anti-PRDX-3 antibody (1 : 1000, Abcam, Cambridge, UK) using a Bondmax Autostainer. PRDX-3 staining was visualised using Alexafluor 488 (green) (Molecular Probes, 2007; Pak et al., 2011; Zhiyu et al., 2011; Miki and Funato, 2012). Ray et al., 2012).
Paisley, UK) and counterstained with DAPI (blue) to visualise nuclei. The PRDX-3-stained prostate TMAs were scanned and quantified using the ArioT system (Applied Imaging, New Milton, UK). Cores containing mixed pathology or only stroma were excluded from the analysis. A threshold intensity of 0.75 was applied to all images to remove pixel densities caused by non-specific staining. After cores were mapped, total pixel count for each core was calculated using the following equation: total pixel intensity = total pixel area/1000. Data were grouped according to pathology, for example, benign, PIN or PCa or subdivided by Gleason grade. N numbers indicate the number of cores analysed. P-values were calculated using a Kruskal–Wallis test.

**Cell culture.** COS Cells derived from Monkey kidney and human prostate PC3 and parental LNCaP cells were purchased from the Cancer Research UK cell bank. Primary benign prostatic hyperplasia fibroblasts were a kind gift from Dr Michael Brown (The Genito-Urinary Cancer Research Group, Cancer Research UK Paterson Institute for Cancer Research). COS cells were routinely cultured in DMEM supplemented with 10% foetal bovine serum (Labtech, Uckfield, UK). All other cell lines were routinely cultured in RPMI media (Gibco, Paisley, UK) with 10% foetal bovine serum. Antiandrogen-resistant LNCaP cells have previously been characterised (Hobisch et al, 2006; Vias et al, 2006) and were grown in media supplemented with either 1 µM hydroxyflutamide alone (LNCaP-OHF), 1 µM OHE and 10 µM R1881 (a non-hydrolysable DHT analogue) (LNCaP-OHF/R1881), 1 µM alone (LNCaP-BIC), 1 µM BIC and 10 µM R1881 (LNCaP-BIC/R1881).

Short-term antiandrogen treatment was performed using parental LNCaP cells grown in the presence of 0 µM, 1 µM or 10 µM bicalutamide. PRDX-3 knockdown was performed on six-well plates of parental LNCaP cells using 0 µM, 50 µM or 100 µM Accell SMARTpool siRNA (Dharmacon, Loughborough, UK) according to the manufacturer’s guidelines. Cells were harvested or used for additional experiments 72 h after transfection.

**Western blotting.** Cells were washed in PBS, pelleted and protein lysate was made as before (Whitaker et al, 2008). Twenty micrograms of total protein, as determined using Bradford assay, were compared using the SDS–PAGE and western blot analysis.

The following antibodies were used for the detection of proteins: mouse anti-PRDX-3 antibody (1:3000), rabbit anti-PRDX-SO2/SO3 (1:3000) and rabbit anti-COX IV (1:5000) (all from Abcam). Mouse anti-actin (1:5000, Abcam) was used as a loading control. Proteins were visualised using anti-mouse or anti-rabbit HRP-conjugated secondary antibodies (1:1000, Dako Cytomation, Ely, UK) and ECL-Plus (GE Healthcare, Amersham, UK). If the signal detected was beyond the dynamic range of film, additional experiments 72 h after transfection.

**Synthesis of cDNA and quantitative real-time PCR.** For RNA, cells were harvested in 1 ml Trizol. From each condition, 5 µg of total RNA was reverse transcribed using the SuperScript III First-Strand synthesis system (Invitrogen, Paisley, UK) with random hexamer primers according to manufacturer’s recommendations. For RT–PCR, primers were designed to recognise PRDX-3; forward 5'-GCCGCTCCTGGATGAGACT-3', reverse 5'-CCAGCTGGGGACACCTCC-3'. Real-time PCR was performed in triplicate in 10-µl reactions containing 5 µl of SYBR Green PCR Master Mix (Applied Biosystems), 2 pmol of primers and 1.5 µl of cDNA as template. The cycling conditions for the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) were 50 °C for 2 min, 95 °C for 10 min, 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Real-time efficiencies were calculated from the slopes in the standard dilution curves. Relative expression levels were calculated on the basis of the difference in Ct values between the test samples and the control using the following formula: 10 mean-Y intercept/slope. Results were normalised with the expression of GAPDH results expressed relative to vehicle-treated cells.

**Gene expression experiments.** Gene expression data were generated as part of a previously published study (Massie et al, 2011). Briefly, gene expression analysis was carried out on Illumina Human HT12 version 4 arrays. All analyses were carried out on R using Bioconductor packages (Gentleman, 2004). Raw intensity data were processed using the BASH and HULK algorithms as implemented in the beadarray (Cairns, 2008; Dunning, 2007). Log2 transformation and quantile normalisation of the data were performed across all sample groups. The differential expression analysis was carried out using the limma package (Smyth, 2005). Points were calculated to represent log-expression relative to the 0 time point. The probability of seeing that if there were not a trend was calculated from 1 000 000 permutations to give the P-value for the hypothesis that there is no change after androgen treatment.

**Confocal microscopy.** Immunofluorescence was performed as previously described (Whitaker et al, 2007). LNCaP cells were probed for PRDX-3 (1:400) in 10% whole-galut serum (Zymed, Paisley, UK) in PBS. For colocalisation, experiments COX IV (1:400) and E-cadherin (1:400, Abcam) were used. Proteins were visualised using AlexaFluor 488 or 594-conjugated secondary antibodies (Molecular Probes) and visualised using a Nikon Eclipse confocal microscope using a ×100 objective. All scale bars represent 10 µM.

**Citrate synthase assay.** Parental LNCaP cells and antiandrogen-resistant cell lines were washed in ice-cold PBS and lysed on ice using Celllytic MT Cell Lysis Reagent (Sigma, St Louis, MO, USA). Ten micrograms total protein was assayed immediately for citrate synthase activity using a Citrate Synthase Assay Kit (Sigma) in accordance with the manufacturer’s instructions. Citrate synthase activity was measured by a change in OD at 412 nm every 10 s for a total of 100's using a Lucyl II spectrophotometer. After plotting A_{412} against time, the linear range of the plot was used to calculate the citrate synthase activity in μmole ml⁻¹ min⁻¹. P-values were determined using a two-way ANOVA test.

**Cell viability assays.** Parental LNCaP and resistant LNCaP-BIC cells were grown in six-well dishes to 60% confluence, washed twice in PBS and incubated with serum-free RPMI for 24 h before treatment. Duplicate wells were dosed with 0–0.001% H₂O₂ for 24 h and cells harvested by scraping into the existing media, centrifuged at 3000 r.p.m. and the media removed. Cells were gently but thoroughly resuspended in ice-cold PBS and cell viability was measured using a ViCell automated cell counter. Duplicate cells were dosed with 0.00033% H₂O₂ for 24 h were harvested and lysed as for western blotting. Human apoptosis proteome profiler antibody arrays (R&D Systems, Abingdon, UK) were performed in accordance with the manufacturer’s instructions using 200 µg total protein. Following siRNA knockdown for 72 h, LNCaP cells were washed twice in PBS and incubated with serum-free RPMI for 24 h before 0.00033% H₂O₂ treatment for 24 h. Duplicate cells were scraped, resuspended and cell viability measured as before. All results were calculated as relative to the no treatment controls. P-values were determined using a two-tailed Student’s t-test.

**RESULTS**

Using a matched tumour and normal TMA containing tissue cores from a variety of organs, we investigated the expression of PRDX-3 using immunofluorescent staining (Figure 1A and Table 2). In this single TMA, PRDX-3 was overexpressed in numerous endocrine
tumours such as the thyroid and ovary and highly expressed in both normal and tumourigenic breast. PRDX-3 was also down-regulated in many non-endocrine tissues such as the kidney and lung although upregulated in the liver and cervix. Using a prostate TMA, we found a highly significant increase in PRDX-3 in PIN and prostate tumours relative to benign prostate (Figures 1B and C) \((P < 0.0001)\). Consistent with previous reports, we found no link between PRDX-3 expression in prostate tumours and Gleason grade (Figure 1D) \((P = 0.5321)\) (Basu et al., 2011).

There was no obvious difference in PRDX-3 subcellular distribution between benign and tumourigenic tissue samples (Figure 1B). Tissue staining for PRDX-3 was punctate, consistent with the previously reported mitochondrial localisation (Araki et al., 1999). To confirm that the majority of PRDX-3 was localised to the mitochondria, PRDX-3 was colocalised with COX IV, a known mitochondrial marker, in LNCaP cells (Figure 2A). PRDX-3 has also been localised to the cell surface in a PCa cell line, and we investigated whether it colocalised with E-cadherin, a known plasma membrane protein, using high-power confocal microscopy (Whitaker et al., 2007) (Figure 2B). A proportion of PRDX-3 was seen localised to the cellular membrane alongside E-cadherin. This colocalisation was only visible in vehicle-treated cells and lost in the presence of R1881, confirming the androgen regulation of cell surface PRDX-3 previously reported (Whitaker et al., 2007). Androgen regulation was confirmed using previously published gene expression data, which supported the weak downregulation of PRDX-3 in response to androgen treatment (Figure 2C) (Massie et al., 2011).

To investigate how the expression changed following treatment with antiandrogens, PRDX-3 expression was studied in a number of prostate cell lines including the antiandrogen-resistant cells (LNCaP-BIC and LNCaP-OHF) and LNCaP-BIC-R1881 and LNCaP-OHF-R1881 cells cultured with the antiandrogens plus 10 pM of synthetic DHT, R1881 (Hobisch et al., 2006; Vias et al., 2006). Consistent with previous data, total PRDX-3 was down-regulated following R1881 treatment of LNCaP cells (Figure 3A) (Whitaker et al., 2007; Massie et al., 2011). Lower expression was also seen in benign fibroblasts and the AR-negative PC3 cells which is consistent with low the PRDX-3 expression in androgen-independent cell lines seen previously (Basu et al., 2011). All of the...
antiandrogen-resistant cell lines exhibited the upregulation of PRDX-3, compared with vehicle only treated wtLNCaP cells. When PRDX-3 mRNA levels were investigated using qRT–PCR there was a reciprocal correlation with the protein expression with PRDX-3 mRNA increasing with androgen treatment and decreasing in the antiandrogen-resistant cell lines (Figure 3B). To determine whether the changes in PRDX-3 expression had any causal effect in antiandrogen resistance or whether they resulted from antiandrogen resistance, we treated LNCaP cells for 3 days with vehicle, 1 μM or 10 μM bicalutamide. Cells treated with bicalutamide quickly became etiolated and showed a more neuroendocrine phenotype consistent with cell stress and the development of resistance (Figure 3D, upper panels) (Vias et al., 2006). PRDX-3 was upregulated in response to bicalutamide in a dose-dependent manner (Figure 3D, lower panel). An antibody that detects the oxidised forms (SO₂ and SO₃) of PRDX-3 demonstrated a marked upregulation in response to bicalutamide treatment indicating an increase in mitochondrial oxidative stress and PRDX-3 activity.

To determine whether PRDX-3 is upregulated in antiandrogen-resistant cells or whether there was an increase in overall mitochondrial number or activity, we probed cell lysates for COX IV, a key enzyme in the mitochondrial electron transport chain that is an effective mitochondrial loading control (Figure 2A). COX IV was markedly overexpressed in all of the antiandrogen-resistant cell lines compared with wtLNCaP cells, consistent with the PRDX-3 results and suggestive of increased mitochondrial number. To determine whether this resulted in an increase in the mitochondrial function, we measured the activity of citrate synthase, a key enzyme of the mitochondrial tricarboxylic acid (TCA) cycle (Figure 4A). Citrate synthase activity was increased by R1881 treatment of wtLNCaP cells, whereas the antiandrogen-resistant cells exhibited three- to five-fold greater citrate synthase activity than the androgen-dependent wtLNCaP cells. Thus, long-term antiandrogen-treated cells have more mitochondria and are much more metabolically active than untreated cells (P = 0.0032) resulting in an increase in mitochondrial enzymes, including PRDX-3.

As PRDX-3 protein increases following antiandrogen treatment, we aimed to establish whether this conferred any survival advantage upon cells that might suggest that PRDX-3 expression might be clinically significant. To induce oxidative stress, both wtLNCaP- and bicalutamide-resistant cells were treated with 0–0.001% H₂O₂ for 24 h and assayed for cell viability (Figure 4B). Long-term antiandrogen-treated LNCaP-BIC cells with raised PRDX-3 levels showed increased resistance to H₂O₂-induced oxidative stress, particularly at lower concentrations (0–0.0005%). Cell lysates from wtLNCaP and LNCaP-BIC treated with and without 0.00033% H₂O₂ were used on an apoptosis focused antibody array to determine the difference in response to oxidative stress (Figure 4C). In these arrays, wtLNCaP and LNCaP-BIC cells with knocked down PRDX-3 (siPRDX3) showed upregulation of a number of pro-apoptotic markers including p21, Fas ligand and cleaved caspase-3, but not Bad. We did not observe any change in the expression of pro-apoptotic proteins in the LNCaP-BIC cells, consistent with the survival advantage seen in Figure 4b. Trail receptor 2 which protects cells against apoptosis was also upregulated in wtLNCaP and LNCaP-BIC siPRDX3 cells but not LNCaP-BIC cells, suggesting deregulation of both pro- and anti-apoptotic pathways. When PRDX-3 was knocked down in wtLNCaP and LNCaP-BIC cells using siRNA, the knocked-down cells were hyper sensitive to treatment with H₂O₂ compared with a scrambled control (P = 0.005 (wtLNCaP), P = 0.009 (LNCaP-BIC)) (Figure 4D).

### DISCUSSION

Although the PRDX proteins all share peroxidase activity, their expression, particularly in tumours, is diverse (Table 1). Previously PRDX-3 has been shown to be upregulated in a variety of tumour tissues (Noh et al., 2001; Choi et al., 2002; Kinnula et al., 2003; Lehtonen et al., 2004; Lin et al., 2007; Kim et al., 2009; Chua et al., 2010; Basu et al., 2011; Ummanni et al., 2012). Using a matched normal/tumour TMA, we have shown that PRDX-3 upregulation in tumours is most pronounced in endocrine-regulated tissues such as the breast, thyroid and prostate (Figure 1A and Table 2) supporting the previous association made between PRDX-3 upregulation and the presence of hormone receptors in the breast tissue (Karihtala et al., 2003). In PCa, PRDX-3 has been shown to be upregulated in needle-core biopsies and a commercial TMA, where the patient treatment and surgical technique could not be determined (Lin et al., 2007; Basu et al., 2011). Our data, gathered from 32 radical prostatectomy samples, confirm upregulation of PRDX-3 in tumours compared with benign and also shows the upregulation of PRDX-3 in PIN, suggesting that its overexpression may be an early event in tumourigenesis. Previous studies have suggested that 33% of adjacent normal prostate tissue may demonstrate some raised expression of PRDX-3, consistent with a field effect seen in other solid tumours (Basu et al., 2011). Although the benign on our TMA was taken from the same patient, whenever possible, it was not taken directly from the adjacent tumour. Any field effect is likely to raise expression in the benign cohort, making any differences in expression between benign and tumour even more significant. Our results are also consistent with a previous study showing a negative correlation between PRDX-3 overexpression and TMPRSS-ERG fusion status in PCa (Ummanni et al., 2012).

The localisation and of the PRDX proteins is varied with PRDX-3 predominantly localised to the mitochondria and, to a lesser extent, the cell surface (Araki et al., 1999; Liu et al., 2005; Whitaker et al., 2007) (Figure 2). The localisation of the mitochondrial proteins with mitochondria and the cellular membrane has previously been reported for p32 gC1qR, a protein with no defined function, this, is thought to act as a generic chaperone (Fogal et al., 2008). Our group has previously reported downregulation of cell surface and total cellular PRDX-3 in response to androgen treatment (Whitaker et al., 2007). We have confirmed this androgen regulation of PRDX-3 using gene expression data.
showing a decrease in the PRDX-3 RNA expression in response to R1881 treatment (Figure 2C). This reduction in PRDX-3 corresponds to a loss of cell surface PRDX-3, consistent with our previous results (Figure 2B) (Whitaker et al., 2007). The localisation of the mitochondrial proteins with mitochondria and the cellular membrane has previously been reported for p32 gC1qR, a protein of unknown function that is being studied for potential therapeutic utility.

The protective role of PRDX-3 in oxidative stress has previously been shown in the cardiovascular system (Araki et al., 1999) and in response to pro-apoptotic drugs, such as auranofin and isothiocyanates in tumour cells (Brown et al., 2008; Cox et al., 2008a). We report that PRDX-3 down regulated by R1881 treatment despite reports that androgen treatment increases fatty-acid synthesis and ROS production (Lin et al., 2010). PRDX-3 is highly upregulated in the antiandrogen-resistant cell lines, indicating that it may have an antiapoptotic function in these cells and promote their survival (Figure 3A). Interestingly, upregulation occurs at the protein but not the mRNA level, suggesting that regulation of PRDX-3 occurs at the translation or protein level and this is consistent with an increase in the oxidised form of PRDX-3 seen after antiandrogen treatment (Figures 3A, B and 4A). Our data suggest that the stabilisation of PRDX-3 via oxidation and its subsequent, slower, recycling back to the reduced and active form may be an additional layer of regulation of peroxiredoxin expression levels in prostate cells. Upregulation of PRDX-3 was relatively rapid, occurring after only 3 days bicalutamide treatment, suggesting that raised PRDX-3 may be part of the mechanism...
leading to antiandrogen resistance rather than an increase occurring as a result of resistance (Figure 4A). The accompanying increase in oxidised PRDX-3 supports the hypothesis that higher levels of oxidative stress exist in cells treated with antiandrogen. Although PRDX-3 oxidation has been linked to apoptosis via Bax/Bak, it may also represent the mechanism by which PRDX-3 can be...
stabilized and recycled by thioredoxin-2 in resistant cells (Wood et al., 2003; Cox et al., 2008a).

PRDX-3 is known to catalyse the reduction of ROS, including \( \text{H}_2\text{O}_2 \), which are produced as a result of cellular metabolism, for example, ATP synthesis. A major site of ROS production is the mitochondria, organelles which can also regulate apoptosis (Rustin and Kroemer, 2007). Bicalutamide-resistant cells that contain higher levels of PRDX-3 also overexpress COX IV and possessed four- to six-fold higher citrate synthase activity (Figure 4A). Citrate synthase is the rate limiting first enzyme in the TCA cycle and represents the metabolic activity via mitochondrial oxidative phosphorylation occurring within a cell. We hypothesise that a subset of prostate cells with greater mitochondrial number can overcome the oxidative stress and pro-apoptotic signals caused by antiandrogen treatment due to higher metabolic capacity and an increased resistance to oxidative stress due to increased PRDX-3.

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**Figure 4. PRDX-3 protects cells from apoptosis.** For the citrate synthase assay, wtLNCaP cells and antiandrogen-resistant cells were lysed on ice and 10 µg total protein assayed immediately for citrate synthase activity. \( P \)-values were determined using a two-way ANOVA test (A). wtLNCaP cells (solid line, squares) and LNCaP-BIC cells (dashed line, circles) were serum starved for 24 h and treated with varying concentrations of \( \text{H}_2\text{O}_2 \) (0.00016–0.001%) for 24 h before measuring cell viability (B). wtLNCaP cells and LNCaP-BIC (BIC) were serum starved and treated as before with (+) or without (−) 0.00033% \( \text{H}_2\text{O}_2 \) and 200 µg of total protein used on an apoptosis protein array. Selected changes are highlighted in the lower panel (C). PRDX-3 knockdown was performed on wtLNCaP and LNCaP-BIC cells using 0, 50 or 100 nM siRNA. Seventy-two hours after transfection cells were harvested, lysed and probed for PRDX-3 or actin (D, right panel). Cells were treated with 0.00033% \( \text{H}_2\text{O}_2 \) for 1 h or 24 h before harvesting and assaying for cell viability as before (D, left panel). All results were calculated as relative to the no treatment controls. \( P \)-values were determined using a two-tailed Student’s t-test.
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levels. Consistent with this, others have shown that mutations in mitochondrial DNA are essential for conferring androgen independence in the LNCaP derivative cell lines C4-2 (Higuchi et al., 2006).

Concomitant with the raised levels of PRDX-3, antiandrogen-resistant cells show an increased resistance to H$_2$O$_2$, particularly at low concentrations, where PRDX proteins are known to be most effective (Lee et al., 2007; Cox et al., 2009). Treatment of bicalutamide-resistant cells with H$_2$O$_2$ failed to induce the pro-apoptotic signals seen in the parental cell lines (Figure 4C). This effect was reversed by knocking down PRDX-3 in LNCaP-BIC cells. Conversely knocking down PRDX-3 in parental LNCaP cells makes them more susceptible to H$_2$O$_2$-mediated apoptosis as previously noted following TNF-α treatment (Cox et al., 2008b). This confirms a previous report that PRDX-3 is responsible for increased resistance to H$_2$O$_2$, and suggests that PRDX-3 overexpression may have a role in antiandrogen resistance (Chen et al., 2008). We suggest that metabolic adaptation and increase in the mitochondrial number in prostate cells may result in resistance to the oxidative stress and pro-apoptotic signals initiated by hormone therapy, as seen in neuronal cells and breast cancer xenografts (Besada et al., 2006; Busija et al., 2008; De Simoni et al., 2008). ROS regulation is a novel mechanism leading to anti-androgen resistance which could be exploited for as therapeutic targets for small-molecule inhibitors or radiotherapy (Trachootham et al., 2009; Zhang et al., 2009).

ACKNOWLEDGEMENTS

We acknowledge the support of The University of Cambridge, Cancer Research UK and Hutchison Whampoa Limited. We also acknowledge the support of the National Institute for Health Research (NIHR) which funds the Cambridge Bio-medical Research Centre, Cambridge UK. We would also like to acknowledge the support of the NCRI (ProMPT) which has funded tissue collections in Cambridge. We are grateful to Zoran Culig (Institute of Medical Research and Humanities) for the help and advice with publishing tools. This work is funded by Prostate Cancer UK, Cancer Research UK and the Medical Research Council.

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