The androgen receptor (AR) is expressed in almost all prostate cancers and the growth and proliferation of prostate cancer cells is initially an androgen-dependent process (1). The time-honored observation by Huggins and Hodges in the 1940s that prostate cancer regresses after castration was the first illustration of the activity of androgen deprivation therapy, which is the standard frontline therapy for advanced prostate cancer today (2, 3). Although prostate cancer generally responds to androgen deprivation therapy, the majority of tumors will eventually grow and are termed castration-resistant prostate cancer (CRPC). Despite castrate levels of testosterone, AR is reactivated in CRPC and drives the proliferation and survival of these tumors. AR reactivation occurs via gain-of-function changes through AR gene amplification, mutations in AR, changes in expression of steroid receptor coactivators, activation through growth factor receptors and other phosphorylation-dependent mechanisms, and local tumor-driven synthesis of androgens (4–8). Gene expression profiling of hormone-responsive tumors versus CRPC has revealed some of these mechanisms. These studies also suggest that in the transition to CRPC, manganese superoxide dismutase (SOD2) is one of the most down-regulated genes (9). SOD2 regulates reactive oxygen species (ROS) by converting superoxide to a less reactive species (ROS). The mechanism of gene expression for steroid hormone receptors depends on an oxidative process, which occurs as a result of localized histone demethylation (10). Therefore, we hypothesized that SOD2 down-regulation in CRPC is mechanistically linked to AR reactivation.

Here, we examine changes in the expression of androgen-responsive genes after knocking down SOD2 in an AR-expressing prostate cancer cell line. We show that knocking down SOD2 leads to the expression of androgen-responsive genes. The expression of these genes is reversible on treatment with...
Translational Relevance

This body of work shows that manganese superoxide dismutase (SOD2) down-regulation, which has been shown to occur in castration-resistant prostate cancer (CRPC), induces androgen receptor (AR) activity and the expression of AR-responsive genes. It is known that the transition from androgen-responsive to CRPC is mediated by a gain of function in AR. Therefore, reversing this process would provide a new avenue of therapy for prostate cancer. SOD2 down-regulation leads to an increase in reactive oxygen species (ROS), and this work also shows that the mechanism of AR activation in this setting is mediated through ROS and, furthermore, that reversal of increased ROS with an antioxidant reverses AR activity. These findings have clear implications for clinical therapy and suggest that antioxidants may have a role in the treatment of patients with CRPC.

N-acetylcysteine (NAC). DNA binding to androgen response elements (ARE) is induced by SOD2 knockdown in a NAC-reversible manner both in a Panomics transcription factor array and by electrophoretic mobility shift assay (EMSA). Furthermore, SOD2 knockdown also induces AR transcription activity, which is reversible with NAC. These findings suggest that SOD2 down-regulation in CRPC directly affects AR activity and AR DNA binding in a ROS-dependent manner. Furthermore, the ROS-dependent mechanism of AR reactivation in CRPC suggests that antioxidants may have therapeutic value for CRPC.

Materials and Methods

Cell lines and drug treatments. The LNCaP cell line was maintained in phenol red–free RPMI 1640 containing 10% fetal bovine serum, 2 mmol/L L-glutamine, and antibiotics. For the gene expression studies, cells were washed with PBS, incubated at 37 °C for 30 min to allow for recovery and conversion of the acetate esters. Cells were washed and resuspended in PBS + 1% fetal bovine serum and read by flow cytometry immediately.

Measurement of superoxide dismutase activity. Two million cells were lysed in 20 mmol/L HEPES buffer containing 1 mmol/L EGTA, 210 mmol/L mannitol, and 70 mmol/L sucrose using Calbiochem Superoxide Dismutase Assay Kit II (EMD Chemicals) according to the manufacturer’s directions. Lysate (10 μL) was mixed with 200 μL tetrazolium salt, the radical detector. The reaction also contained 1 mmol/L potassium cyanide and 20 μL xanthine oxidase to inhibit SOD1 and SOD3 activities. The reaction was incubated at room temperature for 20 min and read at 450 nm using a Genios plate reader (Tecan). One unit of SOD activity is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical.

Measurement of cellular ROS. Cellular ROS was measured by the hydrolysis of the acetate esters of 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H2DCFDA; Invitrogen). One million cells per reaction were washed and resuspended in prewarmed (37 °C) PBS containing 5 μmol/L CM-H2DCFDA and incubated at 37 °C for 15 min to load the dye into the cells. Following the incubation, cells were washed with prewarmed (37 °C) growth medium and resuspended in 10 mL growth medium/million cells and incubated at 37 °C for 30 min to allow for recovery and conversion of the acetate esters. Cells were washed and resuspended in PBS + 1% fetal bovine serum and read by flow cytometry immediately.

Fig. 1. Expression of shSOD2 decreases SOD2 protein and SOD2 enzymatic activity in LNCaP cells. A, Western blot of vector control and shSOD2-expressing LNCaP cells with actin as a loading control. B, quantitation of decrease in SOD2 protein normalized to actin. C, decrease in SOD2 enzymatic activity in shSOD2-expressing cells.
Microarray analysis. Total RNA was isolated from 1 million cells using Trizol (Invitrogen). RNA (20 μg) was used in reverse transcription reaction containing either Cy3-dUTP or Cy5-dUTP, oligo(dT) primer, and SuperScript III, as described previously (12). Each experimental sample was labeled with Cy5-dUTP and Human Universal RNA (Stratagene) was labeled with Cy3-dUTP. Following reverse transcription, RNA was hydrolyzed using NaOH, the Cy3 and Cy5 reactions were mixed, and the probe was cleaned up using a Microcon YM-30 column (Millipore). The probe was hybridized to the National Cancer Institute oligonucleotide arrays and analyzed as previously described (12).

Real-time PCR. RNA was extracted using Trizol. RNA (10 μg) was used in a reverse transcription reaction using an oligo(dT) primer and SuperScript III (Invitrogen). cDNA (100 ng) was then used in a 20 μL Taqman assay using Universal Master Mix and gene-specific Taqman probes both from Applied Biosystems. The gene-specific Taqman mixes used are as follows: FKBP5, Hs00188025_m1; APRIN, Hs00971886_m1; VEGF, Hs00900054_m1; aldol-keto reductase family 1, member C3 (AKR1C3), Hs00366267_m1; 17-β-hydroxysteroid dehydrogenase B 14 (HSD17B) 14, Hs00212233_m1; HSD17B8, Hs00157993_m1; HSD17B2, Hs00157993_m1; prostate-specific antigen (PSA), Hs02576345_m1; TMPRSS2, Hs00237175_m1; and 18S, Hs99999901_m1. PCR was done using a One-Step Real-Time PCR machine (Applied Biosystems).

DNA/protein arrays. Transcription factor–binding site analysis was done using Panomics DNA/protein combo arrays containing 345 consensus binding sites according to the manufacturer’s instructions. Each nuclear protein lysate (15 μg), prepared using Panomics nuclear protein extraction kit, was used in the binding reaction. Following binding and separation of unbound from bound biotinylated transcription factor–binding sites, the probe was hybridized at 45°C overnight. The blot was then washed and detected using a streptavidin-horseradish peroxidase conjugate. Data were quantitated using ImageQuant (GE Healthcare), and a binding site was considered to be regulated only if it showed reciprocal regulation in the shSOD2 and shSOD2 + NAC cell lines.

Electrophoretic mobility shift assay. Nuclear protein extracts were prepared from cell lines using Panomics nuclear extraction kit, following the manufacturer’s instructions. The nuclear protein was quantified using the bicinchoninic acid colorimetric assay (Pierce). Nuclear extract (8 μg) was mixed with 50 nmol/L IR-labeled androgen-responsive element (ARE; LI-COR Biosciences) in a 20 μL reaction containing 10 mmol/L Tris, 50 mmol/L KCl, 1 mmol/L DTT, 1 μg poly(deoxyinosinic-deoxycytidylic acid), 2.5 mmol/L DTT/0.25% Tween 20, and 5 mmol/L MgCl2 and incubated for 30 min at room temperature. Following incubation, the reaction was run on a 6% polyacrylamide gel in Tris-glycine buffer and scanned using an Odyssey IR scanner (LI-COR Biosciences).

Luciferase assay. Empty vector–expressing and shSOD2-expressing LNCaP cells were seeded at 20,000 per well and transfected with TK-luc-ARE3 (150 ng) to measure AR activity and pGL4.75 (15 ng; Promega) as a transfection control using Lipofectamine 2000 (Invitrogen). Twenty-four hours after transfection, medium was changed to phenol red–free, serum-free medium containing 10 nmol/L R1881 or 5 mmol/L NAC, as

Fig. 2. ROS are increased in the shSOD2 cell line compared with vector control cells. ROS activity was measured in triplicate with the intensity shifting from a mean of 1,497 in the vector sample to a mean of 1,681 in the shSOD2 sample (P < 0.05) by flow cytometry.

Fig. 3. Genes regulated by SOD2 show a large overlap with androgen signaling. Venn diagrams showing the number of genes regulated by SOD2 knockdown and R1881 as well as the intersection of these genes.
indicated. Firefly and Renilla luciferase activity was measured using the Dual-Glo Luciferase Assay System (Promega) 24 h later. Firefly luciferase measurements were normalized using the Renilla luciferase measurements. All measurements were done in quadruplicate.

Results

To determine the consequences of SOD2 down-regulation, we infected LNCaP cells with a construct expressing a SOD2 short hairpin RNA and a puromycin resistance gene (shSOD2). shSOD2-expressing LNCaP cells had a 71% decrease in SOD2 protein expression when compared with cells expressing the empty vector (Fig. 1A and B). The function of SOD2 is to neutralize reactive superoxide radicals by converting them to hydrogen peroxide. To determine the degree to which shSOD2-expressing LNCaP cells have an altered ability to neutralize superoxide, we assayed these cells for enzymatic function specific for SOD2. LNCaP cells expressing shSOD2 showed a 2-fold decrease in SOD2 enzymatic activity compared with control cells (Fig. 1C).

Down-regulation of SOD2 protein and enzymatic activity is expected to increase levels of ROS. To determine the change in cellular ROS induced by SOD2 knockdown, we measured ROS activity as determined by hydrolysis of acetate esters susceptible to ROS-mediated cleavage using flow cytometry. The distribution of cells with higher levels of cleaved acetate esters is significantly greater (P < 0.05) in SOD2 knockdown cells compared with control cells (Fig. 2). These results show that...
SOD2 down-regulation in LNCaP prostate cancer cells leads to lower levels of SOD2 enzymatic activity and consequently higher levels of ROS activity.

Next, we determined gene expression changes in LNCaP induced by (a) direct activation of AR with R1881, (b) SOD2 knockdown, and (c) SOD2 knockdown cells treated with NAC (Fig. 3). Gene expression changes that occur with SOD2 knockdown and are ROS mediated would be reversed with NAC treatment. We found that 46% and 38% of genes that are induced 2- and 4-fold, respectively, by R1881 are also induced to the same extent with SOD2 down-regulation. Examination of the shared genes that are induced with R1881 exposure and SOD2 down-regulation reveals both potential mechanisms through which AR may be reactivated and a set of well-recognized AR-responsive genes that are also activated through SOD2 knockdown. Furthermore, many of the gene expression changes that are common to both direct AR activation with R1881 and SOD2 knockdown are reversed in SOD2 knockdown cells by treatment with NAC, suggesting that activation of these androgen-regulated genes in SOD2 knockdown cells is ROS mediated (Fig. 4A). Several genes induced by SOD2 knockdown, such as AKR1C3, HSD17B1, HSD17B8, HSD17B14, and HSD11B2, are intimately involved in steroid metabolism. Some of these genes have been implicated in AR reactivation by converting adrenal androgens to testosterone in the setting of CRPC (13). A subset of genes induced by SOD2 down-regulation is coregulators of steroid hormone receptors. Some of these coregulators, such as NCOA4, specifically activate AR by direct protein-protein interaction (14). Another subset of genes, including interleukin-6 receptor (IL-6R) and RICTOR, is involved in AR activation in the presence of low levels of testosterone through phosphorylation-dependent mechanisms. Numerous studies have shown that IL-6 is implicated in castration-resistant growth and activates AR through a signal transducer and activator of transcription (STAT) 3-dependent mechanism (15, 16). RICTOR overexpression up-regulates the mammalian target of rapamycin/Akt pathway (17), which results in AR activation (18).

Widely studied AR-regulated genes, such as FK506-binding protein 5 (19, 20), α-methylacyl-CoA racemase (21), and APRIN (22, 23), were also up-regulated in a NAC-reversible manner in these microarray studies. To further validate our findings, we analyzed a selected group of genes found to be induced by R1881 in a previously published cDNA microarray study of prostate cancer cells (21). A subset of these genes is also induced by SOD2 knockdown in a NAC-reversible manner (Fig. 4B). Next, for selected genes, we confirmed gene expression changes that were induced by SOD2 knockdown and reversible with NAC treatment using quantitative PCR (Fig. 4C). All seven genes we tested—FKBP5, APRIN, VEGFA, AKR1C3, HSD17B8, HSD17B14, and HSD11B2—were induced by SOD2 knockdown and repressed with NAC treatment. Furthermore, we also tested PSA, which is an androgen-regulated gene that changed less than 2-fold with SOD2 knockdown, and TMPRSS2, which was not on our microarray but is known to drive oncogene expression through an AR-driven regulatory region with a chromosomal translocation (24). Both of these genes are induced by SOD2 knockdown in a NAC-reversible fashion. Notably, we found that several nuclear receptors were among the genes regulated by SOD2 knockdown in a NAC-reversible manner, but not by R1881. Estrogen receptor-α, estrogen-related receptor-β, and retinoid X receptor-α are down-regulated and progesterone receptor is up-regulated with SOD2 knockdown. These changes are all reversible with NAC treatment (Supplementary Fig. S1).

Although we found that induction of androgen-regulated gene expression changes with SOD2 knockdown was largely reversible with NAC, suggesting a ROS-mediated mechanism of AR activation, we wished to further confirm that this mechanism is

**Fig. 5.** Changes in transcription factor binding in response to SOD2 knockdown. Binding of transcription factors was measured using a Panomics array and the fold change in binding activity is shown. An asterisk represents a low level of binding in the control sample, and the value for quantitation was set to 100 over background.
indeed ROS mediated. Therefore, we examined DNA-binding changes of transcription factors induced by SOD2 down-regulation that were also NAC reversible using a Panomics array, which detects the relative activation status of transcription factors (Fig. 5). Nineteen of 345 (5.5%) transcription factors had an increase in DNA binding with SOD2 knockdown that was reversible with NAC treatment. Binding to the ARE increased 5.3-fold with SOD2 knockdown and decreased 4.1-fold with NAC treatment of SOD2 knockdown cells. Importantly, binding to GATA-2 elements increased 8.9-fold with SOD2 knockdown and decreased 8.3-fold with NAC treatment. GATA-2 activity has recently been shown to be absolutely required to drive the expression of AR-regulated genes (25). Binding to the myc-max DNA-binding element in our Panomics array is also consistent with ROS regulation. However, myc is an AR-regulated gene and this effect is probably downstream of AR (26).

To further investigate the effect of SOD2 down-regulation and ROS on AR activity, we did EMSA using ARE oligos (Fig. 6A). Treatment with 10 nmol/L R1881 and SOD2 knockdown induced ARE binding to a similar extent. Treatment of SOD2 knockdown cells with NAC reversed ARE binding, consistent with a ROS-mediated mechanism. These EMSA results in conjunction with the results of the Panomics array show that SOD2 knockdown induces ARE binding in a NAC-reversible manner. This suggests that the induction of androgen-responsive genes we observed are due to direct activation of AR via a ROS-mediated mechanism.

Finally, we directly tested the effect of SOD2 down-regulation on AR transcriptional activity using the luciferase assay (Fig. 6B). Treatment of empty vector–infected LNCaP with R1881 induced ARE-luciferase activity to 7-fold above baseline, whereas SOD2 knockdown cells have activity that is 4-fold over the baseline of empty vector–expressing LNCaP. Furthermore, ARE-luciferase activity is largely reversible in SOD2 knockdown cells on treatment with NAC.

Discussion

Although by definition testosterone is depleted with CRPC, the growth and progression of prostate cancer in this form is still dependent on AR (1, 6). Defining exactly how AR is reactivated in CRPC is the first step in determining how AR should be targeted (27). SOD2 is one of the most potently down-regulated genes in CRPC when compared with hormone-responsive tumors (9). Here, we show that SOD2 down-regulation is directly responsible for AR reactivation in prostate cancer and that this occurs through a ROS-mediated mechanism. SOD2 knockdown induces AR binding to ARE followed by gene expression changes that overlap with gene expression changes that occur with ligand-mediated activation of AR. AR activation that occurs with SOD2 knockdown is reversible with NAC and is therefore mediated directly through ROS.

Our studies reveal several ways in which ROS activates AR. First, we found that several genes involved in steroid metabolism, including AKR1C3, are regulated by SOD2 knockdown. One mechanism of AR reactivation in CRPC is the local conversion of androgen precursors to ligands, such as testosterone, which effectively activate AR. AKR1C3 is overexpressed in CRPC and reduces adrenal androstenedione to testosterone (13). Such gene expression changes lead to an increase in the local concentration of androgens in CRPC (8). We found that all four genes involved in steroid metabolism that are induced by SOD2 knockdown and that we tested by real-time PCR reverse gene expression with NAC.

Second, AR reactivation can be induced by altering the balance of steroid receptor coregulators (28). Five nuclear receptor coregulators are induced in a ROS-reversible manner. Although the ultimate effect of the combination of these expression changes is difficult to discern, the overexpression of one of these genes, NCOA4, has been shown to directly result in an increase in AR activity (14).

Third, multiple signaling pathways have been implicated in ligand-independent or ligand-sensitizing AR activation (29). One of the most widely studied of these is the IL-6 pathway...
IL-6 activates AR in a STAT 3–dependent manner and antibodies to IL-6 reverse castration resistance (16, 30). Furthermore, levels of IL-6R are predictive of biochemical recurrence and metastasis (31, 32). Our finding that IL-6R is induced by SOD2 down-regulation in a NAC-reversible manner suggests that treatment with antioxidants may down-regulate this pathway.

Whether ROS reactivates the expression of androgen-responsive genes through increasing the local availability of androgen ligand, altering the distribution of coregulators to more favorable levels, or through signaling mechanisms such as the IL-6 pathway, our data suggest that it directly activates AR, resulting in increased binding to ARE and stimulation of AR-driven transcription. Interestingly, we also found that binding to GATA-2 DNA motifs also increases with SOD2 knockdown and is reversed with NAC treatment. GATA-2 directly interacts with AR and the GATA-2 transcription factor and DNA-binding motif are absolutely required for optimal expression of androgen-regulated genes (25, 33). Therefore, GATA-2 is another way in which ROS may activate AR in the castration-resistant setting.

We found that VEGFA was strongly induced by both R1881 and SOD2 and was reversible with NAC by quantitative PCR. ROS may therefore mediate an angiogenic switch that also occurs through AR. One possibility we considered is that because VEGFA is activated through hypoxia-inducible factor-1 (HIF-1; ref. 34) and HIF-1 activity may be reversible with antioxidants (35), some of what we have observed may be due to HIF-1 activation. However, HIF-1 activity did not increase with SOD2 knockdown in our Panomics array not by EMSA (data not shown). Furthermore, it has been shown that VEGFA is located in an AR-binding region (36), further supporting that ROS-mediated VEGFA activation in our experiments indeed occurs through AR. Others have shown that SOD2 has tumor suppressor activity in prostate cancer cells that do not express AR (37). However, the vast majority of clinical prostate cancers do express AR and the significance of changes in SOD2 expression in prostate tumors that do not express AR is unclear.

One caveat is that our studies focus on a single AR-expressing cell line, LNCaP. Although further work is required to determine if these results can be generalized to other prostate cancer cell lines, the LNCaP growth rate and relatively high levels of AR expression make this the most amenable model to test our hypothesis. Furthermore, and importantly, it should be noted that the hypothesis that SOD2 down-regulation is involved in AR activation originated from observations made from clinical tumors, not cell lines.

In conclusion, we have found that SOD2 down-regulation, a gene expression change which occurs clinically in patients with CRPC, is responsible for AR reactivation. AR activation in this setting is ROS dependent and results in the expression of genes such as AKR1C3, NCOA4, and IL-6R, which are known to lead to AR activation. Furthermore, AR DNA binding is increased and results in expression of androgen-responsive genes. Finally, AR reactivation in cells with low SOD2 expression is reversible with antioxidant treatment. In general, reexpression of genes that have lost function in cancer for therapeutic purposes is problematic and has not been feasible. However, SOD2 down-regulation in CRPC may be overcome simply by treatment with an antioxidant and may result in silencing AR reactivation. Further work is warranted to determine if this strategy will be of therapeutic value clinically.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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