

# ARTICLES

## Prooxidant–Antioxidant Shift Induced by Androgen Treatment of Human Prostate Carcinoma Cells

Maureen O. Ripple, William F. Henry, Randall P. Rago, George Wilding\*

**Background:** Prostate cancer is a disease associated with aging. Also commonly associated with increasing age is a shift in the prooxidant–antioxidant balance of many tissues toward a more oxidative state, i.e., increased oxidative stress. We hypothesize that androgen exposure, which has long been associated with the development of prostate cancer, may be a means by which the prooxidant–antioxidant balance of prostate cells is altered. **Purpose:** Using established prostate carcinoma cell lines, we studied the effect of androgens on various parameters of oxidative state (e.g., generation of hydrogen peroxide and hydroxyl radicals, lipid peroxidation, and oxygen consumption) and antioxidant defense mechanisms (e.g., the glutathione system and catalase). **Methods:** The androgen-responsive LNCaP and the androgen-independent DU145 prostate carcinoma cell lines were exposed to 5 $\alpha$ -dihydrotestosterone (DHT) and to the synthetic androgen R1881. The cellular proliferation responses were measured by use of a fluorometric assay to quantitate the amount of DNA. The generation of reactive oxygen species was measured by use of 2',7'-dichlorofluorescein diacetate, a dye that fluoresces in the presence of hydrogen peroxide or hydroxyl radicals. Lipid peroxidation was quantitated by use of a chromogen specific for malonaldehyde and 4-hydroxy-2(E)-nonenal. General mitochondrial activity was determined by assaying 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction. A Clark-type electrode was used to assess oxygen consumption per cell. Intracellular glutathione concentrations and the activities of catalase and  $\gamma$ -glutamyl transpeptidase were measured spectrophotometrically. All *P* values resulted from two-sided tests. **Results:** DHT at less than 1 to 100 nM (a concentration range encompassing the physiologic levels of DHT considering all ages) and R1881 at 0.1–1 nM concentrations were effective in inducing in LNCaP cells comparable proliferative responses and changes in oxidative stress. In contrast, neither DHT nor R1881 had any effect on the oxidative stress in DU145 cells. The mitochondrial activity in LNCaP cells, as measured by MTT reduction, was significantly elevated above the levels of the untreated controls by DHT (0.1–1000 nM) and R1881 (0.05–1 nM) (*P* < .001 in both). Oxygen consumption and catalase activity were increased in LNCaP cells in the presence of 1 nM R1881 by 60% and 40%, respectively, over the values in the untreated control cells (*P* < .03 and *P* < .01, respectively). The same concentration

of R1881 resulted in a decrease in intracellular glutathione concentrations and an increase in  $\gamma$ -glutamyl transpeptidase activity in LNCaP cells. Treatment with the oxidizing agents H<sub>2</sub>O<sub>2</sub> and menadione produced an increase in  $\gamma$ -glutamyl transpeptidase activity in LNCaP cells, whereas treatment with the antioxidant compound ascorbic acid (100 mM) reduced the oxidative stress produced in LNCaP cells by 1 nM R1881 and completely blocked the  $\gamma$ -glutamyl transpeptidase activity. **Conclusions:** Physiologic levels of androgens are capable of increasing oxidative stress in androgen-responsive LNCaP prostate carcinoma cells. The evidence suggests that this result is due in part to increased mitochondrial activity. Androgens also alter intracellular glutathione levels and the activity of certain detoxification enzymes, such as  $\gamma$ -glutamyl transpeptidase, that are important for maintenance of the cellular prooxidant–antioxidant balance. [J Natl Cancer Inst 1997;89:40–8]

Prostate cancer is the most commonly diagnosed solid tumor in U.S. men (1). It is associated with aging and occurs in a latent or in a clinical form in 30%–40% of men by age 30–50 years and increases to 75% in men by age 80 (2,3). The cause of this disease is not well understood; however, certain factors are commonly linked to its development. These factors include genetic predisposition and exposure to androgens and other hormones, infectious agents, and environmental and dietary factors (2). The importance of androgens in prostate carcinogenesis is suggested by the observations that prostate cancer rarely occurs in eunuchs or men with a deficiency in 5 $\alpha$ -reductase, the enzyme responsible for converting testosterone to its more active form, 5 $\alpha$ -dihydrotestosterone (DHT) (2,3). Normal prostate development and functional maintenance depend on androgens, and at least 75% of tumors in men with metastatic prostate cancer are androgen dependent at initial diagnosis (3,4).

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See "Notes" section following "References."

The question of how increasing age and androgen exposure are involved in prostate carcinogenesis has been investigated (4), but it has not been entirely resolved. Serum androgen levels, as well as androgen–estrogen ratios, have been demonstrated to decrease with age (5). Also believed to be age dependent is an increase in oxidative stress, a result of decreasing levels of antioxidants and antioxidant enzymes (e.g., glutathione, vitamin A, vitamin C, vitamin E, superoxide dismutase, catalase, and glutathione peroxidase) or of increasing production of reactive oxygen species (e.g., superoxide radicals, hydroperoxy radicals, organic peroxide radicals, singlet oxygen, hydrogen peroxide, and hydroxyl radicals) (6). Some studies (7,8) suggest a link between oxidative stress (i.e., an increase in the prooxidant state) and tumor development in various tissues. Reactive oxygen species, such as superoxide radicals, hydrogen peroxide, and hydroxyl radicals, are capable of causing lipid peroxidation, altering the activity of sulfhydryl (SH)-dependent enzymes, and of damaging DNA and cellular members (9). In addition, alterations in the redox balance of cells have been shown to affect the activation of certain transcription factors and thus can control gene expression (10–14). Low levels of reactive oxygen species have been demonstrated to act as mitogens, and it is proposed that redox alterations may play a key role in a novel signal transduction pathway important for regulating cell growth (15).

The metabolism of certain estrogenic compounds can generate free radicals. Studies by Liehr et al. (16,17) showed that estrogens have the capacity to cycle between reduced and oxidized forms, which allows for the formation of free radicals capable of damaging DNA and protein. It was hypothesized that estrogen-induced lipid peroxidation and lipid peroxide-associated DNA adducts play an important role in the development of kidney tumors in estrogen-treated hamsters (18). It is unknown whether androgen exposure can elicit similar responses. However, a recent prospective epidemiologic study (19) of nearly 48 000 subjects found a statistically significant decrease in risk of developing prostate cancer associated with lycopene intake. Found in tomatoes and certain tomato products, lycopene is a known potent antioxidant (20).

The present study was planned to examine if androgens are capable of altering the prooxidant–antioxidant balance in human prostate carcinoma cell lines and, if so, whether this can provide a mechanism by which androgen exposure may play a role in prostate carcinogenesis. In this study, we determined the levels of hydrogen peroxide and hydroxyl radical formation following androgen treatment. We also measured lipid peroxidation, a possible end product of oxidative stress. In addition, we measured mitochondrial reductase activity and oxygen consumption to assess the general level of mitochondrial activity, since the mitochondria is a major source of reactive oxygen species within cells (6,9). Furthermore, we determined the activity of catalase (an antioxidant enzyme that reduces hydrogen peroxide) following androgen exposure as well as the level of glutathione (an abundant antioxidant) and the level of  $\gamma$ -glutamyl transpeptidase (a key enzyme in glutathione salvage pathway) (6,8). Together, the results of this study may provide a partial representation of the prooxidant–antioxidant balance of prostate carcinoma cells and suggest that androgen treatment has an influence on maintaining such a balance.

## Materials and Methods

### Cell Culture and Harvest

Androgen-responsive, androgen receptor-positive LNCaP and androgen-independent DU145 prostate carcinoma cells were obtained from the American Type Culture Collection, Rockville, MD. They were maintained at 37 °C in an atmosphere of 5% CO<sub>2</sub>–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) plus antibiotics and antimycotics (Sigma Chemical Co., St. Louis, MO). Clones of LNCaP (5B, 5D, 9H, 10B, and 11B) were developed by selecting colonies established from single cells. To select for populations able to grow in the absence of steroid hormones, we maintained these colonies in medium containing 5% charcoal-stripped (treated) FBS (CSS).

For measurement of cell proliferation, reactive oxygen species formation, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) activity, LNCaP cells (1000 or 2500 cells per well) and LNCaP CSS clonal cells (2000 cells per well) were plated in 100  $\mu$ L DMEM plus 1% FBS : 4% CSS per well in 96-well plates. Experiments were conducted in medium containing 1% FBS and 4% CSS in order to limit the adverse growth effects encountered with charcoal-stripped serum that are unrelated to steroid hormone depletion. For example, the growth of androgen-independent DU145 cells is inhibited in medium containing 5% CSS compared with that in 5% FBS; growth in medium containing 1% FBS and 4% CSS is identical to growth in medium containing 5% FBS; this serum mixture is low enough in androgen content to slow the growth of the androgen-responsive LNCaP cells, yet it allows for easy detection of an androgen growth response. At 24 or 72 hours after plating, the cells were treated in sets of six with 0.001–5 nM R1881 (Du Pont NEN, Boston, MA), 0.1–1000 nM DHT (Sigma Chemical Co.), or vehicle control. R1881 is a synthetic androgen that is experimentally useful because it is not metabolized as rapidly as DHT (21–26). Control medium contained the ethanol vehicle. Cells were harvested at various times after treatment. The amount of DNA per well was quantitated with a 96-well fluorometric assay (27) on a CytoFluorometric plate scanner (PerSeptive Biosystems, Framingham, MA) and analyzed with CytoCalc software. The formation of hydrogen peroxide and hydroxyl radical by intact cells was determined by use of a fluorescent probe, 2',7'-dichlorofluorescein diacetate (DCF) (Molecular Probes, Inc., Eugene, OR), as described by Kane et al. (28). DCF fluorescent units per well 45 minutes after addition of DCF were normalized to the DNA fluorescent units of the same well. The data expressed are the average DCF fluorescent units per DNA fluorescent units of six wells  $\pm$  the standard deviation (SD). The general mitochondrial activity was determined by assaying reduction of MTT to formazan. MTT activity was determined by adding 30  $\mu$ L of MTT (5 mg/mL phosphate-buffered saline [PBS]) to the 200  $\mu$ L of medium in each well and incubating the mixture for 4 hours at 37 °C (29). Formazan crystals that formed within the attached cells were dissolved by adding 100  $\mu$ L of dimethyl sulfoxide to each well after the medium was removed by dumping. Formazan production was measured at 515 nm on a 96-well microplate reader (Bio-Tek Instruments, Inc., Winooski, VT). MTT reduction data are expressed as average optical density due to formazan formed in six samples per average DNA fluorescent units  $\pm$  SD (DNA when measured in a separate plate; interplate variation is generally <10%; n = 5 plates).

For measurement of lipid peroxidation, oxygen consumption,  $\gamma$ -glutamyl transpeptidase activity, and glutathione concentration, 10<sup>6</sup> cells were plated in triplicate in 100-mm tissue culture plates containing DMEM plus 5% FBS. Then 24 hours after plating, medium was replaced with DMEM containing 1% FBS and 4% CSS. Three days later, medium was again removed and replaced with DMEM containing 1% FBS and 4% CSS; and the appropriate treatment was given (i.e., R1881, menadione and hydrogen peroxide [Sigma Chemical Co.], ascorbic acid [Life Technologies, Inc.], or ascorbic acid plus R1881). Plates were harvested by trypsinization at various times after treatment, resuspended in DMEM containing 1% FBS and 4% CSS. At that time, cells to be used to measure oxygen consumption were counted. Cells to be used for the other assays were spun at 1000 rpm for 5 minutes at 4 °C. The pellets were resuspended in cold PBS, centrifuged at 1000 rpm for 5 minutes at 4 °C, and resuspended again. Cells were counted in a hemocytometer. Cells were spun once again and resuspended in 1 mL cold PBS. This suspension was then split; 500  $\mu$ L was combined with 500  $\mu$ L 0.5 M sucrose ( $\gamma$ -glutamyl transpeptidase assay) or with 500  $\mu$ L 6% sulfosalicylic acid (glutathione assay) and stored at –80 °C. For measurement of lipid peroxidation, 3  $\times$  10<sup>6</sup> cells were resuspended in 100  $\mu$ L H<sub>2</sub>O.

In addition to measuring hydrogen peroxide and hydroxyl radical formation,

we measured lipid peroxidation and oxygen consumption to assess the oxidative state following androgen treatment. We ascertained antioxidant capacities by determining catalase activity and intracellular glutathione concentration. The synthetic androgen R1881 was used for these studies because of its metabolic stability (21-26).

### Determination of Lipid Peroxidation

LNCaP cells were plated and harvested as described above. They were treated for 18 or 68 hours with 0.05 or 1 nM R1881 or the vehicle control. Lipid peroxidation of membrane preparations was measured by use of a colorimetrically based kit (Calbiochem-Novabiochem International, Inc., San Diego, CA). Membrane preparations from  $3 \times 10^6$  cells containing end products of lipid peroxidation, e.g., malonaldehyde and 4-hydroxy-2(E)-nonenal, were reacted with the chromophore *N*-methyl-2-phenyllindole and with methanesulfonic acid (40 minutes at 45 °C). The samples were cooled on ice and centrifuged at 10 000g for 5 minutes at room temperature, and absorbance of the supernatant at 586 nm and 25 °C was read on a spectrophotometer. The combined concentration of malonaldehyde and 4-hydroxy-2(E)-nonenal was determined from a standard curve. The results are expressed as the average micromolar concentration per microgram protein  $\pm$  SD.

### Determination of Oxygen Consumption

Cell suspensions of 2-3 mg of protein per 1.7 mL 0.0012 M phosphate buffer plus 10 mM glutamine, 10 mM lactate, and 1 mM alanine were aerated with 95% O<sub>2</sub>-5% CO<sub>2</sub> and incubated for 10 minutes at 37 °C. Suspensions were then transferred to a 37 °C closed chamber. Oxygen tension was measured polarographically with a Clark-type electrode (Yellow Springs Instrument Co., Yellow Springs, OH). Results are given as the average nanomoles of oxygen consumed per 10<sup>6</sup> cells per minute  $\pm$  SD for six samples from two separate experiments.

### Determination of Intracellular Glutathione Concentration

Cells were harvested as described. At the time of assay, cell samples were thawed at 4 °C, sonicated, and centrifuged at 10 000g for 10 minutes at 4 °C. Supernatants were saved and assayed for glutathione by use of the Tietze assay (30) as modified by Bump et al. (31). Protein content was determined by use of the Bradford assay (32). Results are expressed as the average glutathione content per milligram of protein of triplicate samples  $\pm$  SD.

### Determination of Catalase Activity

Unless otherwise noted, all chemicals were obtained from Sigma Chemical Co. Cells were plated and harvested as described in the cell culture section with the exception that  $2 \times 10^6$  cells were plated in 100-mm plates and the cell pellet obtained was resuspended in 1 mL 0.007 M phosphate buffer. Catalase activity of the sonicated cells was determined spectrophotometrically by use of the method described by Luck (33). Protein content was determined by use of the Bradford assay (32). Data are expressed as units of catalase activity per microgram of protein (unit = the amount of enzyme that liberates one half of the peroxide oxygen from a hydrogen peroxide solution of any concentration in 100 seconds at 25 °C). Results given are the average of triplicate samples  $\pm$  SD.

### Determination of $\gamma$ -Glutamyl Transpeptidase Activity

At the time of assay, cells were thawed at 4 °C, sonicated, and centrifuged for 10 minutes at 10 000g at 4 °C. The resulting pellet containing the cellular membranes was resuspended by sonication in 300  $\mu$ L 0.1 M Tris-HCl (pH 8.0) at 4 °C.  $\gamma$ -Glutamyl transpeptidase activity of triplicate samples was determined by measuring the release of *p*-nitroaniline from the  $\gamma$ -glutamyl transpeptidase substrate L- $\gamma$ -glutamyl-*p*-nitroanilide spectrophotometrically at 410 nm for 4 minutes at 37 °C (34). Specific activity is defined as micromoles of *p*-nitroaniline released per minute per milligram protein or per cell. Results are expressed as the average  $\gamma$ -glutamyl transpeptidase activity of triplicate samples  $\pm$  SD.

### Statistical Analysis

Experiments conducted in 96-well plates were done as six replicates, whereas those using samples obtained from 100-mm plates were done as triplicates, unless otherwise noted. Each treated group was compared with the untreated control by use of the unpaired, two-tailed Student's *t* statistic and tested at the nominal .05 significance level. Since multiple comparisons were performed, Bonferroni's inequality was implemented for each individual comparison to

control for the overall type 1 error rate. For instance, if eight concentrations of androgen were being compared with the control, statistical significance for an individual dose would be determined by a *P* value of less than .05/8 (i.e., equals .00625). The reported *P* values reflect the data shown. Only those *P* values for analyses that are significant following Bonferroni correction are given.

## Results

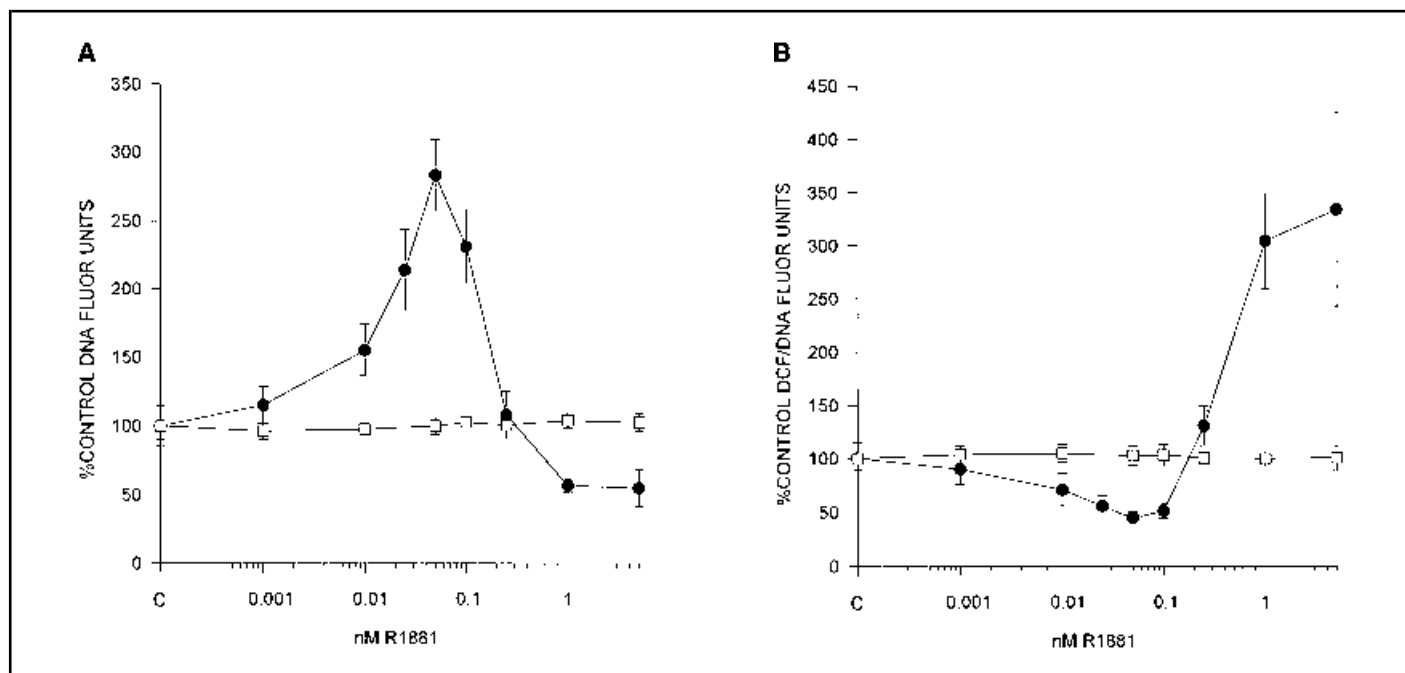
### Growth response and measurement of levels of reactive oxygen species after treatment with R1881 and DHT.

LNCaP, an androgen receptor-positive and androgen-responsive cell line, displayed a characteristic bell-shaped growth response to the synthetic androgen R1881 (Fig. 1, A). DU145 cells were androgen independent, and their growth was not affected by exposure to R1881. The generation of reactive oxygen species measured with the fluorescent probe DCF, which is primarily activated by hydrogen peroxide and hydroxyl radicals (28,35), tended to mirror the effect of R1881 on growth (Fig. 1, B). DCF/DNA fluorescence of DU145 cells was not affected by R1881. LNCaP cells displayed a decrease in DCF fluorescence compared with the control at doses of R1881 that stimulate growth (0.025-0.1 nM; *P* < .001) and a dramatic increase in oxidative stress at doses that inhibit cell growth (1-5 nM; *P* < .001). DCF fluorescence was also induced by DHT (Table 1). Sublines of LNCaP capable of growing in androgen-depleted medium were developed by selecting and maintaining single cells in medium containing 5% CSS. As shown in Table 1, the growth of LNCaP CSS clones 5B, 5D, and 9H was inhibited at much lower concentrations of R1881 and DHT compared with that of the parental line (the same being true for clones 10B and 11B, data not shown). DCF fluorescence was also induced by lower doses of R1881 and DHT in the sublines.

**Kinetics of growth response and reactive oxygen species generation after R1881 treatment of LNCaP cells.** An alteration in reactive oxygen species generation, as measured by DCF fluorescence, was consistently seen before any growth effects were detected. Growth inhibition of LNCaP cells by 1 nM R1881 was not seen until hour 84 (*P*  $\leq$  .02), whereas an increase in DCF fluorescence was seen by hour 60 (*P* < .001) (Fig. 2). Similarly, a decrease in DCF fluorescence was noted 36 hours after treatment with 0.05 nM R1881, but growth stimulation was not seen until 60 hours after treatment.

**MTT reduction by LNCaP cells after androgen treatment.** LNCaP cells exposed to 0.05-1 nM R1881 or 0.1-1000 nM DHT for 68 hours reduced more MTT per DNA than vehicle control-treated cells (*P* < .001) (Fig. 3, A). The androgen-independent cell line DU145, when treated with the same amount of R1881 or DHT (Fig. 3, B) showed no change in MTT reduction per DNA.

**Measurement of lipid peroxidation, oxygen consumption, and glutathione concentration after treatment with R1881.** As shown in Table 2, LNCaP cells treated with 1 nM R1881 for 3 days had nearly double the level of lipid peroxidation of that of vehicle control-treated cells. Exposure to 0.05 nM R1881 had no effect on lipid peroxidation levels. Oxygen consumption per cell was elevated approximately 60% above control levels by exposure to 1 nM R1881 (*P* < .03). The intracellular glutathione concentration per milligram protein was reduced by about 20% in cells treated with 1 nM R1881 compared with untreated control cells (*P* < .003).



**Fig. 1. A)** Growth response of LNCaP and DU145 cells treated with various doses of R1881. LNCaP (●) and DU145 (□) cells were plated in 96-well microtiter plates and were treated 72 hours later with medium containing 0.001-5 nM R1881 or with the vehicle control. Six days after treatment, the cells were harvested, and the DNA was quantitated with a fluorescent DNA assay. Results are expressed as percent of vehicle control  $\pm$  standard deviation ( $n = 6$ ). For some points, the error bars are obscured by the symbol. The growth of LNCaP cells was stimulated above the control level by 0.01-0.1 nM R1881 and was inhibited by 1 and 5 nM R1881. DU145 cells were unaffected by R1881. **B)** The

generation of reactive oxygen species by the same cells after R1881 treatment was determined with a microtiter assay by use of 2',7'-dichlorofluorescein diacetate (DCF), a probe that fluoresces in the presence of hydrogen peroxide and hydroxyl radicals. The level of reactive oxygen species (DCF fluorescence units) was normalized by dividing by the DNA fluorescence units. The DCF fluorescence of LNCaP cells was reduced, compared with the control, by 0.025-0.1 nM R1881 and elevated by 1 and 5 nM R1881. No change was observed in DU145 cells. FLUOR = fluorescence.

**Catalase activity in LNCaP cells treated with 1 nM R1881.** LNCaP cells treated with 0.05-1 nM R1881 for 72 hours displayed a 40% increase in catalase activity at 1 nM R1881 ( $P \leq .01$ ) (Fig. 4). R1881 had no effect on the catalase activity of DU145 cells. The baseline catalase activity of LNCaP cells was almost double that of DU145 cells.

**$\gamma$ -Glutamyl transpeptidase activity of LNCaP cells after treatments with oxidants and with R1881.** The  $\gamma$ -glutamyl transpeptidase activity of LNCaP cells treated with 1 nM R1881 was elevated by greater than 70% above control levels ( $P < .001$ ). Exposure to the oxidants menadione and hydrogen peroxide also resulted in an increase in activity (Table 3). Menadione (0.1 mM) increased  $\gamma$ -glutamyl transpeptidase activity by 70% above control levels ( $P < .007$ ).  $\gamma$ -Glutamyl transpeptidase activity was elevated 50% and 60% by exposure to 0.1 and 0.3 mM  $H_2O_2$ .

**Effect of ascorbic acid on R1881-induced increase in reactive oxygen species generation and  $\gamma$ -glutamyl transpeptidase activity.** The reducing agent ascorbic acid could block the increase in DCF fluorescence per DNA that was seen after treatment of LNCaP cells with 1 nM R1881 (Fig. 5, A). The combination of 1 nM R1881 and 1 mM ascorbic acid for 56 hours brought the level of DCF fluorescence back to that found in vehicle control-treated cells. Higher concentrations of ascorbic acid (2.5-100 nM) lowered the DCF fluorescence by 70%-90% of control levels (all  $P < .001$ ). The elevation in  $\gamma$ -glutamyl transpeptidase activity noted after 1 nM R1881 treatment was unaffected by concomitant treatment with 1 mM ascorbic acid for 45 hours; however, adding 100 mM ascorbic acid nearly eliminated

$\gamma$ -glutamyl transpeptidase activity ( $P < .001$ ) (Fig. 5, B). Treatment with 1, 10, and 100 mM ascorbic acid plus 1 nM R1881 for 48 hours followed by a repeated treatment for 24 hours significantly reduced  $\gamma$ -glutamyl transpeptidase activity compared with that seen after treatment with 1 nM R1881 ( $P < .05$ , data not shown).

## Discussion

In this study, androgen-responsive prostate carcinoma LNCaP cells responded to androgens in various ways, depending on the dose. In terms of growth, a bell-shaped growth response was normally obtained after treatment with 0.001-5 nM of the synthetic androgen R1881 (Fig. 1, A). Treatment with DHT elicited a similar bell-shaped response (data not shown). The androgen-independent prostate carcinoma DU145 cell line did not respond to androgens in terms of growth. DU145 cells do not express androgen receptor messenger RNA (mRNA) (36). Androgens have been shown to exert their effects by binding the androgen receptor, a ligand-activated transcriptional regulatory protein (37). Androgen-androgen receptor complexes are capable of specifically altering the expression of certain genes, including oncogenes, growth factors, and enzymes, by binding to responsive elements in the promoter region (4,38). Other effects noted after androgen exposure are likely secondary and result from concerted changes in androgen-mediated gene expression. The data presented here demonstrate that androgens can alter the prooxidant-antioxidant balance of androgen-responsive cells. The ability of androgens to shift the prooxi-

**Table 1.** Effect of R1881 on DNA levels (measured as fluorescence units) and levels of reactive oxygen intermediates (measured as 2',7'-dichlorofluorescein diacetate [DCF] units) normalized to DNA (DCF/DNA) levels in LNCaP parent cells and in LNCaP clonal cells grown in charcoal-stripped fetal bovine serum (CSS)\*

Cell line and treatment	Concentration nM	% control†	
		DNA ± SD	DCF/DNA ± SD
LNCaP parent cells	0	100 ± 14	100 ± 21
	R1881		
	0.01	177 ± 16	nd
	0.05	251 ± 17	109 ± 19
	1	32 ± 4	273 ± 51
	DHT		
	0.1	163 ± 15	69 ± 9
	1	32 ± 7	nd
	5	30 ± 4	497 ± 109
	100	27 ± 5	400 ± 83
LNCaP clonal cells	Clone 5B		
	0	100 ± 6	100 ± 9
	R1881		
	0.01	111 ± 5	nd
	0.05	27 ± 2	237 ± 26
	1	11 ± 1	454 ± 137
	DHT		
	0.1	102 ± 4	100 ± 15
	1	24 ± 4	nd
	5	12 ± 1	1087 ± 104
	100	10 ± 1	467 ± 78
	Clone 5D		
	0	100 ± 8	100 ± 9
	R1881		
	0.01	100 ± 2	nd
	0.05	53 ± 5	192 ± 19
	1	14 ± 1	447 ± 58
	DHT		
	0.1	97 ± 11	133 ± 9
	1	57 ± 11	nd
	5	26 ± 5	511 ± 6
	100	14 ± 1	670 ± 48
Clone 9H	0	100 ± 9	100 ± 22
	R1881		
	0.01	148 ± 13	nd
	0.05	99 ± 10	102 ± 12
	1	23 ± 1	195 ± 36
	DHT		
	0.1	111 ± 12	101 ± 11
	1	37 ± 4	nd
	5	27 ± 1	492 ± 62
	100	26 ± 2	237 ± 39

\*LNCaP cells and LNCaP CSS clonal cells were plated in 96-well plates and treated with R1881 or 5 $\alpha$ -dihydrotestosterone (DHT); 6 days later, replicate plates were harvested for DNA and DCF determination. The average DNA and DCF fluorescent units  $\pm$  standard deviation (SD) was determined ( $n = 6$ ). The level of reactive oxygen species generation normalized to DNA level was determined by dividing the DCF fluorescence units by the DNA fluorescence units for a given treatment.

†Results are expressed as the percent of the vehicle-treated control. nd = not determined.

dant-antioxidant balance could provide a novel mechanism by which androgens alter gene expression or protein activation.

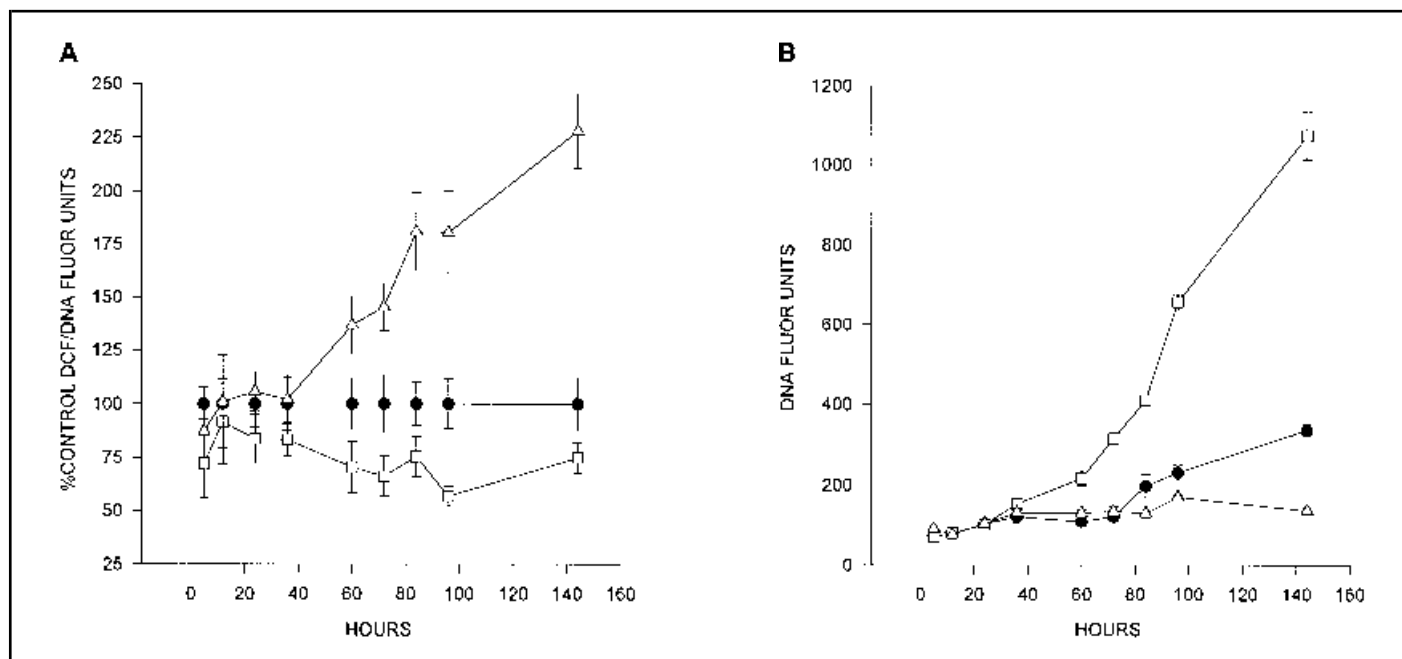
The data presented in Fig. 1, B, and in Table 1 illustrate that reactive oxygen species generation (measured with DCF, a probe that fluoresces in the presence of hydroxyl radicals or hydrogen peroxide) was elevated by physiologically relevant levels of DHT and corresponding concentrations of R1881. The dose at which oxidative stress was elevated appeared to depend on the androgen responsiveness of the cells being tested. Clones of the parental LNCaP line developed from single cells selected and maintained in androgen-depleted medium were more sensitive than the parental cells to the growth-inhibitory effects of androgens. These results are consistent with the results of Konkantis et al. (39), who found that LNCaP clones selected for by growth in medium containing 10% CSS were growth inhibited at lower doses of R1881 and expressed increased levels of andro-

gen receptor mRNA and protein than the parental cells. We also found that an increase in reactive oxygen species generation occurred at lower androgen concentrations in the clones than in the parental cells. In general, DCF fluorescence increased with R1881 or DHT doses that were growth inhibitory. However, other growth-inhibitory substances did not induce DCF fluorescence in these cells (data not shown). Also, since changes in DCF fluorescence per DNA appear before any changes in growth were detected, it is unlikely that the oxidative stress noted after androgen treatment is simply the result of a growth change; in fact, alteration in the oxidative stress may itself act as a growth regulator (15,40). A change in DCF fluorescence did not occur in DU145 cells treated with androgen, suggesting that the oxidative stress noted in LNCaP cells is not merely a non-specific effect.

A shift in the prooxidant-antioxidant balance toward the prooxidant state after androgen exposure is also suggested by the rise in lipid peroxidation level and catalase activity following R1881 treatment. Androgens also increased the rate of MTT reduction in androgen-responsive but not in androgen-independent cells. MTT reduction is a general measure of mitochondrial activity (29). An elevation in mitochondrial activity per cell was further suggested by the increase in oxygen consumption noted after treatment with 1 nM R1881. MTT can also be reduced in the presence of superoxide (15). A general increase in mitochondrial activity could produce oxidative stress as a product of the normally occurring inefficient reduction of oxygen that results in superoxide production (41). Since superoxide is highly reactive, it is normally reduced to hydrogen peroxide by superoxide dismutase (42). The increase in catalase activity seen after treatment with 1 nM R1881 was likely in response to an increased production of hydrogen peroxide (43).

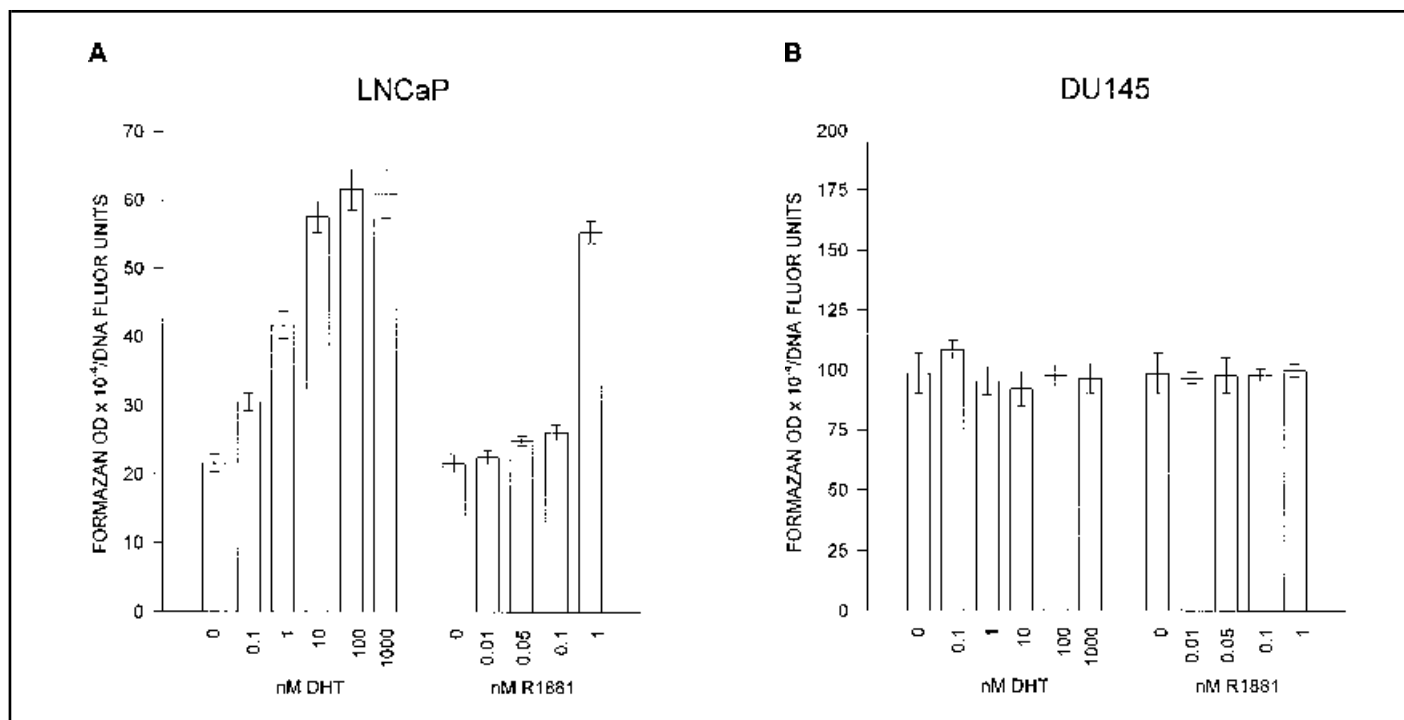
Another indication that the redox state is altered by androgen treatment was demonstrated by the decrease in glutathione that occurred after treatment of LNCaP cells with 1 nM R1881. An abundant tripeptide, glutathione, acts as a reducing agent and antioxidant (44). Reduced glutathione, glutathione peroxidase, glutathione reductase, and NADPH (reduced nicotinamide adenine dinucleotide phosphate) taken together all provide an important cellular defense against oxidative stress (45). In our study, intracellular glutathione concentrations were lower in LNCaP cells treated with 1 nM R1881 at 72-96 hours after treatment but were similar to control levels at earlier time points (data shown only for 72 hours). These results are consistent with a response to increased levels of oxidative stress that occur earlier. The activity of  $\gamma$ -glutamylcysteine synthetase, the rate-limiting enzyme of glutathione synthesis, was unchanged by androgen treatment (data not shown), suggesting that the decrease in glutathione concentration is not the result of reduced production.

$\gamma$ -Glutamyl transpeptidase works in glutathione salvage pathway by cleaving extracellular glutathione and allowing the amino acid components to enter the cell (46).  $\gamma$ -Glutamyl transpeptidase activity of LNCaP cells was elevated by treatment with 1 nM R1881. The  $\gamma$ -glutamyl transpeptidase activity of LNCaP cells was also elevated by treatment with the oxidants menadione and hydrogen peroxide, which agrees with results found in other tissue types (47). The antioxidant ascorbic acid reduced the level of DCF fluorescence detected after exposure of



**Fig. 2.** Time course of R1881 effects on the generation of reactive oxygen species and growth. LNCaP cells were plated in 96-well plates and were treated 72 hours later with R1881 at 0.05 nM (□) and 1 nM (△) or with the vehicle control (●). 2',7'-Dichlorofluorescein diacetate (DCF) fluorescence per DNA per well was determined at various time points. Results are graphed as a percent of vehicle control  $\pm$  standard deviation ( $n = 6$ ) (panel A). For some points, the error bars are obscured by the symbol. DCF fluorescence was statistically elevated above control levels 60 hours after treatment with 1 nM R1881 ( $P < .001$ ) and was lower

than control levels 36 hours after treatment with 0.05 nM R1881. Panel B shows the effect of 0.05 and 1 nM R1881 on growth of LNCaP after 0-140 hours of exposure. DNA content of each well described above was determined with a fluorescent microplate assay. There was no inhibitory effect on growth of cells treated with 1 nM R1881 (△) until 84 hours after treatment ( $P \leq .02$ ). Growth was significantly stimulated above control levels 60 hours after treatment with 0.05 nM R1881 ( $P < .001$ ). Alterations in reactive oxygen species generation appeared before changes in growth. FLUOR = fluorescence.



**Fig. 3.** Ability of LNCaP (A) and DU145 (B) cells to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was measured after 68 hours of exposure to R1881 or 5 $\alpha$ -dihydrotestosterone (DHT). Cells were plated in duplicate 96-well plates. MTT reduction to formazan, a general measure of mitochondrial activity, was determined by use of a colorimetric assay. The formazan optical density was normalized to DNA fluorescence (FLUOR)

units, which was determined in separate but identical plates for every treatment using a DNA fluorescent assay. Results given are the average formazan optical density/DNA fluorescence units  $\pm$  standard deviation ( $n = 6$ ). MTT reduction was elevated in LNCaP cells treated with 0.1-1000 nM DHT or 0.05-1 nM R1881 relative to untreated cells ( $P < .001$ ). MTT reduction in DU145 cells was not affected by androgen treatment.

**Table 2.** Effect of R1881 on lipid peroxidation, intracellular glutathione concentration, and oxygen consumption of LNCaP cells\*

Treatment	Lipid peroxidation, nmol/ $\mu$ g protein <sup>†</sup>	Glutathione, nmol/mg protein <sup>‡</sup>	Oxygen consumption, O <sub>2</sub> nmol/10 <sup>6</sup> cells per min <sup>§</sup>
Vehicle control	0.11 $\pm$ 0.01	22.25 $\pm$ 0.89	3.75 $\pm$ 1.12
R1881			
0.05 nM	0.17 $\pm$ 0.02	20.30 $\pm$ 0.97	nd
1 nM	0.30 $\pm$ 0.03	16.95 $\pm$ 0.49	6.07 $\pm$ 1.69¶

\*LNCaP cells were treated for 3 days with medium containing 0.05 or 1 nM R1881 or the vehicle control. Lipid peroxidation of membrane samples was determined spectrophotometrically by use of a chromogen specific for malonaldehyde and 4-hydroxy-2(E)-nonenal.

<sup>†</sup>Average level of lipid peroxidation  $\pm$  standard deviation (SD) is shown (n = 2).

<sup>‡</sup>Average glutathione content is expressed per milligram of cytosolic protein  $\pm$  SD (n = 3).

<sup>§</sup>Oxygen consumption was determined by use of a Clark electrode. Average oxygen consumption per cell  $\pm$  SD is given (n = 6). nd = not determined.

||Student's *t* test: *P* < .003.

¶Student's *t* test: *P* < .03.

LNCaP to 1 nM R1881. Ascorbic acid and, to a lesser extent,  $\alpha$ -tocopherol (data not shown) are capable of blocking the activation of  $\gamma$ -glutamyl transpeptidase induced by R1881.

Redox control of protein activation through alteration of thiol status is well established in plant systems. For example, the translation of certain light-induced mRNAs in chloroplasts occurs through a series of oxidation-reduction reactions that eventually reduce the disulfide bonds of a group of proteins that bind RNA and activate translation (48). Redox control of mammalian enzymes is a bit more controversial, but certain enzyme activities have been shown to be modified by thiol-disulfide exchange (49,50). The activation of transcription factors, such as nuclear factor  $\kappa$ B, Fos, and Jun, has been shown to be dependent on intracellular glutathione levels (10,13). These same transcription factors, along with serum response factor/ternary complex factor (SRF/TCF), have been shown to modulate redox regulation of

gene expression (51). The ability of androgens to alter the redox balance of cells could potentially provide a mechanism by which androgen exposure can activate or inactivate a variety of proteins or turn on or off a series of genes, without necessarily requiring binding of the androgen-androgen receptor complex to the promoter. Our results suggest that this ability to shift the prooxidant-antioxidant balance occurs only in androgen-responsive cells. The lack of response in DU145 cells could be due to the absence of a functional androgen receptor in these cells, but it may also be due to some other difference between the cell lines, such as the presence or absence of certain metabolic enzymes.

Recent epidemiologic evidence linking a reduced risk of prostate cancer and dietary lycopene intake already suggests a role for antioxidants in protection against prostate carcinogenesis in humans (19). Plasma and tissue concentrations of lycopene, a potent carotenoid antioxidant, have been demonstrated to decrease with increasing age (52). Lycopene has also been shown to significantly suppress spontaneous mammary tumor development in mice (53). An understanding of exactly how androgens mediate the observed changes in the prooxidant-antioxidant balance is required. It is possible that some of the described age-related changes in oxidative state and antioxidant levels are hormonally regulated. If so, understanding how androgens bring about these changes could potentially provide information on how to regulate or prevent certain age- and hormone-associated diseases, including cancer.

**Table 3.**  $\gamma$ -Glutamyl transpeptidase (GGT) activity of LNCaP cells treated with R1881, menadione, or hydrogen peroxide\*

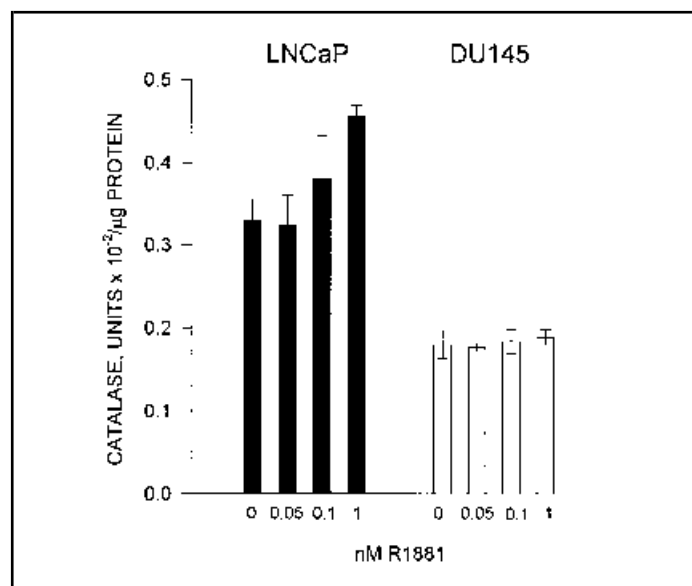
Treatment	Concentration	GGT activity/mg protein <sup>†</sup> (% control)
Control	—	0.14 $\pm$ 0.01 (100)
R1881	1 nM	0.23 $\pm$ 0.01 (168) <sup>‡</sup>
Menadione	0.01 mM	0.18 $\pm$ 0.02 (127)
Menadione	0.1 mM	0.27 $\pm$ 0.04 (193) <sup>§</sup>
Hydrogen peroxide	0.1 mM	0.21 $\pm$ 0.02 (149)
Hydrogen peroxide	0.3 mM	0.23 $\pm$ 0.03 (162) <sup>§</sup>

\*LNCaP cells were treated for 3 days with R1881 (1 nM) or the oxidants menadione (0.01 and 0.1 mM) and hydrogen peroxide (0.1 and 0.3 mM). Membrane preparations were analyzed for GGT activity spectrophotometrically.

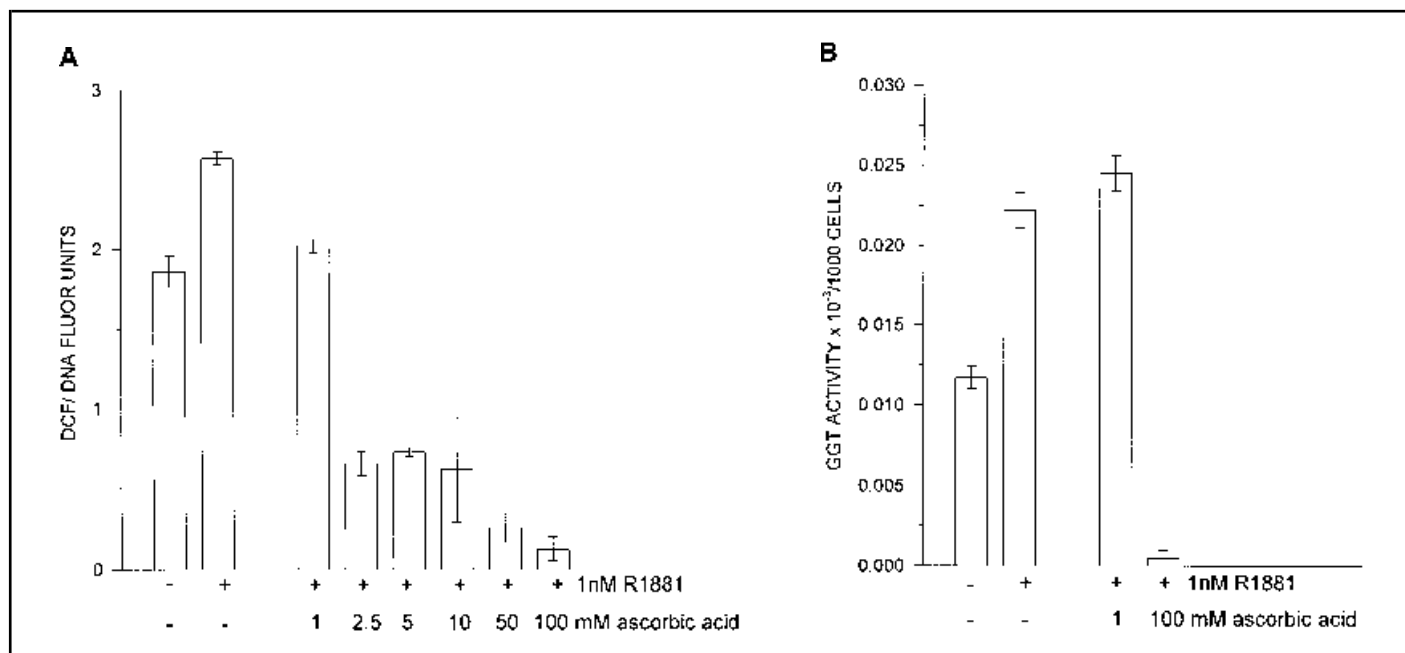
<sup>†</sup>Average activity of triplicate samples  $\pm$  standard deviation is given.

<sup>‡</sup>Student's *t* test: *P* < .001.

<sup>§</sup>Student's *t* test: *P* < .007.



**Fig. 4.** Catalase activity of LNCaP (■) and DU145 (□) cells treated with R1881. Cells were plated in 100-mm plates. The next day, the medium was replaced with medium containing 1% fetal bovine serum (FBS) plus 4% charcoal-stripped FBS; 3 days later, the cells were treated with 0.05-1 nM R1881 or vehicle control. Catalase activity was measured spectrophotometrically. Results expressed are the average catalase activity  $\pm$  standard deviation (n = 3). LNCaP cells exposed to 1 nM R1881 for 72 hours had increased catalase activity compared with control levels (*P* < .01). R1881 had no effect on DU145 catalase activity.



**Fig. 5.** Ascorbic acid blocks the generation of reactive oxygen species associated with R1881 treatment (**A**). Cells were plated in 96-well plates and treated with control medium (–) or medium containing 1 nM R1881 (+) plus 0–100 mM ascorbic acid. Reactive oxygen species generation was measured with a fluorometric assay. The addition of 1–100 mM ascorbic acid lowered 2',7'-dichlorofluorescein diacetate (DCF) fluorescence in a dose-related manner and almost totally blocked DCF fluorescence at the 50–100 mM. These observations are statistically significant (all  $P < .003$ ). The results are expressed as the average  $\pm$  standard deviation ( $n = 6$ ). Ascorbic acid at 100 mM also totally blocked the increase in  $\gamma$ -glutamyl transpeptidase (GGT) seen after treatment

with 1 nM R1881 (**B**). LNCaP cells were plated in 100-mm plates. Four days after plating, the cells were treated with medium containing 1 nM R1881 plus 1 or 100 mM ascorbic acid. The treatment medium was removed the following day and replaced with fresh treatment medium. Twenty-four hours later, the cells were harvested and assayed for GGT activity. Results are expressed as the average activity  $\pm$  standard deviation ( $n = 3$ ). R1881 alone significantly elevated GGT activity by 80% ( $P < .001$ ). Treatment with 1 mM ascorbic acid plus 1 nM R1881 had no additional effect over R1881 alone, whereas adding 100 mM ascorbic acid virtually eliminated all GGT activity. FLUOR = fluorescence.

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## Notes

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