NQO1 Suppresses NF-κB–p300 Interaction to Regulate Inflammatory Mediators Associated with Prostate Tumorigenesis

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Abstract

NADPH reductase NAD(P)H:quinone oxidoreductase 1 (NQO1) is needed to maintain a cellular pool of antioxidants, and this enzyme may contribute to tumorigenesis on the basis of studies in NQO1-deficient mice. In this work, we sought deeper insights into how NQO1 contributes to prostate carcinogenesis, a setting in which oxidative stress and inflammation are established contributors to disease development and progression. In the TRAMP mouse model of prostate cancer, NQO1 was highly expressed in tumor cells. NQO1 silencing in prostate cancer cells increased levels of nuclear IKKα and NF-κB while decreasing the levels of p53, leading to interactions between NF-κB and p300 that reinforce survival signaling. Gene expression analysis revealed upregulation of a set of immune-associated transcripts associated with inflammation and tumorigenesis in cells in which NQO1 was attenuated, with IL8 confirmed functionally in cell culture as one key NQO1-supported cytokine. Notably, NQO1-silenced prostate cancer cells were more resistant to androgen deprivation. Furthermore, NQO1 inhibition increased migration, including under conditions of androgen deprivation. These results reveal a molecular link between NQO1 expression and proinflammatory cytokine signaling in prostate cancer. Furthermore, our results suggest that altering redox homeostasis through NQO1 inhibition might promote androgen-independent cell survival via opposing effects on NF-κB and p53 function.

Introduction

Oxidative stress and the associated pathologic conditions are considered as risk factors in the initiation, development, and progression of many cancers, including prostate cancer (1–3). NAD(P)H:quinone oxidoreductase 1 (NQO1) is a homodimeric flavoprotein that catalyzes the reduction of quinones to hydroquinones and is a key component of cellular antioxidant defense system. NQO1 regulates stability of p53 protein and cell fate decisions in response to endogenous and exogenous stress (4, 5). Mice with targeted disruption of NQO1 were highly susceptible to chemical-induced mouse skin carcinogenesis, suggesting its potential role in carcinogenesis (6, 7). On the other hand, deletion of NQO1 gene in keratinocytes leads to cell death by inhibiting NF-κB activation (8) and NQO1-induced melanoma progression via upregulating NF-κB–p50 (9). NQO1-mediated reduction of quinones to hydroquinones is essential for antitumor activity of chemotherapeutic quinone drugs signifying the importance of NQO1 in cancer therapy (10). A single-nucleotide polymorphism (SNP) in NQO1 cDNA results in a proline to serine substitution (C609T) and production of an unstable protein that undergoes rapid degradation (10). This SNP has been associated with increased risk for various cancers (11, 12). However, association of this SNP with prostate cancer risk is unclear although recent meta-analysis data show no significant association in Caucasians (13). The molecular pathogenesis and consequences of NQO1 changes in prostate cancer have not been explored. Here, we determined the basic function of NQO1 using prostate cancer as a model system.

Prostate cancer is the most commonly diagnosed cancer among men in the United States (14). Cancer progresses from prostatic intraepithelial neoplasia (PIN) to hormone-responsive locally invasive adenocarcinoma, to hormone-independent metastatic carcinoma. Perturbation in redox balance in PIN and prostate cancer has been attributed to elevated oxidative stress (15, 16). However, the role of redox imbalance in the development of androgen-independent prostate cancer is anecdotal and based on increased levels of cytokines and.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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chemokines (17, 18). The C–C chemokine ligand 2 (CCL2), CXCL12, and interleukin-8 (IL8) are major cytokines associated with prostate cancer (19–21).

NF-κB upregulates cytokines and chemokines, providing a critical mechanistic link between inflammation and cancer (22). Several reports establish an inverse correlation between NQO1 and NF-κB activation (23). In addition to IKK-mediated regulation of IKK–NF-κB interaction, its cross-talk with other cell-signaling networks determine cell fate (24). Cross-talk between NF-κB and p53 can play a pivotal role in determining the cellular response to stress (24, 25). IKK in the nucleus plays a key role in NF-κB activation in response to stress or inflammation (26, 27). Interaction between IKK and transcriptional coactivator proteins CREB binding protein (CBP) or p300 has been reported as a mechanism of NF-κB activation to promote cell proliferation and tumor growth (28). Moreover, higher levels of nuclear IKK in human and mouse prostate carcinomas are correlated with prostate cancer progression and metastatic phenotype (29).

We show that NQO1-mediated p53 inhibition activates NF-κB–p300 association, which appears to be associated with nuclear IKK. We report for the first time that NQO1 knockdown LNCaP cells are more resistant to androgen deprivation–induced growth arrest, in which an increased fraction of NQO1 knockdown cells survive under reduced hormone conditions. Together, our results provide a mechanism that implicates NQO1 as an important player in the development of hormone-resistant prostate cancer.

Materials and Methods

Cell culture

RWPE-1, LNCaP, PC3, and DU145 purchased from ATCC were cultured according to the vendor recommendations. C4-2B cells obtained from Dr. T Dorai (New York Medical College, Valhalla, NY) were maintained in T-medium containing 5% FBS and 100 IU/ml penicillin and 100 μg/ml streptomycin. BPH-1 cells obtained from Dr. M.S. Lucia (University of Colorado Denver, Denver, CO) were maintained in RPMI-1640 supplemented with 10% FBS, and penicillin/streptomycin.

Real-time quantitative PCR

RNA was reverse transcribed using the SuperScript VILO cDNA Synthesis Kit according to the manufacturer’s instructions (Invitrogen), and gene expression was analyzed by qPCR on ABI 7300 (Applied Biosystems). β-Actin was used as a reference.

Immunoblotting

Whole cell, cytoplasmic, or nuclear extracts were resolved by SDS-PAGE and immunoblotted. β-Actin and Lamin B1 were used as loading controls. Immunoprecipitation kit from eBioscience was used to determine protein interactions.

NQO1 knockdown

Cells were transduced with lentiviral particles against control [nontargeted control cells (NTC)] or NQO1 (shNQ; Dharmacon) and selected with 1 μg/ml puromycin. Knockdown was verified by qPCR and immunoblot analysis.

Functional assays

Trypan blue, crystal violet staining, and MTT assays were used to determine the growth and survival. Wound scratch was used to determine migration. Peroxy Orange 1 (PO1) was used for H2O2 generation (30).

Microarray analysis

The Agilent 8×60K Whole Human Genome platform was used to compare NQO1 knockdown and nontargeted control LNCaP cells. Data have been deposited in the Gene Expression Omnibus (GEO) database (accession no. GSE58336).

Luciferase assay

Luciferase activity was determined using the Dual-Luciferase Reporter Assay (Promega).

Cytokine array

Antibody-based array was used to determine cytokines in culture supernatant and lysates according to the manufacturer’s protocol (R&D Systems).

Immunofluorescence

Immunostained cells were examined on a Sweptfield confocal system (Prairie Technologies).

Animal experiments

NQO1 and IKKt levels were examined in TRAMP tumors and tissues were obtained from a repository available from previous studies in our laboratory (31, 32). These animals display high-grade PIN (HGPIN) at 28 weeks and poorly differentiated carcinoma by 34 weeks. Blinded pathologist (R.L. Reddick) scored samples. Scoring was based on proportion of cells stained+ intensity of staining. For HGPIN, n = 7 and for carcinoma, n = 8.

Statistical analysis

Data are expressed as mean ± SD. Student t test or one-way ANOVA was used to calculate the P values. Statistically significant differences between experimental results were established as P < 0.05 (GraphPad Prism v5 software).

Results

NQO1 in the prostate

Basal levels of NQO1 protein, transcript, and enzymatic activity were higher in all prostate cancer cells tested (LNCaP, C4-2B, PC3, and DU145) compared with noncancerous cells (RWPE-1 and BPH-1; Fig. 1A–C). We examined basal transcriptional regulation of NQO1 using the NQO1-promoter-luciferase construct containing NRF2-binding sites. Consistent with the transcript level, NQO1 promoter activity was higher in cancer cell lines compared with the noncancerous cell lines (Fig. 1D). Higher expression of NQO1 was observed in cancer cells of bladder, pancreas, and melanocyte origin (Supplementary Fig. S1). Higher levels of NQO1 may be because of adaptive mechanism against persistent oxidative stress in most cancer cells (1). It is interesting to note, however, that the two aggressive prostate cancer cells (PC3 and DU145) have different levels of NQO1. Furthermore, a representative image of a
28-week-old TRAMP animal shows HGPIN, whereas in the 34-week-old animal, the histologic finding was the presence of poorly differentiated carcinoma (Fig. 1E). Immunohistochemical detection of NQO1 showed cytoplasmic staining in the animal with HGPIN with a marked increase in cytoplasmic staining of tumor cells in the animal with poorly differentiated carcinoma (Fig. 1F). The association observed between high NQO1 levels in the absence of p53 in TRAMP tumors (SV40 T-antigen used in generating TRAMP animal inactivates p53 in this model) is in agreement with the mutant p53 DU145 cells.

**Effects of stable inhibition of basal level of NQO1 in prostate cancer cells**

We asked whether higher levels and activities of NQO1 affect growth, proliferation, and migration of prostate cancer cells. To characterize these functions, we generated NQO1-knockdown stable clones of hormone-responsive (LNCaP) and hormone-independent (PC3 and DU145) prostate cancer cells. LNCaP- and PC3-shNQ–transduced cells showed a highly significant reduction in NQO1 mRNA expression, while DU145-shNQ was reduced by approximately 50% compared with the NTC (Fig. 2A). NQO1 silencing in these cells was further confirmed by Western blot analysis (Fig. 2B). Immunocytochemistry was used to examine NQO1 localization and efficiency of stable knockdown in LNCaP cells (Supplementary Fig. S2). Interestingly, stable NQO1 knockdown cells maintained lower basal levels of H2O2 as determined by PO1 (H2O2-specific dye; Fig. 2C) and intracellular reactive oxygen species (ROS; determined by CellROX; data not shown). Although, these findings suggest a critical role of NQO1 in maintaining the balance between ROS production and detoxification, we found small but significant changes in cellular oxidative stress markers, such as NAD(P)H/NAD(P) ratio, total glutathione (GSH), and antioxidant level in a cell type–specific manner with DU145 cells remaining largely unaffected (Supplementary Fig. S3). NQO1 knockdown had a modest differential effect on *in vitro* growth characteristics; with a small but
significant increase in cell proliferation in LNCaP cells and a nonsignificant decrease in PC3 and DU145 cells (Fig. 2D). A similar trend was observed in cell proliferation monitored in long-term culture to clonogenic density (data not shown). In contrast, NQO1 silencing increased cell migration in all cells (Fig. 2E). Our data suggest that under basal growth conditions, inhibition of NQO1 does not largely alter cellular redox state and growth of prostate cancer cells. Cell type–specific effects may be due to alteration in intracellular ROS levels, given that ROS can induce growth arrest, death, and growth/proliferation signals depending on the cellular context of redox adaptation, threshold to stress, and redox-mediated signaling (33). In addition, p53 status may be an important factor to define cell type–specific fate during NQO1-mediated inhibition of ROS (34). These results emphasize the need for a deeper understanding of the role of NQO1 in regulating genes under basal redox homeostasis as well as in oxidative stress-induced conditions in prostate cancer.

**NQO1 knockdown does not alter AR signaling but increases resistance to hormone deprivation–mediated cell death in vitro**

We investigated the effect of NQO1 inhibition on hormone response because oxidative stress, androgen receptor (AR) signaling, and their interactions are regarded as oncogenic drivers in prostate tumorigenesis. However, compared with NTC cells, LNCaP-shNQ cells showed no significant changes in AR signaling as determined by AR-promoter luciferase (Fig. S4). Furthermore, DHT treatment
increased proliferation of both LNCaP-NTC and LNCaP-shNQ cells in a dose-dependent manner (Fig. 3A). Similar to basal growth condition, there was a marginal increase in DHT-induced cell proliferation in LNCaP-shNQ cells. When cells were cultured in charcoal-stripped serum (CSS) media (mimics hormone-deprivation condition), both LNCaP-NTC and LNCaP-shNQ responded with significantly decreased survival (Fig. 3B). However, upon closer observation, we found that under CSS conditions, a population of LNCaP-shNQ cells escaped from hormone dependency. We observed clear differences in morphology with differentiated growth and survival rates over time (days 2–4) under hormone-deprivation condition (Fig. 3B). Live-Dead cell count supported the above observations (Fig. 3B, right). Although long-term culture confirmed increased cell survival in a hormone-independent manner (Fig. 3C), we do not rule out the possibility that increased survival is due to a marginal increase in proliferation rate under CSS conditions. Confluent monolayers of LNCaP-NTC and LNCaP-shNQ cells were switched to CSS medium and observed for wound closure. LNCaP-shNQ cells migrated faster and significantly decreased wound size compared with NTC control at 24 hours (Fig. 3D). These observations suggest that NQO1 repression may induce cellular signaling that supports survival and adaptation to hormone depletion conditions.

Effects of silencing NQO1 on global gene expression

To answer the fundamentally important question regarding how LNCaP-shNQ cells survive under hormone-depleted conditions, we performed genome-wide molecular profiling using the Agilent platform. Three different clones from each LNCaP-NTC and LNCaP-shNQ cells were subject to whole-genome microarray. Expression profiles are shown in clustered heat-maps (Supplementary Fig. S5). Analysis of up- and down-regulated transcripts showed that NQO1 knockdown affected several hundred genes (Supplementary Tables S2 and S3) involved in receptor signaling, inflammation, stress, invasion, migration as well as angiogenesis, suggesting that NQO1 is a top discriminatory gene (Fig. 4A and B). We analyzed the database for targets of signaling pathways and noted a major upregulation in immune and cytokine signaling pathway targets as summarized in Fig. 4C. Of note, genes related to inflammatory signaling, notably target signaling of IL1 (CCL20, CCL12, CCL2, BIRC3, TNFAIP3, IL8, and SAA2) and TNFα (CXC7, CCL2, UBD, CCL2, BIRC3, IL8, and CD70) were among the most differentially overexpressed targets in NQO1 knockdown cells (Fig. 4C and D). In addition, EGFR targets, such as LCN2, GJB2, and IER3, also increased upon NQO1 knockdown (Fig. 4C and D). Many of the deregulated genes, including proinflammatory mediators, have previously been demonstrated to induce prostate tumorigenesis and androgen

Figure 3. Increased survival of NQO1 knockdown LNCaP cells under hormone-deprived condition. A, NTC and shNQ cells were cultured in serum-free medium with or without indicated concentration of DHT for 72 hours. Representative microscopic images show DHT-induced cell survival (left). Graph shows DHT-induced cell proliferation measured by MTT assay (right). Values are mean ± SD from four wells. B, NTC and shNQ cells grown in either FBS- or CSS-supplemented medium were assessed for growth characteristics at various time points. Live-Dead cells were estimated using Trypan blue assay. Bar graphs show the percentage of live and dead cells counted at 72 and 96 hours. Values are mean ± SD from four wells. C, NTC and shNQ cells were cultured in CSS-supplemented medium for 12 days and cells were stained with crystal violet. A representative enlarged microscopy image from each well is shown. D, confluent monolayer of LNCaP cells was switched to CSS-supplemented medium and wound-scratch assay was done as described previously.
We also validated microarray data on gene expression using qPCR. Affected genes and their functions are listed in Table 1.

Link between NQO1 and inflammatory mediators
The link between NQO1 and inflammatory signaling was reinforced in an independent clone following transient knockdown with siRNA smartpool against NQO1 in LNCaP cells. Expression of NQO1, IL8, and CCL2 was analyzed by qPCR. As shown in Fig. 5A, expression of IL8 and CCL2 was significantly increased in NQO1 knockdown cells. To further validate the effect of NQO1 in IL8 expression, pharmacologic inhibitors (dicoumarol and MAC220) that inhibit the catalytic activity of NQO1 were used. Consistent with stable (shRNA) and transient...
Table 1. Expression levels of selected genes in LNCaP (shNQ) cells compared with NTC analyzed by real-time qPCR

<table>
<thead>
<tr>
<th>Genes</th>
<th>Relative levels in shNQ cells (shNQ/NTC)</th>
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<tbody>
<tr>
<td>Chemokines and cytokines</td>
<td></td>
</tr>
<tr>
<td>IL8</td>
<td>3.11</td>
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<tr>
<td>IL32</td>
<td>1.47</td>
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<tr>
<td>IL17C</td>
<td>1.31</td>
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<tr>
<td>IL10RA</td>
<td>1.51</td>
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<tr>
<td>CCL2</td>
<td>5.31</td>
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<tr>
<td>Cell growth and proliferation</td>
<td></td>
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<tr>
<td>BMP7</td>
<td>3.85</td>
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<tr>
<td>AKT3</td>
<td>2.55</td>
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<td>1.83</td>
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<tr>
<td>Stress response</td>
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<tr>
<td>NQO1</td>
<td>0.15</td>
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<tr>
<td>Chromatin reprogramming</td>
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<tr>
<td>NPM2</td>
<td>0.76</td>
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(siRNA) genetic inhibition, both agents increased the expression of IL8 and CCL2 in LNCaP cells (Fig. 5B and C). We used a cytokine array to test whether increased expression leads to higher secreted levels of these cytokines. We found that IL8 induction was clearly observed in conditioned media from LNCaP-shNQ cells (Fig. 5D). Although migration inhibitory factor (MIF) was found in both conditioned media and cell lysates, the IL1 receptor antagonist (IL1Ra) was only detected in cell lysates with slightly higher levels in LNCaP-shNQ cells (Fig. 5D). Supplementation of hormone-deprived media with IL8 partially rescued LNCaP-NTC and LNCaP-shNQ cells from cell death (Fig. 5E). We used siRNA to attenuate IL8 expression in LNCaP-shNQ clones to examine the role of elevated IL8 production and IL8-induced signaling cascade in promoting androgen-independent survival of prostate cancer cells. IL8 knockdown partially abrogated the NQO1 knockdown-mediated increased cell survival (Fig. 5F).

**NQO1-mediated negative regulation of p53 promotes NF-κB activation and IL8 induction**

Regulation of IL8 gene expression by transcriptional activation of NF-κB is widely reported, and many of the IL1β and TNFα signaling targets shown in Fig. 4C and D are regulated by NF-κB. This is consistent with the possibility that NF-κB activation may mediate IL8 expression for increased cell survival and migration, either directly through redox imbalance or indirectly by negative regulation of p53. We found that NQO1 knockdown increased basal constitutive NF-κB luciferase activity by approximately 3-fold (Fig. 6A). Interestingly, treatment with TNFα induced NF-κB activation in LNCaP-NTC but not in LNCaP-shNQ cells. This differential response suggests that NQO1 might be an important determinant of NF-κB activation via the classical pathway. Previously, Ahn and colleagues (8) reported that NQO1-deficient keratinocytes have impaired TNFα-mediated NF-κB activation. We consistently observed higher constitutive nuclear p65 level in LNCaP-shNQ cells (Fig. 6B, right). NF-κB translocation to the nucleus in response to TNFα was only noticeable in LNCaP-NTC but not in LNCaP-shNQ cells. In a parallel experiment, NQO1-specific inhibitor MAC220 also increased basal nuclear p65 levels (Supplementary Fig. S6). Given that cross-talk between NF-κB and p53 is probably most relevant to oxidative stress (24) and NQO1-deficiency induces p53 degradation (4), we hypothesized that NQO1 knockdown-mediated p53 inhibition enhances NF-κB activation by competing for limited pools of p300 or CBP coactivators. We found that NQO1 knockdown resulted in decreased p53 levels and increased nuclear levels of NF-κB and IKKα (Fig. 6C and Supplementary Fig. S7). IKKα has been reported to tip the binding preference from p53 to NF-κB by activating these coactivators (28). Our aforementioned hypothesis was confirmed by the findings that NQO1 knockdown decreased p53 level (Fig. 6C) and increased NF-κB–p300 association (Fig. 6D). Furthermore, when cells were treated with the p33-specific inhibitor PFTα and nuclear lysates were immunoprecipitated with anti-p300 followed by immunoblotting with anti-NF-κB, we found that NQO1 knockdown indeed increased p300 binding to NF-κB and this effect was further enhanced by p53 inhibitor (Fig. 6D). IKKα immunostaining showed no nuclear staining in either HGPIN or poorly differentiated carcinoma samples. There was some IKKα cytoplasmic staining in HGPIN lesions of TRAMP animals (Fig. 6E). In poorly differentiated tumors, IKKα staining was sparse to absent. However, material within the lumen of glands showed positive staining (Fig. 6E). Overall, our data suggest that nuclear IKKα might be involved in increased interaction of NF-κB–p300 and activation of NF-κB.

**Discussion**

NQO1 contributes to anti-inflammation, p53 stability, chemosensitivity, and hence it has tumor-suppressor properties (4, 10, 37). On the other hand, high level of NQO1 expression in cancer cells can create a microenvironment favorable for growth and survival during elevated oxidative stress, via induction of oncogenic signaling pathways. Although, cytoprotective effects of NQO1 have been confirmed by several studies, there is increasing evidence that NQO1 may interact with other molecules to integrate their activities with signaling networks in cancer cell biology. These new roles of NQO1 may be direct effects or consequences of cross-talk between signaling pathways that ultimately determine cell fate. In this study, we attempted to delineate the biologic significance and mechanism through which NQO1 is involved in prostate cancer. We report for the first time, that NQO1 may be protective against prostate cancer because its suppression increases inflammatory mediators that lead to increased resistance to
hormone deprivation–induced cell death. Furthermore, we have defined a molecular mechanism that links NQO1 to IL8 expression via control of IKKα-mediated p300 recruitment to NF-κB.

Accumulating evidence shows that NQO1 is overexpressed in most cancer cells as well as solid tumors (10, 37). Our screening results suggest that basal NQO1 protein levels may be under the control of NRF2, a master regulator that binds to the ARE sites on the NQO1 promoter. In fact, the levels of NQO1 expression in these prostate cancer cells correlated with constitutively higher level of NRF2 activity bearing somatic mutations in NRF2 and KEAP1 genes, suggesting that basal expression is regulated by the KEAP1–NRF2 pathway (38).

Although NQO1 level is induced during cancer initiation and progression, compelling evidence from several studies suggests that NQO1 suppression or deletion leads to adverse biologic consequences. For example, epigenetic downregulation or loss of NQO1 by NQO1/C3, the C609T substituted polymorphic form of NQO1, has been correlated with strong adverse prognosis in various cancers such as hepatocellular carcinoma, breast etc. (39, 40).

Our findings indicate that NQO1 may play a critical role in maintaining redox balance between intracellular ROS and endogenous oxidative stress stimuli. NQO1 has been shown to generate ROS from NQO1-dependent futile redox cycling of certain substrates, including endogenous quinones (10). On the basis of this fact, we speculate that stable inhibition of NQO1 may hinder constitutive ROS generation. However, at the basal level, DU145 cells expressing high levels of NQO1 show significant low levels of H2O2. It is possible that because DU145 cells have the highest basal level of NQO2, it is able to quench H2O2 by converting to less reactive metabolites and therefore display the low levels observed. Furthermore, our data also show high basal levels of GSH. These observations along with published literature that shows constitutive high levels of NRF2 and its target antioxidant genes in DU145 may also contribute to the significant lower basal H2O2 level. Further work in this area is required to establish how specific inhibition of NQO1 decreases intracellular ROS, as this may help provide novel strategies for prostate cancer management (41). Surprisingly, decrease in cellular ROS by NQO1 knockdown increased cell migration. Although elevated ROS levels are directly associated with development and progression of various cancers (2, 3), ROS detoxification by antioxidant supplementation can also promote tumor aggressiveness in some genetic settings (42). Recently, in Nkx3.1-mutant mice, the ROS scavenger N-acetyl cysteine failed to inhibit tumor growth.

Figure 5. NQO1 knockdown–mediated IL8 production in LNCaP cells. A, transient knockdown of NQO1 using specific siRNA increased CCL2 and IL8 expression. B and C, NQO1 inhibitors induced cytokine expression in LNCaP cells. D, conditioned media or cell lysates from NTC and shNQ cells were subjected to cytokine expression profiling using human cytokine expression array. E, GFP-tagged stable NTC and shNQ cells were cultured in CSS-supplemented medium with or without IL8 for 3 days and fluorescence images were recorded. F, stable NTC and shNQ cells were transfected with scrambled (Scr) and IL8 siRNA smartpool (si-IL8). These cells were cultured in CSS-supplemented medium for 9 days. Cell growth was assessed by Trypan blue assay and colony formation assay.

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Figure 6. NQO1-mediated p53 inhibition activates NF-κB–p300 association via IKKa. A, NF-κB luciferase activity was analyzed by the Dual-Luciferase Assay system. B, NTC and shNQ LNCaP cells were treated with TNFα and cytoplasmic and nuclear fractions were prepared. Western blot analysis shows changes in nuclear NF-κB and IKKα. C, NTC and shNQ cells were fixed and immunostained with anti-NQO1, anti-p53, anti-NF-κB p65, and anti-IKKα. Representative images are shown from two independent experiments performed in duplicate wells. D, NTC and shNQ cells were incubated with or without PFTα (20 μmol/L) and lysates were immunoprecipitated with anti-p300 followed by immunoblotting with anti-NF-κB. E, representative IHC staining of IKKα in HGPIN lesions (n = 7) and poorly differentiated carcinoma (n = 8) are shown. Scale bar, 1.0 mm for low magnification and 100 μm for high magnification. F, proposed mechanism of hormone-independent survival of NQO1 knockdown prostate cancer cells. NQO1 suppression reduces endogenous ROS and p53 levels that facilitate increased NF-κB–p300 interaction. These cells appear to adapt to hormone-deprivation condition by the activation of proinflammatory and survival signaling molecules.
NQO1 Regulates Inflammatory Signaling

cysteine increased protumorigenic gene signature, increased prostate epithelial cell proliferation, and promoted the expression of a proproliferative gene signature. More importantly, ROS did not promote cell proliferation in the prostate of Nkx3.1-null mice (43). Furthermore, ROS inhibition by N-acetyl cysteine and vitamin E prevented p53 activation and markedly increased tumor cell proliferation in mouse and human lung tumor cells (34).

Several studies suggest that hormone deregulation, elevated oxidative stress, and chronic inflammation in the prostatic microenvironment are connected through orchestrated ways to cause prostate cancer progression from androgen dependence to castration resistance (15, 18, 44). The exploitation of complex relationships between these factors will facilitate increased efficacy of hormone ablation and allow designing of novel combinatorial therapeutic approaches for advanced disease. Saylor and colleagues (45) measured inflammatory and angiogenic biomarkers in men undergoing androgen deprivation therapy treatment and found induction of inflammatory and angiogenic biomarkers, including IL8, in the treatment group compared with controls. We unexpectedly observed that NQO1 knockdown cells survived in hormone-deprived condition using CSS-supplemented medium. However, multiple approaches failed to show significant differences between LNCaP-NTC and LNCaP-shNQ cells in hormone responsiveness and androgen signaling. These data suggest that the LNCaP-shNQ cells may have acquired changes, which contribute to their ability to survive under hormone-deprived condition. Genome-wide microarray revealed that the absence of NQO1 allows orchestration of inflammatory signaling that sustains cell survival and androgen independence. In this study, we identified a link between NQO1 and genes involved in different signaling pathways (Fig. 4), and were intrigued as to how these transcripts could be deregulated by NQO1 knockdown, which after all is best known as a key antioxidant enzyme. We hypothesized that it could be due to cross-talk with the activity of many signaling pathways and transcription factors that control their expression. Our analysis of targets of signaling pathway and their validation suggest an inverse relationship of NQO1 and proinflammatory targets. In vivo studies from our laboratory and others have found an inverse correlation between NQO1 and proinflammatory markers (46, 47). Here, we report that proinflammatory cytokines, including IL8, are significantly highly expressed in androgen-independent cells, while being barely detectable in androgen-responsive prostate cancer cells (Supplementary Fig. S8). Our observation shows increased IL8 production in LNCaP-shNQ cells and that induction, at least in part, contributes to cell survival under hormone-deprived stress condition. Furthermore, we noted that IL8 silencing partially reversed NQO1 knockdown-mediated survival under hormone-depleted condition, indicating that IL8 may be an important but not exclusive mediator of hormone-independent growth. Given the documented roles of these inflammatory mediators in prostate cancer progression and androgen-independent survival (35, 44, 48), it is possible that NQO1-mediated alterations may play a role in prostate pathology and the increased resistance of polyclonal individuals lacking NQO1 to standard treatments.

In keeping with our observations, Rushworth and colleagues (49) reported increased TNFα and ILβ production upon NQO1 silencing, while NQO1 overexpression inhibited inflammatory responses without affecting NF-κB activation in lipopolysaccharide-activated monocytes. In addition, an increased level of nuclear IKKα, p300 binding to NF-κB, and its transcriptional activation was confirmed in our NQO1 knockdown model system (Fig. 6). Several mechanisms have been proposed to define the role of nuclear IKKα in NF-κB activation. For instance, IKKα is recruited to NF-κB–responsive promoters by interacting with CBP, which contributes to NF-κB–mediated gene expression through phosphorylation of histone H3 (26, 27). IKKα promotes prostate tumor metastasis by downregulating tumor-suppressor Maspin (29). The underlying mechanisms of increased nuclear shuttling of IKKα in NQO1 knockdown LNCaP cells remain unclear. First, loss of NQO1 may result in excessive inflammatory response by releasing the feedback inhibition of NQO1, which in turn activates various cytokine-induced responses, including NF-κB transactivation, by nuclear IKKα activation. Second, in response to NQO1 blockade, p53 degradation is induced. We propose that NQO1 antagonizes the proapoptotic function of p53 and increases the access of shared transcription cofactors, CBP or p300, to NF-κB to induce cytokines and cell survival genes.

Our observations regarding nuclear IKKα in LNCaP cells and lack thereof in TRAMP tissues suggest that p53 status may be an important determinant for the observed inconsistency. As discussed, p53 was inactivated in the creation of this model; therefore, the lack of p53 could explain the difference between our in vitro and in vivo work. We tested this hypothesis in mutant p53 DU145 cells and found that nuclear IKKα was absent in both NTC and shNQ DU145 cells (data not shown). Further studies are on going to understand this mechanism.

In summary, our findings provide substantial insight into the intricate roles of NQO1 in prostate cancer and how NQO1 inhibition leads to the induction of inflammatory mediators in prostate cancer cells. Our data show that NQO1 blockade decreases p53 levels and activates NF-κB and its targets, including IL8, implying it is a crucial step leading to hormone-independent survival. NQO1 knockdown–induced nuclear IKKα may promote NF-κB activation by recruiting p300 to mediate proinflammatory gene expression. These findings may also provide a rationale for the development of new combination treatments using hormone ablation or chemotherapy and modifiers of oxidative stress for prostate cancer management.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References


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