ORIGINAL ARTICLE

Androgens induce oxidative stress and radiation resistance in prostate cancer cells though NADPH oxidase

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Androgen deprivation therapy (ADT) facilitates the response of prostate cancer (PC) to radiation. Androgens have been shown to induce elevated basal levels of reactive oxygen species (ROS) in PC, leading to adaptation to radiation-induced cytotoxic oxidative stress. Here, we show that androgens increase the expression of p22^{phox} and gp91^{phox} subunits of NADPH oxidase (NOX) and ROS production by NOX2 and NOX4 in PC. Pre-radiation treatment of 22Rv1 human PC cells with NOX inhibitors sensitize the cells to radiation similarly to ADT, suggesting that their future usage may spare the need for adjuvant ADT in PC patients undergoing radiation.
Prostate Cancer and Prostatic Diseases (2010) 13, 39–46; doi:10.1038/pcan.2009.24; published online 23 June 2009

Introduction

With the advent of PSA screening, most prostate cancers (PC) are currently diagnosed at a localized stage, for which total-gland radiation or surgical removal are the gold standard therapies. Nevertheless, even with the use state-of-the-art high-dose conformal radiation therapy, treatment failure occurs in 45% of patients, particularly in those with locally advanced disease.[1] It is well established that the use of androgen deprivation therapy (ADT) concurrently with radiation, improves overall survival in patients with locally advanced disease compared with patients who received radiation alone.[2,3,4] Potential reasons for this include, but are not restricted to, the anti-angiogenic[5] and pro-apoptotic[6] effects of ADT, as well as to cyto-reduction and control of micrometastatic disease.[7] We have recently suggested another potential mechanism for the radiosensitizing effect of ADT, namely that ADT enhances the vulnerability of PC cells to toxic oxidative stress induced by radiation.[8] Ionizing radiation exposure of cells/tissues generates multiple highly reactive oxygen species (ROS) from cellular water, which oxidize DNA, proteins and lipids. This translates into toxic oxidative damage in the cancer cells by DNA strand breaks, disruption of plasma, mitochondrial, nuclear, and endoplasmic reticulum membranes and protein cross links, leading to increased membrane permeability and

loss of function, as well as alteration of cellular protein functions and their degradation.

We have showed that androgens increase the basal levels of ROS, which consequently stimulates the activation and the expression of stress molecules and anti-oxidative enzymes to better cope with the shift in redox balance. Thus, upon radiation these cells become less sensitive to toxic levels of ROS compared with androgen-deprived cells and so are less susceptible to treatment by radiation.[8]

One of the major sources of basal ROS generation in PC cells is the NADPH oxidase (NOX) system.[9] NOX are a family of multi-component transmembrane enzymes that transport electrons across biological membranes to generate super-oxide $O_2(-)$ by the reduction of oxygen.[10,11] NOX can be activated by various growth factors,[12] but to the best of our knowledge it is unknown whether androgens can enhance the generation of ROS by NOX in human PC. The present study sought to determine if androgens stimulate ROS generation in PC cells through NOX, and whether inhibition of NOX can increase the response of PC cells to radiation. We found that androgens increase and ADT decreases the *in-vitro* and *in-vivo* expression of the p22^{phox} catalytic subunits of NOX, and the mRNA levels of NOX2 and NOX4 in 22Rv1 human PC cells. Inhibition of NOX by apocynin, sensitized these cells *in-vitro* to radiation to a similar extent as androgen deprivation.

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Materials and methods

Cell lines and xenografts
22Rv1 human PC cells (ATTC, Manassas, VA, USA) were cultured at 37 °C in a typical CO_2 incubator with 5% CO_2

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in air. The culture medium consisted of phenol red-free RPMI-1640 with 2 mM L-glutamine adjusted to contain $1.5 g l^{-1}$ sodium bicarbonate, $4.5 g l^{-1}$ glucose, 10 mM HEPES, 1.0 mM sodium pyruvate (all from Invitrogen, Burlington, ON, Canada) and 10% charcoal-stripped fetal calf serum (CSFCS, Hyclone, UT, USA). CWR22,[13] WISH-PC14 and WISH-PC23[14] human prostate adenocarcinomas were grown as subcutaneous xenografts in castrated and testosterone-supplemented male (CB.17–SCID BEIGE) mice within the stem and progenitor cells (SPC) colony of the Weizmann Institute of Science, Israel in compliance with institutional guidelines. Professor Eshhar (Weizmann Institute) provided frozen samples of these xenografts.

Hormonal treatments

In vitro. Cells were grown for 48–72 h in an androgen-depleted medium comprising of phenol-free medium and 10% CSFCS. The normal value for testosterone in the serum of adult males is 14–35 nM. Thus, to create an androgen-supplemented medium, testosterone (R1881; Sigma, Oakville, ON, Canada) was added to a final concentration of 10 nM. To block the effects of testosterone, the androgen receptor (AR) blocker bicalutamide (AstraZeneca, Macclesfield, Cheshire, UK) was added to a final concentration of 10 μM, mimicking the mean plasma concentration (50.2 μM) in PC patients treated with bicalutamide monotherapy (150 mg daily).[15]

In vivo. CWR22, WISH-PC14 and WISH-PC23 xenografts were grown in 7–10 week old male mice (CB.17–SCID BEIGE) that underwent bilateral orchiectomy or transplanted subcutaneously with 90-day slow-release testosterone pellets (12.5 mg per pellet; Innovative Research of America, Sarasote, FL, USA), as previously described.[16]

Inhibition of NOX

In some experiments, two different compounds were used to inhibit NOX: apocynin (Sigma, Oakville, ON, Canada) and diphenyleneiodonium (DPI, Sigma). Cells were grown for 48–72 h under different hormonal manipulations described above. In this timeframe, cells were treated for the final 24 h with either apocynin at a concentration of 200 μM or DPI at a concentration of 10 μM. As control, we used the reducing agent N-acetylcysteine (NAC, Sigma) at a concentration of 5 mM, which is a general ROS quencher.

In vitro detection of ROS

Both the nitroblue tetrazolium (NBT, Sigma) and dihydroethidium (DHE, Sigma) confocal microscopy assays were used to detect ROS, as we previously described, [8] under different hormonal manipulations with and without treatment with apocynin, DPI or NAC as described above. Briefly, cells were grown to a confluence in 96-well plates and then incubated for 90 min in PBS containing 0.1% NBT. The reduction of NBT by ROS induces a proportional change in the absorption of light at 620 nm in the medium. Results are expressed as mean ± s.d. after controlling for the meta-

bolic activity of the cells in each hormonal condition using the WST-1 assay (Roche, Mississauga, ON, Canada), by the following calculation: (Value sample–Value background)/Value of the samples' metabolic activity.

For the DHE confocal microscopy assay, cells were grown to confluence and then trypsinized and equal numbers of cells were placed on glass coverslips at a density of 10^3 cells mm^{-2}. After 24 h the cells were loaded with 10 μM DHE (Molecular Probes, Invitrogen, Burlington, ON, Canada) for 30 min at 37 °C. Cells were washed and fluorescence was measured using 488 nm argon/crypton laser. Images were analyzed using Image Pro software. Results are expressed as mean ± s.d. after controlling for the metabolic activity of the cells in each hormonal condition using the WST-1 test (Roche) by the following calculation: (Value sample-Value background)/Value of the samples' metabolic activity.

Immunoblot assays

Cells were grown to sub-confluence under the different hormonal conditions described above. After two washes with ice-cold PBS, cells were lysed in 1% Triton X-100/PBS lysis buffer supplemented with an anti-proteases and anti-phosphatases cocktail (Sigma) for 30 min at 4 °C. Following centrifuging at 12 000 g for 15 min and separation insoluble materials and supernatants were dissolved in Laemmli sample buffer. Protein purification from tumor tissues was carried using a protein extraction kit (Biochain Institute, Greenland, NH, USA). Equivalent amounts of protein (30–50 μg) were resolved by sodium dodecyl sulfate polyacrylamide electrophoresis in 8–12% gels (80 V for 20 min; 100 V for 1 h) and transferred by electroblotting (1.5 h at 100 V) to a polyvinylidene fluoride membrane. After blocking nonspecific binding using Tris-buffered saline (TBS) containing 0.05% Tween-20 (TBS-T) and 5% nonfat powdered milk, the blot was incubated with primary antibody against p22^{Phox} (1:1000, Cat #20781, Santa Cruz, CA, USA) and gp91^{Phox} (1:1000, #20782, Santa Cruz) at 4 °C overnight or at room temperature for 1 h. The membrane was then washed multiple times with TBS-T and incubated with appropriate horseradish peroxidase-conjugated secondary antibodies: goat anti-mouse or goat anti-rabbit (Sigma), both at 1:1000 dilutions for 1 h at room temperature. Protein-antibody complexes were detected with an enhanced chemiluminescence kit (Amersham, Arlington Heights, IL, USA) according to the manufacturer's recommended protocol. All quantifications of western blot analysis were carried out by reprobing the blots with 1:1000 mouse anti-β-actin antibody (Sigma) to ensure equal protein loading, followed by scanning and densitometry using Quantity-One software (Bio-Rad, Hercules, CA, USA).

Reverse transcriptase-PCR

Total RNA was isolated using the RNAspin Mini Kit (GE healthcare, Buckinghamshire, UK). cDNA was prepared from total RNA (2 μg) by reverse-transcription using superscript reverse transcriptase (Invitrogen). PCR was performed with the cDNA, TaqDNA polymerase (Promega, Madison, WI, USA) using the primers described by Vaquero et al.[12] Relative amount of cDNA

representing the transcript expression level of mRNA was analyzed following 24, 28 or 36 PCR cycles. The PCR products were separated using 3% agarose gel electrophoresis. Representative images are displayed from at least three repetitions each from three different experiments.

Clonogenic radiation assay

Plated 22Rv1 cells that were hormonally pretreated for 48 h underwent a single dose of 3 Gy γ-radiation using a 137Cs irradiator at 1 Gy/min at room temperature. Plates were then incubated at 37 °C for 14 days. Medium was replaced every 4 to 5 days. At the end of this period, colonies (defined as a cluster of greater than 50 cells) were counted after fixation and staining (methylene blue/50% methanol). Radiation survival was calculated as the plating efficiency of treated cells divided by the plating efficiency of untreated cells as previously described.[17]

Data analysis

Results are derived from at least three separate experiments and are expressed as mean ± s.d. Statistical analysis was done using paired Student's *t*-test with $P < 0.05$ considered significant.
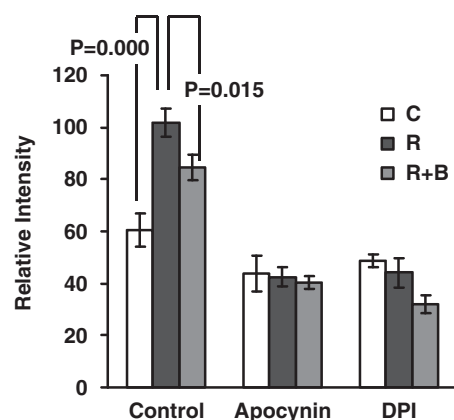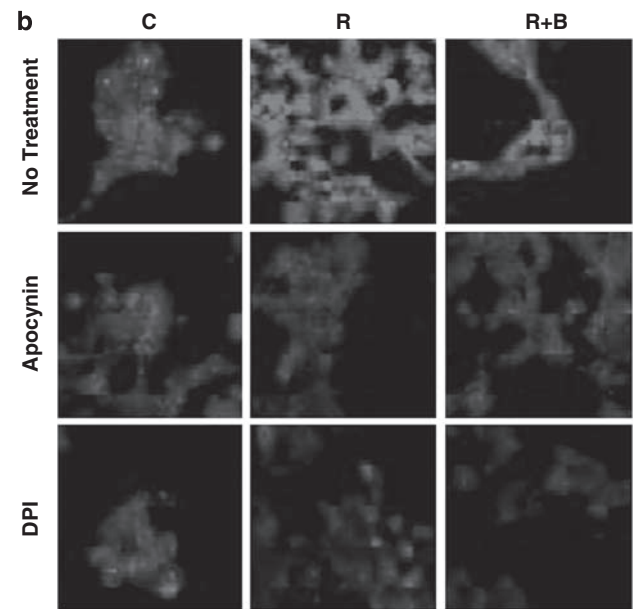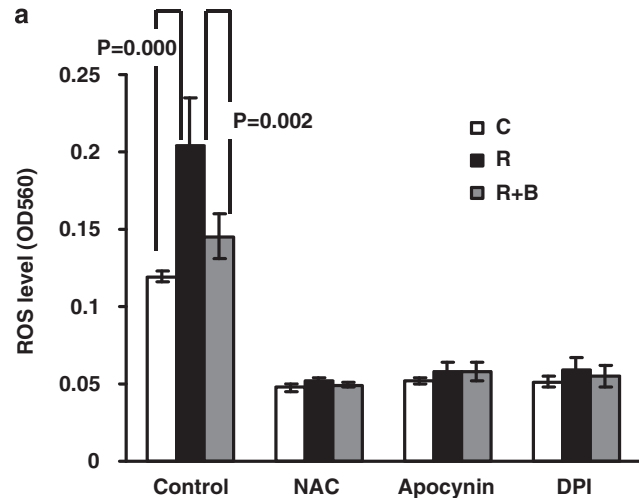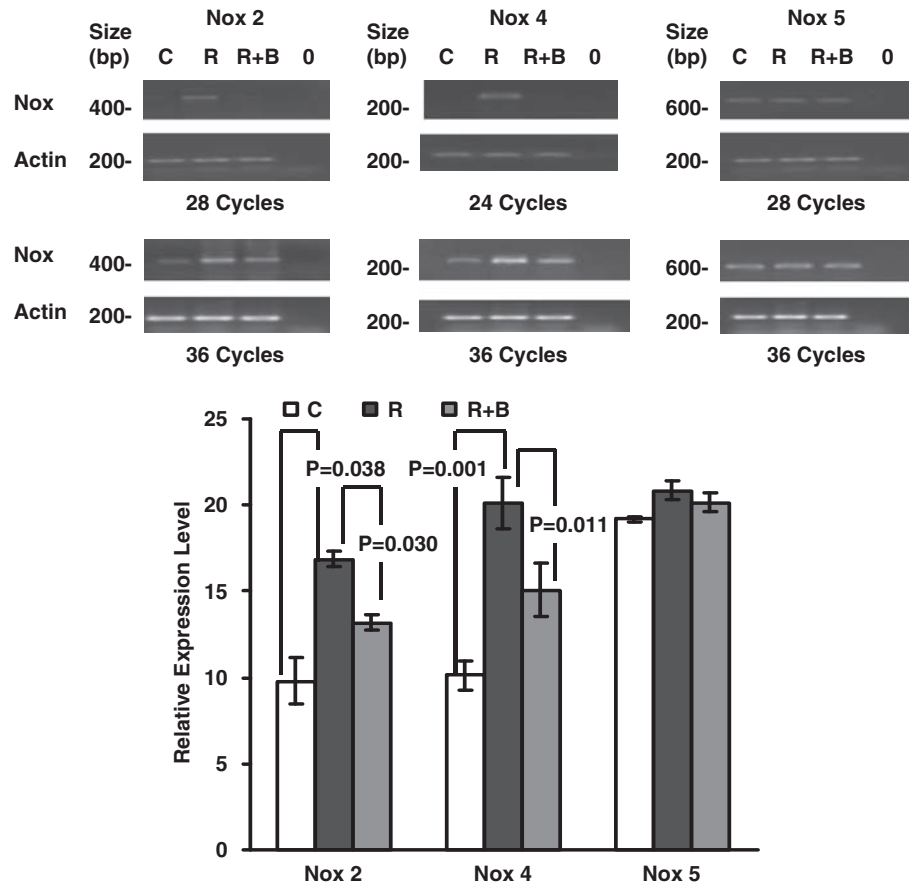
Results

Androgens induce the production of ROS by NOX

In accordance with our previous report,[8] treatment of 22Rv1 cells with physiologic levels of androgens (10^{-8} M R1881) significantly increased the levels of ROS production compared with androgen-deprived cells ($P = 0.0001$ in both the NBT and DHE assays) or cells that were treated with the combination of 10^{-8} M R1881 and 10^{-5} M bicalutamide ($P = 0.002$ and $P = 0.015$ in the NBT and DHE assays respectively) (Figure 1). To test if the effect of androgens on ROS production is mediated through NOX, we concurrently treated the cells with two structurally-unrelated inhibitors of NOX (DPI and apocynin), as well as the general antioxidant *N*-acetyl-cysteine under all hormonal conditions. This led to marked suppression of ROS production in all cases, suggesting that NOX enzymes are indeed a source of ROS production in these cells (Figure 1).

Androgens increase the expression of NOX2 and NOX4

Once we confirmed that androgens induced the production of ROS by NOX, we characterized the specific sub-types of NOX in our model. In the absence of adequate antibodies to NOX1, 3, 4 and 5 we examined the expression of NOX1–5 mRNA. We found that 22Rv1

Figure 1 Effects of androgen on reactive oxygen species (ROS) production in 22Rv1 cells is abolished by inhibitors of NADPH oxidase (NOX), diphenyleneiodonium (DPI) and apocynin, as well as the general antioxidant *N*-acetylcysteine (NAC). (**a**) Nitroblue tetrazolium (NBT) reduction assay. (**b**) Dihydroethidium (DHE) staining top- fluorescent microscopy view (×400) and bottom-calculated relative fluorescent intensity. C = androgen-depleted medium comprising phenol-free medium and 10% charcoal-stripped fetal calf serum (CSFCS). R = same medium as C + 10 nM R1881. R + B = same medium as C + 10 nM R1881 and 10 μM bicalutamide.

Figure 2 Androgens increase the mRNA levels of NOX 2 and NOX4 enzymes compared with treatment with no androgens (C), treatment with R1881 (R) or the combination of R1881 and bicalutamide (R + B). (0)− mRNA control.

cells expressed the mRNA of NOX2, NOX4 and NOX5 (Figure 2). We then examined if the transcript levels of these NOX is regulated by androgens. As shown in Figure 2, androgens could significantly increase the mRNA levels of NOX2 and NOX4 compared with treatment with no androgens ($P = 0.038$ and 0.001 for NOX2 and NOX4 respectively) or the combination of R1881 and bicalutamide ($P = 0.03$ and 0.011 for NOX2 and NOX4 respectively) This was evident even at a very low number of PCR cycles (Figure 2). In addition, we verified the effect of androgens on the protein expression of NOX2 using antibodies specific for NOX2 subunit gp91^{Phox}. Treatment with R1881 significantly increased the expression of gp91^{phox} in 22Rv1 cells compared with androgen deprivation ($P = 0.0001$) or the combination of R1881 and bicalutamide ($P = 0.002$) (Figure 3a). The androgen-regulated effect on the protein expression of gp91^{phox} was also evident *in-vivo* in three different xenograft models of human PC (CWR22, WISH-PC14 and WISH-PC23), in which the tumor bearing mice were either castrated or implanted with slow release testosterone pallets (Figure 3b).

Androgens increase the in-vitro *and* in-vivo *protein expression of p22^{phox}*
The NOX complex consists of the membrane-associated flavocytochrome b$_{588}$ protein, which is composed of gp91^{phox} and p22^{phox} and cytosolic components p47^{phox},

p67^{phox} and p40^{phox}.[10] As we showed that androgens induce the expression of NOX2 and NOX4, we wanted to examine their effect on p22^{phox}, which is essential to all the NOX family sub-members with the exception of NOX5.[10] Treatment with androgens significantly increased the expression of p22^{phox} compared with androgen deprivation ($P = 0.028$) or the combination of R1881 and bicalutamide ($P = 0.016$) (Figure 4a). The androgen-regulated effect on p22^{phox} expression was also evident *in-vivo*, confirmed using three different xenograft models of human PC (CWR22, WISH-PC14 and WISH-PC23), in which the tumor-bearing mice were either castrated or implanted with slow-release testosterone pallets (Figure 4b). These results suggest that the p22^{phox} catalytic subunit of NOX is a target for androgen regulation.

The inhibition of NOX sensitizes 22rv1 cells to radiation
Finally, we examined *in-vitro* if abolishing the increased NOX production of ROS (induced by androgens) by means of short pretreatment with apocynin can eliminate the relative radiation resistance of the cells conferred by preconditioning with R1881. We found that incubation with apocynin and R1881 fully restored the radio-sensitivity compared with incubation with R1881 alone ($P = 0.0001$). Treatment with apocynin induced even better radiosensitization than bicalutamide, although the latter was also very efficient (Figure 5). The radiation sensitization effects of NOX inhibition is likely related to
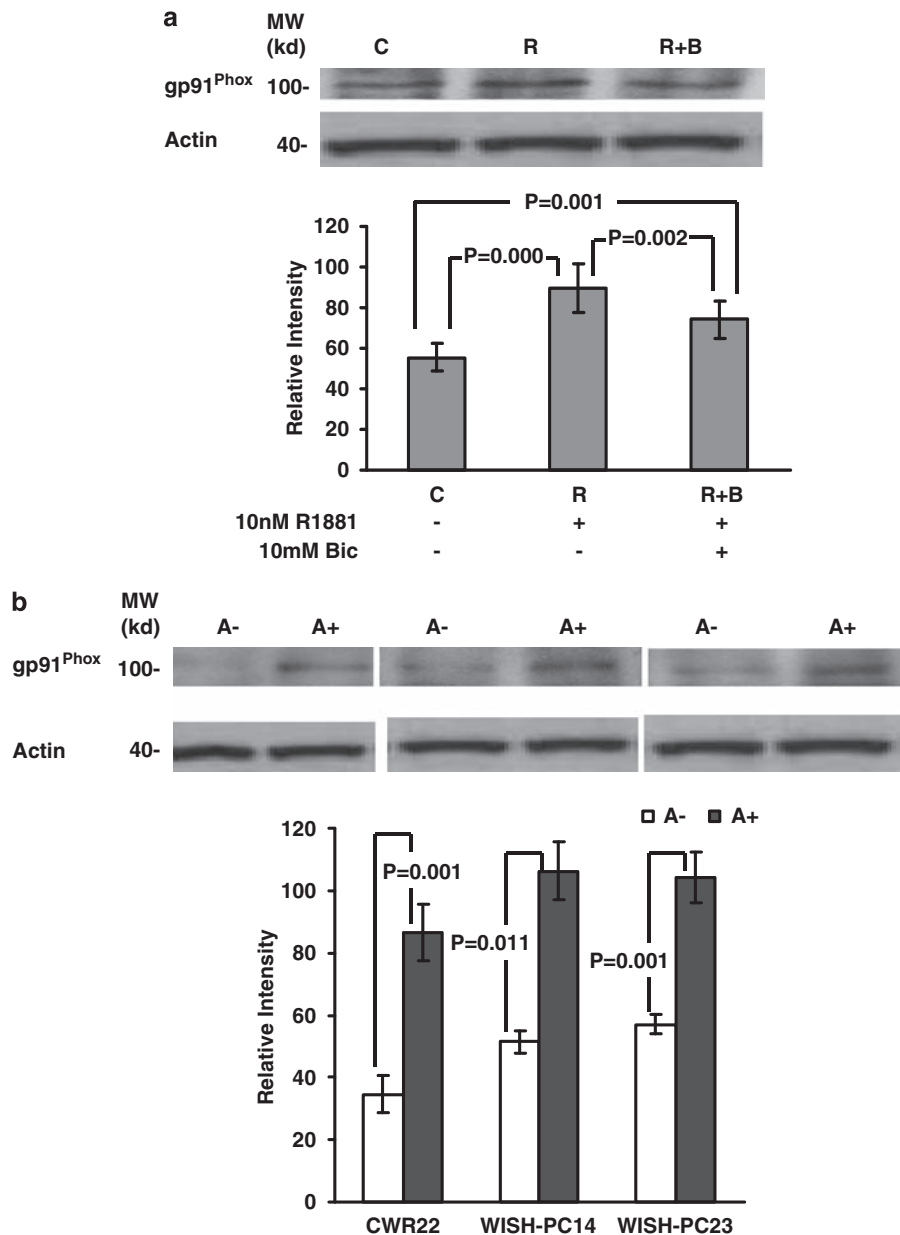
Figure 3 Androgens increase the protein expression of gp91^{phox} in human prostate cancer. (**a**) *In-vitro* (22Rv1 cells). C = androgen-depleted medium comprising phenol-free medium and 10% charcoal-stripped fetal calf serum (CSFCS). R = same medium as C + 10 nM R1881. R + B = same medium as C + 10 nM R1881 and 10 μM bicalutamide. (**b**) *In-vivo*. A + testosterone supplemented, A− castrated SCID mice.

the inhibition of basal production of ROS by NOX, as scavenging ROS using NAC, a non specific ROS scavenger, achieved a similar effect (data not shown). Collectively, these results indicate that reducing the basal levels of ROS in PC cells *in vitro*, using specific NOX inhibitors, can sensitize the cells to radiation across all hormonal conditions and may thus, circumvent the need for androgen deprivation as a means to enhance the tumor response to radiation.

Discussion

The results presented here show that androgens can stimulate NOX to produce ROS in prostate cancer cells. In accordance with the model we previously proposed,[8]

these elevated ROS levels induce adaptation to radiation. Thus, by applying ADT or inhibition of NOX, the sensitivity of androgen-stimulated PC cells to radiation can be restored (Figure 6). As shown here, one of the major sources of ROS generation in PC cells is the NOX system. Accordingly, inhibition of NOX using apocynin increases the response of PC cells to radiation (Figure 5).

Several studies have shown a variable profile of NOX expression in PC. The expression of NOX2, NOX4 and NOX5 by the 22Rv1 human PC cells used in our model is in line with the findings of Kumar *et al.*,[9] who showed an increased expression of NOX2, NOX4 and NOX5 mRNA in PC3, LnCaP and DU145 human PC cells, and linked it to their proliferative and survival capacity. Other studies have also characterized the expression of NOX *in-vitro* in PC and their functional significance. Thus, Brar *et al.*[18]
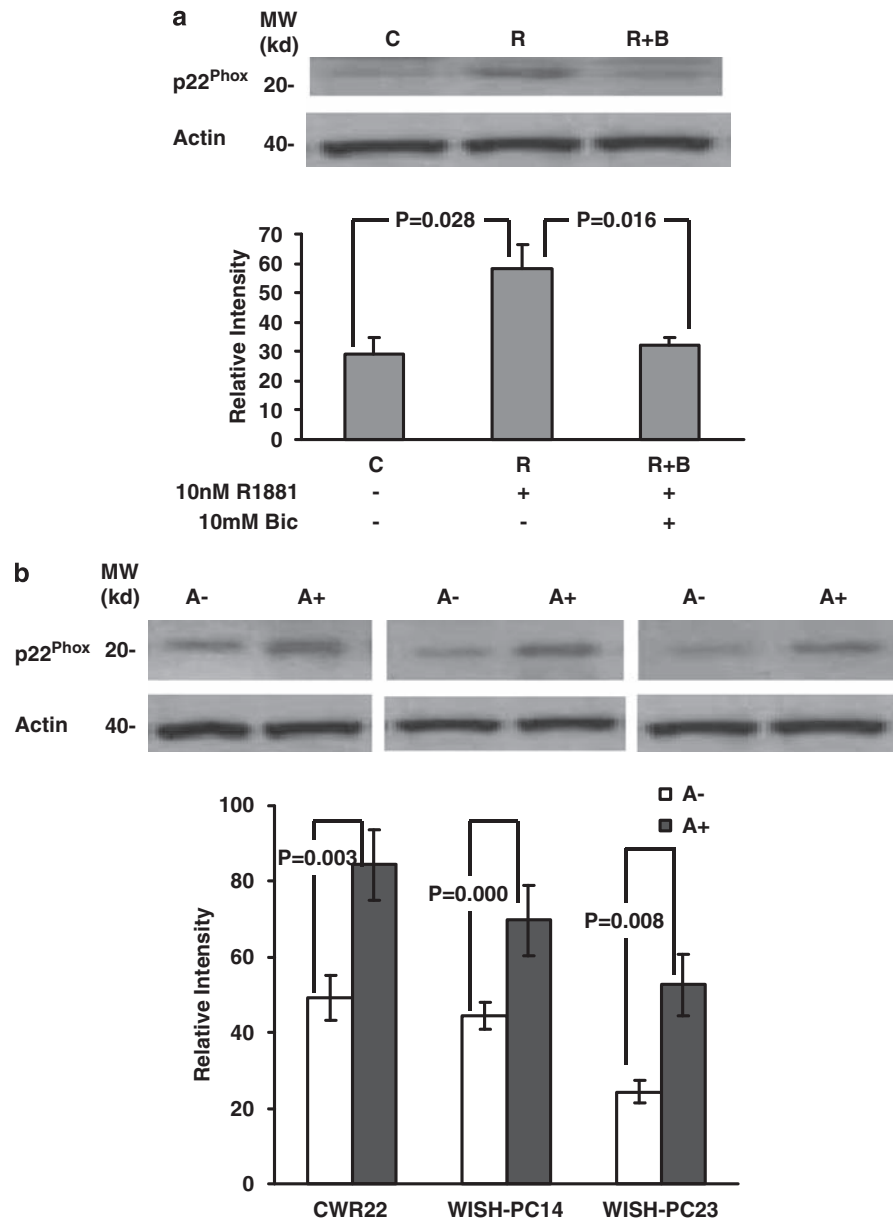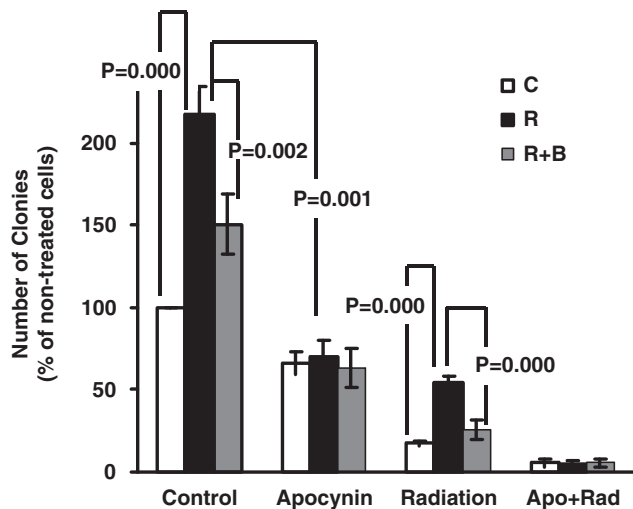
Figure 4 Androgens increase the protein expression of p22[phox] in human prostate cancer. (**a**) *In-vitro* (22Rv1 cells). C = androgen-depleted medium comprising phenol-free medium and 10% charcoal-stripped fetal calf serum (CSFCS). R = same medium as C + 10 nM R1881. R + B = same medium as C + 10 nM R1881 and 10 μM bicalutamide. (**b**) *In-vivo*. A + testosterone supplemented, A- castrated SCID mice.

showed that NOX5 regulates growth and apoptosis in DU145 cells. NOX1 has been shown to be associated with PC[19] and as a potent trigger of angiogenesis in DU145 xenograft model.[20] Tam *et al.*[21] showed hormonal regulation of NOX in the normal ventral rat prostate. In their model, castration up-regulated NOX1, NOX2 and NOX4. Nevertheless, to the best of our knowledge the hormonal effects of androgens and androgen deprivation on the expression of NOX and its ability to generate ROS have not been tested in the human prostate, in general and in PC, in particular. Using the 22Rv1 human androgen-responsive but not dependent cell line, we could document that the mRNA expression of NOX2 and NOX4, but not NOX5, was up-regulated by androgens and downregulated by lack of androgens in the culture media or with the concurrent use of

androgens and androgen-receptor blockers (Figure 2). In addition, the protein expression of NOX2 (gp91[phox]) was increased by androgens and decreased by ADT, both *in-vitro* and *in-vivo* (Figure 3). In the absence of available anti-NOX4 antibodies, we could not directly test the effect of androgens and ADT on NOX4 protein expression. Nevertheless, we could do so for gp22[phox]— an essential catalytic subunit of NOX2, and show that it is regulated by androgens both *in-vitro* and *in-vivo* (Figure 4).

There are several possibilities to explain how androgens induce expression of NOX enzymes. The gene expression of these enzymes may be directly regulated by androgens, although in the rat NOX2 and NOX4 genes do not contain androgen-responsive elements.[21] Alternatively, androgens can induce the expression of

Figure 5 The effect of apocynin on the *in-vitro* response of 22Rv1 cells to a single dose of 3 Gy radiation. C = androgen-depleted medium comprising phenol-free medium and 10% charcoal-stripped fetal calf serum (CSFCS). R = Same medium as C + 10 nM R1881. R + B = same medium as C + 10 nM R1881 and 10 μM bicalutamide.
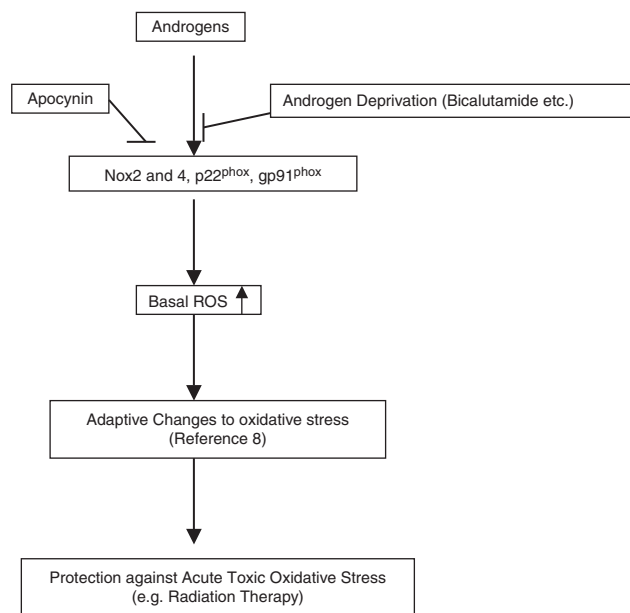
Figure 6 Proposed model for the reversal of androgen-driven adaptation to radiation therapy in prostate cancer cells by apocynin or androgen deprivation therapy. Androgens increase the expression of NOX2, NOX4, p22phox and gp91phox resulting in increased basal production of reactive oxygen species (ROS). The cells in response, develop adaptive molecular machinery to protect from ROS accumulation, which leads to relative resistance to the toxic oxidative stress induced by radiation (as describe in details in reference 8). Inhibition of ROS production by NOX2 and NOX4 using apocynin or androgen deprivation therapy, reduces the basal ROS levels and prevents adaptive changes to oxidative stress, thus sensitizing the cells to the toxic oxidative effects of radiation.

NOX indirectly. One of the potential mediators could be nuclear factor-κB, whose expression levels are regulated by androgens.[22] Nuclear factor-κB have been shown to increase NOX expression.[23] It is interesting to

note that, the positive effect of androgens on NOX activity showed in this study may be unique to PC cells, as Juliet et al.[24] showed that testosterone actually inhibit superoxide production in macrophages, potentially through an inhibitory effect on the expression of the cytosolic subunits of NOX- p67phox and p47phox. In this study we have shown that androgen can increase the basal ROS levels through NOX2 and NOX4, an effect that is blocked by apocynin or androgen receptor blockade. Unlike severe or excessive accumulation of ROS that triggers a pro-apoptotic toxic event in cancer cells,[25,26] reducing the basal ROS levels by means of apocynin or ADT reset the adaptive molecular machinery of the cells rendering it much more vulnerable to toxic oxidative stress as induced by radiation therapy (8 and Figure 6)

The potential future clinical application of apocynin as a radiation sensitizer is appealing. It is a natural organic compound that acts as a NOX inhibitor by preventing the assembly of its subunits.[27,28] Accordingly, it has been shown to be effective in preventing the production of the superoxide in human white blood cells.[29] It does not, however, obstruct the phagocytic or other defense roles of granulocytes including intracellular killing,[26] and so may be widely used as an inhibitor of NOX.[30,31] Obviously, preclinical *in-vivo* studies are needed before the use of apocynin as an alternative to ADT for radiation sensitization in PC can be explored in clinical trails, but our findings establish a proof-of-principle for this concept.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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