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Maintenance of Androgen Receptor Inactivation by S-nitrosylation

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Abstract

Anti-androgens target ligand binding domain of AR and are used as frontline therapeutics to treat patients diagnosed with locally advanced and metastatic prostate cancer. Although initially beneficial as judged with actual tumor mass shrinkage, this therapy invariably fails and the cancer reappears as castration-resistant disease. Here we report that increased intracellular nitric oxide levels leads to growth inhibition of both androgen-dependent and castration-resistant prostate tumors through a mechanism that involves AR function inactivation by S-nitrosylation of a single C601 residue present in the DNA binding domain. AR S-nitrosylation does not impact its subcellular distribution but attenuates its ability to bind AR responsive elements in promoter region of target genes. Mechanistically, AR is transnitrosylated by its partner HSP90 protein. Ubiquitous small molecule nitric oxide donors promote the AR S-nitrosylation and inhibit growth of castration-resistant prostate tumors. These findings reveal a new mechanism of regulating AR function and suggest that sequential targeting of distinct domains of AR may extend therapeutic efficacy for patients with advanced prostate cancer.

Keywords

androgen receptor; castration-resistant prostate cancer; nitric oxide; S-nitrosylation

Introduction

Prostate cancer is the most diagnosed malignancy in males and the second leading cause of male cancer-related mortality in majority of Western countries (1). Patients with cancer confined to the prostate gland have several treatment options, including watchful waiting, surgery, and radiation. Pathologic growth of the prostate is controlled mainly by steroid androgens and locally advanced and metastatic diseases are treated with endocrine therapies aimed to decrease circulating androgen levels via castration, or block androgen receptor (AR) activation with ligand antagonist anti-androgens (2). A limitation of the hormonal therapies is that they offer only a temporary relief in that the disease progresses to castration-resistant prostate cancer (CRPC) characterized by aggressive growth and invasion of distal organs, predominantly the bone (3–5) and to date, there is no cure for CRPC.

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Author contribution

Y.Q., A.D. and Y.D. designed research. Y.Q., A.D. and H.P. performed all experiments.

Y.Q., A.D. and Y.D. analyzed data and wrote the paper.

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Factors involved in the transition of prostate cancer from androgen-dependent to CRPC are not well established and present an obstacle to improving disease outcome. Emerging evidence implicates AR not only in the initiation but also progression of the disease to CRPC. Available work has identified activating mutations in AR, and model transgenic mice harboring mutated AR develop prostatic intraepithelial neoplasia that progress to invasive cancer (6). CRPC lesions exhibit frequent mutations in AR, which allow it to be activated by steroids other than androgens, or even anti-androgens. Also, AR can be transactivated by factors other than androgens, including peptide growth factors (7) that exert their effects on target cells by activating cognate plasma membrane-anchored receptors. These laboratory and clinical results firmly implicate AR in prostate carcinogenesis.

AR is an androgen-dependent transcription factor (8) that manifests its effects on target cells by controlling expression of specific genes through association with protein co-regulators in the form of co-activators and co-repressors (9, 10). In general, multi-protein complex formation is controlled by post-translational changes of partner proteins, and AR has been reported to undergo several post-translational modifications including phosphorylation, ubiquitylation and acetylation that impact its subcellular localization, stability and overall transcriptional activity (11–13). Here, we tested the idea that AR undergoes the S-nitrosylation modification; i.e. covalent attachment of a nitric oxide (NO) group to a cysteine thiol, and determined the effect on AR signal transduction and consequent growth of prostate tumors.

Materials and Methods

Cell Culture and Manipulations

LNCaP, PC3, 22Rv1 and HEK293 cells were obtained from American Type Culture Collection. LNCaP C4-2 cells were obtained from MD Anderson Cancer Center. LNCaP and 22Rv1 cells were maintained in RPMI 1640 supplemented with 10% FBS, 1% HEPES, 1% D-glucose and 1% sodium pyruvate. LNCaP C4-2 cells were cultured in DMEM/Ham's F12 supplemented with 5% FBS, 5 µg/ml insulin, 13.65 pg/ml triiodothyronine, 5 µg/ml apo-transferrin, 0.244 µg/ml α -biotin, and 25 µg/ml adenine (all from Sigma). PC3 and HEK293 cells were maintained in Ham's F12 and DMEM, respectively, supplemented with 10% FBS. Androgen deprivation was accomplished by incubation in phenol red-free culture medium supplemented with 5% charcoal-stripped serum. Stable overexpression of eNOS in LNCaP cells was achieved by transfecting cells with pcDNA3-HA-eNOS. Transfected cells were propagated in the presence of G418 (1 mg/ml) for selection for 4 weeks. Positive clones were pooled and maintained with G418 (100 µg/ml). Transient forced overexpression of AR in PC3 cells was done by transfecting cells with pcDNA3-Flag-AR. Stable knockdown of eNOS was achieved by infection of shRNA constructs (Open Biosystems) in lentiviral pLKO vector plus equal concentration of vesicular stomatitis virus G and Δ 8.9 vector into packaging HEK293T cells. Lentivirus containing medium was harvested, mixed with polybrene and used to infect LNCaP cells. The infected polyclonal cells were selected with 2 µg/ml puromycin for two weeks and maintained in medium containing 1 µg/ml puromycin. Cell growth and doubling time analyses were done with WST-1 reagent.

Real-Time PCR

Cells were cultured in starvation medium for 24 hr followed by stimulation with R1881 (1 nM) in the presence, or absence, of GSNO for 48 hr. Total RNA was isolated using the High Pure RNA Isolation Kit (Roche) and mRNA was reverse-transcribed to cDNA using the iScript Reverse Transcription Supermix (Bio-Rad). PSA (in LNCaP) and FKBP51 (in PC3) gene levels were measured by iQ SYBR Green Supermix (Bio-Rad) and iQ5 thermal iCycler

detection system. GAPDH was used as an internal control. The primer sequences were as follows: PSA (sense) 5'-AGA ATC ACC CGA GCA GGT GC-3'; PSA (antisense) 5'-CAG AGG AGT TCT TGA CCC CA-3'; FKBP51 (sense):5'-CCA AAG CTG TTG AAT GCT GTG A-3'; FKBP51 (antisense):5'-CAA ACT CGT TCA TGA GC A GCT G-3'; GAPDH (sense) 5'-CAT GGG TGT GAA CCA TGA GAA-3'; GAPDH (antisense) 5'-GGT CAT GAG TCC TTC CACGAT-3'.

Western Blotting

Appropriately treated cells were lysed in RIPA buffer and fractionated by SDS-PAGE. Proteins were transferred to a nitrocellulose membrane and immunoblotted with the indicated primary antibody (used at a 1:1000 dilution except anti- β -Actin antibody that was used at a 1:5000 dilution) at 4°C overnight. HRP-conjugated anti-mouse (1:30,000 dilution) or anti-rabbit (1:20,000 dilution) secondary antibodies were used and proteins were visualized by SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific).

Biotin Switch Assay

Protein S-nitrosylation reactions were performed exactly as described before (14, 15). For protein transnitrosylation, purified recombinant HSP90 β and AR were dialyzed into HEN buffer. Equimolar amounts of each protein were exposed, or not, to CysNO (100 μ M) for 30 min at 37°C in the dark. The resultant S-nitrosylated proteins were desalted and then used as NO donors to their un-nitrosylated purified protein counterpart. Resultant samples were subjected to the NO-biotin switch assay to monitor transnitrosylation of each protein.

Chromatin Immunoprecipitation (ChIP)

Cells were subjected to ChIP analysis using Chromatin Immunoprecipitation kit (Millipore), according to the manufacturer instructions. Anti-AR (clone N20) or equal amount of isotype control antibody was added to purified DNA and incubated overnight at 4°C with rotation. Protein G agarose beads were added to pellet protein-DNA complexes followed by sequential washing once with ice-cold low salt, once with high salt, once with LiCl and twice with TE buffers. DNA-protein crosslink was reversed and chromatin DNA was purified and measured by PCR. Primers were as followed: PSA promoter (sense) 5'-TCT GCC TTT GTC CCC TAG AT-3'; PSA promoter (antisense) 5'-AAC CTT CAT TCC CCA GG ACT-3'; FKBP51 promoter (sense) 5'-GAG CCT CTT TCT CAG TTT TG-3'; FKBP51 promoter (antisense) 5'-CAA TCG GAG TGT AAC CAC ATC-3'. DNA products were used for quantitative PCR analyses by iQ SYBR Green Supermix (Bio-Rad) and iQ5 thermal iCycler detection system or separated on agarose gel, stained with ethidium bromide and visualized under UV light.

Electromobility Shift Assay (EMSA)

Sequence of the FKBP51 consensus binding site oligonucleotides was 5'-ACA TTC AGA ACA GGG TGT TCT GTG CTC-3'. Double-stranded oligonucleotides (100 ng) were end labeled with [γ -³²P]-ATP (specific activity, 3000 Ci/mmol) using polynucleotide kinase. For the EMSA reaction, 1 ng of labeled probe was mixed with 5 μ g of nuclear extracts in 20 μ l EMSA buffer containing 20 mM HEPES (pH 7.9), 50 mM KCl, 2.5 mM MgCl₂, 5 mM EDTA, 1 mM DTT, 1 μ g poly(dI-dC), and 8% (v/v) glycerol. Anti-AR-N20 (0.5 μ g) antibody (Santa Cruz) was used for detecting AR-ARE complex supershift.

Animal studies

Severe-combined immune deficient (SCID) mice were purchased from Harlan Laboratories. The Institutional Animal Care and Use Committee preapproved all animal procedures. LNCaP cells (10⁶ in 50 μ l phenol red-free RPMI 1640) were mixed with equal volume of

Matrigel and the mixture was inoculated subcutaneously into the right flank region of mice. Tumor dimensions were determined with caliper and tumor volume (v) was estimated using the equation $v = \pi/6 \times L \times S^2$, where L is the long and S the short lengths of the tumor mass. Tumor growth rate was measured weekly. When tumor size reached 100–200 mm³, animals were randomly divided into three treatment groups: 1, Control; 2, LHRH; and 3, GSNO. Control mice were injected with vehicle PBS. Animals were treated daily with LHRH (100 µg/kg body weight) subcutaneously for 14 days, or with GSNO (10 mg/kg body weight) intraperitoneally for 7 days. At the end point, mice were sacrificed and tumor tissues were dissected and analyzed for PSA levels by Western blotting. Experiments were repeated three times and each data point represents values from 5 to 10 animals.

Results

Elevated NO expression attenuates prostate tumor growth

Deregulated nitric oxide (NO) levels have been implicated in human disease, most notably those of the cardiovascular and immune systems. Less, however, is appreciated regarding potential role of ubiquitous NO in tumorigenesis (16). NO has been reported to exert pro- and anti-growth effects in a manner that is dependent upon cellular localization and expression level of NO synthases (NOSs) and exposure duration to NO (17). NOSs expression is increased in prostate cancer (18), and increased expression of eNOS in prostate cancer tissues highly correlates with decreased patient survival (19). Impact of elevated expression of NOSs or NO in CRPC remains unclear. We determined effect of model NO-donor diethylenetriamine NONOate (DETA-NO) on the growth rate of prostate cancer cells. Growth of androgen-dependent LNCaP (Fig. 1A) as well as castration-resistant 22Rv1 (Fig. 1B) cells that endogenously express AR was significantly attenuated in response to treatment with DETA-NO. Similar treatment of PC3 cells that do not express AR with DETA-NO for up to 24 hr did not evidence a statistically significant effect on the cell proliferation (Fig. 1C), consistent with previous results (20). Treatment of PC3 cells with DETA-NO for 48 hr, however, exhibited a significant decrease in the cell proliferation. The treatment with DETA-NO showed no effect on the LNCaP cell apoptosis, as assessed with PARP cleavage (Supplementary Fig. S1). These results support the conclusion that, at concentrations used here, DETA-NO targets the proliferation machinery of prostate cancer cells.

To test that intracellular NO impacts the cell growth, we generated a LNCaP cell line that stably overexpressed endothelial NOS (LNCaP-eNOS; Supplementary Fig. S2A). LNCaP-eNOS cells evidenced a prolonged doubling time of 95.7 hr, in comparison to the faster doubling time of 55.2 hr for the control empty-vector (LNCaP-EV) cells (Fig. 1D). Conversely, the stable knockdown of endogenous eNOS expression with shRNA (LNCaP-sheNOS; Supplementary Fig. S2A) accelerated the cell doubling time to 22.7 hr (Fig. 1D). Moreover, androgen-induced proliferation of LNCaP-eNOS cells was significantly reduced compared to LNCaP-EV cells (Fig. 1E). Together, these results establish exogenously supplied and endogenously produced NO as proliferation suppressor of AR-expressing prostate cancer cells.

Effects of modulating NO levels on LNCaP tumor growth in animals were measured next. LNCaP-EV, LNCaP-eNOS and LNCaP-sheNOS cells were inoculated subcutaneously in the flanks of severe-combined immune deficient (SCID) mice. Results show that while knockdown of endogenous eNOS accelerated the tumor growth rate, forced overexpression of eNOS inhibited it, in comparison to control LNCaP-EV tumors (Fig. 2A). Analysis of tumor tissue showed that expression of AR-controlled prostate-specific antigen (PSA) was reduced in LNCaP-eNOS, but increased in LNCaP-sheNOS samples (Supplementary Fig. S2B), implying an effect of endogenously synthesized NO on AR function.

GSNO inhibits castration-resistant prostate tumor growth

We used LNCaP and LNCaP-derived C4-2 cells as models of androgen-dependent and ensuing castration-resistant, respectively, prostate cancer to elucidate possible distinguishing effects of NO on the tumor growth. Tumor-bearing SCID mice were randomly divided into three groups that were treated with vehicle (Veh), luteinizing hormone-releasing hormone (LHRH), the frontline hormone deprivation therapy for advanced prostate cancer, or S-nitrosoglutathione (GSNO), the most abundant form of physiological NO-donor in mammalian cells (21). Consistent with clinical outcomes, treatment with LHRH suppressed growth of parental LNCaP tumors but showed no effect on the growth rate of LNCaP C4-2 tumors (Fig. 2B). Remarkably, the treatment with GSNO inhibited growth of both LNCaP and LNCaP C4-2 tumors (Fig. 2B), but showed no effects on the overall health of animals as measured with intact body weight and physical activity. Furthermore, pathology review of heart, lung, spleen and liver tissue sections indicated lack of GSNO toxicity, consistent with previous results (22). Co-treatment of animals harboring LNCaP C4-2 tumors with LHRH and GSNO did not further decrease the tumor growth rate in comparison to treatment with individual LHRH or GSNO, suggesting they act upon common effector(s). These results establish that endogenously produced as well as exogenously added NO inhibits growth of both androgen-dependent and castration-resistant prostate tumors.

NO attenuates AR activity

LNCaP cell growth is dependent upon AR signaling, and we tested the idea that NO impacts AR activity. Androgen-induced activation of endogenous AR, as measured with PSA expression, was significantly impaired upon treatment with escalating doses of GSNO (Fig. 3A). Confirmatory results showed that androgen-induced AR activation, as measured with AR-response element (ARE)-controlled luciferase reporter, was also reduced in LNCaP cells treated with GSNO or DETA-NO (Supplementary Fig. S3A), and mirroring results were obtained in LNCaP-eNOS cells (Supplementary Fig. S3B). Concordantly, the knockdown of endogenous eNOS expression increased PSA levels, but had no effect on total AR protein expression (Fig. 3B). These results suggest that NO represses the inducible activation of AR in prostate cancer cells.

AR-null prostate cancer PC3 cells were used to illustrate whether AR is directly regulated by NO. AR activity was measured by detection of endogenous AR-regulated FKBP51 gene. FKBP51 mRNA was detected only in cells expressing AR and treated with R1881 (Fig. 3C), justifying use of this assay to measure AR activation. Significantly, the androgen-induced FKBP51 mRNA levels were dose-dependently decreased upon treatment with GSNO in PC3-AR cells (Fig. 3C), suggesting the effect of NO is directly through modulating AR signaling. Similarly, PSA gene levels were attenuated in LNCaP cells treated with GSNO (Fig. 3D).

Activated AR translocates to the nucleus and binds to ARE in the regulatory regions of AR-target genes. Treatment with GSNO (Supplementary Fig. S4A, S4B) or forced overexpression of eNOS (Supplementary Fig. S4C) showed little effect on the androgen-induced AR nuclear translocation in LNCaP cells, consistent with previous results (20). However, the forced overexpression of eNOS (Fig. 3E), like the treatment with GSNO (Fig. 3F and Supplementary S5A, S5B), significantly reduced the androgen-mediated AR binding to ARE in the PSA promoter, as determined by chromatin immunoprecipitation (ChIP). Also, GSNO dose-dependently inhibited the basal AR binding to PSA ARE in LNCaP cells (Fig. 3G), confirming that NO inhibits AR activity through interference of its binding to promoter elements in target genes.

AR modification by S-nitrosylation

We hypothesized that NO regulates AR activity through S-nitrosylation that involves the covalent attachment of NO group to a free cysteine thiol, and protein S-nitrosylation has been suggested to mediate NO effects on target cells (21, 23, 24). Biotin switch assay (25) was performed using prostate cancer cells treated with escalating doses of NO-donor S-nitrosocysteine (CysNO), a cell-permeable and byproduct of endogenous GSNO (26). Results establish the post-translational S-nitrosylation of AR in both LNCaP (Fig. 4A) and 22Rv1 (Fig. 4B) cells. In addition to full-length AR, the 22Rv1 cells express splice-variants that encode truncated proteins lacking ligand-binding domain (AR-V) and our results show that both forms of AR were modified by S-nitrosylation (Fig. 4B). Remarkably, the S-nitrosylation of AR inversely correlates with its transcriptional activity and is regulated by androgen: treatment with R1881 promoted a reciprocal decrease (2-fold) of AR S-nitrosylation content and increase (3-fold) of PSA levels (Supplement Fig. S6A). Forced overexpression of eNOS resulted in more basal level SNO-protein expression (Supplement Fig. S6B) in comparison to control LNCaP-EV cells, which was further supported by the measurement of SNO-AR in LNCaP tumors (Supplement Fig. S6C).

To identify the cysteine residue(s) corresponding to S-nitrosylation site(s) of AR, S-nitrosylation site identification (27) was performed. Purification efficiency of AR protein was confirmed by Coomassie blue staining (Supplement Fig. S7A) and Western blotting (Supplement Fig. S7B), and purified AR protein S-nitrosylation was demonstrated using the biotin switch assay (Supplement Fig. S7C). LC-MS/MS analysis (22, 28) revealed three cysteine residues (C518, C601 and C784) as putative S-nitrosylation sites in AR (Supplement Fig. S7D). Replacement of the individual residues with serine followed by subjugation of the mutated AR protein to biotin switch assay indicated C601 as the primary candidate S-nitrosylation site in AR (Fig. 4C). Notably, both full-length AR and AR-V proteins contain the C601 residue and are modified by S-nitrosylation (Fig. 4B).

We hypothesized that the mutation of S-nitrosylated cysteine residue in AR increases AR activity. However, androgen-induced FKBP51 gene expression confirmed that C601S mutation abolished AR activation (Fig. 4D). C601 resides in the second zinc finger of AR DNA binding domain (DBD), and mutated C601S AR lacked transcriptional activity (Fig. 4D), most likely as a result of its inability to coordinate Zn^{+2} and bind promoter elements in target genes. In addition to cysteine thiol, Zn^{+2} may coordinate two cysteine thiols and two histidine imidazole nitrogens to form a transcriptionally-competent zinc finger domain (29) and we replaced C595 and C601 with histidines (C595/601H) in an attempt to maintain AR binding ability to ARE. ChIP assays were performed using AR-null PC3 cells ectopically expressing wild-type or C595/601H mutated forms of AR (Fig. 5A and Supplement Fig. S8A). Treatment with GSNO significantly reduced the wild-type AR binding to ARE (in FKBP51 promoter), and the binding of mutated C595/601H AR form was absent. We used electromobility shift assay (EMSA) to confirm the effect of GSNO treatment on AR binding to ARE using nuclear extracts from the PC3 cells expressing wild-type or C595/601H forms of AR. Mirroring the findings of the ChIP assay (Fig. 5A), results show that treatment with GSNO significantly decreased the binding of AR to ARE and, again, the binding of C595/601H AR was absent (Fig. 5B and Supplement Fig. S8B). In agreement with these results, treatment with GSNO impaired ability of estrogen receptor (ER) to bind EREs and consequent gene transcription (30) and the S-nitrosylation of p50 subunit of NF- κ B inhibited its binding to target DNA (31). Together, these findings evidence the AR S-nitrosylation which, in turn, impairs its ability to bind ARE that is required for target gene expression.

HSP90 transnitrosylates AR

A remaining question is just how NO promotes the AR S-nitrosylation. One possibility is that NOS directly binds to AR leading to increased local NO levels and AR S-nitrosylation. Co-immunoprecipitation experiments of endogenous AR and eNOS (that is expressed in LNCaP cells at higher levels than iNOS or nNOS) proteins disputed this idea. Another possible mechanism is that eNOS regulates AR S-nitrosylation indirectly; through the binding of common partner HSP90 protein that has been shown to activate eNOS (32) and has been shown to be S-nitrosylated in endothelial cells (33). In epithelial prostate cells, HSP90 associates with AR and this complex is maintained in the cytoplasm. Co-immunoprecipitation results confirmed that HSP90 β (the constitutively-expressed form of HSP90) forms a complex with both AR and eNOS (Fig. 6A). Recent reports have suggested a novel mechanism of NO group transfer between partner proteins as a general signal transduction mechanism, termed transnitrosylation (34–36). We hypothesized that AR becomes S-nitrosylated as a result of NO transfer from S-nitrosylated HSP90. To test this idea, we incubated purified AR or HSP90 β proteins with CysNO to form SNO-AR or SNO-HSP90 β , respectively. To measure potential transnitrosylation, each of these SNO-proteins was mixed with their unlabeled counterpart; i.e., SNO-HSP90 β with AR (Fig. 6B), or SNO-AR with HSP90 β (Fig. 6C). We performed the biotin switch assay and found that AR was transnitrosylated by SNO-HSP90 β (Fig. 6B). Distinctly, SNO-AR was unable to transnitrosylate HSP90 β (Fig. 6C), implying the transfer of NO is unidirectional. The transfer of NO from SNO-HSP90 β to AR was observed using purified proteins, suggesting the reaction is thermodynamically favorable.

We examined feasibility of AR transnitrosylation by SNO-HSP90 β under in situ conditions. PC3 cells ectopically expressing Flag-AR alone (Fig. 6D) or together with Myc-HSP90 β (Fig. 6E) were treated, or not, with CysNO, followed by biotin switch assay. Relative levels of SNO-AR and SNO-HSP90 β were used to calculate the relative redox potential (ΔE°) and the associated change in Gibbs free energy (ΔG°), as described (35). The results show a ΔE° of 22.38 ± 3.11 mV and a ΔG° of -2.16 ± 0.3 kJ/mol, confirming a net energy gain for the transfer of NO from SNO-HSP90 β to AR and supporting the conclusion that the transnitrosylation reaction occurs spontaneously in cells.

Discussion

AR plays a central role in the initiation and progression of prostate cancer and serves as a primary target for developing drugs to treat patients with advanced disease. AR consists of three demarcated domains: a variable N-terminus domain that is involved in receptor transactivation and cofactor recruitment, a centrally located DBD that contains two zinc finger motifs, and a C-terminus that encompasses LBD. Although initially effective in the treatment of locally advanced and metastatic diseases, anti-androgens that target LBD have failed to achieve sustained inhibition of AR signaling, or to cure prostate cancer (4). Recently developed next generation anti-androgen MDV3100 (37) evidenced added survival benefit to patients with CRPC, but the clinical improvements remain short-lived (4, 38), providing rationale to find other targets in AR to better treat patients with advanced disease.

Metastatic and CRPC lesions express AR deletion mutant forms (collectively referred to as AR-V) that lack the ligand-binding domain (39, 40), and recent work shows AR-V to be transcriptionally active (41, 42). Emerging clinical outcomes suggest that expression of AR-V is increased in CRPC and metastatic lesions, and may facilitate progression of prostate cancer in patients undergoing hormonal therapy (40, 43). In animals, interference of AR-V activation attenuates growth of model CRPC tumors (41). Hence, in addition to full-length AR, AR-V may serve as drug targets to inactivate the receptor. Our results show that S-nitrosylation of C601, located in AR DBD and shared by full-length AR and AR-V, inhibits

AR function and growth of CRPC tumors. The sequential targeting of distinct domains in AR may provide a more sustained benefit to patients diagnosed with metastatic and CRPC diseases.

Our data show that C601 is a primary target of NO, and that S-nitrosylation at this site inhibits AR activity. C601 is located in the second zinc finger motif of the AR DBD and is required for AR binding to AREs. The S-nitrosylation of C601 effectively inhibits AR transcriptional activity, likely as a result of NO destroying zinc-sulfur clusters (44). In the absence of androgen stimulation, AR is expressed in the cytoplasm in complex with HSP90. Available evidence shows that HSP90 binds eNOS and undergoes the S-nitrosylation modification (32), and our results show that SNO-HSP90 transnitrosylates AR. Hence, HSP90 may contribute to maintenance of AR inactivation by a two-step mechanism; sequestration in the cytoplasm and S-nitrosylation. Androgen-induced activation of AR, therefore, may involve AR signal disinhibition as a result of AR dissociation from HSP90 in the cytoplasm, translocation to the nucleus and denitrosylation. Indeed, our preliminary results support the idea that stimulation with androgen provokes a decrease in nuclear AR S-nitrosylation content and a concomitant increase in PSA expression.

The preclinical studies show that systemic treatment of animals harboring xenograft prostate tumors with exogenously supplied NO (i.e. GSNO) inhibits growth of both androgen-dependent and castration-resistant tumors. Remarkably, results of a non-randomized Phase II trial show that treatment of patients who failed primary therapy for clinically localized prostate cancer with low-dose and sustained delivery of NO-donor nitroglycerin significantly inhibited disease progression as measured with a decrease in PSA doubling time (45), implying NO abundance inhibits prostate cancer in men. Results of perturbing endogenous levels of NO on the tumor growth rate support the conclusion that NO acts directly on the cancer cells; forced overexpression of eNOS in the cancer cells inhibits tumor growth while knockdown of endogenous eNOS expression accelerates it. However, it remains feasible that the cell-permeable NO impacts other constituents in the tumor mass, such as vascular cells and supporting stroma.

In summary, our results show that increased levels of intracellular NO promotes AR inactivation by S-nitrosylation of a single C601 residue present in the AR DBD and inhibits growth of AR-positive androgen-dependent and castration-resistant prostate tumors. In the absence of androgen stimulation, AR is maintained in a transcriptionally inactive state as a result of being sequestered in the cytoplasm and transnitrosylated by HSP90. Sequential targeting of individual AR domains with pharmacologically distinct agents may provide a better treatment outcome to more effectively abrogate aberrant and sustained AR signaling in advanced prostate cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Effect of NO and eNOS on prostate cancer cell growth

Effect of DETA-NO on (A) LNCaP (A), 22Rv1 (B) and PC3 (C) cell proliferation. Equal number of cells were incubated for 24 hr in appropriate culture medium containing 10% FBS in the presence or absence of DETA-NO. For each time point, viable cells were determined after mixing with WST-1 and data are expressed as fold change in absorbance at A_{450} for which the value of untreated cells that were incubated for 8 hr was defined as 1. *, $P < 0.05$ compared with corresponding not-treated (NT) control cells, and #, $P < 0.05$ compared to non-treated control cells at 8 hr. (D) Doubling times of LNCaP cells stably overexpressing eNOS or sheNOS were calculated by counting viable cells at 24 hr interval. *, $P < 0.05$ compared to control LNCaP-EV cells. (E) Forced overexpression of eNOS attenuates the androgen-induced LNCaP cell proliferation. LNCaP-eNOS and LNCaP-EV cells were treated with DHT (10 nM) or R1881 (1 nM) for 96 hr and viable cell numbers

were counted. *, $P < 0.05$ compared to control LNCaP-EV cells, and #, $P < 0.05$ compared with corresponding LNCaP-eNOS cells. In all panels, the Tukey post-hoc test following a one-way ANOVA was performed to compare groups. All results represent three independent experiments.

Figure 2. Impact of eNOS expression and treatment with GSNO on prostate tumor growth

(A) Effect of modulating eNOS levels on LNCaP tumor growth. LNCaP-EV, LNCaP-eNOS and LNCaP-sheNOS cells were inoculated subcutaneously into SCID mice and tumor volumes were measured weekly using a caliper. Each point represents the average tumor size from 5–10 animals. *, $P < 0.05$ compared to corresponding control (EV) tumor volume. (B) GSNO inhibits LNCaP C4-2 tumor growth. LNCaP and LNCaP C4-2 cells were inoculated subcutaneously into SCID mice. When tumors formed (about 100 to 200 mm³), randomly-grouped animals were treated daily with LHRH (100 µg/kg body weight) subcutaneously for 14 days, GSNO (10 mg/kg body weight) intraperitoneally for 7 days, or vehicle PBS. Tumor volume changes were measured and compared to values before treatment. A Bonferroni post-hoc test following a two-way ANOVA was performed to compare the groups. *, $P < 0.05$ compared to corresponding control (Veh) tumors. All results represent three independent experiments.

Figure 3. NO regulates AR activity

(A) GSNO attenuates PSA expression. LNCaP cells were stimulated, or not, with R1881 (1 nM) in the presence or absence of GSNO for 48 hr. Cell lysates were fractionated on SDS-PAGE and proteins were immunoblotted with the indicated antibodies. (B) Knockdown of eNOS expression increases PSA levels. Lysates from GFP (–) or sheNOS (+) LNCaP cells were fractionated on SDS-PAGE and proteins were immunoblotted with the indicated antibodies. (C) GSNO inhibits R1881-induced FKBP51 gene expression. PC3 cells were transfected with pcDNA3 empty-vector (EV) or pcDNA3-Flag-AR (AR) cDNAs and treated, or not (NT), with R1881 (100 pM) in the presence or absence (CN) of GSNO for 48 hr. FKBP51 mRNA levels were determined by real-time PCR. A Tukey post-hoc test

following a one-way ANOVA was performed to compare groups. *, $P < 0.05$ compared to R1881-stimulated PC3-AR cells without GSNO treatment. (D) GSNO inhibits PSA mRNA expression. LNCaP cells were treated with GSNO for 48 hr in the presence or absence of R1881 (1 nM). PSA mRNA levels were determined by quantitative PCR, as described in C. *, $P < 0.05$ compared with corresponding non-treated cells. (E) Forced overexpression of eNOS attenuates AR binding to ARE (of PSA promoter). Control LNCaP-EV (–) and LNCaP-eNOS (+) cells were starved for 24 hr and then stimulated with R1881 (1 nM) overnight. ChIP assays using anti-AR or control IgG antibodies were performed. Amplified DNA was subjected to quantitative analysis by PCR. (F) GSNO suppresses androgen-induced AR binding to PSA ARE. LNCaP cells were treated with GSNO (100 μ M) for 24 hr, followed by overnight stimulation with R1881 (1 nM). Cell lysates were subjected to ChIP analysis using anti-AR (upper panel), or isotype IgG (lower panel) antibodies. Precipitated DNA was subjected to quantitative PCR amplification. Results are presented as percentages of the input samples. (G) GSNO suppresses AR binding to PSA ARE. LNCaP cells were treated with increasing doses of GSNO (0–500 μ M) for 48 hr, followed by ChIP, as described in E. For panels E, F and G, *, $P < 0.05$.

Figure 4. AR is S-nitrosylated on C601

(A) S-nitrosylation of AR. LNCaP cells were lysed and mixed with escalating doses of CysNO and AR S-nitrosylation was determined with biotin switch assay (top). Equal amounts of cell lysate were immunoblotted with anti-AR antibody to show the equal protein loading (bottom). (B) S-nitrosylation of endogenous AR. LNCaP and 22Rv1 cells were lysed and mixed with CysNO (100 μ M) and AR S-nitrosylation was determined with biotin switch assay (top). Equal amounts of cell lysate were immunoblotted with anti-AR antibody to show the equal protein loading (bottom). *, full length AR and **, AR splice variant that lacks ligand-binding domain. (C) S-nitrosylation of AR is localized to the C601 residue. AR-null HEK293 cells were transiently transfected with cDNAs encoding Flag-AR (wild-type [WT], C518S, C601S, or C784S) and subjected to S-nitrosylation analysis as described in A. (D) AR C601S is devoid of transcriptional activity. PC3 cells were transfected with pcDNA3-Flag-AR (wild-type [WT], C518S, C601S, C784S), starved for 24 hr and then stimulated, or not, with R1881 (1 nM) for 16 hr. FKBP51 mRNA levels were measured by real-time PCR and normalized by GAPDH and AR levels. A Tukey post-hoc test following a one-way ANOVA was performed to compare groups. *, $P < 0.05$ in comparison to corresponding controls not treated with R1881.

Figure 5. NO attenuates binding of AR to ARE

(A) AR C595/601H is impaired in binding to ARE. PC3 cells ectopically expressing Flag-AR (wild-type [WT], C595/601H) were stimulated, or not, with R1881 (1 nM) in the presence or absence of GSNO (100 μ M) for 48 hr. ChIP using anti-AR, or control IgG was performed. Amplified DNA was fractionated on agarose gel and visualized under UV light after staining with ethidium bromide. (B) PC3 cells were transfected as above and stimulated, or not, with R1881 (1 nM) in the presence or absence of GSNO (100 μ M) for 48 hr. 32 P-labeled oligonucleotides containing FKBP51 promoter sequence were incubated with nuclear extract proteins (5 μ g), fractionated by EMSA on 5% polyacrylamide gel, and visualized by autoradiography. N20, binding mixture contained 0.5 μ g of anti-AR antibody. CR denotes binding samples containing 100-fold excess unlabeled oligonucleotides. All results represent three independent experiments.

Figure 6. AR is transnitrosylated by HSP90

(A) HSP90β forms a complex with AR and eNOS. LNCaP cells were lysed and subjected to immunoprecipitation with anti-HSP90β or isotype control (IgG) antibodies. Protein complexes were immunoblotted with the indicated antibodies. (B, C) Purified HSP90β (B) or AR (C) protein was exposed to CysNO (100 μM) and resultant S-nitrosylated protein was desalted and then mixed with un-nitrosylated purified protein counterpart. Biotin switch assay was performed to detect the protein transnitrosylation. Input amounts of AR and HSP90β were verified in each reaction. (D, E) Transnitrosylation of AR in situ. PC3 cells were transfected with pcDNA3-Flag-AR alone (D) or together with pCMV-Myc-HSP90β (E). Cells were exposed to CysNO (200 μM), and cell lysates were subjected to the biotin switch assay.