Peroxiredoxin 1 Interacts with Androgen Receptor and Enhances Its Transactivation

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Abstract
Although hypoxia is accepted as an important microenvironmental factor influencing tumor progression and treatment response, it is usually regarded as a static global phenomenon. Consequently, less attention is given to the impact of dynamic changes in tumor oxygenation in regulating the behavior of cancer cells. Androgen receptor (AR) signaling plays a critical role in prostate cancer. We previously reported that hypoxia/reoxygenation, an in vitro condition used to mimic an unstable oxygenation climate in a tumor, stimulates AR activation. In the present study, we showed that peroxiredoxin 1 (Prx1), a member of the peroxiredoxin protein family, acts as a key mediator in this process. We found that the aggressive LN3, C4-2, and C4-2B prostate cancer cell lines derived from LNCaP possess constitutively elevated Prx1 compared with parental cells, and display greater AR activation in response to hypoxia/reoxygenation. Although the cell survival-enhancing property of Prx1 has traditionally been attributed to its antioxidant activity, the reactive oxygen species–scavenging activity of Prx1 was not essential for AR stimulation because Prx1 itself was oxidized and inactivated by hypoxia/reoxygenation. Increased AR transactivation was observed when wild-type Prx1 or mutant Prx1 (C52S) lacking antioxidant activity was introduced into LNCaP cells. Reciprocal immunoprecipitation, chromatin immunoprecipitation, and in vitro pull-down assays corroborated that Prx1 interacts with AR and enhances its transactivation. We also show that Prx1 is capable of sensitizing a ligand-stimulated AR. Based on the above information, we suggest that disrupting the interaction between Prx1 and AR may serve as a fruitful new target in the management of prostate cancer.

Introduction
Microenvironmental hypoxia develops during tumor growth, and is known to influence cancer progression and treatment response. Previous studies have shown a strong correlation between tumoral pO2 and tumor control and progression in patients with solid tumors, including prostate cancer (1–6). Hypoxia is usually considered a global phenomenon, defined as an overall reduced oxygen availability or partial pressure below critical levels (7). However, tumor vasculature is architecturally and functionally abnormal (8, 9); the vessels are chaotic, tortuous, and highly branched. Ultrastructurally, numerous “openings” (endothelial fenestrations, vesicles, and transcellular holes), widened interendothelial junctions, and discontinuous or absent basement membranes have been described. Aberrant blood vessels can be shut down or reopened temporarily at any time, and the same defects can cause reversal of blood flow. Consequently, local blood flow and oxygenation within a tumor are not homogeneous; these variables are changing dynamically (10, 11). Additionally, regional angiogenesis may also contribute to local oxygenation changes. Although hypoxia is generally accepted as an important microenvironmental factor influencing the survival and proliferation of cancer cells, little is known about the effect of dynamic oxygenation changes on the behavior of cancer cells.

Prostate cancer is the most common noncutaneous cancer and is the second leading cause of cancer death in men from the U.S. (12). Androgen signaling via the androgen receptor (AR) plays a critical role in the development, progression, and treatment response of prostate cancer (13, 14). The binding of androgen to AR causes a translocation of AR to the nucleus where AR activates the expression of a battery of androgen-responsive genes (15). Because prostate cancer arises in an androgen-stimulated environment, androgen-deprivation therapy (ADT) is used to treat advanced prostate cancer. Although initially effective in blocking tumor growth, ADT eventually fails and results in a high rate of relapse. Recurrent prostate cancer is commonly referred to as “androgen-independent.” Despite this clinical androgen independence, recurrent tumors continue to express AR and AR-regulated genes. Immunohistochemical studies of both frozen and formalin-fixed, paraffin-embedded tissues have shown similar levels of AR expression in primary and recurrent cancer tissues (16–18). Androgens were shown to be present in recurrent tumors at levels that may be sufficient to activate AR (16). These findings suggest that the state of “androgen-independence” may be somewhat of a misnomer. Prostate cancer that recurs during ADT may continue to depend on AR activity for survival and growth.

Studies with castrated rat, transgenic mouse and xenograft models suggested that androgen deprivation itself generates a state of hypoxia in the prostate tissue (19–21). Androgen deprivation has been shown to cause endothelial cell death, degeneration of capillaries, and vasconstriction in the prostate. These findings imply that the inherently hypoxic and unstable oxygenation climate of a tumor might be further disturbed by androgen deprivation. Recently, we investigated the effect of an unstable oxygenation milieu on AR activity (22). Hypoxia/reoxygenation, an in vitro condition used to mimic dynamic changes in tumor oxygenation, was found to increase AR binding to the androgen-responsive element (ARE), prostate-specific antigen (PSA) expression, and ARE reporter gene activity. AR activation by hypoxia/reoxygenation was also observed when AR was introduced exogenously into AR-null prostate cancer cells. These findings provide evidence that the AR-stimulatory effect of an unstable oxygenation climate is likely to contribute to resistance to ADT, and the emergence of recurrent prostate cancer.
Peroxiredoxin 1 (Prx1) is a member of the redox-regulating peroxiredoxin protein family. Prx1 has been shown to be elevated in several cancers, and its ability to enhance the survival and progression of cancer cells has been suggested (23–26). However, no information is available on the possible role of Prx1 in prostate cancer. Considering the effect of an unstable oxygenation condition on AR activity, and the redox changes that are likely to be exacerbated by ADT, we postulated that Prx1 might modify the activity of AR and influence the malignant progression of prostate cancer. Several lineage-related cell models have been developed in an attempt to delineate the molecular basis of prostate cancer progression. The LNCaP human prostate cancer cell line has been widely used because it is one of the limited androgen-sensitive cell lines. LNCaP cells possess a functional AR, and secrete PSA in vitro and in vivo. The C4-2 and C4-2B lines were established by Chung and coworkers (27, 28), and LN3 was established by Pettaway and coworkers (29) from LNCaP. The LN3 cell line was developed by repeated lymph node metastasis of LNCaP cells grown orthotopically in the prostate gland of athymic mice. The C4-2 and C4-2B cell lines were established by cocultivating LNCaP cells with stromal cells in vivo and in vitro. The LN3, C4-2, and C4-2B cells are able to grow in a castrated host, and display increased tumorigenic and metastatic potential compared with the parental LNCaP cells (27–31). In the present study, we found that Prx1 is elevated constitutively in these aggressive LNCaP derivatives compared with the parental cells. We therefore investigated whether Prx1 plays a role in regulating AR activation by hypoxia/reoxygenation. We provided evidence that Prx1 directly interacts with AR and enhances its transactivation in response to hypoxia/reoxygenation. To the best of our knowledge, this is the first report showing the unique function and regulatory mechanism of Prx1 in prostate cancer.

Materials and Methods

Cell culture. LNCaP and DU145 cells were obtained from the American Type Culture Collection. C4-2 and C4-2B cells were provided by Dr. Leland Chung (Department of Urology, Emory University School of Medicine, Atlanta, GA). LN3 and PC3-AR (which stably overexpresses the full-length human AR) were provided by Dr. Curtis Pettaway (Departments of Urology and Cell Biology, University of Texas M. D. Anderson Cancer Center, Houston, Texas) and Dr. Shuyuan Yeh (Department of Urology, University of Rochester, Rochester, NY), respectively. All cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 2 mmol/L of glutamine, 100 units/mL of penicillin, and 100 μg/mL of streptomycin at 37 °C in an atmosphere of 5% CO2 and 95% air. In some experiments, cells were switched to deoxygenated medium was prepared prior to each experiment by culturing cells in RPMI 1640 before hypoxia treatment as reported previously (22). In brief, deoxygenated medium was prepared prior to each experiment by equilibrating the medium with a hypoxic gas mixture containing 5% CO2, 85% N2, and 10% H2 at 37 °C. The oxygen concentration in the hypoxic chamber and the exposure medium was maintained at <0.05%. Oxygen concentration was monitored continuously using an oxygen indicator (Forma Scientific). All experiments were done at 70% to 80% confluency at a medium pH between 7.2 and 7.4.

Plasmid and adenoviral expression vectors. The pCR3.1 plasmids containing wild-type Prx1 or mutant Prx1 (pCR C52S) lacking antioxidant activity, and the construction of adenoviral vectors, Ad.Con, Ad.Prx1, and Ad.C52S, were described previously (32). The adenoviral vectors were used at a multiplicity of infection of 5.

Retroviral short hairpin RNA expression constructs and retroviral infection. For the expression of short hairpin RNA (shRNA) targeting Prx1, the pSilencer 5.1 system (Ambion) was used. Only one of the three target sequences tested effectively knocked-down Prx1 expression. Scrambled sequences were designed to generate a scramble shRNA construct. Both sequences were checked by searching the genome database (BLAST) to ensure that they shared no significant sequence homology with other human genes. The sequences of hairpin oligonucleotides targeting the human Prx1 gene that we chose to use were: 5'-GATCGGTTTCTAC- TTTGCTATCTATTGCAAGATATGACGAGAATTTTTGTTTGAAGA-3' (top strand) and 5'-AGCTTTTCGAAAGGATCCTCCACATGAGGTTTTTTGAAA-3' (bottom strand). The scrambled sequences used were: 5'-GATTCCCCGTTCCTCGACGGTG- CATCTTCAGGATATGACGAGAATTTTTTGGAAA-3' (top strand) and 5'-AGCTTTTCGAAAGGATCCTCCACATGAGGTTTTTTGAAA-3' (bottom strand). Each pair of oligonucleotides was annealed, and cloned into the pSilencer vector. The sequence accuracy of the constructs was confirmed using an ABI 3700 capillary sequencer (Applied Biosystems). Phoenix packaging cells were transfected with Prx1- or scramble-shRNA expression vector using LipofectAMINE 2000 reagents (Invitrogen). Culture supernatants were collected 48 h after transfection and filtered. LN3 cells were infected with the filtered Prx1- or scramble-shRNA viral preparations in the presence of 4 μg/mL of polybrene (Sigma). Fresh viral suspensions were added to the cells every 8 h during the subsequent 48 h. Infected cells were selected in a growth medium containing 2 μg/mL of puromycin for 10 days, and used for experiments.

Cell growth assays. Cells were seeded at 2 × 104 per well in six-well plates in the appropriate medium and allowed to grow for 24 h. Cells were washed with PBS and switched to a phenol-free medium containing 2% CS-FBS (Hyclone). Triplicate wells were counted on days 1, 2, 3, and 4 using a hemocytometer.

Western blot analysis. Equal amounts of protein were analyzed in duplicate by SDS-PAGE. Protein concentrations were measured by the method of Lowry et al. (33). The following monoclonal antibodies were used: anti-Prx1 (Lab Frontier), anti–Prx-SO2/3H (Lab Frontier), anti-AR (Upstate), and anti–β-actin (Sigma). Immunoreactive proteins were detected with secondary antibody and visualized using an enhanced chemiluminescence detection system (Amer sham Bioscience).

Determination of Prx1 oxidation status. Two-dimensional electrophoresis was carried out as described previously (32). Nonlinear gradient strips (3–10% pH; Amer sham Biosciences) were used for isoelectric focusing. The immobilized pH gradient strips were then equilibrated in SDS buffer and placed on top of a 12% SDS-PAGE gel. After migration, the gels were transferred to nitrocellulose membrane and probed with anti-Prx1 (Lab Frontier) or anti–Prx-SO2/3H (Lab Frontier) antibody to determine Prx1 oxidation status.

Transfection and luciferase assay. An aliquot of 3 × 105 cells was placed in a six-well plate and transfected with DNA using Fu gene (Roche). The pSG5hAR expression vector was used to express AR (22). The ARE reporter plasmid containing three repeats of the ARE was used to assess the luciferase reporter as described previously (22). The total amount of plasmid DNA was normalized to 1 μg/well by the addition of empty plasmid. Luciferase activities were measured by using the Luciferase Assay System (Promega), and normalized by protein concentration and transfection efficiency. All transfection experiments were done in triplicate wells and experiments were repeated at least thrice.

Nuclear extract preparation and electrophoresis gel shift assay. Nuclear extract was prepared as described previously (22). Cells were harvested, washed twice with PBS, resuspended in a hypotonic buffer [10 mmol/L HEPES-KOH (pH 7.9), 1.5 mmol/L MgCl2, 10 mmol/L KCl and 0.1% NP40] and incubated on ice for 10 min. Nuclei were precipitated by 3,000 × g centrifugation for 10 min at 4 °C. After washing once with the hypotonic buffer, nuclei were lysed in a lysis buffer [50 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, and 1% Triton X-100], incubated on ice for 30 min and precleared by 20,000 × g centrifugation for 15 min at 4 °C. For electrophoresis gel shift assays (EMSA), 10 μg of nuclear protein extract was incubated in 20 μL of a solution containing 10 mmol/L of HEPES (pH 7.9), 80 mmol/L of NaCl, 10% glycerol, 1 mmol/L of DTT, 1 mmol/L of EDTA, 100 μg/mL of poly(deoxyinosinic-deoxycytidylic acid), and radiolabeled

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9295

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Pxr1 Interacts with AR and Enhances Its Activity
double-stranded oligonucleotide containing the AR consensus binding motif 5'-CTAGAAGTCTGATACCGAGGTCCT-3' (Santa Cruz Biotechnology). ARE-binding complexes were resolved on a 4% non-denaturing polyacrylamide gel containing 2.5% glycerol in 0.25x Tris-borate/EDTA at room temperature, and the gels were autoradiographed.

**RNA isolation and reverse transcription-PCR analysis.** Total RNA was isolated using TRIzol Reagent (Invitrogen), dissolved in DNase I buffer and incubated for 15 min with RNase-free DNase I (20 units/mL) in the presence of RNasin. RNA concentration was determined by UV absorption measurement and samples with comparable A260/A280 ratios were used for reverse transcription-PCR (RT-PCR) analysis. The PCR primer pair sequences were: for PSA, forward 5'-GACAGGTCTTGTAGCCTCTC-3' and reverse 5'-CACCAGAGGGTCTTGTG-3'; for prx1, forward 5'-GTCCACCGGAGTATGCCCTTC-3' and reverse 5'-CCCTCTAAGGAGATCATTCATC-3'; and for β-actin, forward 5'-GAGTGCCTTGGAGTTTACCTGA-3' and reverse 5'-GATGCAGATTCCAGCAAGCGCT ATTTG-3'. PCR was carried out using a thermal cycler (Perkin-Elmer 9600). The amplified products were separated by electrophoresis on a 1.5% agarose gel, stained with ethidium bromide, and photographed under UV illumination.

**Immunoprecipitation assay.** Immunoprecipitation was carried out with 1 mg of cell lysate and 1 μg of anti-AR (Upstate) or anti-Prx1 (Lab Frontier) antibody overnight at 4°C. After the addition of 25 μL of Protein G-agarose (Santa Cruz Biotechnology), lysates were incubated for an additional 4 h. Rabbit IgG (Santa Cruz Biotechnology) or mouse IgG (Santa Cruz Biotechnology) was used as a negative control. The beads were washed thrice with the lysis buffer, separated by SDS-PAGE, and immunoblotted with AR or Prx1 antibody. The protein bands were detected using an enhanced chemiluminescence system (Amersham Biosciences).

**Chromatin immunoprecipitation assay.** Chromatin immunoprecipitation (ChiP) assays were carried out following a procedure described previously (22). After cross-linking with formaldehyde, cells were lysed in buffer containing protease inhibitors. Nuclei were isolated and chromatin was sheared to an average length of 250 to 500 bp using sonication. The sheared chromatin was precleared with salmon sperm DNA/protein A-Sepharose and precipitated with an antibody specific for AR (Upstate) or Prx1 (Lab Frontier). Rabbit IgG was used as a control to monitor nonspecific interactions. Immune complexes were adsorbed onto salmon sperm DNA/protein A-Sepharose beads. After an extensive wash to reduce background, Ab/AR/DNA or Ab/Prx1/DNA complexes were eluted. After precipitation, DNA was resuspended in water and PCR was carried out to amplify the proximal ARE-containing region of the PSA promoter using a primer pair. The intervening region of the PSA gene was amplified as a control. Gels representative of three independent experiments.

**Results**

**Expression of Prx1 is elevated in the LNCaP derivatives, LN3, C4-2, and C4-2B cell lines.** The LNCaP-lineage progression cell models, LN3, C4-2, and C4-2B, are less sensitive to androgen, and display an increased metastatic potential compared with the parental cells. Unlike LNCaP, all three are able to grow in a castrated host. Because Prx1 has been suggested to confer an aggressive survival phenotype, we postulated that Prx1 might influence AR activity and promote the malignant progression of prostate cancer. As a first step to test this hypothesis, we examined Prx1 expression in LN3, C4-2, and C4-2B cells. As shown in Fig. 1, our results revealed that Prx1 expression was increased in all three progeny cell lines compared with the parental LNCaP cells. The elevated expression of Prx1 seems to be controlled at the level of message abundance.

**AR stimulatory effect of hypoxia/reoxygenation is greater in LN3, C4-2, and C4-2B than in parental LNCaP cells.** We next examined whether elevated levels of Prx1 might influence AR activation by hypoxia/reoxygenation. LNCaP and LN3 cells were exposed to 4 h of hypoxia, and the ARE-binding activities of AR were examined at 0, 0.5, 2, or 6 h during reoxygenation. As shown in Fig. 2A, although the AR stimulatory effect of hypoxia/reoxygenation was observed in both LNCaP and LN3 cells, the degree of AR stimulation by hypoxia/reoxygenation was greater in LN3 than in LNCaP cells. The ARE-binding activity of AR reached a maximum at 2 h during reoxygenation for both cell lines. Because PSA is one of the best-characterized AR target genes, PSA mRNA accumulation was compared between LNCaP and LN3 cell lines using semiquantitative RT-PCR. Consistent with the greater ARE-binding activity, the accumulation of PSA mRNA by hypoxia/reoxygenation was more pronounced in LN3 than in LNCaP cells (Fig. 2B). Increased PSA mRNA accumulation was not due to increased AR protein level. No appreciable change in AR protein level was observed by hypoxia/reoxygenation (Fig. 2C). Activated AR translates its target genes by binding to the ARE-containing

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**Figure 1.** Elevated levels of Prx1 expression in LNCaP lineage progression cell models. A, equal amounts of protein extract from LNCaP, LN3, C4-2, and C4-2B cells were separated on 12% SDS-PAGE. Western blot analysis was done using an anti-Prx1 antibody as a probe. β-Actin (Sigma) was used as a loading control. Blots representative of three independent experiments. B, RT-PCR analysis of prx1 mRNA with PCR primer pairs specific for the human prx1 gene. The β-actin gene was amplified as a control. Gels representative of three independent experiments.
Prx1 Interacts with AR and Enhances Its Activity

Figure 2. Effect of hypoxia/reoxygenation on AR-ARE binding activity and AR transactivation. A, LNCaP or LN3 cells were exposed to 4 h of hypoxia, and EMSA was done at the indicated times during reoxygenation. The ARE-containing oligonucleotides (Santa Cruz Biotechnology) radiolabeled with γ-32P ATP (MP Biomedicals) were used as a probe. Location of the AR-ARE complex (arrow). To confirm the specificity of the AR-ARE binding, a molar excess of the unlabeled ARE was used as a competitor. B, LNCaP or LN3 were exposed to 4 h of hypoxia, and RT-PCR analysis was carried out using a PCR primer pair specific for the human PSA gene. The β-actin gene was amplified as a control. C, LNCaP or LN3 were exposed to 4 h of hypoxia, and Western blot analysis was carried out using an anti-AR antibody (Santa Cruz Biotechnology) as a probe. β-Actin antibody was used as a loading control. D, LNCaP, LN3, C4-2, or C4-2B cells were transfected with an ARE-luciferase construct (top). The pRL-TK vector containing the Renilla luciferase gene was cotransfected to normalize the transfection efficiency. At 24 h after transfection, cells were treated with 4 h hypoxia/6 h reoxygenation, and luciferase activity was determined. Normalized mean luciferase activity obtained from cells transfected with ARE-luciferase without hypoxia exposure was set at 1.0. Columns, mean ratio of the normalized luciferase activities; bars, SD. **, P < 0.01, compared with the respective non–hypoxia-treated control. *, P < 0.05, compared with hypoxia-treated LNCaP. Western blot analysis was done using an anti-AR antibody as a probe (bottom). β-Actin antibody was used as a loading control.

promoter region. LNCaP, LN3, C4-2, and C4-2B cells were transfected with an ARE-luciferase construct, and luciferase reporter activities were determined as a measure of AR transactivation by hypoxia/reoxygenation. Our results showed that increases of luciferase activity by hypoxia/reoxygenation were significantly higher in LN3, C4-2, and C4-2B when compared with that seen in LNCaP cells (Fig. 2D), although increases of luciferase activities were observed in all cell lines. The level of AR expression seemed to be comparable among the four cell lines; hypoxia/reoxygenation did not change the AR protein level.

**Prx1 increases AR transactivation by hypoxia/reoxygenation.** To test the role of Prx1 directly, Prx1 was introduced exogenously into LNCaP cells using an adenoviral vector carrying the Prxl gene (AdPrx1). As shown in Fig. 3A, the ARE-binding activity of AR was increased in cells transfected with Prx1 compared with that in the Ad.Con-infected control. Consistent with the enhanced ARE-binding activity, increased accumulation of PSA mRNA was observed in LNCaP cells infected with AdPrx1. The effect of Prx1 on AR transactivation was also examined by transfecting an ARE-luciferase construct. As shown in Fig. 3B, a significant increase of the reporter activity was observed in the Prx1-overexpressing cells, thus confirming that the enhanced AR activity by Prx1 was not limited to the PSA gene. We next examined the effect of reducing endogenous Prx1 expression on AR activity.

To do this, LN3 cells were infected with a retroviral vector containing scramble-shRNA (scramble) or Prx1-shRNA (shPrx1). As shown in Fig. 3C, the level of Prx1 was markedly reduced in shPrx1-infected cells, but not in scramble shRNA-infected cells. The Prx1 expression level of mock-infected LN3 cells is shown for comparison. When scramble- or shPrx1-infected cells were exposed to hypoxia/reoxygenation, the accumulation of PSA mRNA was significantly reduced in shPrx1-infected cells. This result is consistent with the findings from Prx1-overexpressing cells. To further validate the functional significance of Prx1 in increasing AR activity, Prx1 was introduced into PC3-AR cells which stably express the wild-type AR. As shown in Fig. 3D, Prx1 overexpression increased the ARE-luciferase activity in response to hypoxia/reoxygenation in PC3-AR cells. Essentially identical results were obtained when Prx1 was overexpressed in the DU145 cells. Because DU145 cells are AR-null, the ARE-luciferase construct was cotransfected with the pSG5shAR vector containing the wild-type AR.

**Prx1 increases ligand-stimulated AR transactivation.** To examine whether Prx1 regulates ligand-stimulated AR activity independent of hypoxia/reoxygenation, LNCaP cells were switched to a CS-FBS medium to deplete residual androgens present in the regular FBS medium. Cells were exposed to a CS-FBS medium supplemented with 10 nmol/L of a synthetic androgen, R1881. It
was shown that prostate tissue contains ~10 nmol/L of dihydrotestosterone (16). A dominant tissue androgen in the prostate tissue is dihydrotestosterone. Our results show that Prx1 increased the ARE-binding activity of a ligand-stimulated AR (Fig. 4A). To test whether Prx1 sensitizes AR to low levels of androgen, we exposed the cells to a lower concentration of R1881. We chose to use 2 nmol/L of R1881 because prostate cancer tissues that recurred during ADT have been shown to contain approximately this level of dihydrotestosterone (16). Our results revealed that Prx1 overexpression significantly increased the ARE reporter activity at a subphysiologic level of R1881 (Fig. 4B). The activity of the ARE-luciferase reporter at 10 nmol/L of R1881 is shown for comparison.

**Figure 3.** Prx1 increases the AR stimulatory effect of hypoxia/reoxygenation. A, LNCaP cells were infected with Ad.Con or Ad.Prx1 (top). At 24 h after infection, cells were exposed to 4 h of hypoxia. Cells were reoxygenated for 2 h, and EMSA was done as described above. Molar excess of the unlabeled ARE was used as a competitor. Prx1 Western blot was done to monitor the expression levels of Prx1 after adenovirus infection. β-Actin was used as a loading control. Ad.Con- or Ad.Prx1-infected LNCaP cells were treated with 4 h hypoxia/6 h reoxygenation (bottom). Total RNA was isolated, and RT-PCR analysis was carried out to amplify the PSA gene. β-Actin gene was amplified as a control. B, Ad.Con- or Ad.Prx1-infected LNCaP cells were transfected with the ARE-luciferase construct. At 24 h after transfection, cells were treated with 4 h hypoxia/6 h reoxygenation, and luciferase activities were measured. Normalized mean luciferase activity obtained from Ad.Con-infected cells without hypoxia exposure was set at 1.0. Columns, mean ratio of the normalized luciferase activities; bars, SD. **, P < 0.01, compared with non–hypoxia-treated control. ##, P < 0.01, between hypoxia-treated Ad.Con and Ad.Prx1 samples. C, LN3 cells were infected with retroviral vectors containing scramble shRNA (Scramble) and Prx1 shRNA (shPrx1), respectively (top). Western blot analysis was done to monitor the levels of Prx1 expression using an anti-Prx1 antibody as a probe. Mock-infected LN3 cells (Mock). β-Actin was used as a loading control. Scramble- or shPrx1-infected LN3 cells were treated with 4 h hypoxia/6 h reoxygenation (bottom). Total RNA was isolated, and RT-PCR analysis was carried out to evaluate the accumulation of PSA mRNA. β-Actin was amplified as a control. D, Ad.Con- or Ad.Prx1-infected PC3-AR cells were transfected with the ARE-luciferase construct (left). Cells were treated with 4 h hypoxia/6 h reoxygenation, and luciferase activity was determined. Columns, mean ratio of the normalized luciferase activities; bars, SD. **, P < 0.01, compared with non–hypoxia-treated control. ##, P < 0.01, between hypoxia-treated Ad.Con and Ad.Prx1 samples. Ad.Con- or Ad.Prx1-infected DU145 cells were cotransfected with wild-type AR containing pSG5hAR and the ARE-luciferase construct (right). Cells were treated with 4 h hypoxia/6 h reoxygenation, and luciferase activity was determined. Columns, mean ratio of the normalized luciferase activities; bars, SD. **, P < 0.01, compared with non–hypoxia-treated control. ##, P < 0.01, between hypoxia-treated Ad.Con and Ad.Prx1 samples.
To evaluate the effect of endogenous Prx1 knock-down on ligand-stimulated AR activity, scramble- or shPrx1-infected LN3 cells were exposed to 10 nmol/L of R1881. Our results clearly showed that the accumulation of PSA mRNA was much lower in shPrx1-infected cells compared with scramble-shRNA infected cells (Fig. 4C). Next, we examined the consequence of Prx1 knock-down on the growth-inhibitory effect of androgen deprivation. As shown in Fig. 4D, when cells were grown in an androgen-depleted medium containing CS-FBS, the growth rate of shPrx1-infected cells was significantly reduced compared with that of scramble shRNA-infected cells, showing that the growth-inhibitory effect of androgen deprivation is increased by Prx1 knock-down.

The AR stimulatory function of Prx1 is independent of its antioxidant activity. The ability of Prx1 to sensitize a ligand-stimulated AR indicated that the antioxidant activity of Prx1 may not be necessary for its AR stimulatory function in response to hypoxia/reoxygenation. When we examined the oxidation status of Prx1 in LNCaP and LN3 cells, we found that the active site Cys52 residue in Prx1 was oxidized and inactivated by hypoxia/reoxygenation. Figure 5A shows the two-dimensional Western blots when probed with a Prx1 antibody (top). Oxidation of the active site Cys52-SH to Cys52-SO2/3H was further confirmed by using a Prx SO2/3H antibody, which specifically recognizes the oxidized Cys52 (bottom). The oxidation status of Prx1 was maintained for at least 6 to 8 h after reoxygenation (data not shown). To test the effectiveness of an antioxidant activity-null Prx1 in modifying AR function, LNCaP cells were transfected with a plasmid containing a mutant Prx1 generated by replacing the catalytic site Cys52 with Ser52 (pCR C52S). As shown in Fig. 5B, regardless of whether the wild-type Prx1 (pCR Prx1WT) or the mutant Prx1 was overexpressed, the ARE-luciferase activity was consistently increased. The effect of hypoxia/reoxygenation on AR activity in mock-transfected cells with an empty plasmid (pCR3.1) is shown for comparison. The enhancement of AR activity in mutant Prx1-overexpressing cells was comparable to that seen in wild-type Prx1-overexpressing cells. This result clearly showed that the antioxidant activity of Prx1 was not essential for the AR-stimulatory function of Prx1.

Prx1 interacts with AR and is recruited to the PSA promoter. Because accumulating evidence suggests that Prx1 interacts with various proteins to modulate their activities (32, 34–36), we tested whether Prx1 interacts with AR. Immunoprecipitation and reciprocal immunoprecipitation experiments were carried out Western blot when probed with a Prx1 antibody (top). Oxidation of the active site Cys52-SH to Cys52-SO2/3H was further confirmed by using a Prx SO2/3H antibody, which specifically recognizes the oxidized Cys52 (bottom). The oxidation status of Prx1 was maintained for at least 6 to 8 h after reoxygenation (data not shown). To test the effectiveness of an antioxidant activity-null Prx1 in modifying AR function, LNCaP cells were transfected with a plasmid containing a mutant Prx1 generated by replacing the catalytic site Cys52 with Ser52 (pCR C52S). As shown in Fig. 5B, regardless of whether the wild-type Prx1 (pCR Prx1WT) or the mutant Prx1 was overexpressed, the ARE-luciferase activity was consistently increased. The effect of hypoxia/reoxygenation on AR activity in mock-transfected cells with an empty plasmid (pCR3.1) is shown for comparison. The enhancement of AR activity in mutant Prx1-overexpressing cells was comparable to that seen in wild-type Prx1-overexpressing cells. This result clearly showed that the antioxidant activity of Prx1 was not essential for the AR-stimulatory function of Prx1.
mean luciferase activity obtained from cells without hypoxia exposure was transfection, cells were treated with 4 h hypoxia/6 h reoxygenation. Normalized analysis of Prx1 was carried out to monitor the levels of Prx1 expression after...

Figure 5. The antioxidant activity of Prx1 is not essential for AR transactivation by hypoxia/reoxygenation. A, LN3 cells were treated with 4 h hypoxia/2 h reoxygenation. The oxidation status of Prx1 was analyzed by two-dimensional gel electrophoresis followed by Western blot using a Prx1 antibody (top). The Prx1 antibody recognizes both oxidized (Ox) and reduced (Re) forms of Prx1. Note the acidic shift of oxidized Prx1 after hypoxia/reoxygenation. Oxidation status of the active site Cys52 was evaluated using Prx-SO2/3H antibody (bottom). B, LNCaP cells were transfected with pCR vectors containing wild-type Prx1 (pCR Prx1WT) or mutant Prx1 lacking antioxidant activity (pCR C52S; top). Empty vector (pCR3.1) was transfected as a negative control. After 24 h of transfection, cells were treated with 4 h hypoxia/6 h reoxygenation. Normalized mean luciferase activity obtained from cells without hypoxia exposure was set at 1.0. Columns, mean ratio of the normalized luciferase activities; bars, SD. *p < 0.05, compared with the respective non–hypoxia-treated control. **p < 0.01 compared with the respective non–hypoxia-treated control. A, B, Our results reveal recruitment of Prx1 to the ARE region in response to hypoxia/reoxygenation. The results obtained from DNA that was PCR-amplified from chromatin extracts before immunoprecipitation (input) are shown for comparison. The specific recruitment of AR to the same ARE region was also confirmed in cells treated with hypoxia/reoxygenation. Neither Prx1 nor AR were recruited to the nonspecific region (intervening) which does not contain an ARE site. No signal was detected in DNA samples obtained from the corresponding IgG samples.

We also examined whether Prx1 interacts directly with AR. To this end, we carried out pull-down experiments using purified Prx1 and in vitro–translated AR. The cell-free experiments showed a direct interaction between Prx1 and AR (Fig. 6C). When pull-down assays were carried out using a mutant Prx1 protein (C52S) or in vitro oxidized Prx1 (Ox), similar interactions between AR and these Prx1 proteins lacking antioxidant activity were observed. This result further corroborates the idea that the antioxidant function of Prx1 is not essential for its binding to the ARE.

Discussion
Recent advances in early detection and treatment have led to a steady decline in prostate cancer mortality. Despite this improvement in survival outcome, prostate cancer is still the second leading cause of cancer death in men in the U.S. An estimated 218,890 new cases and 27,050 deaths will occur from prostate cancer this year alone (12). The androgen signaling through AR plays a central role in the development and progression of prostate cancer. In this study, we showed that Prx1 is intimately involved in increasing AR activity in the context of an unstable oxygenation milieu of a tumor. We show that Prx1 interacts physically with AR, and enhances AR transactivation in response to hypoxia/reoxygenation in prostate cancer cells. We also show that Prx1 is capable of sensitizing ligand-stimulated AR activity. These findings suggest that Prx1 may play an important role in mediating the abnormal activation of AR in a subset of prostate cancer cells which are capable of maintaining survival in an unstable oxygenation condition and a reduced level of androgen. In fact, the inherently abnormal and unstable oxygenation of a tumor might be further perturbed by androgen deprivation. An unstable oxygenation can be exacerbated by vasculature destruction after castration, or during neoadjuvant ADT in conjunction with radiation therapy, or in recurrent tumors during ADT. Prostate cancer that recurs during ADT represents a lethal phenotype and poses serious clinical problems. There is mounting evidence to suggest that AR signaling plays an important role in prostate cancer recurrence (16–18, 37). The importance of the current study is underlined by the possibility that disrupting the interaction between Prx1 and AR may serve as a new therapeutic target in a wide range of clinical conditions.

The cell survival–enhancing function of Prx1 has traditionally been attributed to its antioxidant activity. However, despite the initial biochemical characterization of Prx1 as an antioxidant enzyme, the physiologic significance of its peroxidase function is unclear because Prx1 is highly susceptible to over-oxidation/inactivation during reactive oxygen species detoxification (38, 39). When the catalytic Cys is over-oxidized, the peroxidase activity is reoxygenation. In order to examine whether Prx1 is recruited to the ARE of the PSA promoter in the natural chromatin milieu, ChIP assays were carried out using a Prx1 antibody. To control for possible nonspecific interactions and DNA contamination, samples precipitated with rabbit IgG were analyzed in parallel. As shown in Fig. 6B, our results reveal recruitment of Prx1 to the ARE region in response to hypoxia/reoxygenation. The results obtained from DNA that was PCR-amplified from chromatin extracts before immunoprecipitation (input) are shown for comparison. The specific recruitment of AR to the same ARE region was also confirmed in cells treated with hypoxia/reoxygenation. Neither Prx1 nor AR were recruited to the nonspecific region (intervening) which does not contain an ARE site. No signal was detected in DNA samples obtained from the corresponding IgG samples.
lost. Recent studies suggest that the catalytic site over-oxidation of Prx1 may be physiologically significant because it may facilitate the structural and functional switching of Prx1 from an antioxidant enzyme to a molecular chaperone (32, 40, 41). This hypothesis is consistent with the behavior of Prx1 in interacting with various growth-regulating proteins to modulate their activities. The association of Prx1 with c-Abl, c-Myc, macrophage-inhibiting factor, and glutathione S-transferase π has been previously shown (32, 34–36). The current study shows that the interaction of Prx1 with AR is independent of the antioxidant function of Prx1. As studies of Prx1 progress, the spectrum of molecules interacting with Prx1 is likely to expand. The functional consequence of these interactions would need to be tested and interpreted in a cell type– and tissue context–dependent manner.

Although Prx1 is thought to be expressed primarily in the cytosol, several lines of evidence have suggested a localization of Prx1 to the nucleus. Using immunoelectron microscopy, Prx1 has been shown to be expressed in the nucleus as well as in the cytoplasm in the rat kidney (42). The presence of Prx1 in the nucleolus of hepatic parenchymal cells has also been shown in the rat liver using an immunogold technique (43). Immunohistochemical analysis of oral cancer tissue revealed Prx1 staining in the nucleus (25). In addition, nuclear translocation of Prx1 has been reported in cultured cells (34). Collectively, these findings strongly suggest a potential role of Prx1 in the nucleus. Our study, showing the role of Prx1 in regulating AR activity, provides evidence supporting a physiologic function of Prx1 in the nucleus.

It is of note that Prx1 is elevated at the message level in the phenotypically aggressive LNCaP derivatives, LN3, C4-2, and C4-2B cells, which were established by different procedures. Although future studies will be necessary to elucidate the molecular basis of Figure 6. Prx1 interacts with AR, and is recruited to the PSA promoter with AR. A, LN3 cells were treated with 4 h hypoxia/2 h reoxygenation. Equal amounts of protein were used for immunoprecipitation (IP) with a Prx1 antibody (top). The immunoprecipitates were probed for the presence of AR by Western blot. Prx1 Western blot was done as a loading control. Rabbit IgG immunoprecipitation was done as a negative control. Aliquots of protein were subjected to reciprocal immunoprecipitation using an AR or rabbit IgG antibody (bottom). The immunoprecipitates were probed for the presence of Prx1 by Western blot. AR Western blot was done as a loading control.

B, LN3 cells treated with 4 h hypoxia/2 h reoxygenation were processed for ChIP assays using an anti-Prx1 antibody (top). Primer pairs used to amplify the ARE-containing region (ARE) and the non–ARE-containing region (Intervening) are described in Materials and Methods. Results obtained from DNA that was PCR-amplified from chromatin extracts before immunoprecipitation (Input). PCR results obtained after immunoprecipitation with rabbit IgG (Rabbit IgG). The gels shown are representative of three independent experiments. Aliquots of samples were processed for ChIP assays using an anti-AR antibody (bottom). The ARE region and intervening regions were amplified with the respective primer pairs. C, equal amounts of purified wild-type Prx1 (WT), in vitro oxidized Prx1 (Ox), or mutant Prx1C52S (C52S) were incubated with in vitro–translated, [35S]methionine-labeled AR. Physical interaction of Prx1 and AR was tested using a Prx1 antibody pull-down assay. The proteins were separated by SDS-PAGE, and the presence of AR was visualized by autoradiogram with X-ray film (35S-AR) and Western blot. The presence of Prx1 was detected by Western blot using an anti-Prx1 antibody.
Prx1 up-regulation in these LNCaP progeny cell lines, it is tempting to speculate on the possible existence of altered molecular mechanisms that are shared among the three cell lines compared with the parental LNCaP cells. Recently, we have cloned and characterized the human prx1 promoter, and identified NF-E2–related factor 2, or Nrf2, as one of the key transcription factors responsible for prx1 up-regulation (44). The activation of Nrf2 seems to be dependent on a number of regulatory mechanisms to increase Nrf2 protein level and/or promote its stabilization and accumulation in the nucleus. Phosphorylation of Nrf2 by various kinases, including mitogen-activated protein kinase, has been implicated as an important mechanism in regulating Nrf2 transactivation (45–47). Considering that mitogen-activated protein kinase signaling by epidermal growth factor receptor, HER-2/neu tyrosine kinase, and other growth factor signaling pathways are known to increase the activation of Nrf2 (48, 49), cross-talk mechanisms may exist between the Nrf2 and AR signaling pathways acting in concert to promote the malignant progression of prostate cancer.

The hypoxic and unstable oxygenation milieu of a tumor has been proposed to function as a microenvironmental pressure that selects for a subset of cancer cells with an increased ability to survive and proliferate. The current study revealed that elevated expression of Prx1 enhances AR transactivation by hypoxia/reoxygenation and sensitizes a ligand-stimulated AR, thereby providing a survival advantage to cancer cells subjected to an adverse microenvironment. Future research will be aimed at determining whether aberrant Prx1 up-regulation could be suppressed by genetic and/or pharmacologic approaches, and whether reducing Prx1 expression levels could suppress prostate cancer progression or reverse resistance to ADT in preclinical models. It may be worthwhile to investigate whether the Nrf2-Prx1 axis is dysregulated in human prostate cancer tissues, and whether it correlates with disease progression and poor clinical outcome.

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