Effect of Antioxidants on Androgen-Induced AP-1 and NF-κB DNA-Binding Activity in Prostate Carcinoma Cells

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Background: **Previous studies have suggested that male hormones (androgens) and certain forms of oxygen (reactive oxygen species) are linked to the development of prostate cancer. We hypothesized that androgens contribute to prostate carcinogenesis by increasing oxidative stress. We further hypothesized that antioxidants reduce prostate cancer risk by modulating androgen effects on cellular processes.** ***Methods:*** **To test these hypotheses, we looked for 1) a change in the level of reactive oxygen species in the presence of androgens, 2) androgen-induced binding activity of transcriptional activators AP-1 and NF-κB, whose activities are known to be altered during cell proliferation, and 3) the effect of antioxidants on androgen-induced transcription factor binding.** ***Results:*** **Physiologic concentrations (1 n*M*) of 5α-dihydrotestosterone or 1–10 n*M* R1881, a synthetic androgen, produced sustained elevation of AP-1 and NF-κB DNA-binding activity in LNCaP cells, an androgen-responsive human prostate carcinoma cell line. Androgen-independent DU145 cells (another human prostate carcinoma cell line) were unaffected by R1881 treatment. AP-1-binding activity increased 5 hours after 1 n*M* R1881 treatment; NF-κB DNA-binding activity increased after 36 hours. Both activities remained elevated for at least 120 hours. Nuclear AP-1 and NF-κB protein levels were not elevated. Antioxidant vitamins C plus E blocked both androgen-induced DNA-binding activity and production of reactive oxygen species.** ***Conclusion:*** **Physiologic concentrations of androgens induce production of reactive oxygen species and cause prolonged AP-1 and NF-κB DNA-binding activities, which are diminished by vitamins C and E. [J Natl Cancer Inst 1999; 91:1227–32]**

Androgens, such as testosterone, are necessary for normal growth and development of the prostate *(1–3)* and may also play a role in prostate carcinogenesis *(4).* Whether they act as initiators, as has been proposed for estrogen metabolites in breast cancer *(5,6),* or as promoters *(7–9)* is debatable. However, most investigators agree that androgens play a role in either the development or the progression of prostate cancer. Prostate cancer is a disease of aging *(10).* The oxidative stress hypothesis of aging postulates that oxidative damage to critical molecules accumulates over the life span and eventually impairs function *(11).* We hypothesize that androgens contribute to the age-associated increase in prostate cancer by increasing oxidative stress.

Earlier, we *(12)* linked androgen exposure to production of reactive oxygen species (ROS) in cultured prostate cancer cells. ROS (e.g., superoxide radical, hydrogen peroxide, and hydroxyl radical) cause macromolecular damage and may play important roles in tumor development and aging *(11,13,14).* ROS are also important regulatory molecules acting as intracellular second messengers and regulators of protein function *(15–18).*

Studies of dietary antioxidants provide encouraging data regarding prostate cancer prevention. Data from the Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study *(19)* show that long-term α-tocopherol supplementation reduces prostate cancer incidence by 32% and prostate cancer mortality by 41% in cigarette smokers. Similar evidence links intake of lycopene, a potent carotenoid, and selenium supplementation with reduced prostate cancer risk *(20,21).* Understanding how antioxidants act to reduce cancer risk will aid in developing prevention strategies.

This study examined the effect of androgens and antioxidants on transcription factor binding at AP-1 and NF-κB DNA-binding sites. AP-1 and NF-κB are ubiquitous protein complexes that mediate cellular response to various external signals by binding to distinct DNA sites *(22–24).* Knowing that androgen treatment influences the intracellular oxidation state in cultured cells and that AP-1 and NF-κB are redox regulated, we sought to determine if androgens stimulate the binding activity of these transcription factors and if antioxidants can influence the effect.

Materials and Methods

Cell Culture and Harvest

Androgen-responsive, androgen receptor-positive LNCaP and androgen-independent DU145 human prostate carcinoma cells were obtained from American Type Culture Collection (Manassas, VA). Cells were maintained at 37 °C in an atmosphere of 5% carbon dioxide and 95% air in Dulbecco's modified Eagle medium (DMEM) supplemented with 5% heat-inactivated fetal bovine serum (FBS) (Life Technologies, Inc. [GIBCO BRL], Gaithersburg, MD) and 1× antibiotic–antimycotic (Sigma Chemical Co., St. Louis, MO, from which all chemicals, unless otherwise noted, were obtained). Cells were passaged weekly, and medium was replaced every 3 or 4 days. LNCaP cells of passages 50–90 and DU145 cells of passages 60–70 were used for all experiments.

Experiments were conducted in medium containing 1% FBS and 4% charcoal-stripped FBS (CSS) to limit the adverse growth effects noted with 5% CSS, which are unrelated to hormone depletion *(12).* For 96-well assays, 4000 cells were plated per well in 100 μL of DMEM plus 1% FBS and 4% CSS. Then 4 days later, cells were treated with 100 μL of DMEM plus 1% FBS and 4% CSS containing a 2× concentration of the specified treatment. The final concentration of treatment was 0, 25, 50, 100, or 500 μ*M* vitamin C (ascorbic acid) and vitamin E (α-tocopherol succinate) in 1 n*M* R1881, a synthetic androgen (Du Pont NEN, Boston, MA), or vehicle control medium (containing 0.01% ethanol).

Cells harvested for electrophoretic mobility-shift assay were plated at 10^6cells per 100-mm tissue culture plate in medium containing 5% FBS. The following day, medium was replaced with fresh medium containing 1% FBS and 4% CSS. Cells were treated 3 or 4 days later with fresh medium containing R1881 or the vehicle control. After the appropriate treatment period, cells were washed, scraped from the plate, resuspended in cold Kreb's Ringer

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See "Notes" following "References."

buffer, and centrifuged for 5 minutes at 4 °C at 1000 rpm. The cell pellet was frozen at −80 °C.

Measurement of ROS and DNA Content

We harvested 96-well plates for estimation of cellular peroxides and hydroxyl radical levels using 2′,7′-dichlorofluorescein diacetate (DCF) (Molecular Probes, Inc., Eugene, OR) after 24, 72, and 96 hours of treatment as described elsewhere *(25)*. Fluorescent units were measured in each well after a 45-minute incubation with DCF (1 μg/0.1 mL) at 37 °C by use of an LS 50B Luminescence Spectrometer (The Perkin-Elmer Corp., Norwalk, CT). The same plates were frozen at −80 °C, and the DNA content per well was determined after thawing by a fluorometric assay using the dye Hoechst 33258 *(26)*. The DNA fluorescence units were used as a measure of cell growth, and the DCF fluorescence units normalized to DNA (DCF/DNA) were used as a measure of ROS levels.

Electrophoretic Mobility-Shift Assay

Frozen cell pellets were processed to obtain cytoplasmic and nuclear extracts in the presence of aprotinin (2 μg/mL), leupeptin (2–5 μg/mL), and pepstatin A (5 μg/mL) *(27)*. Each cell pellet was resuspended in a 5× volume of Buffer A (10 m*M* HEPES [pH 7.9], 1.5 m*M* $MgCl_2$, 10 m*M* KCl, 1 m*M* dithiothreitol, and 1 m*M* polymethylsulfonyl fluoride) and was incubated on ice for 15 minutes. Nuclear pellets were obtained after centrifugation (14 000 rpm at room temperature for 10 seconds) in a microcentrifuge; supernatant was saved as cytoplasmic extract after a second centrifugation (13 000 rpm at 4 °C for 10 minutes). The nuclear pellet was resuspended in 2x original pellet volume of Buffer C (20 m*M* HEPES [pH 7.9], 25% glycerol, 1.5 m*M* $MgCl_2$, 420 m*M* NaCl, 0.2 m*M* ethylene diamine tetraacetate [EDTA], 1 m*M* dithiothreitol, and 1 m*M* polymethylsulfonyl fluoride). Cells were incubated on ice for 30 minutes and centrifuged (14 000 rpm at 4 °C for 30 minutes) in a microcentrifuge, and the supernatant was saved as nuclear extract. All extracts were stored at −80 °C. The protein content was measured with the use of the bicinchoninic acid assay (Pierce Chemical Co., Rockford, IL).

Nuclear extracts, 2–6 μg, were brought to 4 μL with water and combined with 5 μL of mix—two parts H_2O, one part poly(dI–dC), and two parts 5× gel shift buffer (375 m*M* NaCl, 75 m*M* Tris–HCl [pH 7.5], 37.5% glycerol, 7.5 m*M* EDTA, 7.5 m*M* dithiothreitol, 50 m*M* 1.5% Nonidet P-40, and 100 μg/mL bovine serum albumin). They were then incubated for 20 minutes on ice. Oligonucleotides (Promega Corp., Madison, WI) end labeled with ^{32}P were added and incubated with the samples for 20 minutes at room temperature. Protein–DNA complexes were run through a 1.5-mm 4.5% acrylamide gel in 0.25× Tris–borate–EDTA buffer. Gels were run at 175 V for 1.5 hours, dried, and imaged with the use of PhosphorImager and ImageQuant analysis software (Molecular Dynamics, Sunnyvale, CA).

Immunoblots

Nuclear extracts, 25–30 μg of protein per lane, were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis with the use of 4%–12% Bis–Tris gels (NOVEX, San Diego, CA) and transferred to Immobilon-P membranes (Millipore Corp., Bedford, MA) with the use of a semidry system. Immunoblots were blocked overnight at 4 °C in 5% dried milk (wt/vol) in phosphate-buffered saline (PBS) (pH 7.4) plus 0.05% Tween 20. Blots were incubated with polyclonal primary antibodies to c-Fos, c-Jun, p50, and p65 (all from Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or androgen receptor (gift of Dr. C. Chang, University of Rochester, Rochester, NY) in PBS plus 0.05% Tween 20 for 1–2 hours at room temperature. The blots were washed and exposed to a horseradish peroxidase-labeled secondary antibody (Amersham Pharmacia Biotech, Arlington Heights, IL) for 45 minutes at room temperature. Washes and luminescent detection were carried out by use of the ECL-plus detection system according to the instructions of the manufacturer (Amersham Pharmacia Biotech). Densitometric analyses of scanned x-ray films were performed with the use of Adobe Photoshop and National Institutes of Health image software.

Statistical Analyses

Experiments were conducted in 96-well plates ($n = 6$). Each treatment was compared with either the vehicle control treatment or the R1881 treatment by use of the unpaired, two-tailed Student's *t* test. Sample sizes were adequate to detect statistically significant differences. Western and immunoblot analyses were done at least twice ($n \geq 2$). The mean density of specific bands from scanned x-ray films was compared by use of a paired, two-tailed Student's *t* test. All data were tested for statistical significance at the nominal .05 significance level. The reported *P* values are two-sided and reflect the data shown. All experiments were repeated at least two times.

RESULTS

Measurement of ROS and Cell Growth After Androgen and Antioxidant Treatment

Growth (DNA per well) and ROS levels as measured by DCF/DNA of LNCaP cells were unaffected by treatment with 1 n*M* R1881 for 24 hours (Fig. 1, A and B). At 96 hours, growth was 40% lower (Fig. 1, D; $P<.0001$) and ROS levels were 86% higher in R1881-treated cells than in untreated control cells (Fig. 1, C; $P<.001$).

LNCaP cells treated with the vehicle control plus 500 μ*M* vitamins C plus E for 24 hours displayed a greater decrease in DCF/DNA than cells treated with R1881 plus the antioxidants (38% versus 21%) (Fig. 1, A; both $P<.001$). After 96 hours of treatment, a decrease in DCF/DNA compared with the vehicle control was observed with 50, 100, and 500 μ*M* vitamins C plus E in both control and R1881-treated cells (Fig. 1, C). In the vehicle control- plus antioxidant-treated cells, an 11%–22% decrease in DCF/DNA occurred ($P = .034$, 50 μ*M*; $P = .026$, 100 μ*M*; $P<.001$, 500 μ*M*). In the R1881-treated cells incubated with 100 or 500 μ*M* vitamins C plus E, DCF/DNA was 21% and 57%, respectively, of cells treated with R1881 alone ($P = .003$, 100 μ*M*; $P<.0001$, 500 μ*M*). Only the highest dose of vitamins C plus E (500 μ*M*) was associated with a decrease in growth (18% for vehicle control-treated cells [$P<.0001$] and 44% for R1881-treated cells [$P = .0017$]) compared with cells not treated with antioxidant (Fig.1, D).

AP-1 DNA-Binding Activity in Cells Treated With R1881

Treatment of LNCaP cells with 0.001–10 n*M* R1881for 96 hours resulted in increased DNA-binding activity to the AP-1 consensus sequence with 0.25, 1, and 10 n*M* R1881 (Fig. 2, A; 0.25-n*M* data not shown). Increased binding activity was also noted with 1 n*M* 5α-dihydrotestosterone (DHT). All of the doses that increased binding activity were previously shown to be associated with elevated levels of DCF-determined ROS content *(12)*. Increased DNA-binding activity to the AP-1 site was first noted 5 hours after treatment with 1 n*M* R1881 (Fig. 2, B). Binding activity increased with time, peaking at 96 hours, and remained elevated for at least 120 hours (Fig. 2, C). Androgen-independent DU145 cells are unresponsive to R1881 based on DCF or DNA measures and to R1881-induced alterations in DNA-binding activity to AP-1 sites (data not shown).

AP-1 DNA-Binding Activity in Cells Treated With R1881 and Antioxidants

Treatment of LNCaP cells with equimolar concentrations of vitamins C plus E abrogated R1881-induced binding to the AP-1 site (Fig. 2, D). Treatment with 50, 100, or 500 μ*M* vitamins C plus E for 24 hours diminished binding at the AP-1 site in both vehicle control-treated and R1881-treated LNCaP cells. The 500-μ*M* dose produced the largest decrease in binding activity at 24 hours and the only clear decrease in AP-1 DNA-binding activity at 96 hours (96-hour data not shown).

NF-κB DNA-Binding Activity in Cells Treated With R1881

DNA binding of nuclear extracts of LNCaP cells to the NF-κB consensus sequence was elevated by 1 and 10 n*M* R1881 after 96 hours of treatment (Fig. 3, A). Doses of 0.001–0.1 n*M* R1881 had no stimulatory effect on DNA binding. NF-

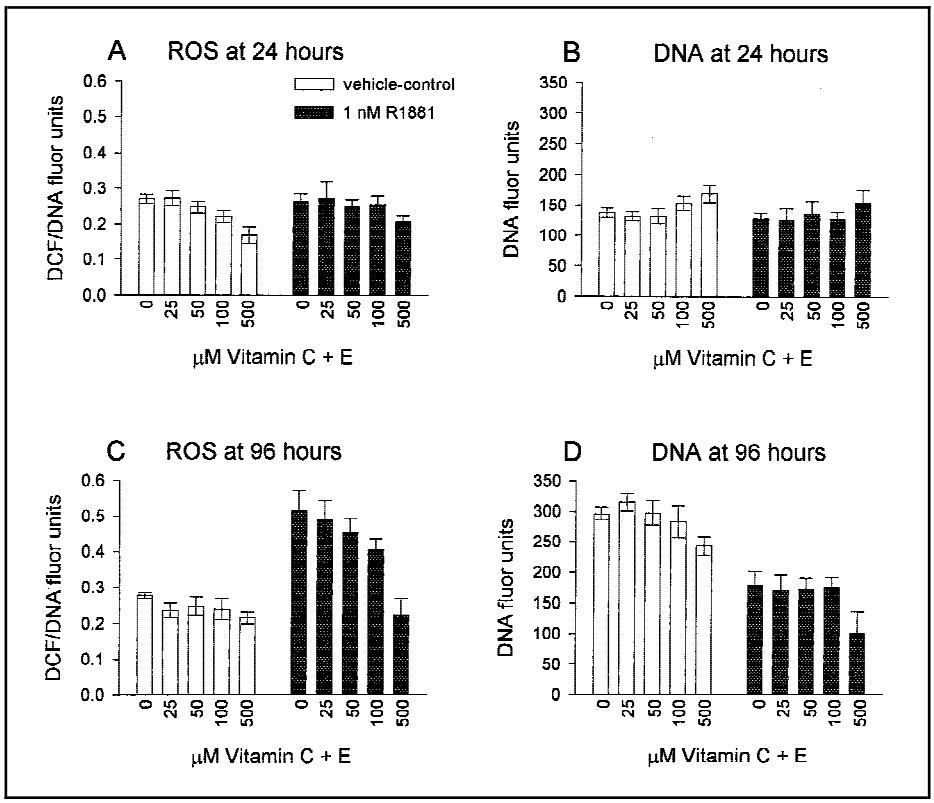

Fig. 1. Effect of vitamins C plus E (25, 50, 100, and 500 μ*M* each) on LNCaP human prostate carcinoma cells treated with vehicle control (**open bars**) and 1 n*M* R1881, a synthetic androgen (**solid bars**). **Panels A** and **B** show data at 24 hours. **Panels C** and **D** show data at 96 hours. Hydrogen peroxide and hydroxyl radical levels, as estimated by 2′,7′-dichlorofluorescein diacetate (DCF) fluorescence normalized to DNA (DCF/DNA), were lowered by the 500-μ*M* antioxidant treatment at 24 hours (**panel A;** $P<.001$). Cell growth, as measured by DNA fluorescence, was not inhibited (**panel B**). By 96 hours, R1881 treatment increased reactive oxygen species (ROS) levels (**panel C;** $P<.001$). Antioxidant treatments of 25–500 μ*M* lowered the DCF/DNA compared with vehicle control treatment (**panel C**). A decrease in DCF/DNA was observed in cells treated with R1881 plus antioxidants ($P = .003$ for 100-μ*M* and $P<.0001$ for 500-μ*M* treatments). At 96 hours, R1881 lowered DNA readings by 40% (**panel D;** $P<.0001$). Only the highest dose of antioxidants reduced DNA readings after 96 hours ($P<.0001$ for vehicle control-treated cells; $P = .0017$ for R1881-treated cells). All data represent the average ± standard deviation of six separate wells.

κB DNA-binding activity was induced by 1 n*M* R1881 at 36 hours (Fig. 3, B). At 72 and 96 hours, very strong binding activity of the slower migrating complex was observed (upper arrow, Fig. 3, B). Binding activity remained elevated for at least 120 hours.

NF-κB DNA-Binding Activity in Cells Treated With R1881 and Antioxidants

Equimolar concentrations of vitamins C plus E lowered the androgen-induced DNA binding at the NF-κB site after 96 hours of treatment (Fig. 3, C). Only the 500-μ*M* dose influenced DNA-binding activity to the NF-κB site. This dose increased the binding activity of the faster migrating NF-κB DNA–protein complex in cells not treated with R1881. A 24-hour treatment with vitamins C plus E did not influence DNA-binding activity at the NF-κB site (data not shown).

Protein Levels of c-Jun, c-Fos, p50, p65, and Androgen Receptor in LNCaP Cells Treated With R1881

Nuclear extracts from LNCaP cells treated with 0–1 n*M* R1881 for 24 and 96 hours showed no increase in AP-1 (c-Jun and c-Fos)-associated proteins, NF-κB (p50 and p65)-associated proteins, or androgen receptor (Table 1). After 24 and 96 hours, 1 n*M* R1881 caused a marked decrease in the protein level of c-Fos ($P = .047$, 24 hours; $P = .0005$, 96 hours).

Discussion

The ability of physiologic levels of androgens to alter the oxidative state of prostate cells may have implications for control of normal cellular processes and prostate carcinogenesis. An increase in the prooxidant state is linked to tumor development in various tissues *(13,14)*. ROS-induced injuries to DNA, proteins, and lipids may be important carcinogenic mechanisms *(13,15,28)*. Damage associated with ROS has been documented in prostate cancer *(29);* however, whether androgens cause prostatic oxidative damage *in vivo* is unknown. ROS are also important regulatory molecules. Just as the phosphorylation state can dictate protein activity, the redox state can regulate protein function *(16,30)*. ROS are now recognized as messengers in cell-signaling pathways *(17,31)* and apoptosis (i.e., programmed cell death) *(32,33)*. Androgen-induced ROS could potentially influence any of these functions.

We observed that androgens administered at doses previously found to induce oxidative stress *(12)* can increase the DNA-binding activity of LNCaP nuclear proteins to both AP-1 and NF-κB sites. In contrast, R1881 and DHT had no effect on AP-1 and NF-κB DNA-binding activities or oxidative state in androgen-independent DU145 cells. AP-1 and NF-κB DNA-binding activities are known to be sensitive to ROS *(34–36)*. DNA binding of AP-1 and NF-κB and of several other ROS-sensitive transcription factors is activated by reducing agents and inhibited by oxidizing agents *in vitro*, but the effects are often reversed in intact cells, which is suggestive of a more complex form of cellular regulation *(36–38)*. DNA-binding activity induced in LNCaP cells by hydrogen peroxide at AP-1 sites and by tumor necrosis factor-α at NF-κB sites was similar in magnitude to that caused by androgen treatment (data not shown).

AP-1, a ubiquitous transcriptional activator, mediates responses to external signals by regulating the expression of genes involved in growth, differentiation, and stress responses *(22,38)*. It is composed of members of the Jun and Fos families that form homodimers (c-Jun:c-Jun) and heterodimers (c-Jun:c-Fos) that bind to a specific DNA response element. Neoplastic transformation may be induced simply by altering the expression or structure of c-Jun or c-Fos *(22)*. Regulation of AP-1 activity is complex and involves mechanisms that increase the levels of Jun and Fos proteins as well as those that stimulate the activity of preexisting Jun and Fos *(22,39)*. Redox regulation of AP-1-binding activity occurs through a conserved cysteine residue in the DNA-binding domain of c-Fos and c-Jun proteins *(34)*. Western blot analyses of protein levels in nuclear extracts of

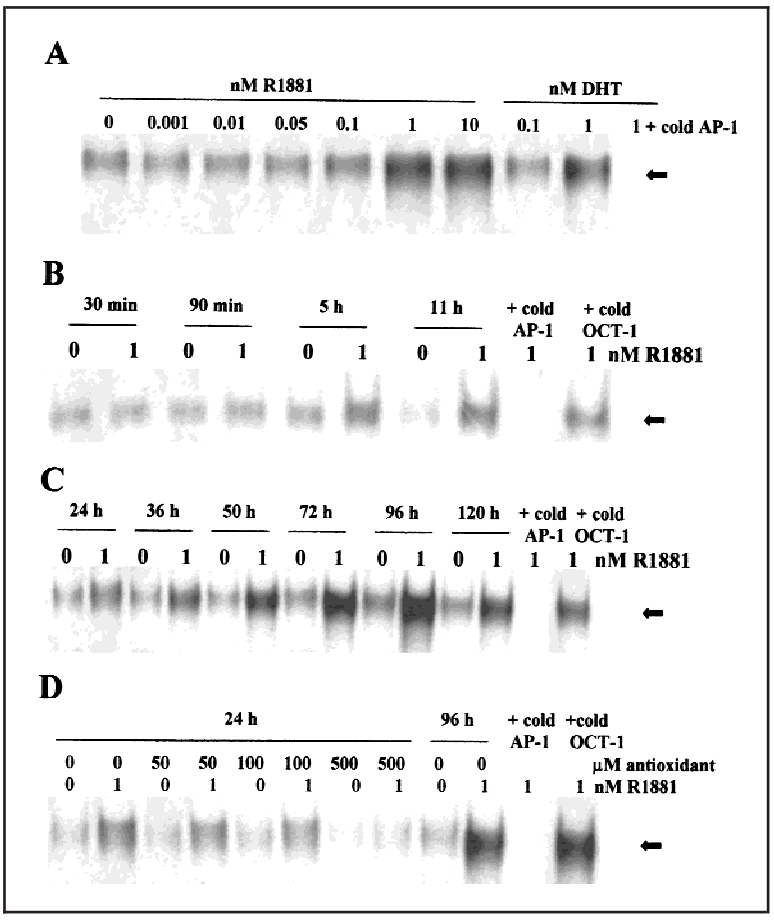

Fig. 2. Nuclear protein binding to ^{32}P-labeled AP-1 consensus oligonucleotides measured by electrophoretic mobility-shift assay. **Arrow** denotes the specific AP-1 protein–DNA complex. Nuclear extracts were obtained from LNCaP cells (a human prostate carcinoma cells line) treated with 0.001–10 n*M* R1881 (a synthetic androgen) or 0.1–1 n*M* 5α-dihydrotestosterone (DHT) for 96 hours and incubated with labeled AP-1 oligonucleotides. DNA-binding activity was elevated after treatment with 1 and 10 n*M* R1881 and with 1 n*M* DHT (**panel A**). Excess unlabeled AP-1 oligonucleotides (= cold AP-1) competed away binding, thereby suggesting specificity. Nuclear extracts harvested at various time points after treatment with vehicle control or 1 n*M* R1881 displayed an increase in binding activity to AP-1 sites by 5 hours (**panel B**). Binding activity continued to rise and reached a maximum at 96 hours (**panel C**). Excess unlabeled OCT-1 (a transcription factor) oligonucleotide, used as a nonspecific competitor, had no effect on binding activity. Co-treatment with vehicle control or 1 n*M* R1881 and a mix of 50–500 µ*M* vitamins C plus E lowered protein binding to AP-1 sites after 24 hours of treatment (**panel D**). A substantial antioxidant effect on DNA-binding activity is seen following treatment with 500 µ*M* vitamins C plus E.

LNCaP cells showed no androgen-induced increase in the level of c-Fos or c-Jun. In fact, c-Fos levels were statistically significantly lower in LNCaP cells treated with 1 n*M* R1881, suggesting that the increased DNA binding is due to a change in activity and not protein level.

There is evidence that transcriptional activation by nuclear receptors, like the androgen receptor, is influenced by interactions with other transcription factors *(40–42)*. Androgen receptor-induced tran-

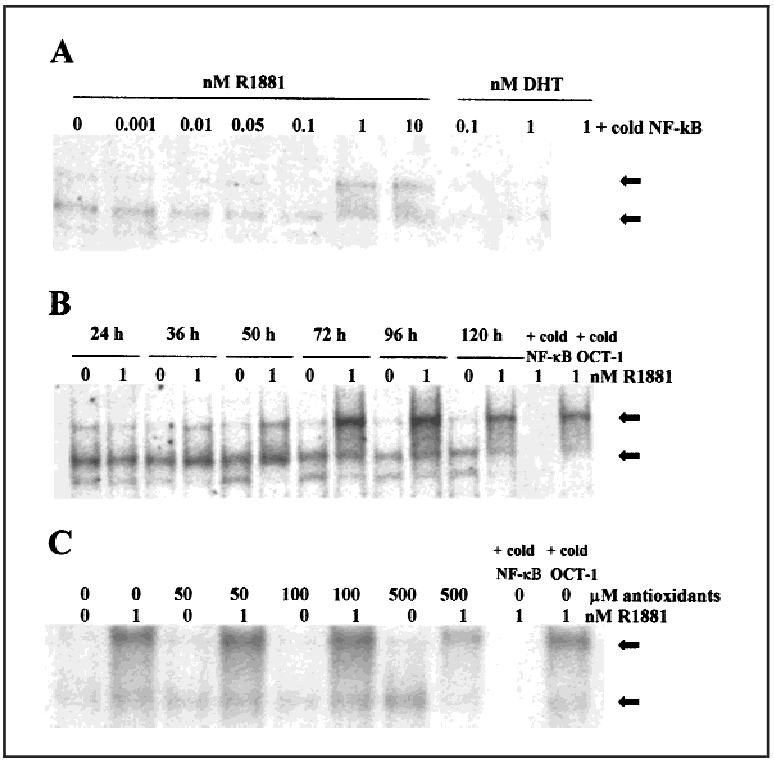

Fig. 3. Nuclear protein binding to ^{32}P-labeled NF-κB consensus oligonucleotides measured by electrophoretic mobility-shift assay. **Arrows** denote NF-κB-specific DNA-binding complexes. Nuclear extracts were obtained from LNCaP cells (a human prostate carcinoma cell line) treated with 0.001–10 n*M* R1881 (a synthetic androgen) or 0.1–1 n*M* 5α-dihydrotestosterone for 96 hours and incubated with labeled NF-κB oligonucleotides. DNA-binding activity was elevated after treatment with 1 and 10 n*M* R1881 (**panel A**). Excess unlabeled NF-κ9 oligonucleotides (= cold NF-κB) competed away binding, thereby suggesting specificity. Nuclear extracts harvested at various time points after treatment with vehicle control or 1 n*M* R1881 displayed an increase in binding activity to NF-κB sites by 36 hours (**panel B**). Binding activity continued to rise and reached a maximum at 72 hours. Excess unlabeled OCT-1 (a transcription factor) oligonucleotide, used as a nonspecific competitor, did not affect binding activity. Co-treatment of vehicle control- or 1 n*M* R1881-treated cells with vitamins C plus E lowered protein binding to NF-κB sites after 96 hours of treatment at the 500-µ*M* dose (**panel C**). At this dose, vitamins C plus E appeared to increase the binding activity of the faster migrating complex (**lower band**) in the vehicle control-treated cells.

Table 1. Relative levels of AP-1 and NF-κB proteins in nuclear extracts of LNCaP prostate carcinoma cells treated with the synthetic androgen R1881 compared with untreated cells*

Time, h	Treatment, n*M* R1881	c-Jun	c-Fos	Androgen receptor	p50	p65
24	0	1	1	1	1	1
24	1	1.15	0.53	0.89	2.14	1.64
96	0	1	1	1	1	1
96	0.01	0.88	0.83	0.89	n.a.	n.a.
96	0.05	0.88	0.88	0.91	n.a.	n.a.
96	0.1	0.95	0.94	1.01	n.a.	n.a.
96	0.25	1.04	1	2.06	n.a.	n.a.
96	1	0.77	0.31	0.68	0.81	0.77

*n.a. = not assayed.

scriptional activity can be inhibited by members of the Jun and Fos families *(43,44)* or can be enhanced by c-Jun *(45)*, depending on the conditions. Conversely, androgen receptor inhibits c-Jun-induced AP-1 transcriptional activity in several cell lines transfected with androgen receptor, c-Jun:c-Fos, and an AP-1-responsive reporter vector *(43,44,46)*. These varying results may derive from inherent differences in the cell lines used, the presence of other co-activators or co-repressors, the level of androgen, and, perhaps most importantly, in the ratio of androgen receptor to c-Jun/c-Fos.

c-Jun can interact with the DNA- and ligand-binding domains of the androgen receptor *(44)*. Whether this interaction occurs *in vivo* and whether it regulates endogenous AP-1 or androgen receptor-induced transactivation is unknown. Our attempts to immunoprecipitate LNCaP androgen receptor with a c-Jun antibody were unsuccessful (data not shown).

Like AP-1, NF-κB is a ubiquitous transcription factor involved in growth, differentiation, and stress response *(23,24)*. It is a dimer of members from the Rel/NF-κB family (e.g., p65:p50). NF-κB exists in an inactive form bound to IκB proteins in the cytoplasm and thus does not require *de novo* protein synthesis for activation *(23)*. Upon activation, IκB is degraded and the active NF-κB complex is translocated to the nucleus *(38)*. Redox regulation of NF-κB is thought to be modulated in part through a conserved cysteine residue in the p50 subunit and through IκB release *(37,47)*. Western blot analyses indicated no substantial change in p50 or p65 protein levels in the nuclear extracts of LNCaP cells treated with R1881. Other Rel/NF-κB family members may be involved in the DNA-binding response. Another explanation for androgen-induced increases in NF-κB DNA-binding activity is that the proteins themselves are altered (e.g., by oxidative modulation of sulfhydryl groups, phosphorylation status, etc.).

The ability of treatment with vitamins C plus E to diminish androgen-induced AP-1 DNA-binding activity suggests that oxidative stress contributes to the regulation of this activity. Vitamins C plus E blocked the rise in DCF fluorescence induced in LNCaP cells by R1881. DCF fluorescence decreased after 24 hours, but this decrease occurred only in cells treated with the highest doses of antioxidants (100 or 500 μ*M*). The antioxidant effect on DCF was greater at 96 hours. However, vitamins C plus E decreased DNA binding to the AP-1 site in both the vehicle control-treated and the R1881-treated cells most effectively after 24 hours. Only the 500-μ*M* antioxidant treatment substantially decreased androgen-induced DNA binding to the AP-1 site at 96 hours after treatment. The differential regulation of AP-1 by specific types of ROS may explain these results. Hydrogen peroxide and superoxide radical are known to elevate AP-1 *(36,38)*. DCF fluorescence, which does not detect superoxide radicals, increases after 36–48 hours of treatment with 1 n*M* R1881 *(12)*, while AP-1 DNA-binding activity is elevated after 5 hours of treatment. Hydrogen peroxide, the product of superoxide radical dismutation by superoxide dismutase enzymes, may rise in response to an earlier elevation in superoxide radical levels.

Androgen-induced NF-κB activity is elevated much later than AP-1 DNA-binding activity in LNCaP cells. Unlike AP-1, NF-κB is not sensitive to superoxide radical and is most potently induced by hydrogen peroxide *(18,35,38)*. In accordance with our DCF data, NF-κB DNA-binding activity is elevated after 36 hours of androgen treatment. NFκB DNA-binding activity is decreased only in cells treated for 96 hours with the highest dose of antioxidants, suggesting that the ROS involved in androgen-induced AP-1 and NF-κB regulation are not identical.

Androgen-induced NF-κB and AP-1 DNA-binding activity and DCF fluorescence remain elevated for prolonged periods. Other antioxidants (*N*-acetyl cysteine, sodium selenite, and α-tocopherol acetate) were less effective at reducing DCF fluorescence or altering DNA-binding activity (data not shown). The DCF assay may be a useful tool for optimizing antioxidant combinations that regulate androgen-induced redox-sensitive transcription factor activity. Some of the downstream events following androgen exposure are likely related to changes in the activities of redox-sensitive transcription factors. Defining the pathways involving androgens, oxidative stress, and prostate carcinogenesis and determining the capacity for antioxidants to favorably intervene appear to be topics worthy of further pursuit.

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