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# Forum Original Research Communication

# Testosterone Regulation of Homocysteine Metabolism Modulates Redox Status in Human Prostate Cancer Cells

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## ABSTRACT

**Clearance of homocysteine *via* the transsulfuration pathway provides an endogenous route for cysteine synthesis and represents a quantitatively significant source of this amino acid needed for glutathione synthesis. Men have higher plasma levels of total homocysteine than do women, but the mechanism of this sex-dependent difference is not known. In this study, we investigated regulation by testosterone of cystathionine β-synthase (CBS), which catalyzes the committing step in the transsulfuration pathway. We report that testosterone downregulates CBS expression *via* a posttranscriptional mechanism in the androgen-responsive prostate cancer cell line, LNCaP. This diminution in CBS levels is accompanied by a decrease in flux through the transsulfuration pathway and by a lower intracellular glutathione concentration. The lower antioxidant capacity in testosterone-treated prostate cancer cells increases their susceptibility to oxidative stress conditions. These results demonstrate regulation of the homocysteine-clearing enzyme, CBS, by testosterone and suggest the potential utility of targeting this enzyme as a chemotherapeutic strategy.** *Antioxid. Redox Signal.* 9, 1875–1881.

Prostate cancer is the most common malignancy in men and is the second leading cause of cancer death in men in the United States (20). Although the etiology of this disease is not well understood, several factors are correlated with a higher risk of malignant transformation. These factors include, but are not limited to, genetic predisposition, aging, androgen exposure, certain prostate infections, and environmental and dietary factors (3, 26, 34, 46). It has been suggested that persistent oxidative stress and free radical damage may be a common feature and an underlying cause for tumor initiation (1, 2, 5, 7, 21, 40). Epidemiologic studies report a marked decrease in the risk for prostate cancer among men consuming antioxidant-rich diets (6, 16, 46).

Reactive oxidative species (ROS) are generated as by-products of mitochondrial respiration and cellular metabolism in aerobic organisms. Under normal conditions, low-to-moderate levels of ROS participate in signal transduction and control of redox-sensitive transcriptional factors and enzymes (9, 12). However, at elevated concentrations, ROS can cause protein oxidation, DNA damage, and lipid peroxidation (10, 22, 23). Cellular levels of ROS are kept in balance by the concerted action of antioxidant enzymes and glutathione, a major redox buffer. Glutathione is an abundant Glu-Cys-Gly–containing tripeptide, which confers its protective effect by functioning as a reductant, a free radical scavenger, and a nucleophile (30).

Glutathione is synthesized from cysteine in two sequential reactions catalyzed by γ-glutamylcysteine ligase and glutathione synthetase, respectively (Fig. 1). Cysteine, a limiting reagent for glutathione production, is supplied from the diet or synthesized endogenously in the transsulfuration branch of homocysteine metabolism. Cystathionine β-synthase (CBS) catalyzes the first and committing step in the transsulfuration pathway. The reaction involves a condensation between homocysteine and serine to give cystathionine, which is further converted to cysteine by the action of cystathionine γ-lyase. It is estimated that in liver cells, the transsulfuration pathway con-

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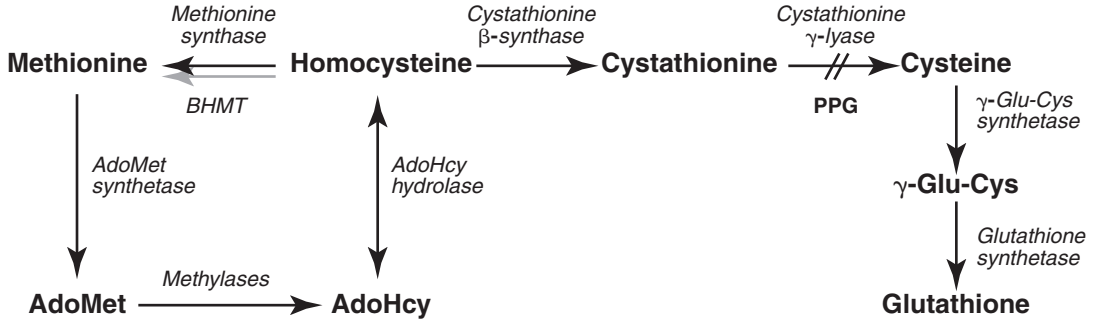


**FIG. 1. Homocysteine metabolism in mammals.** BHMT, betaine homocysteine methyltransferase; PPG, propargylglycine, an inhibitor of γ-cystathionase used in this study.

tributes approximately half the cysteine that is used for glutathione synthesis (32), with the remainder being derived presumably *via* transport of circulating cysteine.

It has been reported that physiological levels of androgens are capable of inducing oxidative stress (manifested as lower glutathione levels) that could result partially from increased mitochondrial activity (39). We previously described tissue- and sex-specific differences in murine (42) and human CBS activity (43). In human kidney, CBS activity is ~35% higher in women than in men (43), but the molecular basis for this difference in sex-dependent regulation is not known. The present study was designed to investigate whether human CBS activity is regulated by androgens and whether this in turn affects the redox capacity in androgen-sensitive human prostate cancer cells.

We demonstrate that testosterone treatment leads to lower CBS activity in prostate cancer cells and that this is associated with a diminished flux through the transsulfuration pathway and with lower glutathione levels. Furthermore, we report that these metabolic changes are associated with increased vulnerability to oxidative stress. Together, these studies reveal an additional mechanism for regulation of homocysteine metabolism and thereby, redox capacity, by androgens and provide insights into the physiology of androgen-responsive prostate cancer cells.

## MATERIALS AND METHODS

### *Cell culture*

The androgen-responsive human prostate adenocarcinoma cell line, LNCaP, was obtained from American Type Culture Collection (at passage number 22). All experiments were performed on cells that were between 23 and 36 passages. Androgen-unresponsive LNCaP-derived cell line C-81 was obtained at passage 129 from the laboratory of Dr. Ming-Fong Lin (University of Nebraska Medical Center). LNCaP cells were grown in RPMI-1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% dialyzed fetal bovine serum (FBS, HyClone) and an antibiotic–antimycotic mixture (Invitrogen) of 100 units/ml sodium penicillin G, 100 μg/ml streptomycin sulfate, and 0.25 μg/ml amphotericin B. Cultures were maintained at 37°C in a 5% $CO_2$ atmosphere. To study the effect of androgens, LNCaP cells were placed in RPMI-1640 medium lacking phenol red (Invitrogen) and supplemented with 10% heat-inactivated (56°C, 30 min) dextran-coated charcoal stripped FBS (HyClone, Logan, VT). A sterilized stock solution of dihydrotestosterone (DHT) dissolved in dimethylsulfoxide (DMSO), or the vehicle alone, was aliquoted into the cell-culture medium at the concentrations indicated in the figure legends. The medium was replaced with fresh aliquots (with or without corresponding treatments) every 3 days. For each experiment, cells from the same passage were used for control and treatment conditions. Because of the slow effect of DHT on CBS levels, 100 n*M* DHT was used in most experiments to ensure availability of the androgen at least at low concentrations, during the extended time course of the experiments.

### *Western blot analysis*

Cells were harvested by trypsinization, washed twice with ice-cold phosphate-buffered saline (PBS), and after centrifugation, were resuspended in an equal volume of lysis buffer (0.1 *M* sodium phosphate, pH 7.4, containing 0.1% Triton-X100, 10 μl/ml protease inhibitor cocktail, 25 μg/ml tosyllysine chloromethylketone, 25 μg/ml phenylmethylsulfonyl fluoride, 27 μg/ml aprotinin, and 10 μg/ml leupeptin) and placed on ice for 20 min and then stored at −80°C until further use. The lysate was centrifuged at 4°C for 20 min at ~14,000 *g*, and the protein concentration in the supernatant was determined by the Bradford assay (Bio-Rad, Hercules, CA). Western blot analysis of CBS and actin protein levels was performed as described previously (42).

### *Northern blot analysis and CBS promoter activity studies*

RNAqueous kit (Ambion, Austin, TX) was used to isolate total RNA. A probe for CBS was generated by PCR amplification of an ~1–kb fragment by using the expression construct pGEX.CBS as a template and the following primers: 5′-GCCAAGGAGCCCCTGTGCATGCGGCCCGATGCTCCG-3′ (sense) and 5′-GTCACCATTCCCAGGATTACCCC-3′ (antisense). Rediprime II Random Prime Labeling Kit (Amersham Biosciences, Piscataway, NJ) was used to label the probe with α-[$^{32}$P]dCTP (Amersham; specific activity, ~3,000 Ci/mmol). RNA concentrations were estimated from absorbances at 260/280 nm, and 5–10 μg total RNA from each sample was used per analysis.

Northern analyses were performed by using NorthernMax reagents (Ambion) and following the manufacturer's protocol. Quantification was performed by densitometric analysis by using Quantity One BioRad image software. To account for variations in loading, the band intensities were normalized to the 18S ribosomal RNA in each sample visualized with ethidium bromide staining.

A reporter gene construct in which the full-length CBS −1b promoter drives luciferase expression (481 bp; −4,046/−3,565) was generously provided by Dr. J. Taub (Wayne State University). The pRSV-$\beta$ galactosidase control vector was obtained from Promega. Both constructs were transiently transfected into 60–80% confluent LNCaP cells by using Lipofectin (Invitrogen). In brief, 1–1.5 $\mu$g of plasmid DNA was premixed with 20 $\mu$l of the transfection reagent and then added to six-well plates. Cells were transferred to medium ± DHT 24 h after transfection and then harvested after 24–48 h of incubation. Luciferase and $\beta$-galactosidase activities were determined by using the respective assay systems (Promega, Madison, WI). To account for differences in transfection efficiency between plates, luciferase activity was normalized to $\beta$-galactosidase activity.

### *CBS activity, metabolite levels and flux measurements*

CBS activity was measured at 37ºC by using the ninhydrin assay, as described (42). For flux measurements, cells were incubated in $DHT^+$ or $DHT^-$ medium for 6 days, with the final medium change being performed 1 day before initiation of the experiment. One hour before the experiment, cells were transferred to fresh $DHT^-$ medium. Flux measurements were conducted as described previously (31). In brief, experiments were initiated by addition of propargylglycine (2.5 m*M* final concentration), and cells were collected after 0-, 3-, and 7-h incubation periods. Flux was expressed as $\mu$moles of cystathionine formed per hour per gram of protein. Metabolite levels were determined as described previously (32, 37). All values reported in this study represent the mean ± SD.

### *Cell-viability studies*

LNCaP cells of the same passage were grown for 6 days in six-well plates in $DHT^+$ or $DHT^-$ medium. During the course of these studies, we observed a consistently higher cell confluency in 100 n*M* DHT-treated cells (two- to threefold more cells in $DHT^+$ medium than in $DHT^-$), which may be explained by the effect of testosterone on the cell cycle (19, 33, 38). To normalize for the higher cell density in DHT-treated cells, one plate from each condition was sacrificed for cell-number quantification. In brief, cells were trypsinized, diluted with 3 ml PBS, and resuspended by passing through a 22-gauge syringe needle several times. The number of cells in an equal volume of suspension from each well was counted by using a cell-count chamber. To measure cell viability, the remaining plates received a fresh 2-ml aliquot of $DHT^-$ medium, to which freshly prepared t-BuOOH in DMSO was added immediately before the experiment. Control plates received an equivalent volume of vehicle only. After a 12-h incubation with t-BuOOH, the viability of cells was assessed by using the 3-[4,5-dimethyl thiazol-2-yl]-2,5-diphenyl tetrazolium bromide dye method (29) and expressed as a percentage of untreated controls.

## RESULTS

### *Testosterone downregulates CBS in human prostate cancer cell line*

To determine whether testosterone regulates CBS in human prostate, we used an androgen-responsive human prostate adenocarcinoma cell line, LNCaP. We compared CBS protein levels and activity in LNCaP cells grown in medium depleted of androgenic compounds, and supplemented with DHT or with vehicle alone. CBS levels were downregulated in response to DHT treatment (Fig. 2A and B). With testosterone treatment, CBS levels decreased to ~50% of the untreated control value in a time-dependent manner, reaching a new steady-state level after 6 days (Fig. 3). The response of CBS expression to DHT was observed even at concentrations as low as 5 n*M* (Fig. 3B).

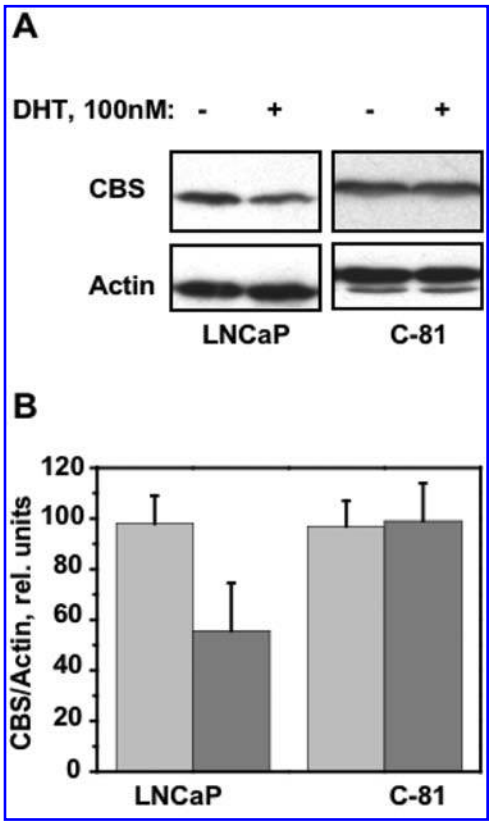


**FIG. 2. Testosterone downregulates CBS protein levels in the androgen-responsive human prostate adenocarcinoma cell line, LNCaP.** (**A**) Androgen-responsive or -unresponsive LNCaP cells were grown for 6 days in phenol red-deficient RPMI medium containing 10% heat-inactivated charcoal stripped FBS in the presence or absence of 100 n*M* DHT. CBS and actin (an equal loading control) were detected in cell extracts by Western analysis, as described under Methods. The Western blots are representative of four independent experiments. (**B**) Quantitative analysis of the CBS response to DHT in the androgen-responsive LNCaP and androgen-unresponsive C-81 cells. The CBS signal in each lane (**A**) was first normalized to actin in the same lane before comparison with DHT-untreated control values. The data represent the average of four independent experiments ± SD.

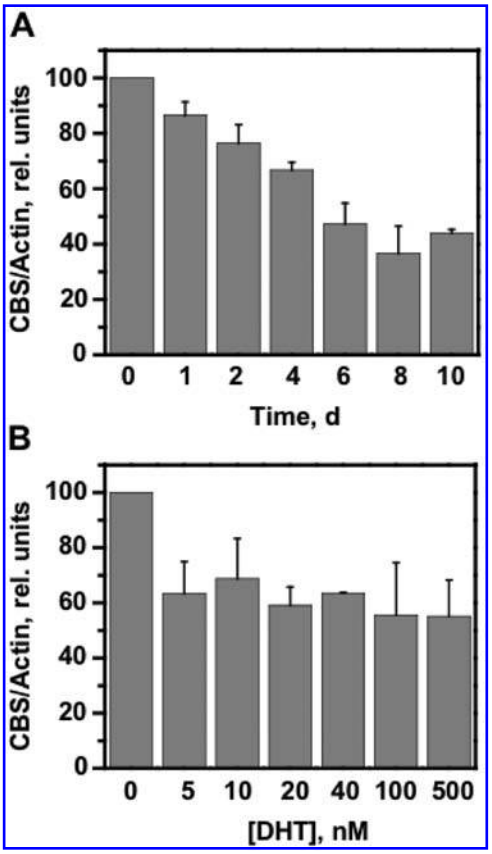


**FIG. 3. Time and concentration dependence of testosterone-induced changes in CBS expression.** (**A**) Quantitative summary of time-dependence studies in LNCaP cells. Androgen-responsive LNCaP cells were grown with 100 n*M* DHT for 0, 1, 2, 4, 6, 8, or 10 days. CBS and actin (an equal loading control) were detected in cell extracts by Western analysis, as described under Methods. (**B**) Quantitative summary of concentration-dependence studies in LNCaP cells. Androgen-responsive LNCaP cells were grown with the indicated concentrations of DHT for 6 days. CBS and actin (an equal loading control) were detected in cell extracts by Western analysis, as described under Methods. In both **A** and **B**, the CBS signal in each lane was first normalized to actin in the same lane before comparison with DHT-untreated control values. In both **A** and **B**, the data summarize three independent experiments; each data point represents mean of at least two measurements ± SD.

As a negative control, we used an androgen-unresponsive LNCaP-derived cell line, C-81 (19), which exhibited no changes in CBS expression after DHT treatment (see Fig. 2). The DHT-induced decrease in CBS protein levels in the androgen-responsive cells was accompanied by an ~30% decrease in CBS activity [from 122 ± 18 ($n = 4$) to 82 ± 19 ($n = 4$) nmol cystathionine formed per milligram protein per hour in untreated and DHT-treated cells, respectively).

### *DHT regulation of CBS occurs posttranscriptionally*

To assess whether DHT regulates CBS at a transcriptional level, Northern analysis was performed. However, no difference in CBS mRNA levels was observed between DHT-treated and untreated LNCaP cells (Fig. 4). As an alternative approach, the effect of DHT on CBS promoter activity was monitored by using a luciferase reporter assay. Again, the results in the presence and absence of DHT were indistinguishable within experimental error (data not shown). Taken together, these results exclude regulation of DHT at a transcriptional level or by a decrease in mRNA stability, or both, and suggest instead a translational or posttranslational mechanism. We recently demonstrated that AdoMet binding stabilizes the CBS protein (36). Intracellular AdoMet concentration in DHT-treated LNCaP cells (0.18 ± 0.02 nmol/mg protein; $n = 3$) were 60% lower as compared with untreated controls (0.41 ± 0.03 nmol/mg protein; $n = 3$). To determine whether the decrease in AdoMet levels was responsible for the lower CBS levels, LNCaP cells were grown in medium supplemented with DHT and 0.3 m*M* AdoMet, DHT alone, or AdoMet alone. AdoMet supplementation was found to increase intracellular AdoMet levels by approximately fourfold (data not shown). However, CBS levels were unresponsive to addition of exogenous AdoMet in DHT-treated cells (Fig. 4B). These results indicate that DHT-depen-

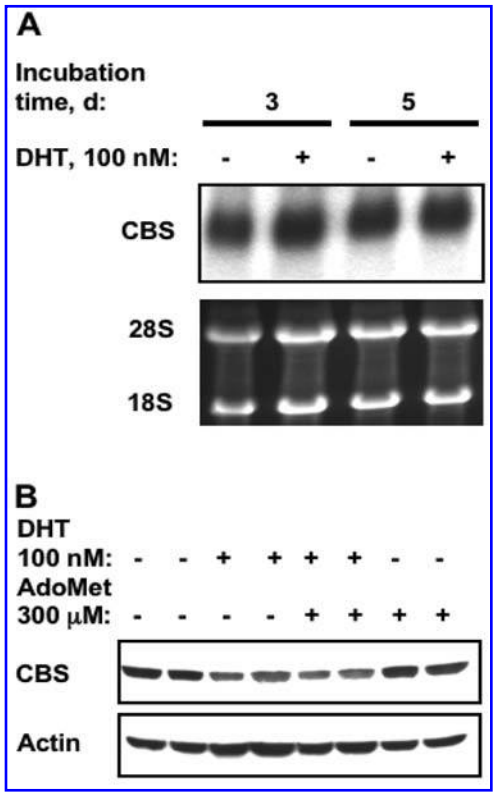


**FIG. 4. Regulation of CBS expression by testosterone in prostate cells does not occur at the transcriptional level.** (**A**) CBS mRNA levels were determined by Northern blot analysis in cells grown for 3 and 5 days, in medium containing 0 or 100 n*M* DHT. Equal loading of samples was ensured by ethidium bromide staining of total rRNA levels in each sample. (**B**) Testosterone-dependent downregulation of CBS levels in prostate cells is not mediated by AdoMet. LNCaP cells were grown for 6 days in phenol red-deficient RPMI medium containing 10% heat-inactivated charcoal stripped FBS and supplemented with 100 n*M* DHT, 300 $\mu M$ AdoMet, or both. CBS and actin (an equal loading control) were detected in cell extracts by Western analysis, and the blot is representative of two independent experiments.

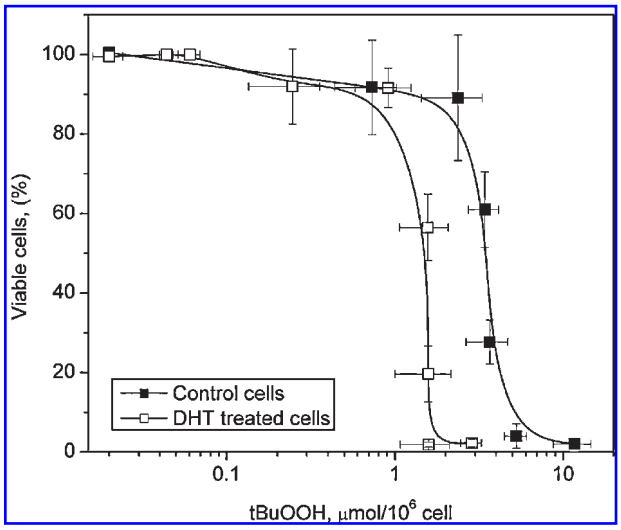


**FIG. 5. The effect of DHT-dependent regulation of CBS on cell viability under oxidative stress.** Cell viability at increasing concentrations of t-BuOOH was determined in cells grown in $DHT^+$ (*open symbols*) or $DHT^-$ (*solid symbols*) medium, as described under Methods. Viability is represented as a percentage of viable cells in t-BuOOH-untreated controls, and each data point is the average of five independent determinations.

dent downregulation of CBS levels in LNCaP cells is not directly mediated by AdoMet.

### *Effect of CBS downregulation on the transsulfuration flux in LNCaP cells*

Because the transsulfuration pathway is important for provision of cysteine, which limits glutathione synthesis, the influence of DHT on glutathione levels was monitored. Intracellular glutathione concentrations were found to be 40% lower [from 119 ± 15 to 75 ± 20 nmol/mg protein ($n = 7$)] in DHT-treated cells. A corresponding decrease in the flux of homocysteine through the transsulfuration pathway was observed [from 1.9 ± 0.2 to 1.4 ± 0.07 μmol/g protein/h ($n = 4$) in untreated and DHT-treated cells respectively].

### *DHT-induced CBS downregulation reduces LNCaP cell viability under oxidative stress conditions*

The efficacy of cellular response to oxidative stress is dependent, in part, on the activity of the transsulfuration pathway (32, 36). We therefore assessed the effect of the DHT-induced decrease in CBS levels and the transsulfuration flux in LNCaP cells on the cellular response to oxidative stress. DHT diminished the viability of LNCaP cells challenged with t-BuOOH. Thus, the $LC_{50}$, decreased from 3.4 to 1.5 μmol/$10^6$ cells t-BuOOH for untreated cells *versus* DHT-treated cells (Fig. 5).

## DISCUSSION

Although a sex-dependent difference in circulating levels of homocysteine is known to exist, with women exhibiting lower plasma homocysteine than men (4, 27), the molecular basis underlying this difference is not known. Recent studies from our laboratory have suggested a link between sex-dependent differences in renal CBS activity and plasma homocysteine levels in mice (42, 43). Curiously, a wide range in the pattern of sex-dependent regulation is observed, with females having higher (in *Drosophila*, rat, and human) or lower (in mouse) CBS activity than males or being indistinguishable from them (in rabbit, hamster, and guinea pig) (43). Studies with mice have implicated testosterone (rather than estrogen) as the sex hormone that regulates CBS, because castration, but not ovariectamization, alters renal CBS levels (43). The difference in the pattern of sex-dependent regulation of CBS in mice *versus* men limits the use of mice as a model organism in this context. In this study, we used a human prostate cancer cell line to test the hypothesis that testosterone regulates the activity of human CBS by using an androgen-responsive human prostate cancer cell line.

Testosterone treatment downregulates CBS activity in the prostate cancer cell line, LNCaP, and this is associated with an approximate twofold decrease in steady-state levels of CBS. DHT-dependent regulation of CBS is not seen in an androgen-unresponsive derivative of the same cell line (see Fig. 2). Androgens serve as ligands for the androgen receptor (AR), and the complex functions as a transcription factor that binds to androgen-response elements in the promoter region of target genes. The resulting transcriptional response is influenced by additional *cis*- and *trans*-acting factors that interact with the androgen-receptor complex (13, 46). In addition, androgen-dependent signaling pathways can initiate secondary effects *via* mechanisms other than direct transcriptional regulation.

Androgens control expression of various genes involved in proliferation, differentiation, secretion, and apoptosis, and therefore are required for normal prostate development and function (8). At initial diagnosis of prostate cancer, 75% of tumors in men are androgen dependent (41, 45) and can be treated by surgical or pharmacologic reduction of circulating androgen levels or by blocking androgen signaling. However, in many cases, the disease returns in a few years in an androgen-independent or hormone-refractory form (25). Despite its name, most cases of androgen-independent prostate cancer exhibit functional AR signaling, even when levels of testosterone are greatly diminished by androgen-ablation treatment. AR pathways can be superactivated through genomic amplification of AR, by mutations in AR or in coactivators, or by activation of AR by alternative ligands. With very few treatment options being effective, the prognosis at this stage of the disease is very poor (11).

Interestingly, the DHT-induced diminution in CBS levels in LNCaP androgen-dependent cancer cells was not accompanied by a corresponding decrease in CBS mRNA levels (see Fig. 4). These results indicate that regulation of CBS by DHT in the human prostate cancer cell line, LNCaP, is not exerted at the transcriptional level. This is consistent with the absence of an androgen-response element consensus sequence in the most commonly used promoter for the human CBS gene, −1b (14, 15). Thus, DHT regulates CBS posttranscriptionally but by a mechanism that is presently not known.

To address the physiologic significance of CBS regulation by testosterone, its effect on the glutathione pool was examined. Diminution of CBS levels was paralleled by a reduction

in CBS activity and accompanied by an ~40% decrease in glutathione levels. The flux through CBS was ~35% lower in DHT-treated cells *versus* untreated controls, providing a plausible mechanistic link between diminution of the glutathione pool and androgen regulation of CBS. It should be noted, however, that whereas the magnitude of changes observed in the glutathione pool correlates well with regulation of CBS levels and activity, additional loci of DHT regulation (cysteine transport and consumption or glutathione turnover or both) could also play a role. The activity of γ-glutamylcysteine synthetase (the rate-limiting enzyme in GSH biosynthesis) has been previously reported to be unchanged in response to androgen treatment of LNCaP cells. However, the activity of γ-glutamyl transpeptidase, which cleaves extracellular glutathione and is a component of the glutathione cycle, is increased by androgen treatment (39). Furthermore, in the PC3 prostate cancer cell line, androgens are reported to increase the activity of glutathione reductase, which converts oxidized glutathione back to its reduced form (35). Together, these findings suggest that the observed decrease in glutathione levels in response to androgen treatment [present study and (39)] results from a CBS-related decrease in cysteine availability, rather than from downregulation of glutathione synthesis enzymes, acting downstream of CBS. Our results indicate that the transsulfuration pathway plays a quantitatively important role in the maintenance of the glutathione pool size in prostate cancer cells.

We tested the role of CBS in glutathione-based antioxidant defense in prostate cancer cells by comparing their sensitivity (±DHT treatment) to oxidative challenge (see Fig. 5). LNCaP cells showed a 2.3–fold increase in sensitivity to t-BuOOH. Interestingly, oxidative damage, which is perceived as a potential underlying cause of tumor development (7, 46), is also used in a controlled manner for cancer treatment for the same reason (*i.e.*, it is cytotoxic). Increased glutathione levels are associated with *in vitro* radioresistance (24) and resistance to arsenic trioxide treatment (28). However, these cells can be sensitized to cytotoxic agents by pretreatment with selenite (17, 18) or the glutathione synthesis inhibitor L-butathione-sulfoximine (24, 28, 38). Further, butathione-sulfoximine–induced increase in ROS levels was shown to overcome a multidrug-resistance phenotype through the activation of receptor tyrosine kinase signaling pathways (44). Our results suggest that selective targeting of CBS in combination with drugs that cause oxidative stress could be a useful chemotherapeutic strategy for diminishing the viability of prostate cancer cells.

In an earlier study, we found that CBS protein levels were 16–fold higher in moderately metastatic human prostate cell line, DU145, compared with another androgen-unresponsive highly metastatic cell line, PC3 (47). These results predicted that DU145 cells would have a higher redox capacity, and indeed, these cells exhibit a 2.5-fold higher $IC_{50}$ for arsenic trioxide than does the PC3 cell line (28). These observations underscore the importance of androgen regulation of CBS activity in setting tumor antioxidant capacity, which, in turn, could influence certain anticancer treatments.

Although it remains to be determined whether CBS levels increase with loss of androgen dependence during prostate cancer progression *in vivo*, our studies suggest a mechanism for changes in cellular redox status that is androgen related. We hypothesize that in benign prostate tissue and during the initial androgen-dependent malignant stage, CBS activity is downregulated by androgens, leading to a more prooxidant environment. Loss of androgen sensitivity would result in an increase in CBS activity, leading to higher levels of glutathione and a more reducing environment that is perhaps favorable for faster-growing metastatic cells. Studies on the kinetics of androgen-sensitive sulfur metabolic and redox changes during progression of prostate cancers are warranted and could suggest new targets for intervention.

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## ABBREVIATIONS

ROS, reactive oxygen species; CBS, cystathionine β-synthase; DHT, dihydrotestosterone; t-BuOOH, tertiary butylhydroperoxide.

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