

# Hyperthermia-Induced Proteasome Inhibition and Loss of Androgen Receptor Expression in Human Prostate Cancer Cells

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## Abstract

**Prostate cancer is the second leading cause of death in men in western countries and is usually treated by surgery and/or radiotherapy. More recently, hyperthermia has been introduced into clinical trials investigating a possible effect in the first-line treatment of prostate cancer. However, the molecular mechanisms of hyperthermia are not completely understood. In this study, we investigated the effects of hyperthermia on proteasome function and its significance for signal transduction, cell death and androgen receptor (AR) expression in PC-3, LnCaP, and DU-145 human and TRAMP-C2 murine prostate cancer cells. Hyperthermia caused apoptosis and radiosensitization and decreased 26S proteasome activity in all three human cell lines to about 40% of untreated control cells. 20S proteasome activity was not affected by heat. Heat treatment inhibited constitutive and radiation-induced activation of nuclear factor  $\kappa$ B caused by stabilization of I $\kappa$ B. Although stabilization of AR by proteasome inhibitors has been reported previously, AR protein levels in LnCaP cells decreased dramatically after heat. Our data suggest that inhibition of proteasome function and dependent signal transduction pathways might be a major molecular mechanisms of heat-induced apoptosis and radiosensitization. Hyperthermia abrogates AR expression in androgen-dependent cells and might thus promote malignant progression of prostate cancer. (Cancer Res 2005; 65(11): 4836-43)**

## Introduction

In western countries, prostate carcinoma is the most common form of cancer and the second leading cause of death in males (1). Whereas early forms of prostate cancer respond well to surgery or radiotherapy, patients with more advanced tumor stages will relapse and are usually not treated with curative intent. A significant increase in disease-free survival can be achieved by combination of surgery or radiotherapy with androgen-ablative treatment. Continuation of androgen-ablative treatment may delay recurrence in those that fail, but it is not curative because tumors become androgen independent. The exact mechanisms of progression to an independent state are unclear. However, there is evidence (2) that mutation of the androgen receptor (*AR*) gene and p53 mutations can be early events in prostate cancer carcinogenesis. Selection of this preexisting tumor cell population, as opposed to adaptation to hormone withdrawal, may therefore be possible.

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In any event, loss of androgen dependence seems associated with increased resistance to radiotherapy and chemotherapy, more aggressive behavior, and poor prognosis (3). To solve these problems, there is an ongoing research effort aimed at identifying new and effective forms of treatment for prostate cancer (1).

Hyperthermia is the oldest documented tumor treatment modality. Numerous *in vitro* studies have shown the dose- and temperature-dependent radiosensitizing effect on cancer cells (reviewed in ref. 4). Although clinical studies with hyperthermia have yielded variable results, this can often be attributed to variations in the heating method, poor standardization of patients, and other uncontrolled variables. A major clinical problem that prevents hyperthermia from entering standard treatment regimens is the lack of a reliable method for real-time thermodosimetry. This is mainly because normal and tumor vascularization cause a highly dynamic efflux of heat, making thermodosimetry extremely complex and at present impossible to predict in real time. In spite of these problems, some studies have shown encouraging results. For example, the combination of heat and irradiation has been reported to increase the number of complete responses up to 6-fold when compared with radiation alone (5), although the mechanism by which hyperthermia operates remains unclear.

A tumor entity that might allow comparatively easy clinical application of hyperthermia is carcinoma of the prostate. Because of its limited tumor diameters and vascularization, relatively superficial tumor location and easy transurethral, transrectal, or transperianal accessibility, prostate cancer has become a major target of hyperthermia, although knowledge about clinical outcome and side effects is still poor. At present, it is not possible to predict which patient will benefit from hyperthermia, and preclinical studies are necessary to understand the molecular mechanisms that might determine response.

It has been recently reported that prostate cancer cells in general show elevated constitutive DNA-binding activity of the transcription factor nuclear factor  $\kappa$ B (NF- $\kappa$ B). NF- $\kappa$ B has been reported to be a negative regulator of AR expression (6). In addition, we and others have shown that inhibition of NF- $\kappa$ B induces apoptosis in prostate cancer cells (7). NF- $\kappa$ B is a heterodimer or homodimer of the subunits p50, p52, p65/RelA, c-Rel, and Rel-B. It is sequestered preformed in the cytosol by inhibitor molecules of the I $\kappa$ B family (I $\kappa$ B $\alpha$ , I $\kappa$ B $\beta$ , I $\kappa$ B $\gamma$ , Bcl-3, p100, and p105). Activation of this pathway is normally achieved by phosphorylation of one of the most important inhibitors, I $\kappa$ B $\alpha$ , at two serine sites (Ser<sup>32</sup> and Ser<sup>36</sup>) by I $\kappa$ B kinases. This marks I $\kappa$ B $\alpha$  for polyubiquitination and subsequent degradation by the 26S proteasome. Degradation of I $\kappa$ B $\alpha$  frees NF- $\kappa$ B for translocation to the nucleus and activation of its target genetic programs (reviewed in ref. 8). The 26S proteasome is a protease of 2 MDa responsible for the controlled ATP- and ubiquitin-dependent degradation of most short-lived proteins (9) and 70% to 90% of all long-lived proteins (9, 10), including key

molecules in signal transduction, cell cycle control, and immune responses (11). We hypothesized that hyperthermia might affect 26S proteasome activity in prostate cancer cells, with consequent changes in NF- $\kappa$ B signal transduction and in tumor cell survival, radiosensitivity, and androgen dependency.

## Material and Methods

**Cell culture.** Cultures of PC-3 (European Collection of Animal Cell Cultures, Salisbury, United Kingdom), DU-145, and LnCaP (DMSZ, Braunschweig, Germany), and human and TRAMP-C2 (a kind gift from Dr. Norman Greenberg, Baylor University, Houston, TX; ref. 12) murine prostate carcinoma cells were grown in 75-cm<sup>2</sup> flasks (Falcon, Bedford, MA) at 37°C in a humidified atmosphere at 5% CO<sub>2</sub>/95% air. DMEM (PC-3 and DU-145, TRAMP-C2, Cell Concepts, Freiburg, Germany) and RPMI 1640 (LnCaP, Cell Concepts) were used supplemented with 10% heat-inactivated FCS and 1% penicillin/streptomycin (Life Technologies, Gaithersburg, MD). Medium for TRAMP-C2 cells was additionally supplemented with insulin (5  $\mu$ g/mL) and dihydrotestosterone (10<sup>-8</sup> mol/L).

**Heat shock treatment.** For hyperthermia treatment, 5  $\times$  10<sup>6</sup> cells were plated into Petri dishes. After overnight incubation at 37°C, dishes were sealed with parafilm and placed into an incubator preheated to 44°C for 1 hour before being returned to 37°C for the indicated times.

**Irradiation and clonogenic assays.** Hyperthermia treated (1 hour at 44°C) and control cells (1 hour at 37°C) were trypsinized, counted, and diluted to a final concentration of 10<sup>6</sup> cells/mL. The cell suspensions were immediately irradiated at room temperature with a <sup>137</sup>Cs laboratory irradiator (IBL 637, CIS Bio International, Gif/Yvette Cedex, France) at a dose rate of 77.5 cGy/min. Corresponding controls were sham irradiated. Colony-forming assays were done immediately after irradiation by plating an appropriate number of cells into culture dishes, in triplicate. After 14 days, cells were fixed and stained with 1% crystal violet, and colonies containing >50 cells were counted. The surviving fraction was normalized to the surviving fraction of the corresponding control, and survival curves were fitted by use of a linear-quadratic model (13). The  $D_q$  and  $D_o$  were calculated from the survival curves using the single-hit multitarget model of radiation dose survival. The  $D_q$  is the point at which the shoulder region of the curve transforms into an exponential region and the injury from room temperature converts from sublethal to lethal damage, whereas  $D_o$  represents the intrinsic radiosensitivity of cells.

**Cell extraction and electrophoretic mobility shift assay for nuclear factor  $\kappa$ B.** For preparation of total cellular extracts, normal and treated cells were dislodged mechanically, washed with ice-cold PBS, and lysed in TOTEX buffer [20 mmol/L HEPES, ref. 14; 0.35 mmol/L NaCl; 20% glycerol; 1% NP40; 0.5 mmol/L EDTA; 0.1 mmol/L EGTA; 0.5 mmol/L DTT; 50  $\mu$ mol/L phenylmethylsulfonyl fluoride (PMSF); and 90 trypsin inhibitor units/mL aprotinin] for 30 minutes on ice. The lysates were centrifuged at 12,000  $\times$  g for 5 minutes. Protein concentration in resultant supernatants was determined with the bicinchoninic acid (BCA) protocol (Pierce, Rockford, IL). Fifteen micrograms of protein from the resulting supernatant were incubated for 25 minutes at room temperature with 2  $\mu$ L of bovine serum albumin (BSA, 10  $\mu$ g/ $\mu$ L), 2  $\mu$ L of dIdC (1  $\mu$ g/ $\mu$ L), 4  $\mu$ L of Ficoll buffer (20% Ficoll 400, 100 mmol/L HEPES, 300 mmol/L KCl, 10 mmol/L DTT, and 0.1 mmol/L PMSF), 2  $\mu$ L of buffer D+ (20 mmol/L HEPES, 20% glycerol, 100 mmol/L KCl, 0.5 mmol/L EDTA, 0.25% NP40, 2 mmol/L DTT, and 0.1 mmol/L PMSF), and 1  $\mu$ L of [ $\gamma$ -<sup>32</sup>P] ATP-labeled oligonucleotide (Promega, Madison, WI, NF- $\kappa$ B: AGTTGAGGGGACTTCCAGG). For a negative control, unlabeled oligonucleotide was added to 50-fold excess. Gel analysis was carried out in native 4% polyacrylamide/0.5 $\times$  Tris-borate EDTA gels. Dried gels were placed on a phosphor screen for 24 hours and analyzed on a phosphor imager (IPR 1500, Fuji, Duesseldorf, Germany).

**Immunoblotting.** Cells were lysed in radioimmunoprecipitation assay buffer [50 mmol/L Tris-HCl (pH 7.2), 150 mmol/L NaCl, 1% NP40, SDS, 10 mmol/L PMSF, aprotinin, sodium vanadate]. Protein concentrations were determined using the BCA protocol (Pierce) with bovine serum albumin (Sigma, St. Louis, MO) as standard. Ten micrograms of protein were

electrophoresed in a SDS gel (0.1% SDS/10% polyacrylamide) and blotted to polyvinylidene difluoride (PVDF) membranes at 4°C. After blocking with Blotto-buffer (TBS, 0.1% Tween 20, 5% skim milk) for 1 hour at room temperature, the membranes were incubated with a polyclonal antibody against murine  $\kappa$ B $\alpha$  (0.5  $\mu$ g/mL, Invitrogen, San Diego, CA), a monoclonal antibody against the human AR (PharMingen, San Diego, CA, 2  $\mu$ g/mL), a monoclonal mouse-anti-human antibody against HSP90 $\alpha$  (Stressgen, Victoria, Canada), a monoclonal mouse-anti-human antibody against HSP70 (Stressgen), a monoclonal mouse-anti-human antibody against HSP27 (Stressgen) or a polyclonal rabbit-anti-human antibody against CHIP (Abcam, Cambridge, MA) for 1 hour at room temperature. A secondary horseradish peroxidase-conjugated goat-anti-mouse antibody (Serotec, 1:10,000) or a goat-anti rabbit antibody (1:20,000, DAKO, Glostrup, Denmark) and the Enhanced Chemiluminescence Plus system (Amersham, Arlington Heights, IL) were used for visualization. Equity of loading was verified using a mouse monoclonal antibody against  $\alpha$ -tubulin (1:5,000, Oncogene, Uniondale, NY).

**Two-dimensional difference gel electrophoresis.** The two-dimensional difference gel electrophoresis (DIGE) technique was done as described in (15). Briefly, cells were washed twice with 0.25-fold PBS and proteins were solubilized in lysis buffer [30 mmol/L Tris-HCl, 2 mmol/L thiourea, 7 mmol/L urea, 4% CHAPS (pH 8.5)]. Protein concentrations were estimated using the two-dimensional Quant kit (Amersham Bioscience, Freiburg, Germany). Each sample and an internal standard consisting of equal parts of each sample were labeled with Cy3 (37°C control), Cy5 (44°C), and Cy2 (internal standard) using 8 pmol dye per  $\mu$ g protein (Amersham Bioscience), respectively, for 30 minutes on ice in the dark. Labeling reaction was quenched by addition of L-lysine (10 mmol/L, 2  $\mu$ L per 240 pmol of dye). Fifty micrograms of two different samples and 50  $\mu$ g of the internal standard were mixed, diluted to a total volume of 350  $\mu$ L with rehydration buffer (4% CHAPS, 8 mmol/L urea, 1% pharmalytes, 13 mmol/L DTT), subjected to isoelectric focusing (IPGphor, Immobiline Dry Strip, nonlinear, 18 cm, pH 3-10; Amersham Bioscience), reduced with DTT (0.5%) for 10 minutes, alkylated with iodacetamide (4.5%) for 10 minutes, and finally separated by SDS-PAGE (12% acrylamide, 0.1% SDS) thrice for each sample. Gels were scanned (Typhoon 9610, Amersham Bioscience) and analyzed using the DeCyder Software package version 5.0 (Amersham Bioscience). Significant changes in expression were defined as a 1.5-fold change with a  $P < 0.05$  using Student's  $t$  test.

**Proteasome function assays.** Proteasome function was measured as described previously (16), with some minor modifications. To obtain crude cellular extracts, cells were washed with PBS, then with buffer I [50 mmol/L Tris (pH 7.4), 2 mmol/L DTT, 5 mmol/L MgCl<sub>2</sub>, 2 mmol/L ATP], and pelleted by centrifugation (1,000  $\times$  g, 5 minutes, 4°C). Glass beads and homogenization buffer [50 mmol/L Tris (pH 7.4), 1 mmol/L DTT, 5 mmol/L MgCl<sub>2</sub>, 2 mmol/L ATP, and 250 mmol/L sucrose] were added and cells were vortexed for 1 minute. Beads and cell debris were removed by centrifugation at 1,000  $\times$  g for 5 minutes and 10,000  $\times$  g for 20 minutes at 4°C. Protein concentration was determined by the Micro BCA protocol (Pierce) with BSA (Sigma) as standard. To measure 26S proteasome activity, 20  $\mu$ g protein of crude cellular extracts of each sample were diluted with buffer I to a final volume of 200  $\mu$ L in quadruplicates. For assessment of 20S proteasome activity, 20  $\mu$ g of protein was diluted to a final volume of 200  $\mu$ L in a buffer consisting of 50 mmol/L Tris/HCl (pH 7.9), 0.5 mmol/L EDTA, and 0.035% SDS in quadruplicates. The fluorogenic proteasome substrate SucLLVY-MCA (chymotrypsin-like, Sigma) was dissolved in DMSO and added in a final concentration of 80  $\mu$ mol/L in 1% DMSO. Proteolytic activity was continuously monitored by measuring the release of the fluorescent group 7-amido-4-methylcoumarin in a fluorescence plate reader (Spectra Max Gemini XS, Molecular Devices, Sunnyvale, CA, 37°C) at 380/460 nm.

**Determination of apoptosis.** In addition to monitoring apoptosis using morphologic criteria, apoptotic cells were detected with an *In situ* Cell Death Kit (Boehringer Mannheim, Mannheim, Germany). The manufacturer's protocol was followed with some minor modifications. Briefly, attached and detached cells were collected, centrifuged, fixed in ice-cold 75% ethanol, washed with PBS, and pelleted by centrifugation for 5 minutes at 500  $\times$  g. Cells were permeabilized by resuspension in a solution of 0.1% Triton X-100

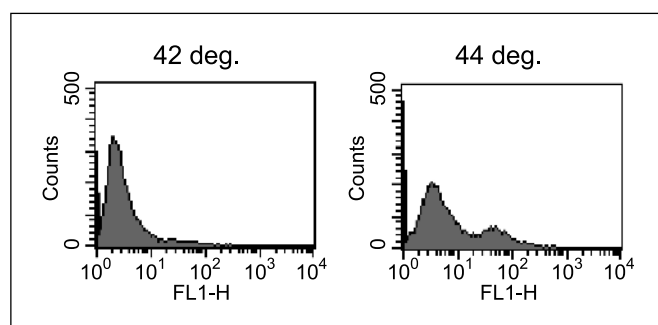
and 0.1% sodium citrate and incubation for 2 minutes on ice. Cells were washed twice in PBS, resuspended in terminal deoxynucleotidyltransferase-mediated nick end labeling (TUNEL) reaction mixture, and incubated for 60 minutes at 37°C. After three washes with PBS, fluorescence was measured at 518 nm in a flow cytometer (FACScan, Becton Dickinson, Mountain View, CA) and analyzed with the CellQuest software (Becton Dickinson).

**Statistics.** Numerical data in proteasome function assays and clonogenic survival assays represents means  $\pm$  SE from at least three independent experiments. Unless otherwise stated, gel shift assays and Western blot assays show representative results from at least three independent experiments. Measurements were compared using a two-sided Student's *t* test. Statistical significance was considered for *P*s < 0.05.

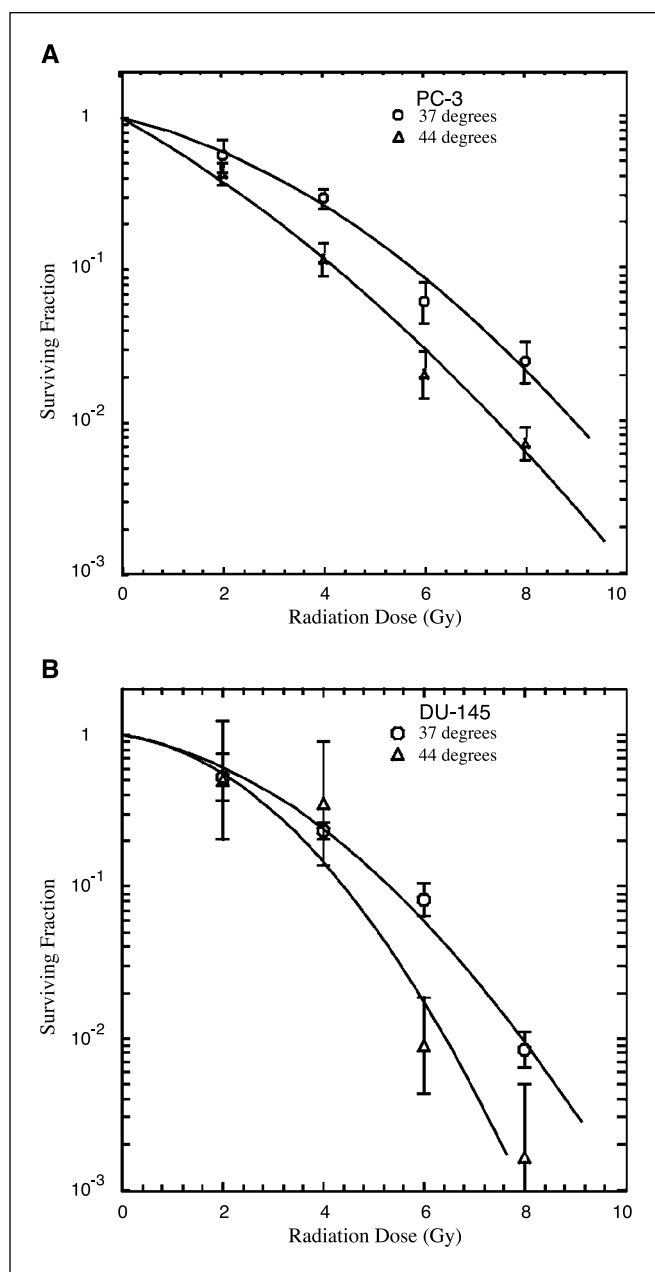
## Results

**Heat shock down-regulates nuclear factor  $\kappa$ B DNA-binding activity.** Hyperthermia has been reported to cause apoptosis in many cell lines including PC-3 prostate cancer cells (17). Additionally, hyperthermia leads to radiosensitization of surviving cancer cells (18). Both observations were confirmed in our present study. Twenty-four hours after a 1-hour heat treatment at 44°C, PC-3 cells showed morphologic signs of apoptosis with membrane blebbing and chromatin condensation that was confirmed by TUNEL staining (Fig. 1). Surviving PC-3 (Fig. 2A) and DU-145 (Fig. 2B) cells were radiosensitized, as shown by a left shift of the survival curves at 44°C.

Constitutive activity of the antiapoptotic transcription factor NF- $\kappa$ B is high in many prostate cancer cell lines, including PC-3 cells, and specific inhibition of NF- $\kappa$ B using dominant-negative I $\kappa$ B $\alpha$  constructs can induce apoptosis in such cells (7). We therefore hypothesized that the proapoptotic effect of hyperthermia on PC-3 cells might be through alterations in the DNA-binding activity of this transcription factor. Electrophoretic mobility shift assay was used to examine NF- $\kappa$ B activity in extracts from cells 1.5 hours after completion of hyperthermia treatment (44°C for 1 hour) and/or irradiation (20 Gy). DU-145 (Fig. 3A), PC-3 (data not shown), and LnCaP (data not shown) cell lines were used to allow more general conclusions to be drawn. Although constitutive baseline levels of NF- $\kappa$ B activity differed between the cell lines, hyperthermia almost completely inhibited constitutive and radiation-induced NF- $\kappa$ B activity in all three cell lines. Inhibition was not prevented by blocking protein translation with cycloheximide (25  $\mu$ g/mL, for 30 minutes), excluding induction of an endogenous inhibitor of NF- $\kappa$ B as a mechanism, although pretreatment of cells with the drug did elevate baseline and

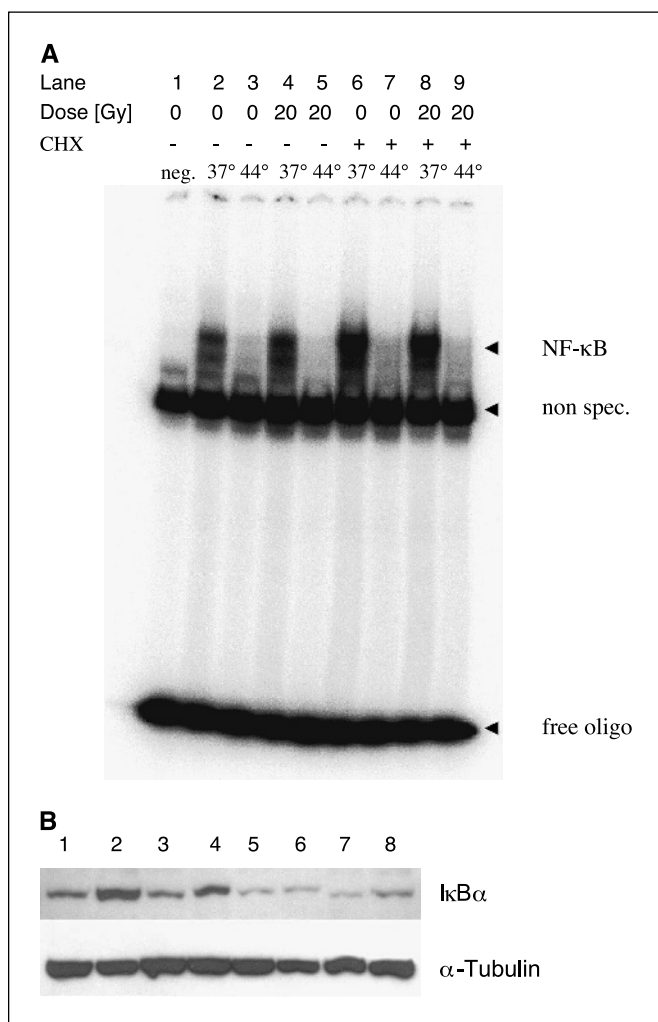


**Figure 1.** Heat-induced apoptosis in PC-3 prostate cancer cells. Flow-cytometric analysis of PC-3 prostate cancer cells. Incubation at 44°C for 1 hour leads to TUNEL-positive staining cells 24 hours after heat shock (right). In contrast, no significant increase of the apoptotic fraction was observed after treatment at 42°C for 1 hour (left).



**Figure 2.** Hyperthermia treatment sensitizes PC-3 and DU-145 prostate cancer cells to ionizing radiation. Clonogenic assay was done with PC-3 (A) and DU-145 (B) cells. Cells were irradiated at room temperature, plated into culture dishes and subsequently treated with 37°C or 44°C for 1 hour. After 14 days, cells were fixed with ethanol and stained with crystal violet. Colonies consisting of >50 cells were counted and normalized against the corresponding unirradiated control. Points, means from three independent experiments each carried out in triplicates. Heat treatment sensitized both cell lines to ionizing radiation indicated by a left shift of the survival curves. PC-3, 37°C:  $\alpha = 0.18$ ,  $\beta = 0.037$ ,  $\alpha/\beta = 4.8$  Gy $^{-1}$ ,  $D_0 = 1.584 \pm 0.23$ ,  $D_q = 2.125 \pm 0.66$  and 44°C:  $\alpha = 0.43$ ,  $\beta = 0.25$ ,  $\alpha/\beta = 1.7$  Gy $^{-1}$ ,  $D_0 = 1.407 \pm 0.075$ ,  $D_q = 1.007 \pm 0.28$ . DU-145, 37°C:  $\alpha = 0.13$ ,  $\beta = 0.056$ ,  $\alpha/\beta = 2.4$  Gy $^{-1}$ ,  $D_0 = 1.255 \pm 0.13$ ,  $D_q = 2.378 \pm 0.79$  and 44°C:  $\alpha = 0.1$ ,  $\beta = 0.1$ ,  $\alpha/\beta = 1$  Gy $^{-1}$ ,  $D_0 = 0.7068 \pm 0.17$ ,  $D_q = 3.402 \pm 0.9$ .

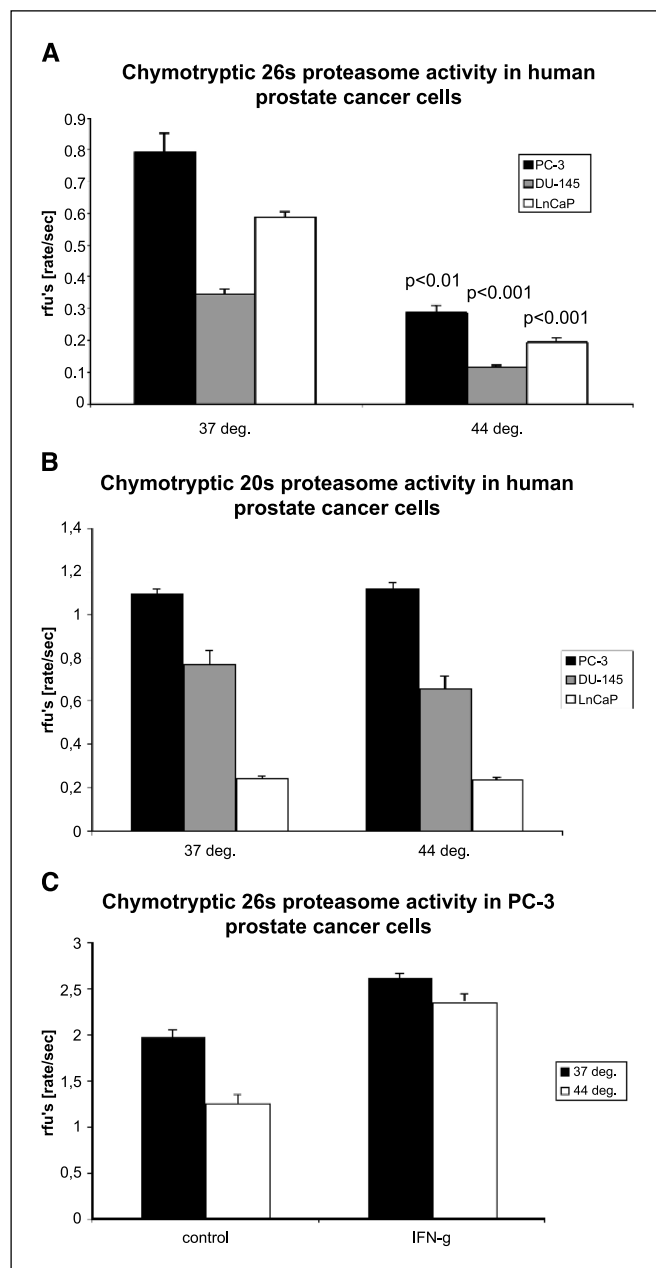
radiation-induced NF- $\kappa$ B activity (Fig. 3A). Western blotting of protein extracts from the same cells and same time point revealed stabilization of I $\kappa$ B $\alpha$  by hyperthermia treatment (Fig. 3B), indicating an inhibitory effect of heat either on I $\kappa$ B-kinase activity, ubiquitination of I $\kappa$ B $\alpha$ , or proteasome function.



**Figure 3.** Hyperthermia down-regulates constitutive and radiation-induced NF-κB activity. **A**, representative gel shift experiment ( $n = 3$ ) using cytosolic protein extracts from DU-145 human prostate cancer cells. Constitutive NF-κB DNA-binding activity (*lane 2*). Irradiation with 20 Gy caused an increase of NF-κB DNA-binding activity after 90 minutes (*lane 4*). Preincubation of the cells with cycloheximide (CHX, 25 μg/mL, 30 minutes before start of heat treatment) increased constitutive and radiation-induced NF-κB DNA-binding activity (*lanes 6 and 8*). Treatment of the cells with hyperthermia (1 hour at 44°C) abrogated constitutive and radiation induced NF-κB DNA-binding activity (*lanes 3 and 5*) independent of protein *de novo* synthesis (cycloheximide treatment: *lanes 7 and 9*). *Lane 1*, negative control; unlabeled oligonucleotide was added in 50-fold molar excess to demonstrate the specificity of the binding reaction. **B**, Western blot analysis ( $n = 3$ ) for IκBα protein levels in total cellular lysates from PC-3 cells. *Lane 1*, control; *lane 2*, 1 hour 44°C; *lane 3*, 20 Gy; *lane 4*, 20 Gy/1 hour 44°C; *lane 5*, cycloheximide; *lane 6*, cycloheximide/1 hour 44°C; *lane 7*, cycloheximide/20 Gy; *lane 8*, cycloheximide/20 Gy/1 hour 44°C. Hyperthermia treatment stabilized IκBα.

**Heat shock impairs 26S proteasome function.** Specific inhibitors of proteasome function have been shown to induce apoptosis in human prostate cancer cells. The mechanism remains unclear, but it has been reported to be independent of p53, the *c-jun*-NH<sub>2</sub>-kinase kinase pathway, bcl-2 (19), Bcl-X(L), Bax, Bad, Bak, or cytochrome *c* (20). Because functional 26S proteasome activity is an obligatory precondition for activation of the NF-κB signal transduction pathway, we hypothesized that hyperthermia might affect NF-κB activation by altering 26S proteasome cleavage activity. Using a fluorogenic assay, heat shock was found to decrease 26S proteasome activity to  $36.2 \pm 3\%$  (PC-3,  $P < 0.01$ ),

$33.4 \pm 8.4\%$  (DU-145,  $P < 0.001$ ), and  $45 \pm 3.4\%$  (LnCaP,  $P < 0.001$ ), respectively, in the three prostate cancer cell lines (Fig. 4A). This effect was also observed when cells were pretreated with cycloheximide (25 μg/mL for 30 minutes, data not shown). The function of the 26S proteasome is dependent on ubiquitin and ATP, which reflects its activity *in vivo*, but proteolytic activity resides in



**Figure 4.** Hyperthermia inhibits 26S proteasome function. Results of proteasome function assays for the chymotryptic cleavage activity of the fully functional regulated 26S proteasome ( $n = 3$ ). Release of the fluorescent group 7-amido-4-methylcoumarin from the proteasome substrate SucLLVY-MCA was continuously monitored in a fluorescence plate reader for 30 minutes (excitation/emission, 380/460 nm at 37°C). Heat treatment of human prostate cancer cell lines led to rapid inhibition of 26S proteasome function in PC-3, DU-145, and LnCaP prostate cancer cells. This effect was independent of protein *de novo* synthesis as showing by a lack of an effect of cycloheximide and restricted to 26S proteasome function (A). The activity of the 20S core unit remained unchanged (B). Loss of chymotryptic 26S proteasome function was prevented by preincubation with IFN-γ (100 IU/mL), which leads to expression of immunoproteasomes (C).

the 20S core unit. This can be assessed by addition of SDS to the 26S complex. To determine whether the thermosensitive component of the 26S proteasome is located in the 19S regulatory units or in the 20S core, we repeated the experiments in the absence of ATP and the presence of SDS (0.035%). In all three cell lines, 20S activity was not significantly altered by hyperthermia treatment suggesting that the thermosensitive proteasome units are located in the 19S caps (Fig. 4B). IFN- $\gamma$  treatment is known to alter the structure of the proteasome resulting in expression of so-called immunoproteasomes. In these complexes, the constitutively expressed  $\beta$ -subunits  $\beta$ 1,  $\beta$ 2, and  $\beta$ 5 are replaced by LMP2, MECL-1, and LMP7 (21). Additionally, there is an increase in proteasomes containing the 11S heteroheptamer activator complex (PA28  $\alpha/\beta$ ; refs. 22, 23). Preincubation of PC-3 cells with IFN- $\gamma$  (100 units/mL) for 24 hours prevented heat-induced decrease in chymotryptic 26S proteasome activity, also suggesting that hyperthermia acts on the 19S regulatory subunits of the 26S proteasome (Fig. 4C).

**Heat shock down-regulates androgen receptor protein levels.** NF- $\kappa$ B has been recently reported a negative regulator of AR expression (6, 24). Because heat shock down-regulated NF- $\kappa$ B in all three cell lines, we hypothesized that it might increase AR expression. To test this, cells were incubated for 1 hour at 44°C and thereafter at 37°C for an additional 90 minutes. Total cellular protein was separated by SDS-PAGE and blotted to PVDF membranes. As expected, immunoblotting using a monoclonal antibody against human AR could not detect any AR expression in PC-3 and DU-145 cells, whereas LnCaP cells, on the contrary, showed strong expression. Heat shock treatment for 1 hour at 44°C did not cause accumulation of AR protein in PC-3 and DU-145 cells even after subsequent incubation at 37°C for 24 hours (not shown). Surprisingly, AR expression was completely abrogated in LnCaP cells by 90 minutes of heat treatment (Fig. 5A). Comparable loss of AR expression was also observed in murine TRAMP-C2 murine prostate cancer cells underlining the general nature of this observation (Fig. 5B).

The extent of AR down-regulation in LnCaP cells was dependent on the temperature and duration of heat shock treatment (Fig. 5C-D) and did not recover within 24 hours (data not shown). The AR is in complex with heat shock proteins, which codetermine binding affinity of steroids to this receptor as well as its stability (25). To rule out changes in chaperone expression as a cause for loss in AR (37°C/44°C: 0.15-fold  $\pm$  0.08,  $n$  = 4,  $P$  = 0.0012, two-sided Student's  $t$  test) expression, HSP90, HSP70, and HSP27 protein levels were examined by immunoblotting. Loss of AR was associated by only slight changes in HSP90 $\alpha$  [37°C/44°C: 1.62-fold  $\pm$  0.35,  $n$  = 3, not significant (NS)], HSP27 [37°C/44°C: 1.34-fold  $\pm$  0.56, NS), HSP70 (37°C/44°C: 1.36-fold  $\pm$  0.87,  $n$  = 3, NS), or CHIP protein levels (37°C/44°C: 1.45-fold  $\pm$  0.19,  $n$  = 4,  $P$  = 0.041) normalized to  $\alpha$ -tubulin expression immediately after 1 hour of heat shock (Fig. 5D). The loss of AR could be explained by its precipitation and loss during subsequent protein preparation. To examine if this were the case, membrane as well as cytosolic protein fractions were tested for AR protein expression. After 1 hour at 44°C, AR protein was not detectable in any fraction. Furthermore, attempts to refold insoluble proteins using guanidiniumhydrochloride did not result in detection of AR protein (Fig. 5E). The observed loss of AR expression was not prevented by inhibition of transcriptional activity, inhibition of proteasome function or of calpain I and II before heating (Fig. 5F). To examine the involvement of proteases other than the proteasome and calpains in the effect, we incubated LnCaP cells with a panel of protease inhibitors including the

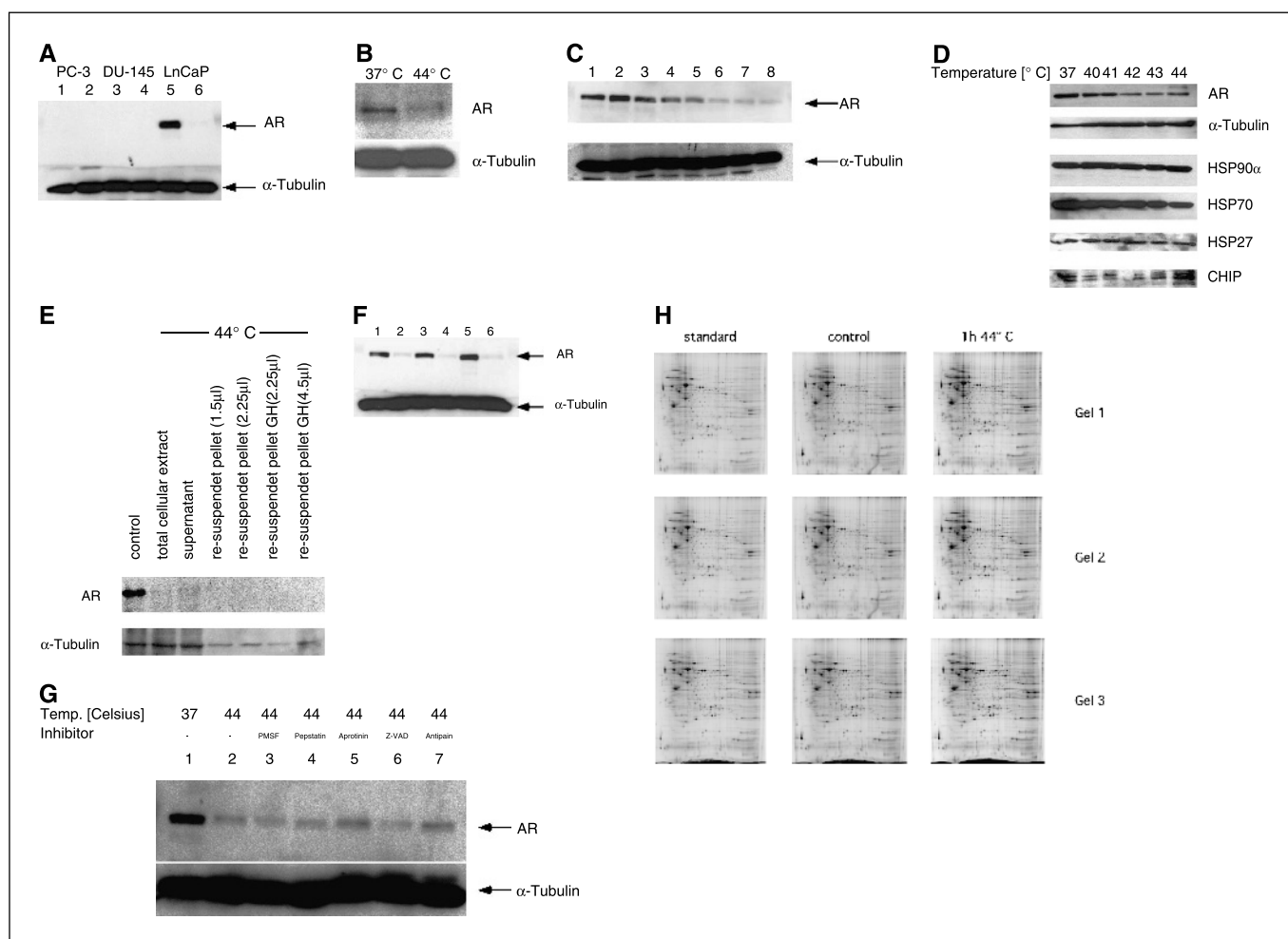
nonspecific caspase inhibitor Z-VAD-FMK (20  $\mu$ mol/L), PMSF (50  $\mu$ mol/L), pepstatin (1.5  $\mu$ mol/L), antipain (100  $\mu$ mol/L), or aprotinin (1.5  $\mu$ mol/L) for 1 hour before and during heat shock treatment. None of these treatments prevented heat-induced disappearance of AR protein (Fig. 5G). AR has a half-life of >3 hours in the absence and >6 hours in presence of androgens (26). However, heat shock may decrease transcription and translation and alter protein half-life at the same time. To rule out a general transcriptional shutdown, we studied total cellular extracts from LnCaP cells by two-dimensional DIGE technique. Using this approach we could detect and compare expression of >1,500 proteins (MW, 70-10 kDa;  $pI$ , 3-10) in a quantitative manner (15). However, we were not able to detect significant changes in protein expression pattern between cells treated for 1 hour at 44°C if compared with control cells (Fig. 5H; all gels were normalized against the internal standard, a minimum of a 1.5-fold change with a  $P$  < 0.05 using Student's  $t$  test was considered as statistical significant).

## Discussion

Although the molecular basis of hyperthermia is poorly understood, it is frequently used alone or in combination with radiotherapy in the treatment of prostate cancer (27-30). In this study, we investigated the effect of hyperthermia on NF- $\kappa$ B activity, proteasome function, and cell death as well as AR expression in human prostate cancer cell lines.

We recently showed that human PC-3 prostate cancer cells exhibit high constitutive expression of the antiapoptotic transcription factor NF- $\kappa$ B (7). Inhibition of NF- $\kappa$ B by transduction with an I $\kappa$ B superrepressor gene induced apoptosis in PC-3 cells (7). Here we show that constitutive high activity of NF- $\kappa$ B was also found in DU-145 and LnCaP human prostate cancer cells. Treatment of all three cell lines with heat shock down-regulated both constitutive and radiation-induced activation of NF- $\kappa$ B. Down-regulation of NF- $\kappa$ B after hyperthermia treatment has also been reported by other groups (31-33). Whereas Curry et al. (32) attributed this to inhibition of the I $\kappa$ B-kinases, we found that hyperthermia inhibited 26S proteasome function, providing an alternative pathway for heat-induced NF- $\kappa$ B inhibition through blocking I $\kappa$ B $\alpha$  degradation. Impairment of proteasome function by heat is in agreement with the findings of Kückelkorn et al. (34) and a recent publication of Mattson et al. (33) showing not only NF- $\kappa$ B inhibition but also increased activator protein activity after heat shock, which could be explained by inhibition of proteasome-dependent degradation of c-Fos and c-Jun. Because 20S proteasome activity remained unchanged after heat shock, our data suggest that the thermosensitive component of the proteasome is located in the 19S regulatory unit rather than in the 20S core unit. This was further supported by the observation that IFN- $\gamma$ , which replaces 19S regulatory subunits with PA28  $\alpha/\beta$  structures, prevented heat-induced inhibition of proteasome cleavage activity.

Down-regulation of NF- $\kappa$ B DNA-binding activity by heat-induced proteasome inhibition offers a molecular mechanism by which hyperthermia could operate in cancer cells to induce apoptosis. Proteasome inhibition is known to induce apoptosis in most cancer cells (19, 20, 35-39) as well as to sensitize surviving cells to ionizing radiation (36, 40). The effect of NF- $\kappa$ B down-regulation as a mechanism of radiosensitization of cancer cells is controversial (7, 40, 41). However, proteasome inhibition affects multiple pathways, many of which could account for the well-established radiosensitizing effect of hyperthermia (5, 42, 43). There is



**Figure 5.** Hyperthermia treatment abrogates expression of AR protein in androgen-dependent prostate cancer cells. **A**, Western blot analysis of total cellular lysates from AR-negative PC-3 (lanes 1 and 2) and DU-145 (lanes 2 and 4), and AR-positive LnCaP human prostate cancer cells (lanes 5 and 6). Equity of loading was confirmed using an anti- $\alpha$ -tubulin antibody. Heat treatment did not induce AR expression in PC-3 and DU-145 cells. In contrast, LnCaP cells completely lost AR expression after treatment at 44°C for 1 hour. **B**, Western blot analysis of total cellular lysates from AR-positive TRAMP-2 murine prostate cancer cells. Lane 1, control cells; lane 2, 60 minutes at 44°C. Equity of loading was confirmed using an anti- $\alpha$ -tubulin antibody. Heat treatment caused a decrease in AR expression. **C**, Western blot analysis of total cellular lysates from AR-positive LnCaP human prostate cancer cells incubated for 0 (lane 1), 15 (lane 2), 30 (lane 3), 45 (lane 4), 60 (lane 5), 75 (lane 6), 90 (lane 7), and 120 minutes (lane 8) at 44°C. Equity of loading was confirmed using an anti- $\alpha$ -tubulin antibody. Heat treatment causes a time-at-44°C-dependent decrease of AR expression. **D**, Western blot analysis of total cellular lysates from AR-positive LnCaP human prostate cancer cells, incubated for 1 hour at stated temperature for AR, HSP90 $\alpha$ , HSP70, HSP27, and CHIP. Equity of loading was confirmed using an anti- $\alpha$ -tubulin antibody. While HSP90 $\alpha$ , HSP27, and CHIP protein levels did not change, HSP70 levels decreased with increasing temperatures. **E**, Western blot analysis of extracts from total cellular lysates from AR-positive LnCaP human prostate cancer cells. Lane 1, control cells. Lanes 2-7, cells were treated for 1 hour at 44°C and allowed to recover for 90 minutes at 37°C. No AR expression was found in crude extracts (lane 2) or extracts precleared by centrifugation (lane 3). AR remained undetectable in resuspended pellets of insoluble material from total cellular extracts (lanes 4 and 5) even after refolding with guanidine hydrochloride (lanes 6 and 7). **F**, Western blot analysis of total cellular lysates from AR-positive LnCaP human prostate cancer cells. Preincubation with cycloheximide (25  $\mu$ g/mL for 30 minutes, lanes 3 and 4) or MG-132 (50  $\mu$ mol/L, 30 minutes, lanes 5 and 6) did not affect loss of AR expression following hyperthermia, excluding gene induction in general as well as proteasomal or calpain-mediated proteolysis of the AR as a possible mechanism. Equity of loading was confirmed using an anti- $\alpha$ -tubulin antibody. **G**, Western blot analysis of total cellular lysates from AR-positive LnCaP human prostate cancer cells. Cells were pretreated with PMSF (lane 3), pepstatin (lane 4), aprotinin (lane 5), Z-VAD-FMK (lane 6), and antipain (lane 7) for 1 hour. Protease inhibition with none of the protease inhibitors used prevented heat-induced (lane 2) abrogation of constitutive AR expression (lane 1). Equity of loading was confirmed using an anti- $\alpha$ -tubulin antibody. **H**, two-dimensional DIGE analysis of lysates from LnCaP cells. Protein expression of cells treated for 1 hour at 44°C (Cy5, left) was compared with protein expression of control cells (Cy3, middle), standardized to the corresponding internal standard (Cy2, right).

increasing evidence that impairment of nucleotide excision repair (NER) rather than nonhomologous end-joining of DNA-double strand breaks is crucial for heat-induced radiosensitization (44). This is of special interest in the light of our observation of heat-induced proteasome inhibition because proteasome inhibitors like MG-132 have been shown to inhibit NER (45).

Intriguingly, we also found that heat down-regulated AR expression in LnCaP cells. Survival of most tumor cells that originate from the prostate depends on the presence of androgen.

Thus, prostate cancer is usually controlled by androgen ablation for many years. The appearance of tumor cell populations lacking androgen dependence following androgen ablation is however inevitable and responsible for failure of this treatment. The mechanisms underlying development of androgen independence are unclear, but in some cases may be caused by down-regulation of AR expression or mutation of the *AR* gene combined with p53 mutations, which are thought to be early events during carcinogenesis occurring in a small subset of cancer cells (46-48).

The expression of the AR is a tightly regulated process that involves recruitment of several transcription factors (6, 24, 49).

Binding of NF- $\kappa$ B to the promoter region of the AR gene has been reported to repress AR gene expression (6). Heat-induced down-regulation of NF- $\kappa$ B might therefore be expected to result in increased AR protein expression. In AR-negative PC-3 cells, which are known to have minimal expression of functional AR mRNA and protein (50, 51), we could not detect any increase in AR protein levels in PC-3 cells, or in DU-145 cells, over a period of 24 hours after heat treatment. Furthermore, heat rapidly down-regulated AR expression in LnCaP human prostate cancer cells that normally express high levels of AR mRNA and protein (52), and AR expression did not recover over a period of 24 hours. Precipitation of AR during preparation was excluded as a mechanism using guanidine hydrochloride to refold insoluble material. A general translational shutdown after heat shock was excluded using quantitative two-dimensional electrophoresis. Loss of AR expression was dependent of the duration of heat treatment, occurred at temperatures above 41°C. In our study, the decrease in AR protein levels could not be prevented by inhibition of calpain I and II, or inhibition of the 26S proteasome. This is in accordance with the observation of Cardozo et al. who postulated degradation steps additional to proteasome-dependent cleavage to be responsible for AR degradation (53). On the other hand, these findings are, at least in part, contrary to results of an earlier study reporting that the 26S proteasome is responsible for degradation of AR because it was inhibited by MG-132 treat-

ment (54). Alternative pathways were also examined. Hyperthermia is known to activate lysosomal enzyme activity (55), which is mainly based on cathepsins (56) and hydrolysis of the AR is mediated by cathepsin D (57). However, preincubation of LnCaP cells with a panel of protease inhibitors blocking cysteine proteases and chymotrypsin (PMSF), cathepsin D (pepstatin), kallikrein, plasmin and trypsin (aprotinin), caspases (Z-VAD-FMK), and cathepsin A (Antipain) failed to prevent heat-induced abrogation of AR protein, leaving the mechanism of heat-induced loss of AR elusive.

Loss of AR expression in LnCaP cells was dependent of duration of hyperthermia treatment as well as on temperature. If the loss of AR in LnCaP cells reflects the behavior of androgen dependent prostate cancer cells *in vivo*, our study raises the question as to whether poorly done clinical hyperthermia lacking good thermosimetry and insufficient cell killing would drive the rapid acquisition of androgen independency, in particular in cells that have mutations affecting apoptotic pathways. Further studies are necessary to explore the clinical significance of our findings.

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## References

- Sweeney P, Pisters LL. Ad5CMVp53 gene therapy for locally advanced prostate cancer: where do we stand? *World J Urol* 2000;18:121-4.
- Elo JP, Visakorpi T. Molecular genetics of prostate cancer. *Ann Med* 2001;33:130-41.
- Tso CL, McBride WH, Sun J, et al. Androgen deprivation induces selective outgrowth of aggressive hormone-refractory prostate cancer clones expressing distinct cellular and molecular properties not present in parental androgen-dependent cancer cells. *Cancer J* 2000;6:220-33.
- Kampinga HH, Dikomey E. Hyperthermic radiosensitization: mode of action and clinical relevance. *Int J Radiat Biol* 2001;77:399-408.
- Overgaard J. The current and potential role of hyperthermia in radiotherapy. *Int J Radiat Oncol Biol Phys* 1989;16:535-49.
- Supakar PC, Jung MH, Song CS, Chatterjee B, Roy AK. Nuclear factor  $\kappa$ B functions as a negative regulator for the rat androgen receptor gene and NF- $\kappa$ B activity increases during the age-dependent desensitization of the liver. *J Biol Chem* 1995;270:837-42.
- Pajonk F, Pajonk K, McBride WH. Inhibition of NF- $\kappa$ B, clonogenicity, and radiosensitivity of human cancer cells. *J Natl Cancer Inst* 1999;91:1956-60.
- Bauerle PA, Baltimore D. NF- $\kappa$ B: ten years after. *Cell* 1996;87:13-20.
- Ciechanover A. The ubiquitin-proteasome proteolytic pathway. *Cell* 1994;79:13-21.
- Lee DH, Goldberg AL. Selective inhibitors of the proteasome-dependent and vacuolar pathways of protein degradation in *Saccharomyces cerevisiae*. *J Biol Chem* 1996;271:27280-4.
- Rolfé M, Chiu MI, Pagano M. The ubiquitin-mediated proteolytic pathway as a therapeutic area. *J Mol Med* 1997;75:5-17.
- Greenberg NM, DeMayo F, Finegold MJ, et al. Prostate cancer in a transgenic mouse. *Proc Natl Acad Sci U S A* 1995;92:3439-43.
- Albright N. Computer programs for the analysis of cellular survival data. *Radiat Res* 1987;112:331-40.
- Dubray B, Mosseri V, Brunin F, et al. Anemia is associated with lower local-regional control and survival after radiation therapy for head and neck cancer: a prospective study. *Radiology* 1996;201:553-8.
- Friedman DB, Hill S, Keller JW, et al. Proteome analysis of human colon cancer by two-dimensional difference gel electrophoresis and mass spectrometry. *Proteomics* 2004;4:793-811.
- Glas R, Bogyo M, McMaster JS, Gaczynska M, Ploegh HL. A proteolytic system that compensates for loss of proteasome function. *Nature* 1998;392:618-22.
- Li WX, Franklin WA. Radiation- and heat-induced apoptosis in PC-3 prostate cancer cells. *Radiat Res* 1998;150:190-4.
- Dewey WC, Hopwood LE, Sapareto SA, Gerweck LE. Cellular responses to combinations of hyperthermia and radiation. *Radiology* 1977;123:463-74.
- Herrmann JL, Briones F Jr, Brisbay S, Logothetis CJ, McDonnell TJ. Prostate carcinoma cell death resulting from inhibition of proteasome activity is independent of functional Bcl-2 and p53. *Oncogene* 1998;17:2889-99.
- Kitagawa H, Tani E, Ikemoto H, Ozaki I, Nakano A, Omura S. Proteasome inhibitors induce mitochondria-independent apoptosis in human glioma cells. *FEBS Lett* 1999;443:181-6.
- Gaczynska M, Rock KL, Spies T, Goldberg AL. Peptidase activities of proteasomes are differentially regulated by the major histocompatibility complex-encoded genes for LMP2 and LMP7. *Proc Natl Acad Sci U S A* 1994;91:9213-7.
- Harris JL, Alper PB, Li J, Rechsteiner M, Backes BJ. Substrate specificity of the human proteasome. *Chem Biol* 2001;8:1131-41.
- Li J, Rechsteiner M. Molecular dissection of the 11S REG (PA28) proteasome activators. *Biochimie* 2001;83:373-83.
- Supakar PC, Roy AK. Role of transcription factors in the age-dependent regulation of the androgen receptor gene in rat liver. *Biol Signals Recept* 1996;5:170-9.
- Fang Y, Fliss AE, Robins DM, Caplan AJ. Hsp90 regulates androgen receptor hormone binding affinity *in vivo*. *J Biol Chem* 1996;271:28697-702.
- Syms AJ, Norris JS, Panko WB, Smith RG. Mechanism of androgen-receptor augmentation. Analysis of receptor synthesis and degradation by the density-shift technique. *J Biol Chem* 1985;260:455-61.
- Algan O, Fosmire H, Hynynen K, et al. External beam radiotherapy and hyperthermia in the treatment of patients with locally advanced prostate carcinoma. *Cancer* 2000;89:399-403.
- Hurwitz MD, Kaplan ID, Svensson GK, Hynynen K, Hansen MS. Feasibility and patient tolerance of a novel transrectal ultrasound hyperthermia system for treatment of prostate cancer. *Int J Hyperthermia* 2001;17:31-7.
- Diederich CJ, Nau WH, Burdette EC, Bustany IS, Dearnoff DL, Stauffer PR. Combination of transurethral and interstitial ultrasound applicators for high-temperature prostate thermal therapy. *Int J Hyperthermia* 2000;16:385-403.
- Tucker RD, Huidobro C, Larson T, Platz CE. Use of permanent interstitial temperature self-regulating rods for ablation of prostate cancer. *J Endourol* 2000;14:511-7.
- Pritts TA, Wang Q, Sun X, et al. Induction of the stress response *in vivo* decreases nuclear factor- $\kappa$ B activity in jejunal mucosa of endotoxemic mice. *Arch Surg* 2000;135:860-6.
- Curry HA, Clemens RA, Shah S, et al. Heat shock inhibits radiation-induced activation of NF- $\kappa$ B via inhibition of I- $\kappa$ B kinase. *J Biol Chem* 1999;274:23061-7.
- Mattson D, Bradbury CM, Bisht KS, Curry HA, Spitz DR, Gius D. Heat shock and the activation of AP-1 and inhibition of NF- $\kappa$ B DNA-binding activity: possible role of intracellular redox status. *Int J Hyperthermia* 2004;20:224-33.
- Kuckelkorn U, Kneuhl C, Boes-Fabian B, Drung I, Kloetzel PM. The effect of heat shock on 20S/26S proteasomes. *Biol Chem* 2000;381:1017-23.
- Zhang XM, Lin H, Chen C, Chen BD. Inhibition of ubiquitin-proteasome pathway activates a caspase-3-like protease and induces Bcl-2 cleavage in human M-07e leukaemic cells. *Biochem J* 1999;340:127-33.
- Pajonk F, Pajonk K, McBride W. Apoptosis and radiosensitization of Hodgkin's cells by proteasome inhibition. *Int J Radiat Oncol Biol Phys* 2000;47:1025-32.

37. McDade TP, Perugini RA, Vittimberga FJ Jr, Callery MP. Ubiquitin-proteasome inhibition enhances apoptosis of human pancreatic cancer cells. *Surgery* 1999;126:371-7.
38. Adams J, Palombella VJ, Sausville EA, et al. Proteasome inhibitors: a novel class of potent and effective antitumor agents. *Cancer Res* 1999;59:2615-22.
39. An B, Goldfarb RH, Siman R, Dou QP. Novel dipeptidyl proteasome inhibitors overcome Bcl-2 protective function and selectively accumulate the cyclin-dependent kinase inhibitor p27 and induce apoptosis in transformed, but not normal, human fibroblasts. *Cell Death Differ* 1998;5:1062-75.
40. Russo SM, Tepper JE, Baldwin AS Jr, et al. Enhancement of radiosensitivity by proteasome inhibition: implications for a role of NF- $\kappa$ B. *Int J Radiat Oncol Biol Phys* 2001;50:183-93.
41. Jung M, Zhang Y, Lee S, Dritschilo A. Correction of radiation sensitivity in ataxia telangiectasia cells by a truncated I $\kappa$ B- $\alpha$ . *Science* 1995;268:1619-21.
42. Sapareto SA, Hopwood LE, Dewey WC. Combined effects of X irradiation and hyperthermia on CHO cells for various temperatures and orders of application. *Radiat Res* 1978;73:221-33.
43. Dewey WC, Sapareto SA, Betten DA. Hyperthermic radiosensitization of synchronous Chinese hamster cells: relationship between lethality and chromosomal aberrations. *Radiat Res* 1978;76:48-59.
44. Kampinga HH, Dynlacht JR, Dikomey E. Mechanism of radiosensitization by hyperthermia ( $\geq 43$  degrees C) as derived from studies with DNA repair defective mutant cell lines. *Int J Hyperthermia* 2004;20:131-9.
45. Wang QE, Wani MA, Chen J, et al. Cellular ubiquitination and proteasomal functions positively modulate mammalian nucleotide excision repair. *Mol Carcinog* 2005;42:53-64.
46. Navone NM, Labate ME, Troncoso P, et al. p53 mutations in prostate cancer bone metastases suggest that selected p53 mutants in the primary site define foci with metastatic potential. *J Urol* 1999;161:304-8.
47. Navone NM, Troncoso P, Pisters LL, et al. p53 protein accumulation and gene mutation in the progression of human prostate carcinoma. *J Natl Cancer Inst* 1993;85:1657-69.
48. Marcelli M, Ittmann M, Mariani S, et al. Androgen receptor mutations in prostate cancer. *Cancer Res* 2000;60:944-9.
49. Mora GR, Mahesh VB. Autoregulation of androgen receptor in rat ventral prostate: involvement of *c-fos* as a negative regulator. *Mol Cell Endocrinol* 1996;124:111-20.
50. Lin MF, Meng TC, Rao PS, Chang C, Schonthal AH, Lin FF. Expression of human prostatic acid phosphatase correlates with androgen-stimulated cell proliferation in prostate cancer cell lines. *J Biol Chem* 1998;273:5939-47.
51. Jarrard DF, Kinoshita H, Shi Y, et al. Methylation of the androgen receptor promoter CpG island is associated with loss of androgen receptor expression in prostate cancer cells. *Cancer Res* 1998;58:5310-4.
52. Suzuki H, Sato N, Watabe Y, Masai M, Seino S, Shimazaki J. Androgen receptor gene mutations in human prostate cancer. *J Steroid Biochem Mol Biol* 1993;46:759-65.
53. Cardozo CP, Michaud C, Ost MC, et al. C-terminal Hsp-interacting protein slows androgen receptor synthesis and reduces its rate of degradation. *Arch Biochem Biophys* 2003;410:134-40.
54. Sheflin L, Keegan B, Zhang W, Spaulding SW. Inhibiting proteasomes in human HepG2 and LNCaP cells increases endogenous androgen receptor levels. *Biochem Biophys Res Commun* 2000;276:144-50.
55. Yamauchi N, Watanabe N, Maeda M, et al. Mechanism of synergistic cytotoxic effect between tumor necrosis factor and hyperthermia. *Jpn J Cancer Res* 1992;83:540-5.
56. Hasselgren PO, Fischer JE. The ubiquitin-proteasome pathway: review of a novel intracellular mechanism of muscle protein breakdown during sepsis and other catabolic conditions. *Ann Surg* 1997;225:307-16.
57. Mordente JA, Choudhury MS, Tazaki H, Mallouh C, Konno S. Hydrolysis of androgen receptor by cathepsin D: its biological significance in human prostate cancer. *Br J Urol* 1998;82:431-5.