Androgen Induces Adaptation to Oxidative Stress in Prostate Cancer: Implications for Treatment with Radiation Therapy[1]

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Abstract

Radiation therapy is a standard treatment for prostate cancer (PC). The postulated mechanism of action for radiation therapy is the generation of reactive oxygen species (ROS). Adjuvant androgen deprivation (AD) therapy has been shown to confer a survival advantage over radiation alone in high-risk localized PC. However, the mechanism of this interaction is unclear. We hypothesize that androgens modify the radioresponsiveness of PC through the regulation of cellular oxidative homeostasis. Using androgen receptor (AR)$^+$ 22rv1 and AR$^-$ PC3 human PC cell lines, we demonstrated that testosterone increased basal reactive oxygen species (bROS) levels, resulting in dose-dependent activation of phospho-p38 and pAKT, and increased expression of clusterin, catalase, and manganese superoxide dismutase. Similar data were obtained in three human PC xenografts; WISH-PC14, WISH-PC23, and CWR22, growing in testosterone-supplemented or castrated SCID mice. These effects were reversible through AD or through incubation with a reducing agent. Moreover, testosterone increased the activity of catalase, superoxide dismutases, and glutathione reductase. Consequently, AD significantly facilitated the response of AR$^+$ cells to oxidative stress challenge. Thus, testosterone induces a preset cellular adaptation to radiation through the generation of elevated bROS, which is modified by AD. These findings provide a rational for combined hormonal and radiation therapy for localized PC.

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Introduction

In 2005, there were an estimated 234,460 new cases of prostate cancer (PC) diagnosed in the United States, and 27,350 men succumbed to this disease [1]. Widespread prostate-specific antigen (PSA) screening has led to earlier diagnosis of PC. Radiation is a common viable treatment option for localized PC. However, substantial heterogeneity in patient response to radiation exists. Using the current regimen of high-dose conformal radiation, treatment failure occurs in 45% of patients with locally confined disease [2]. It is likely that, in addition to individual patient risk factors, intrinsic differences in cellular radiosensitivity exist, explaining the diversity in responses. Escalating the dose of radiation to ultrahigh levels is one experimental approach used to overcome treatment failure. Although treatment outcome directly correlates with risk factors such as PSA, Gleason score, and clinical stage, radiation dose escalation improves cancer control even for intermediate-risk to high-risk patients [3]. However, results are still unsatisfactory, and an increased rate of rectal toxicity has been reported [3,4].

Combining radiation therapy and androgen deprivation (AD) therapy is another tactic used to combat treatment failure. Several phase 3 randomized controlled trials demonstrated an overall survival benefit for patients with locally advanced disease who received combined radiation and AD compared to patients who received radiation alone [5–7]. Nevertheless, the specific contribution of AD therapy in the majority of intermediate-risk PC patients remains controversial [8]. From a clinical perspective, there is concern that the net benefit of the combined approach stems mainly from hormonal effects *per se*. It is also unknown whether AD therapy obviates the need for dose escalation or whether it is even necessary when higher radiation doses are used. Unfortunately, adequately powered trials, which are designed to address these issues, have not been reported. The potential of AD therapy for cytoreduction and control of micrometastatic disease has been postulated [9]. *In vivo* studies using androgen-sensitive Shionogi tumors illustrate that overall cell killing increases when AD is

Abbreviations: 4-HNE, 4-hydroxynoneal; 8-OHdG, 8-hydroxy-2′-deoxyguanosine; AD, androgen deprivation; AR, androgen receptor; bROS, basal reactive oxygen species; CSFCS, charcoal-stripped fetal calf serum; DHE, dihydroethidium; GR, glutathione reductase; MnSOD, manganese superoxide dismutase; NAC, *N*-acetylcysteine; PC, prostate cancer; PSA, prostate-specific antigen; SOD, superoxide dismutase

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added to radiation [10]. *In vitro*, androgens were shown to block apoptosis induced by the combination of Fas antibodies or tumor necrosis factor α (TNFα) and irradiation in LNCaP PC cells [11]. Furthermore, AD therapy may facilitate radiation through its antiangiogenic effect [12] and through the reduction of HIF-1α levels [13], which has been shown experimentally to increase *in vitro* radiosensitivity in HCT116 and PC3 tumors [14].

In this study, we examined a novel mechanism by which androgens may play a role in the therapeutic effect of radiation on PC. We examined cellular sensitivity to oxidative stress challenge in the presence and in the absence of androgens.

The major therapeutic feature of radiation is the induction of toxic oxidative damage in targeted cancer cells. Reactive oxygen species (ROS) are generated from cellular water by high-energy deposition during radiation. These free radicals mediate multilevel cell injury: disruption of plasma, mitochondrial, nuclear, and endoplasmic reticulum membranes by lipid peroxidation, and induction of protein cross links, leading to increased membrane permeability and loss of function. ROS oxidize amino acid residues (mainly cysteine), altering normal protein function and degradation processes. Most importantly, ROS oxidize mitochondrial and nuclear DNA, resulting in strand breaks. However, ROS are formed constantly as byproducts of normal enzymatic metabolic reactions. Thus, to prevent overwhelming oxidative damage, cells maintain a basal redox balance between prooxidative and antioxidative reactions.

Despite numerous studies on androgens and PC, there have been few reports on the effects of AD on oxidative stress in PC cells. Ripple et al. [15] demonstrated that physiological levels of androgens are capable of increasing oxidative stress in LNCaP cells, and they suggested that this is partly due to increased mitochondrial activity. Interestingly, Sun et al. [16] postulated that PSA, rather than testosterone, is the factor responsible for the effect of testosterone on ROS generation because the androgen receptor (AR) blocker flutamide, as well as anti-PSA antibody, can block the effect. These studies have not been reported using PC patient samples.

Serum testosterone levels in the aging PC patient population widely vary. From the fourth decade, a period believed to be related to PC initiation and progression [17], circulating testosterone levels start to drop significantly [18]. It has been shown that androgen levels affect individual tumor phenotypes differently [19]. This finding may indicate that a patient's own androgenic milieu models his individual tumor features and response to therapy.

In this study, we examined the hypothesis that AD sensitizes PC cells to oxidative stress, thereby enhancing radiation response. We demonstrate that, in the presence of androgens, AR-expressing human PC cells acquire relative resistance to oxidative stress. This adaptation process stems from the fact that androgens increase basal reactive oxygen species (bROS) levels. In turn, this stimulates the activation and the expression of stress molecules and antioxidative enzymes to better cope with the shift in redox balance. Then, on subsequent challenges with oxidative stress–based therapies, such as radiation or photodynamic therapy, these cells become less sensitive to toxic levels of ROS compared to androgen-deprived cells and are, therefore, less susceptible to treatment.

Materials and Methods

Cell Lines and Xenografts

Both 22rv1 and PC3 human PC cell lines were purchased from the American Type Culture Collection (Manassas, VA) and cultured at 37°C in a humidified atmosphere under 5% CO_2 in air. The 22rv1 culture medium consisted of phenol red-free RPMI 1640 with 2 mM L-glutamine adjusted to contain 1.5 g/l sodium bicarbonate, 4.5 g/l glucose, 10 mM HEPES, 1.0 mM sodium pyruvate, and 10% charcoal-stripped fetal calf serum (CSFCS). PC3 culture medium was composed of phenol red-free DMEM with 2 mM L-glutamine adjusted to contain 1.5 g/l sodium bicarbonate and 10% CSFCS. CWR22 [20], WISH-PC14, and WISH-PC23 [21] human prostate adenocarcinomas were grown as subcutaneous xenografts in SCID mice within the SPC colony of the Weizmann Institute of Science, in compliance with institutional guidelines. Prof. Eshhar (Weizmann Institute of Science) provided paraffin blocks of LuCAP-35 xenografts grown in castrated and testosterone-supplemented male SCID mice [22].

Hormonal Treatments

In vitro Cells were grown in an androgen-depleted medium composed of phenol-free medium and 10% CSFCS. The normal values for testosterone in the serum of adult males are 14 to 35 nM. Thus, testosterone (R1881; Sigma, St. Louis, MO) was added to a final concentration of 10^{-10} to 10^{-8} M to create an androgen-supplemented medium. To block the effects of testosterone, the AR blocker bicalutamide (AstraZeneca, Macclesfield Cheshire, UK) was added to a final concentration of 10^{-5} M, mimicking the mean plasma concentration (50.2 μM) in PC patients treated with bicalutamide monotherapy (150 mg daily) [23]. In several experiments, 10^{-5} M bicalutamide was added to the androgen-depleted medium to exclude the effects of the drug itself. To induce different hormonal effects, cells were preincubated in these media for 48 to 72 hours before further manipulation.

In vivo To induce AD or androgen supplementation, tumors were grown in 7- to 10-week-old male mice (CB.17-SCID BEIGE) that underwent bilateral orchiectomy or that were transplanted subcutaneously with 90-day slow-release testosterone pellets (12.5 mg/pellet; Innovative Research of America, Sarasote, FL), as previously described [24].

Measurement of Global Metabolic Activity

WST-1 assay (Roche, Quebec, Canada) was used to assess cellular metabolic activity according to the manufacturer's instructions.

Detection of bROS In Vitro

We used two methods for the detection of bROS.

Nitroblue tetrazolium (NBT) assay Cells were grown to confluence in 96-well plates. R1881 and/or bicalutamide was added 48 hours before the experiments. ROS production was detected by NBT assay (Sigma). NBT is a yellow water-soluble powder that, on reduction by ROS, changes to dark-blue insoluble formazan. Cells were incubated for 90 minutes in phosphate-buffered saline (PBS) containing 0.1% NBT. Formazan was dissolved by sonication in 50% acetic acid, and optical absorbance was determined at 560 nm. After the subtraction of assay blanks, the results were normalized to the metabolic activity of cells in each hormonal condition, as determined by WST-1 assay.

Confocal microscopy 22rv1 cells were grown in a cell culture for 72 hours under different hormonal conditions, as specified above. The cells were then either incubated or not incubated with 5 mM NAC (Sigma) for 3 hours, followed by PBS wash and trypsinization. Equal numbers of cells were then placed onto glass cover slips at a density of 10^3 cells/ml. After 24 hours, the cells were loaded with 5 μM dihydroethidium (DHE; Molecular Probes, Burlington, Ontario, Canada) for 30 minutes at 37°C. After two PBS washes, fresh medium was added. DHE is oxidized by ROS to yield ethidium, the fluorescence of which was measured with a Zeiss SM510 confocal fluorescence microscope (Carl Zeiss, Toronto, Ontario, Canada; excitation = 475 nm, emission = 610 nm). Images were analyzed with Image Pro software (Media Cybernetics, Silver Spring, MD). The results were expressed as mean ±1 SD after background subtraction and normalization to metabolic activity, as above.

Immunoblot assays Cells were grown to subconfluence under the different hormonal conditions described above. When indicated, cells were preincubated with H_2O_2 at 1 mM for 30 minutes. After two washes with ice-cold PBS, cells were lysed in a 1% Triton X-100/PBS lysis buffer supplemented with antiproteases and antiphosphatases cocktail (Sigma) for 30 minutes at 4°C. Centrifuging at 12,000*g* for 15 minutes separated insoluble materials. Supernatants were dissolved in Laemmli sample buffer. Protein purification from tumor tissues was performed using a protein extraction kit (Biochain Institute, Inc., Greenland, NH). Equivalent amounts of protein (30–50 μg) were resolved by sodium dodecyl sulfate–polyacrylamide electrophoresis in 8% to 12% gels (80 V for 20 minutes; 100 V for 1 hour) and transferred by electroblotting (1.5 hours at 100 V) to a PVDF 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 p38 (1:1000; Sigma); phospho-p38 (pp38) (1:1000; Sigma); phospho-AKT(ser473) (1:1000; Cell Signaling, Danvers, MA); anti-global AKT (1:1000; Cell Signaling); mouse anti–4-hydroxynoneal (4-HNE) (1:10; Oxis, Inc., Foster City, CA); rabbit anti-catalase (1:2000; Chemicon International, Temecula, CA); rabbit anti–manganese superoxide dismutase (MnSOD), DD-17 (1:1000; Sigma); rabbit anti-HSP27, H-77 (1:250; Santa Cruz Biotechnology, Santa Cruz, CA); mouse anti-HSP70 (1:1000; Santa Cruz Biotechnology); mouse anti-HSP90 (1:1000; StressGen Biotechnologies, Ann Arbor, MI); and rabbit anti-clusterin, H-330 (1:100; Santa Cruz Biotechnology) in TBS–5% bovine serum albumin (BSA) at 4°C overnight or at room temperature for 1 hour. 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 dilution for 1 hour at room temperature. Protein–antibody complexes were detected with an enhanced chemiluminescence kit (Amersham, Arlington Heights, IL) according to the manufacturer's recommended protocol. All quantifications of Western blot analysis were performed by reprobing the blots with 1:1000 mouse anti–β-actin antibodies (Sigma) to ensure equal protein loading, followed by scanning and densitometry using Quantity-One software (Bio-Rad, Hercules, CA).

Neutral Red Viability Assay

Cells were seeded in triplicate in 96-well plates. After 24 hours of treatment with H_2O_2 at different concentrations (200–1500 μM), the cells were incubated for 2 hours with neutral red (Sigma) diluted to a final concentration of 40 μg/ml in the medium. Cells were rinsed with fixation solution consisting of 0.5% formaldehyde and 0.1% $CaCl_2$. After a quick wash with PBS, the cells were treated with extraction solution (1% glacial acetic acid and 50% ethanol) for 10 minutes at room temperature. Absorbance was measured by a scanning multiwell spectrophotometer ELISA reader (Tecan Systems, San Jose, CA) using an excitation of 570 nm. After the subtraction of assay blanks, the results for each hormonal condition were expressed as the mean ± 1 SD of the H_2O_2 concentration that induced 10%, 20%, or 30% cell deaths relative to cells that were not incubated with H_2O_2 (for each hormonal condition).

Clonogenic Radiation Assay

Clonogenic radiation assay (0–4 Gy) with 22rv1 cells that were hormonally pretreated for 48 hours was performed as previously described [25].

pAKT and p38 Activity Assays

pAKT and pp38 kinase activities were determined using specific nonradioactive kits (Cell Signaling) according to the manufacturer's instructions. Briefly, whole-cell lysates were immunoprecipitated overnight at 4°C with immobilized anti-AKT or anti-pp38 primary antibodies. For pAKT kinase assay, cell lysates/immobilized antibodies were incubated for 30 minutes at 30°C with ATP/GSK-3 fusion protein. For p38 kinase assay, cell lysates/immobilized antibodies were incubated for 30 minutes at 30°C with ATP/ATF-2 fusion protein. Reactions were terminated by adding sodium dodecyl sulfate sample buffer and by heating to 95°C for 5 minutes. Samples were then subjected to Western blot analysis, as described above. Blots were developed using anti–phospho-GSK-3 α/β primary antibody to determine pAKT activity and using anti–phospho-ATF-2 primary antibody to determine pp38 activity.

Immunohistochemistry

Paraffin-embedded formalin-fixed human PC xenograft samples from testosterone-supplemented or castrated SCID mice (WISH-PC14, WISH-PC23, and LuCAP-35) were pretreated with proteinase K (DakoCytomation, Glostrup, Denmark) for 5 minutes at room temperature and washed with PBS-T. Samples were blocked in 10% normal rabbit serum (Sigma-Aldrich, St. Louis, MO) diluted in 2% BSA-T for 30 minutes before 1 hour of incubation with goat anti–8-hydroxy-2′-deoxyguanosine (8-OHdG) antibodies (1:200; Chemicon International). Detection was performed with biotinylated rabbit anti-goat IgG antibodies (1:500; Vector Laboratories, Burlingame, CA), followed by the suppression of endogenous peroxidase activity with 30% H_2O_2 solution, wash with PBS-T (×2), incubation with ABC reagent (Vector Laboratories), and development with Vector NovaRED Substrate Kit (Vector Laboratories). Slides were counterstained with hematoxylin and eosin (Sigma-Aldrich).

Superoxide Dismutase (SOD) Activity Assay

SOD activity was measured with a commercial assay (BIOXYTECH SOD525; Oxis, Inc.). Cell lysates from 22rv1 cells growing for 72 hours in 75-cm^2 flasks in the presence of 10^{-8} M R1881 with or without 10^{-5} M bicalutamide or without hormonal manipulation (control) were prepared without using proteinase inhibitors in the lysate buffer and were then subjected to the assay in accordance with the manufacturer's protocol.

Catalase Activity Assay

Lysates from 22rv1 cells growing in 75-cm^2 flasks in the presence of 10^{-8} M R1881 with or without 10^{-5} M bicalutamide or without hormonal manipulation (control) for 72 hours were tested for catalase activity with a commercial kit, in accordance with the manufacturer's instructions (Cayman Chemicals, Ann Arbor, MI).

Glutathione Reductase (GR) Activity Assay

GR activity was analyzed by measuring the rate of NADPH oxidation to $NADp^+$, accompanied by a change in absorbance at 340 nm proportional to GR activity. A commercial GR activity kit (Cayman Chemicals) was used according to the manufacturer's instructions.

Global Antioxidative Capacity Assays

The total antioxidative potential of 22rv1 cells following 72 hours of preconditioning under different hormonal manipulation was examined using two calorimetric assays according to the manufacturer's instructions. BIOXYTECH AOP-490 assay (Oxis, Inc.) measures the reduction of Cu^{2+} to Cu^+ by the combined action of all antioxidants present in the sample, whereas ImAnOx assay (ALPCO Diagnostics, Salem, NH) measures the reaction of antioxidants in the sample with a defined amount of exogenously provided H_2O_2, with residual H_2O_2 depending inversely on antioxidative capacity. All measurements were then normalized to the metabolic activity of individual samples using WST-1 assay to control for potential bias stemming from the difference in metabolic activity caused by different hormonal stimulations.

Data Analysis

Normally distributed data were analyzed using analysis of variance (ANOVA). When needed, Box-Cox transformation was used to reach normality. Differences between cells treated with androgen supplementation and cells treated with AD or controls were tested by two-sided Student's *t* test and Dunnett's test at the 5% significance level. One-way ANOVA with "Cell" factor was employed for NBT and DHE data. Two-way ANOVA with "Cell" and "Treatment" factors and Cell × Treatment interaction term was employed for neutral red viability experiments and clonogenic survival of data. These results were confirmed by secondary nonparametric analysis using Mann-Whitney test (CT = control cells growing in a culture medium with CSFCS and without phenol red; T = cells growing in the same medium but with the addition of 10 nM R1881; T + B = cells growing in the same medium but with the addition of both 10 nM R1881 and 10 μM bicalutamide; results are derived from at least three experiments and are expressed as mean ± SD).

Results

Effect of AD on Oxidative Stress Response

To avoid bias stemming from the removal of essential growth factor, 22rv1 human PC cells that express the AR and manifest androgen responsiveness without growth dependency were selected as prototypes for all our *in vitro* experiments. We first attempted to test whether androgen supplementation (within the serum range of adult males, ~ 10^{-8} M) have a protective effect against oxidative stress challenge. We initially challenged the cells directly with toxic levels of H_2O_2 and determined their survival using neutral red viability assay. Following 24 hours of incubation with H_2O_2 at different doses (200–1500 μM), 22rv1 cells preconditioned with 10^{-8} M R1881 were found to be significantly more resistant than control cells grown in the absence of R1881 ($P < .05$) (Figure 1*A*). The addition of bicalutamide to the conditioned medium partially restored sensitivity ($P < .05$).

We then exposed the cells to γ-radiation using a single dose of 1 to 4 Gy, representative of the 1.8- to 2-Gy daily clinical fraction given during curative radiation. Except for the 1-Gy radiation dose, preconditioning with R1881 induced a significant protective effect ($P < .001$) (Figure 1*B*). Again, the addition of bicalutamide significantly restored the radiosensitivity of cells grown in the presence of R1881 ($P < .001$).

These results suggest that the relative resistance to oxidative challenge that androgens conferred in 22rv1 cells is induced through the AR because coincubation with both R1881 and bicalutamide could reverse the effect.

Effect of Androgens on bROS

To determine the primary effect of androgens on the generation of ROS, we first measured ROS levels in 22rv1

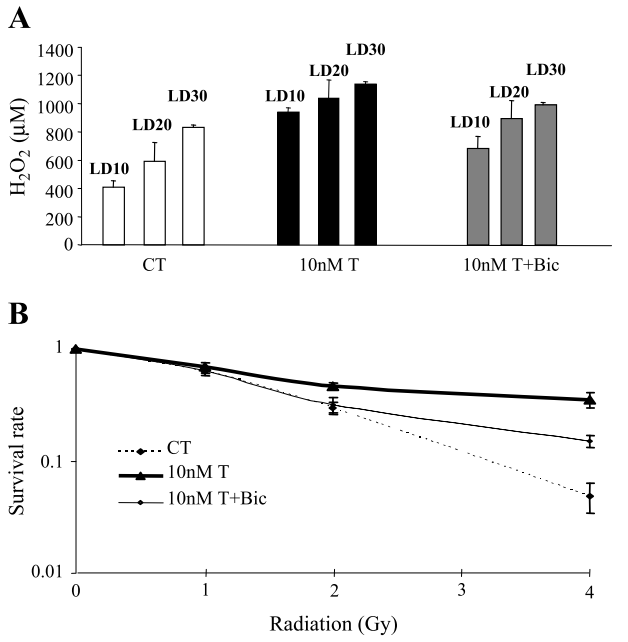

Figure 1. *Androgens induce relative resistance to oxidative stress challenge in 22rv1 human PC cells that can be reversed by AD. (A) Survival of 22rv1 cells in response to 24 hours of incubation with increasing doses of hydrogen peroxide, as determined by neutral red viability assay. LD = lethal dose. (B) Survival of 22rv1 cells in response to γ-radiation, as determined by colony formation assay. CT = control cells growing in a culture medium with CSFCS and without phenol red; T = cells growing in the same medium but with the addition of 10 nM R1881; T + Bic = cells growing in the same medium but with the addition of both 10 nM R1881 and 10 μM bicalutamide. Results are derived from at least three experiments and are expressed as mean ± SD.*

cells treated with increasing doses of R1881. To avoid potential misinterpretation of the net effects of androgens on the production of ROS, as androgens act as prime growth factors in androgen-responsive and androgen-dependent PC cells (hence inducing metabolic activity higher than that of androgen-deprived cells), ROS measurements were normalized to the metabolic activity of cells under different hormonal manipulations. We found that treatment of 22rv1 cells with R1881 increased bROS levels in a dose-dependent manner (Figure 2*A*). Bicalutamide could significantly block this phenomenon at all levels of R1881 supplementation used ($P < .05$). The highest levels of bROS were recorded in cells supplemented with 10^{-8} M R1881, equivalent to the serum range of adult males.

We then elected to use 10^{-8} M R1881 to further test whether the increased induction of bROS following androgen stimulation is AR-mediated. It was found that R1881 could increase bROS only in PC cells expressing the AR. This was demonstrated by two assays: NBT reduction (Figure 2*B*) and fluorescent staining with DHE that is oxidized by ROS to yield fluorescence (Figure 2*C*). R1881 had no effect on bROS levels in PC3 cells not expressing the AR (Figure 2*B*). Moreover, the addition of bicalutamide to R1881 significantly reduced ($P = .01$) the bROS level (Figure 2, *B* and *C*) in 22rv1 cells to an extent similar to that obtained in cells not treated with R1881 or in cells treated with bicalutamide only (Figure 2*C*). Collectively, these results demonstrate that androgens induce elevated levels of bROS in an AR-dependent manner, independent of increased metabolic rate. Androgen depletion of the culture medium prevents increased bROS levels, whereas AR blockade (bicalutamide) can reverse this phenomenon in cells supplemented with R1881.

To further validate that the increase in bROS is androgen-regulated not only *in vitro* and in a single model, we confirmed *in vivo* that the androgenic milieu of the host dictates the basal redox status, using three models of human PC. This

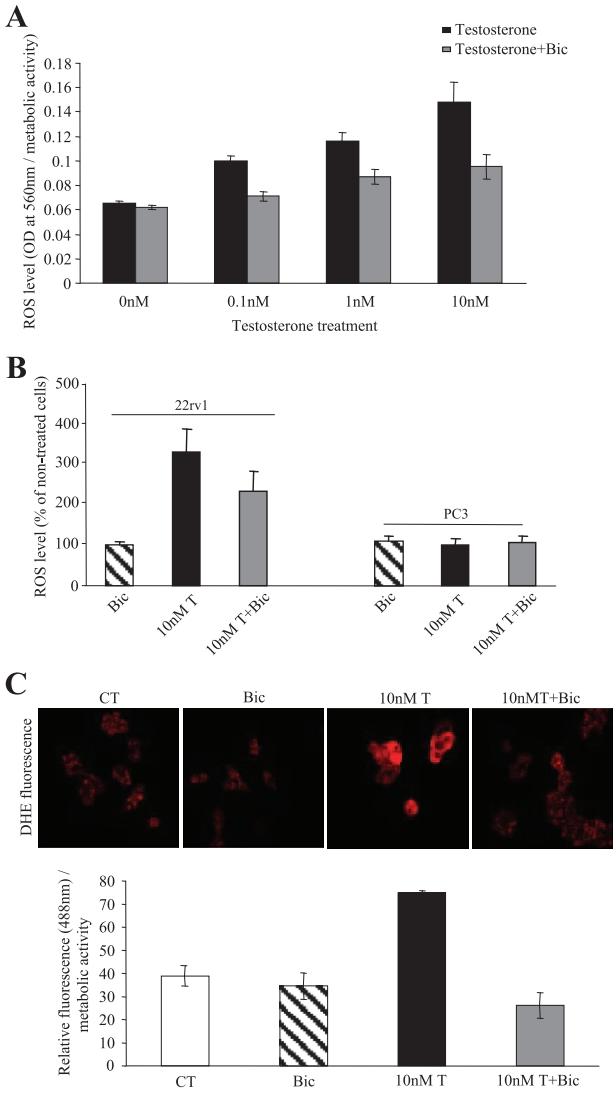

Figure 2. *Androgens increase bROS levels in an AR-dependent manner. (A) Dose-dependent effect of R1881 on ROS levels in 22rv1 cells, as measured by NBT reduction assay. Addition of 10 μM bicalutamide antagonizes this effect. (B) The androgen-induced increase in bROS, as measured by NBT reduction assay, is evident only in PC cells expressing the AR (22rv1), and not in PC3 cells not expressing the AR. (C) Upper figure: DHE fluorescence measurements (representative microscopic view of the DHE fluorescence of 22rv1 cells in the presence and in the absence of R1881 and/or bicalutamide). Lower figure: Quantification of bROS levels (as measured by confocal fluorescent microscopy) following normalization to cells' metabolic activity (measured using WST-1 assay) under different hormonal manipulations. CT = control cells growing in a culture medium with CSFCS and without phenol red; T = cells growing in the same medium but with the addition of 10 nM R1881; T + Bic = cells growing in the same medium but with the addition of both 10 nM R1881 and 10 μM bicalutamide. Results are derived from at least three experiments and are expressed as mean ± SD.*

showed that tumors grown in the absence of endogenous or exogenous androgens had a reduced expression of peroxidation-induced 4-HNE protein adducts compared to tumors growing in the presence of continuous testosterone supplementation (Figure 3*A*). These adducts are formed as a result of covalent links with highly reactive aldehydes such as 4-HNE that are generated by lipid peroxidation of polyunsaturated fatty acids, correlating with the oxidative status of the cells. Similarly, human PC xenografts growing in male SCID mice supplemented with testosterone had intense staining for 8-OHdG compared to the same tumors growing in castrated animals (Figure 3*B*). This staining identifies modified DNA formed by attacks of hydroxyl radicals.

Effect of Androgens on the Expression and Activity of Stress-Related Molecules

The adaptive response of cells to oxidative stress, in general, and to radiation, in particular, involves the induction of a set of proteins and signaling molecules that help combat further challenge [26]. Therefore, we elected to examine the effects of androgen supplementation at different doses *versus* AD on intracellular levels of several key stress molecules that had been previously linked to adaptive response to oxidative stress–based therapies [26,27]. Accordingly, the activated form of AKT–pAKT(ser473) was significantly upregulated by R1881 compared to cells that were grown in the absence of R1881 ($P = .004$) or in the presence of the combination of R1881 and bicalutamide ($P = .03$) (Figure 4*A*). These differences were accentuated when the cells were stimulated with toxic doses of ROS following hormonal preconditioning. 22rv1 cells grown in the presence of 10^{-8} M R1881 and subsequently exposed to 1 mM H_2O_2 expressed significantly higher levels of the activated form of AKT1 than cells that were preconditioned with 10^{-5} M bicalutamide ($P = 4 \times 10^{-5}$) or with the combination of 10^{-8} M R1881 and 10^{-5} M bicalutamide ($P = .007$). We clearly demonstrate that the expression levels of pAKT(ser473) were increased with androgen supplementation; we did not, however, observe increased kinase activity.

We found that the activated form of p38 (pp38) was also significantly upregulated by R1881 compared to cells that were grown in the absence of R1881 ($P = .004$) or in the presence of the combination of R1881 and bicalutamide ($P = .04$) (Figure 4*B*). As in the case of pAKT expression, exposure to toxic levels (1 mM) of H_2O_2 following androgen preconditioning heightened the expression of pp38 (Figure 4*B*). Moreover, the catalytic activity of pp38 was significantly increased by R1881 ($P = .0001$) and was inhibited by the addition of bicalutamide ($P = .0001$) (Figure 4*C*).

It has been previously suggested that clusterin, a molecular chaperone, might contribute to resistance to oxidative stress–mediated damage, especially in the presence of androgens [28]. We therefore examined the expression of clusterin in cells either deprived of androgen or treated with R1881 in the presence or in the absence of bicalutamide. Androgen increased the expression of clusterin in a dose-dependent manner, which could be reduced to control levels by the addition of bicalutamide (Figure 4*D*). Clusterin expression was significantly higher in 22rv1 cells preconditioned with 10^{-9} or 10^{-8} M R1881 than in cells grown also in the presence 10^{-5} M bicalutamide ($P = .003$ and $P = .04$, respectively). No effect of androgen supplementation or AD on the expression of clusterin was seen in PC3 cells not expressing the AR (data not shown), further supporting the androgen-regulated expression of this stress protein. *In vivo*, compared to tumors grown in castrated hosts, AR^+ androgen-responsive xenografts grown in the presence of continuous testosterone supplementation expressed higher levels of pAKT, pp38, and clusterin (Figure 5).

Finally, heat shock proteins HSP70, HSP27, and HSP90 have been shown by several authors to play a role in protecting cells from oxidative stress [27] and radiation [29]. However, we could not demonstrate, either *in vitro* or *in vivo*, any differential effect of androgen on the level of expression of any of these proteins. Thus, we speculate that androgens could facilitate the expression of these stress molecules in PC cells only once oxidative stress has started to become a threat. To test this, we preconditioned 22rv1 cells with 10^{-8} M R1881, with 10^{-8} M R1881 + 10^{-5} M bicalutamide, or without hormones (control) for 48 hours. This was followed by an overnight incubation with 10 to 50 μM H_2O_2 for each condition. Although the expression of HSP70, HSP90, and HSP27

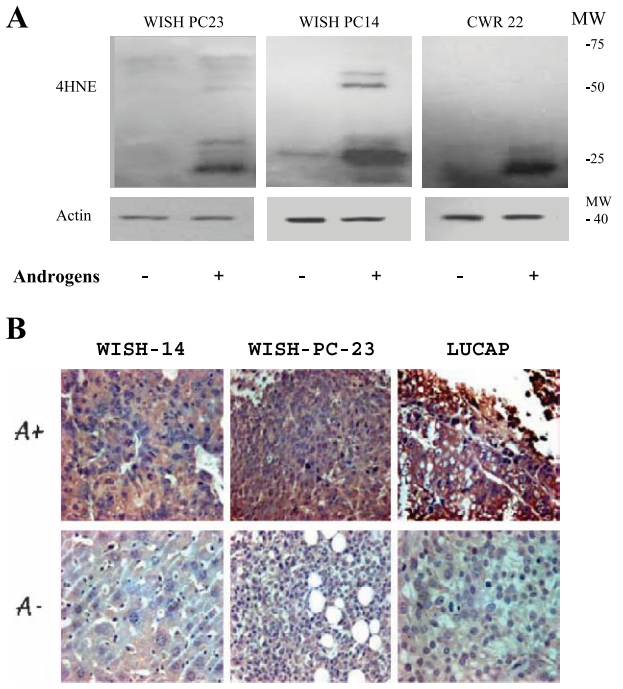

Figure 3. *Androgens increase bROS levels in an AR-dependent manner.* In vivo *effect on human PC xenografts. (A)* In vivo *expression of oxidative stress–induced lipid peroxidation as detected by the presence of 4-HNE protein adducts, judging by immunoblotting with anti–4-HNE antibodies. WISH-PC23, WISH-PC14, and CWR22 xenografts were grown as subcutaneous tumors in castrated (androgen$^-$) or testosterone-supplemented (androgen$^+$) SCID mice. (B)* In vivo *expression of 8-OHdG in WISH-PC14, WISH-PC23, and LuCAP-35 human PC xenografts grown as subcutaneous tumors in castrated (A^-) or testosterone-supplemented (A^+) SCID mice (original magnification, ×400).*

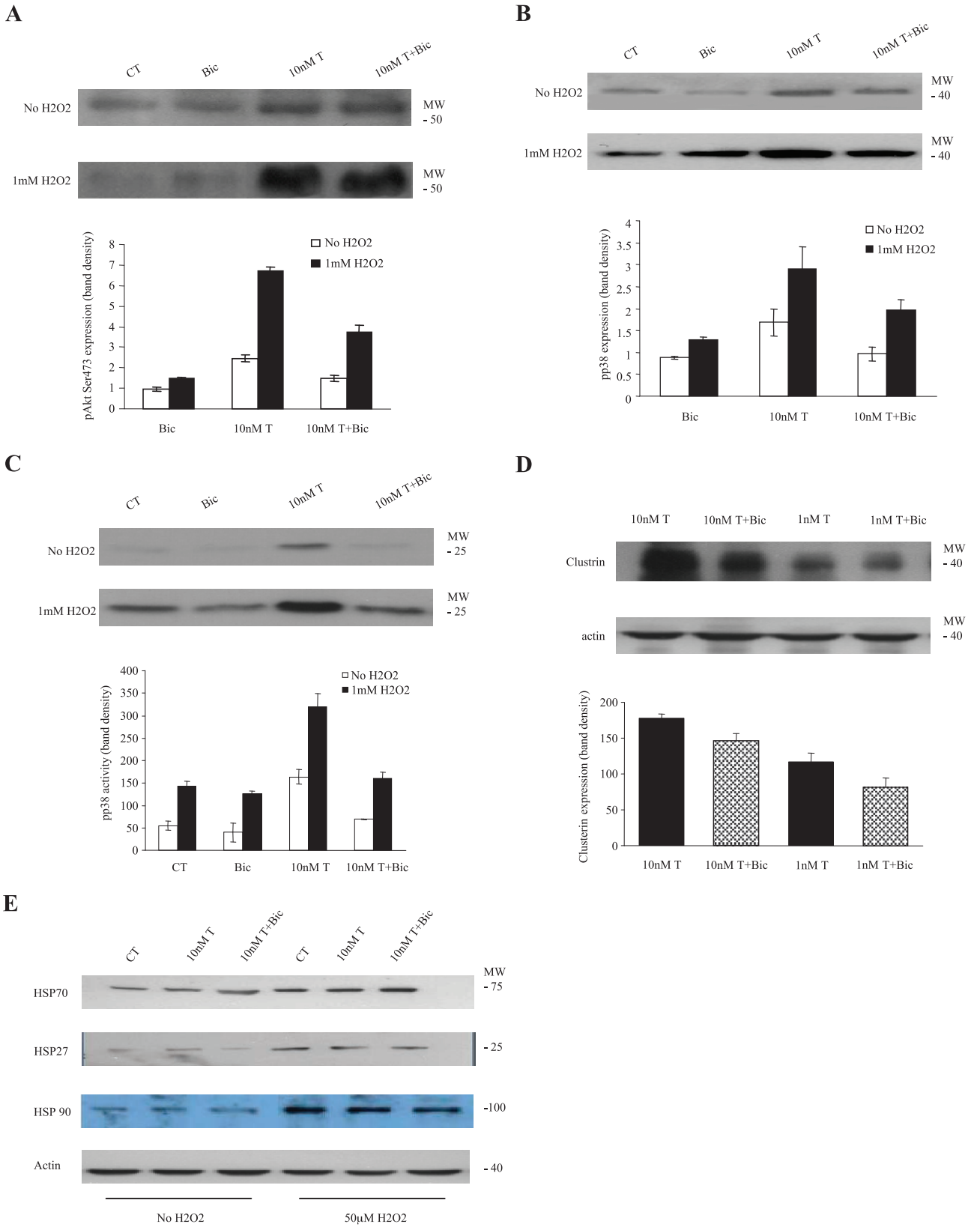

Figure 4. *Androgens induce the activation and expression of stress proteins in 22rv1 cells that can be reverted by AD. (A) pAKT(ser473) expression. (B) pp38 expression. (C) pp38 activity as determined by the phosphorylation of the ATF-2 client protein. (D) Clusterin expression. (A–D) Lower panels show relative expression in graphs. Results are derived from a minimum of three experiments and are expressed as mean ± SD. (E) The expression of HSP70, HSP27, and HSP90 in 22rv1 cells is not differentially affected by androgen supplementation or deprivation, but is increased in response to oxidative stress (overnight incubation with 50 μM H_2O_2). CT = control cells growing in a culture medium with CSFCS and without phenol red; Bic = cells growing in the same medium but with the addition of 10 μM bicalutamide; T = cells growing in the same medium but with the addition of 10 nM R1881; T + Bic = cells growing in the same medium but with the addition of both 10 nM R1881 and 10 μM bicalutamide. Results are derived from at least three experiments and are expressed as mean ± SD.*

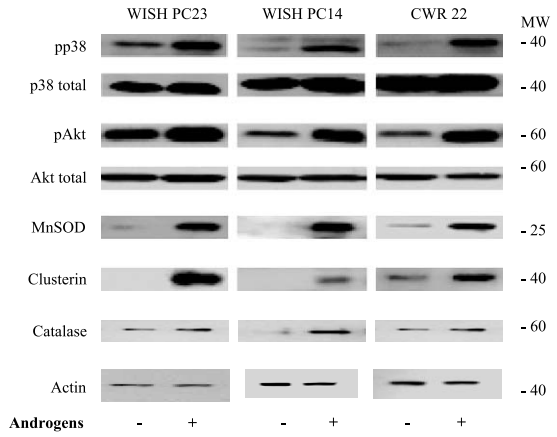

Figure 5. In vivo *effects of androgen supplementation and deprivation on the expression of stress molecules and antioxidative enzymes. Androgens induce* in vivo *activation of the stress molecules pAKT and p38, and the expression of clusterin, the antioxidative enzyme MnSOD, and catalase, as documented in three human PC xenografts: WISH-PC23, WISH-PC14, and CWR22. These tumors were grown in castrated (androgen$^-$) or testosterone-supplemented (androgen$^+$) SCID mice. Note that the global expression of pAKT and p38 is unchanged by androgen supplementation or deprivation, as opposed to their activated (phosphorylated) forms.*

was increased by oxidative challenge, this did not depend on the presence of androgens (Figure 4*E*).

Effect of Androgens on the Expression and Activity of Antioxidative Enzymes

We next examined whether AD causes a reduced expression and/or activity of several antioxidative enzymes. We first elected to measure the antioxidative capacity of the cells in a different hormonal milieu and found that androgens did increase antioxidative capacity in a dose-dependent manner (Figure 6*A*). Furthermore, blocking the AR with bicalutamide could reduce antioxidative capacity, but this did not reach statistical significance ($P = .057$) (Figure 6*B*).

Two key enzymes, MnSOD and catalase, were also investigated to examine whether androgens regulate the expression of antioxidative enzymes. Expression of both enzymes was significantly increased in androgen-supplemented cells than in AD cells ($P = .04$ for MnSOD and $P = .004$ for catalase, respectively), and blocking of the AR with bicalutamide significantly reduced their expression levels ($P = .036$ and $P = .004$ for MnSOD and catalase, respectively) (Figure 6*C*). Likewise, *in vivo*, all three AR$^+$ xenografts that grew in the

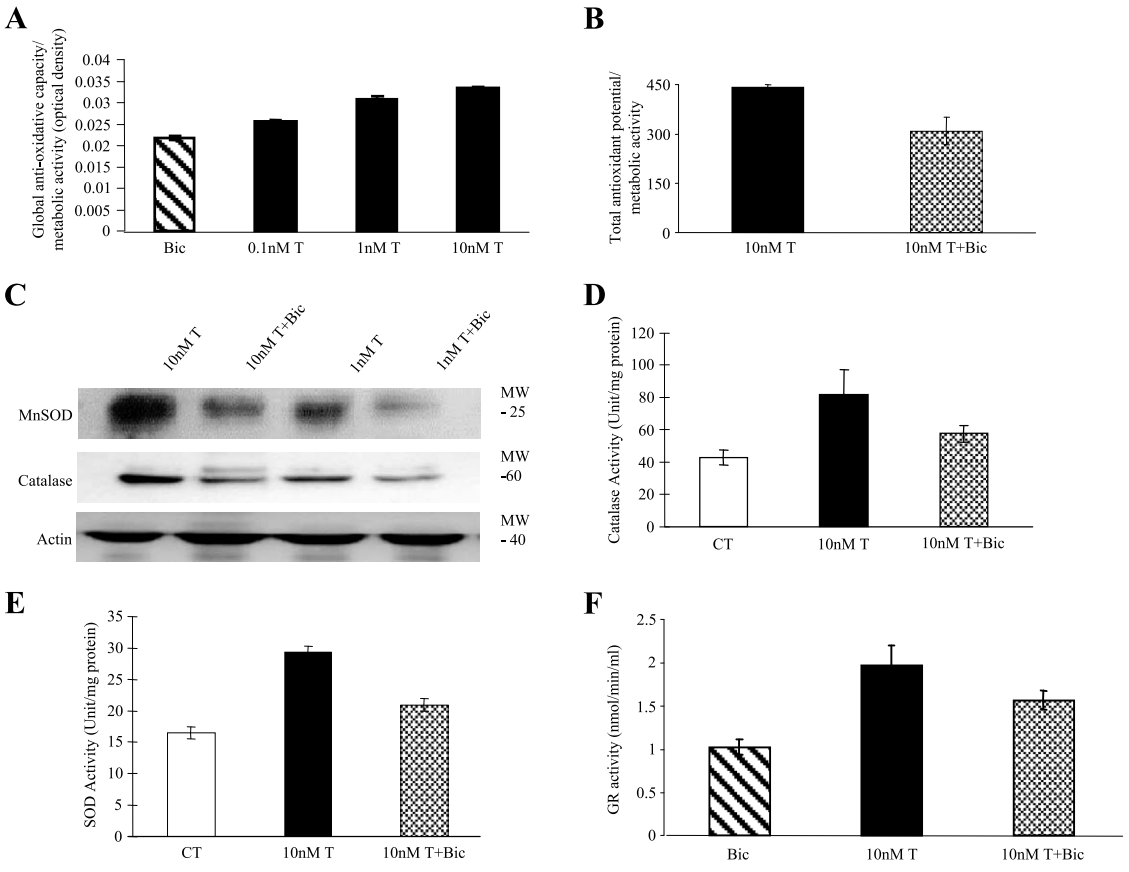

Figure 6. *Androgens increase the antioxidative capacity of 22rv1 cells. (A) Dose-dependent effect of R1881 on the antioxidative capacity of the cells. (B) AD, using 10 μM bicalutamide, can reduce the antioxidative capacity of 22rv1 cells that are supplemented with 10 nM R1881. (C) The expression and activity of catalase and MnSOD (D and E) in 22rv1 cells are significantly increased by androgen supplementation and are reduced by AD. (F) The activity of GR in 22rv1 cells is significantly increased by androgen supplementation and is reduced by AD. Results are derived from at least three experiments and are expressed as mean ± SD. CT = control cells growing in a culture medium with CSFCS and without phenol red; Bic = cells growing in the same medium but with the addition of 10 μM bicalutamide; T = cells growing in the same medium but with the addition of 10 nM R1881; T + Bic = cells growing in the same medium but with the addition of both 10 nM R1881 and 10 μM bicalutamide.*

presence of continuous testosterone support expressed higher levels of both enzymes compared to the same tumors grown in castrated hosts (Figure 5). Furthermore, the activity of both enzymes was significantly increased in cells treated with R1881 compared to AD controls ($P = .015$ for MnSOD and $P = .022$ for catalase) (Figure 6, *D* and *E*). The addition of bicalutamide to R1881 in the culture medium reduced the activity of both enzymes, but this was not statistically significant. Compared to AD, physiological levels of androgens significantly increased the activity of GR ($P = .032$) (Figure 6*F*). Collectively, these results suggest that androgens induce an increase in the antioxidative capacity of AR-expressing PC cells, irrespective of their metabolic rate and in an androgen-regulated manner that could be reversed by AR blockade. This probably results from a dual effect: increased expression of antioxidative enzyme and enhancement of their activity.

Effect of Elevated Levels of bROS on Adaptive Response to Oxidative Stress

Following the finding that androgens induce elevated levels of bROS in an androgen-regulated manner, we examined whether this is the primary mechanism responsible for acquired adaptation to oxidative stress challenge. To test this, we incubated the cells following separate hormonal preconditioning with the reducing agent *N*-acetylcysteine (NAC) in an attempt to quench bROS. The levels of bROS were reduced to an equal extent under all conditions of hormonal support by short-term preincubation with 5 mM NAC (Figure 7*A*). Using this protocol, the differential effects of R1881 on the expression of clusterin, pAKT(ser473), and pp38 were no longer evident (Figure 7, *B*–*D*). In fact, there was no difference in the expressions of these molecules between control cells (no androgen), cells preconditioned with R1881, and cells preconditioned with R1881 and bicalutamide. Collectively, this suggests that the induction of elevated levels of bROS by androgens is the primary factor responsible for the increased expression of these stress proteins. Similarly, we also demonstrated that the expression of MnSOD and catalase was directly dependent on bROS levels. Short-term preincubation of the cells with 5 mM NAC abolished the differential effects of androgen supplementation and AD on the expression of these enzymes (data not shown). Intuitively, the androgen-induced increase in bROS levels could also stimulate increased antioxidative enzymatic activity; however, we did not test this, as incubation with reducing agents such as NAC would naturally reduce the activity of antioxidative enzymes.

Finally, we demonstrated that abolishing increased levels of bROS (induced by androgens) by short-term pretreatment with NAC eliminates the relative radioresistance of cells that were preconditioned with R1881, bringing it to the same level as cells that were preconditioned with AD (Figure 7*E*), whereas cells that were androgen-deprived were almost three times more sensitive to 3-Gy γ-radiation than the same cells following pretreatment with 10 nM R1881 (mean surviving fraction, 17.55% *vs* 49.3%; $P < .001$). The addition of bicalutamide to R1881 increased cell killing by two-fold ($P = .0036$). However, preconditioning also with NAC almost equalized radiosensitivity across all hormonal treatments (6%, 8.7%, and 8.1% surviving fraction for 10 μM bicalutamide, 10 nM R1881, and 10 μM bicalutamide + 10 nM R1881, respectively). Thus, by reducing bROS levels under all hormonal conditions, the net effect of NAC itself was to sensitize the cells to radiation.

Discussion

The major finding here is that AD alters the redox environment of PC cells, thereby increasing their sensitivity to radiation killing. This may strengthen the biologic rational for adjuvant hormonal therapy, in combination with radiation, in PC. We demonstrated that androgens induce adaptation to oxidative stress challenge in PC cells. The development of cellular adaptation to stress requires the acquisition of relative tolerance to it following exposure to lower levels of the same stressor. Accordingly, adaptation of cells to oxidative stress generally involves changes in the expression and/or the activity of numerous cellular proteins that will prime the cells with the ability to withstand more severe oxidative attacks. Indeed, the generation of ROS leads to the stimulation of various signaling pathways, such as mitogen-activated protein kinases (MAPKs), and other stress-responsive protein kinases [30]. In particular, stress-responsive signal transduction pathways are strictly regulated by the intracellular redox state [31].

The framework of this study focused on proteins that are recognized as playing an important role in the response of tumor cells to radiation. Accumulating evidence suggests that the phosphatidylinositide 3-kinase (PI3-K)–AKT signaling pathway is a major contributor to radiation resistance. As seen in patients with head and neck cancer who were treated with radiation, a significant association was found between positive pAKT immunostaining of tumors and local control [32]. Furthermore, inhibition of AKT phosphorylation at ser473 has recently been shown to sensitize tumors to radiation, both *in vitro* and *in vivo* [33]. Inhibition of AKT1 with siRNA reduces tumor cell radiation survival, whereas overexpression of exogenous AKT1 in AKT1 siRNA-treated cells restores radiation sensitivity to baseline level [34]. Baron et al. [35] showed that androgens can stimulate AKT phosphorylation through a direct interaction between the AR and the p85α regulatory subunit of class I(A) PI3-K. Here, we suggest that the androgen-induced phosphorylation of AKT at the ser473 position is linked also to the fact that androgens increase cellular levels of bROS that, by themselves, trigger AKT phosphorylation, thereby enhancing cell survival following oxidative injury. Notably, the expression levels of nonphosphorylated AKT were not affected, *in vitro* (data not shown) or *in vivo* (Figure 5), by androgens.

Activation of the p38 pathway is strongly related to stress, with p38 being often referred to as a stress-activated protein kinase. Low levels of oxidative stress tend to activate p38 transiently, as opposed to sustained activation of the protein, which supports cell survival and not apoptosis [30]. The activation of p38 kinase has been reported to protect cells

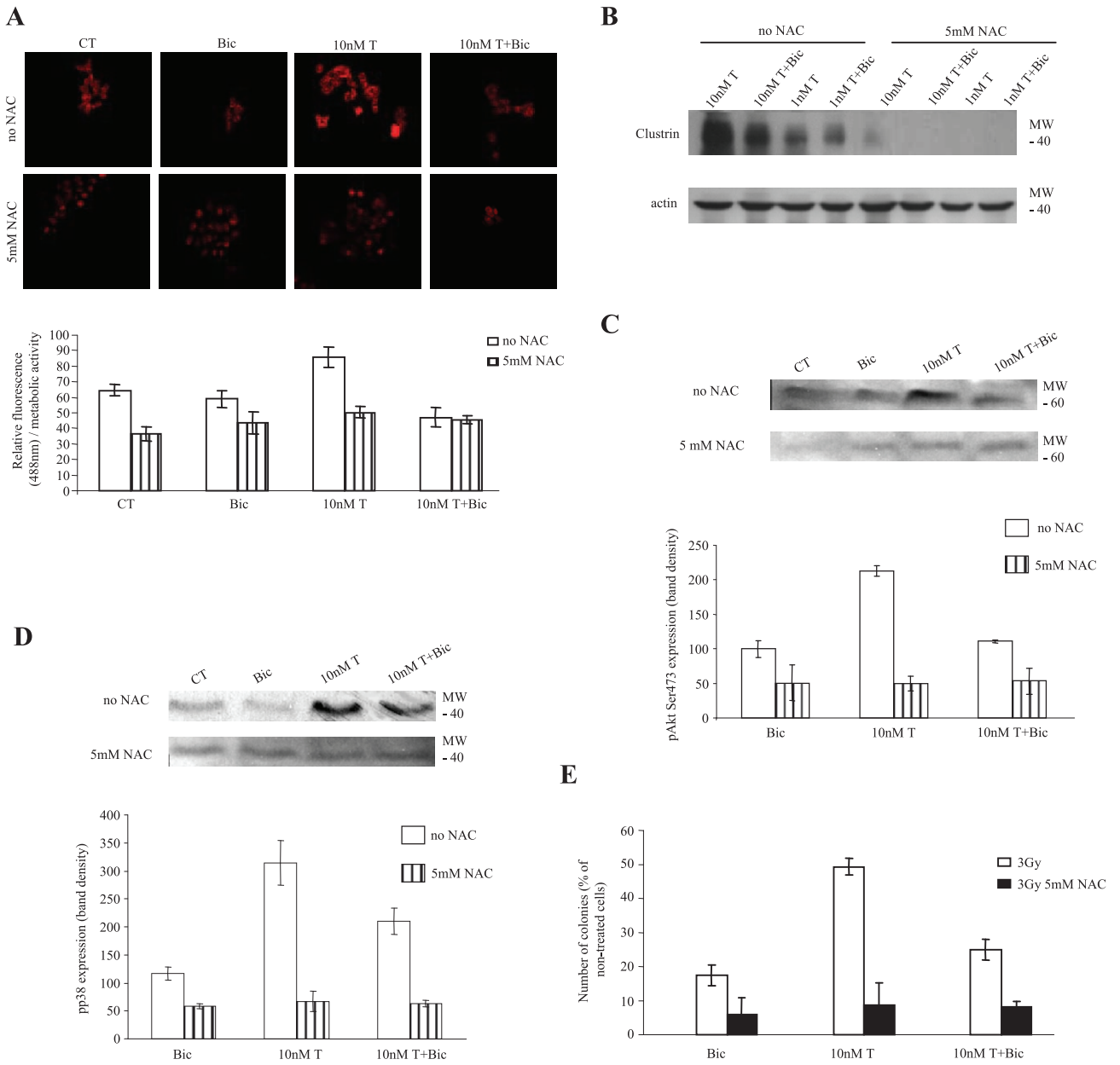

Figure 7. *The androgen-induced activation and expression of stress molecules and antioxidative enzymes stem from their induction by elevated bROS. (A) Incubation with 5 mM NAC reduced bROS under all hormonal conditions. Upper figure: DHE fluorescent test (representative microscopic view of the DHE fluorescence of 22rv1 cells in the presence and in the absence of R1881 and/or bicalutamide). Lower figure: Quantification of bROS levels (as measured with confocal fluorescent microscopy) following normalization to the cells' metabolic activity under different hormonal manipulations. Combined preincubation of 22rv1 cells with both R1881 and the reducing agent NAC erases the differential effects of different hormonal treatments on the expression of clusterin (B), pAKT(ser473) (C), and pp38 (D). (E) Combined preincubation of 22rv1 cells with both R1881 and the reducing agent NAC abolishes relative resistance to radiation, which is conferred to cells by androgens. Results show the survival of 22rv1 cells in a colony formation assay following a single treatment with 3-Gy radiation. CT = control cells growing in a culture medium with CSFCS and without phenol red; Bic = cells growing in the same medium but with the addition of 10 μM bicalutamide; T = cells growing in the same medium but with the addition of 10 nM R1881; T + Bic = cells growing in the same medium but with the addition of both 10 nM R1881 and 10 μM bicalutamide. Results are derived from at least three experiments and are expressed as mean ± SD.*

from TNFα-induced apoptosis, which plays a role in apoptotic response to radiation [36]. It has also been shown that p38 kinase can mediate AKT activation following UV radiation [37], which also triggers oxidative challenge. Likewise, several studies showed that an increased activity of p38 confers a survival advantage on cells exposed to oxidative stress, and that this can be induced by oxidative preconditioning of the cells. Our results suggest that it is primarily the increase in the oxidative status of the cells, rather than a direct androgen-regulated effect, that triggers the increased expression of pp38 and its activity because preconditioning with NAC (to reduce bROS levels) could ameliorate differences in the expression and activity of pp38 across different hormonal manipulations (Figure 7*D*). Interestingly, Takada et al. [38] showed that alteration of the redox status of SK-OV-3 human ovarian cancer cells by MnSOD over-

expression induced resistance to radiation by potentiating the p38–MAPK pathway through ROS generation. The same mechanism may be implicated here, as the levels of both bROS and MnSOD were elevated in PC cells *in vitro* and *in vivo* due to androgen stimulation. Interestingly, the expression levels of nonphosphorylated p38 were not influenced *in vitro* (data not shown) or *in vivo* (Figure 5) by androgens; thus, androgens (through their effect on bROS) appear to exert their effect on the p38–MAPK pathway through the activated form of the kinase.

Clusterin is a stress-associated cell survival molecule. Gleave and Miyake [39] demonstrated that suppression of clusterin levels using antisense oligonucleotides in PC cells enhances killing following treatment with androgen ablation, radiation therapy, and chemotherapy. Overexpression of clusterin confers resistance to toxic oxidative membrane damage in PC cells, especially in the presence of androgens [28]. Our results clearly demonstrate that androgens increase the expression levels of clusterin *in vitro* and *in vivo*, as evident in four human PC models (22rv1, CWR22, WISH-PC14, and WISH-PC23). An opposite phenomenon has been reported in an immunohistochemical study using archival radical prostatectomy specimens from 128 patients, some of whom received neoadjuvant hormonal therapy [40]. The clusterin expression in malignant prostate tissues was significantly higher in patients who underwent preoperative neoadjuvant hormonal therapy. However, this may reflect stress-induced expression of clusterin resulting from hormonal deprivation of androgen-dependent PC, rather than the fact that clusterin is an androgen-repressed gene. Indeed, inhibition of castration-induced apoptosis by calcium channel blockers in androgen-dependent Shionogi tumors prevented clusterin gene upregulation, suggesting that clusterin is not directly androgen-repressed but rather is regulated by apoptotic stimuli [41]. In addition, it has been suggested that the increased expression of clusterin in specimens from patients who underwent AD mirrors part of the subsequent adaptive increase in the expression of antiapoptotic genes [40].

The induction of heat shock proteins coincides with the acquisition of tolerance to different kinds of cellular stress, including oxidative stress [27]. Plaks et al. [27] showed in H5V mouse embryonic endothelial cells that the acquisition of an adaptive phenotype to oxidative stress is associated with the expression of HSP70 and HSP27. We could not demonstrate that androgens, by themselves, increase the expression of either HSP70 or HSP27 *in vitro* or *in vivo*. Although we clearly demonstrate that androgens significantly increase bROS levels, this may not be a sufficient trigger for the induction of HSP70 and HSP27 expression. Interestingly, even preconditioning with both androgens and H_2O_2 could not induce the differential expression of these heat shock proteins; however, as expected, this did increase the global expression of these heat shock proteins (as well as that of HSP90) under all hormonal conditions (Figure 4*E*). HSP90 is a molecular chaperone involved in mediating cellular response to stress and has a relatively high selectivity for several key molecules such as Raf-1 and AKT, which are associated with protection against radiation-induced cell death. In view of this, HSP90 inhibitors have been used in several tumor models, including PC, to reduce the levels of multiple radioprotective regulatory proteins, thereby increasing radiation sensitivity [42].

Another potential mechanism by which oxidative stress–based therapies may be effective when combined with hormonal therapy is reduction in the expression and activity of antioxidative enzymes because less ROS can be detoxified. In the present study, we measured the expression and activity of MnSOD and catalase (Figures 5 and 6, *C*–*E*), as well as the activity of GR (Figure 6*F*). ROS, including superoxide, are inevitably generated by a normal cellular metabolism, primarily in the mitochondria as byproducts of the electron transport chain. SOD, a group of metal-containing enzymes, has a vital antioxidant role, catalyzing the dismutation of superoxide—an anion very reactive to oxygen and H_2O_2. Once formed, H_2O_2 must be reduced to water to prevent the formation of toxic hydroxyl radicals. This is executed principally by catalase. Thus, SODs and catalase constitute the first enzymatic line of defense in the detoxification of products resulting from the basal cellular production of ROS. Supplementation of the culture medium with R1881 enhanced the antioxidative capacity of the cells (Figure 6, *A* and *B*), increased the expression of MnSOD and catalase (Figure 6*C*), and increased the activity of catalase, SOD, and GR (Figure 6, *C*, *D*, and *F*). Glutathione (GSH) is the most abundant intracellular antioxidant, and intracellular redox status is often measured by the ratio of GSH (reduced) to oxidized glutathione (GSSG) [43]. Increased levels of intracellular GSH correlate with radioresistance *in vitro* [44]. Accordingly, Husbeck et al. [45] radiosensitized human PC cells by GSH depletion with selenite, thereby decreasing GSH/GSSG ratio. In the present study, we demonstrated that AD decreases the activity of GR (Figure 6*F*), which is responsible for the replenishment of GSH by the reduction of GSSG. This could be another important mechanism by which androgens induce adaptation to oxidative stress generated by ionizing irradiation.

In summary, we believe that AD therapy facilitates oxidative stress–based therapies of PC by a dual mechanism (Figure 8). Firstly, it may set lower levels of bROS in PC cells, resulting in the reduced expression and activity of adaptive stress molecules. Secondly, it may also inhibit the expression and activity of antioxidative enzymes and thus reduce cellular antioxidative capacity. The effect of AD on the expression and activity of antioxidative enzymes can be AR-mediated and/or through reduced expression of bROS.

Although outside the scope of this study, we recognize that another potential mechanism by which androgens can induce adaptation to oxidative stress–based therapies may be the induction of DNA repair systems [26,46], supporting the practice of periodic continuation of AD even after the completion of the radiation treatment course.

A potential future application of these findings is the ability to use the expression profiles of pAKT, clusterin, pp38, MnSOD, catalase, and GR as oxidative stress–related tumor adaptation markers to triage patients with localized PC into subgroups for refined preradiation prognosis criteria. Interestingly, studies with human lymphocytes *in vitro* suggest

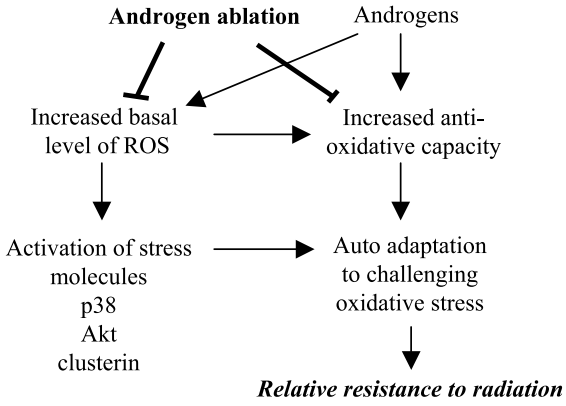

Figure 8. *Proposed model of androgen-driven adaptation for radiation therapy in PC. Androgens induce higher bROS in PC cells, resulting in both the increased expression and activity of stress molecules (pAKT and pp38 protein kinases) and clusterin, and the increased activity of antioxidative enzymes stimulating cellular antioxidant capacity. AD therapy reduces bROS levels, thus overturning the increased activity of stress molecules and antioxidative enzymes and facilitating oxidative stress–based therapies such as radiation.*

that the human population is heterogeneous in its adaptive response to ionizing radiation, which might, at least in part, be genetically determined [47]. It is tempting to speculate that the application of AD erases, to some extent, these differences by overcoming adaptive molecular barriers. A future strategy would be to detect those PC patients who need adjuvant AD with radiation, sparing it from all others.

In conclusion, we have demonstrated for the first time that alteration of the intracellular redox state by androgens in PC cells alters response to irradiation through the overexpression and increased activity of several stress kinases and antioxidative enzymes. AD can antagonize these effects, rendering AR-expressing PC cells more vulnerable to radiation. These findings elucidate the clinical observation that concomitant AD and radiation of localized PC confer therapeutic benefits.

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