Oxidative Stress Is Inherent in Prostate Cancer Cells and Is Required for Aggressive Phenotype

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Abstract

Reactive oxygen species (ROS) and the coupled oxidative stress have been associated with tumor formation. Several studies suggested that ROS can act as secondary messengers and control various signaling cascades. In the present studies, we characterized the oxidative stress status in three different prostate cancer cells (PC3, DU145, and LNCaP) exhibiting various degrees of aggressiveness and normal prostate cells in culture (WPMY1, RWPE1, and primary cultures of normal epithelial cells). We observed increased ROS generation in cancer cells compared with normal cells, and that extramitochondrial source of ROS generator, NAD(P)H oxidase (Nox) systems, are associated with the ROS generation and are critical for the malignant phenotype of prostate cancer cells. Moreover, diphenylidonium, a specific Nox inhibitor, blocked proliferation, modulated the activity of growth signaling cascades extracellular signal-regulated kinase (ERK)1/ERK2 and p38 mitogen-activated protein kinase as well as AKT protein kinaseB, and caused cyclin B-dependent G2/M cell cycle arrest. We also observed higher degrees of ROS generation in the PC3 cells than DU145 and LNCaP, and that ROS generation is critical for migratory/invasiveness phenotypes. Furthermore, blocking of the ROS production rather than ROS neutralization resulted in decreased matrix metalloproteinase 9 activity as well as loss of mitochondrial potential, plausible reasons for decreased cell invasion and increased cell death. Taken together, these studies show for the first time, the essential role of ROS production by extramitochondrial source in prostate cancer and suggest that therapies aimed at reducing ROS production might offer effective means of combating prostate cancer in particular, and perhaps other malignancies in general. [Cancer Res 2008;68(6):1777–85]

Introduction

Prostate cancer is the most frequently diagnosed cancer among men in the developed world. An estimated 218,890 new cases will be diagnosed and 27,050 deaths will result from prostate cancer in the United States in 2007 (Cancer Facts and Figures 2007, American Cancer Society, 2007). Although the mechanisms that drive prostate cancer have not been completely understood, age, race, and family history of the prostate cancer have been shown to be the potential factors closely associated with this fatal disease (1). Association between prostate cancer risk and oxidative stress has been well-recognized. There is considerable evidence suggesting oxidative stress contributes to the etiology and pathogenesis of the prostate cancer (2, 3). Given that the mitochondria are a major source of reactive oxygen species (ROS), altered mitochondrial bioenergetics might underlie the development of prostate cancer. In addition to mitochondria, ROS generated through extramitochondrial NAD(P)H oxidase (Nox) system/s (4, 5) are also implicated in the mitogenic signaling. Furthermore, high levels of ROS have been detected in several human cell lines as well as in different human tissue (6, 7). It is believed, and some supporting evidence suggests, that increased ROS generation could be a result of oncogenic transformation (8). Inherent oxidative stress may affect several functions in cancer cells or tumor tissue, such as cell proliferation, promotion of mutations and genetic instability, alterations in cellular sensitivity to anticancer agents, invasion, and metastasis (7, 9). Maintenance of an appropriate level of intracellular ROS is important in keeping the redox balance and cellular signaling (10, 11). However, the actual effects of oxidative stress may depend on the cellular genetic background, the types of ROS involved, and the levels and duration of the ROS.

In the present study, we set out to determine role of extramitochondrial ROS generator, Nox system, by using the specific inhibitor diphenylidonium and accumulation of ROS by an antioxidant, N-Acetyl-l-cysteine (NAC), in the pathobiology of the prostate cancer cells. For these studies, we evaluated ROS levels and mechanisms associated with ROS generation in commonly used prostate cancer cells (LNCaP, DU145, and PC3 cells) and normal prostate cells in culture (WPMY1, RWPE1, and primary cultures of normal epithelial cells). We also evaluated the effects of either blocking ROS generation or neutralizing ROS on characteristics of these cells. Results of our studies showed an inherent increased ROS generation in all three prostate cancer cells with a degree of ROS generation directly proportional to aggressive phenotype. Moreover, we observed that ROS generation rather than ROS accumulation was essential for aggressive phenotype of prostate cancer cells, including dysregulated growth, colony formation, cell migration, and invasion. These results suggested that targeting ROS production rather than ROS neutralization might offer a novel mechanism in combating prostate cancer and perhaps other malignancies.

Materials and Methods

Chemicals and reagents. 5-(and-6)-chloromethyl-2,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H2DCFDA), 3, 3′-dihexylxocarbocyanine iodide, Mitroprobe JC-1 (M34152), and dihydroethidium were obtained from Molecular Probe (Invitrogen). All the primary antibody such as extracellular signal-regulated kinase (ERK), pp-ERK, P38 mitogen-activated protein kinase (MAPK), pp-P38 MAPK, AKT, pp AKT, and cyclin B1 were obtained from Cell Signaling. All the chemical reagents such as

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NAC, carbonylcyanide m-chloro-phenylhydrazone (CCCP), 3-(4-5-dimethylthiazol-2-yl) 2,5 diphenyltetrazolium bromide (MTT), propidium iodide, and diphenyliodonium were procured from Sigma.

**Cell culture and treatments.** Human prostate carcinoma cell lines, LNCaP, DU145, PC3, and normal prostate cell lines WPMY1 (Stroma) and RWPE1 (Epithelial) were obtained from American Type Cell Culture (ATCC) and were cultured according to the guidelines of ATCC. Prostate epithelial cells were maintained in the prostate epithelial cell basal medium (Cambrex). For each experiment, equal numbers of cells were seeded in respective medium, and same concentration of DMSO was applied to control cells where indicated. In most of the experiment, 20 μmol/L of diphenyliodonium (stock in DMSO) and 1 mmol/L of NAC (stock in deionized water) were used for the treatment of the cells.

**Determination of H₂O₂.** To visualize intracellular H₂O₂ levels, log-phase cells were grown on 8 chamber slides, incubated with 10 mmol/L of CM-H₂DCFDA in warm PBS, and detected according to the methods described by Dolado et al. (12). Intracellular ROS were visualized by using an inverted fluorescence microscope (Leica RX-DA). For ROS quantification, cells were treated with the above dye (CM- H₂DCFDA in HBSS) and were scanned for the 60 min in the microplate reader (Cytofluor multiwell plate reader; PerSeptive Biosystem) at λex = 490 ± 20 and λem = 520 ± 20 nm. Where indicated, cells were pretreated with NAC and diphenyliodonium for 1 h before ROS measurement.

**Determination of superoxide.** To visualize superoxide in prostate cancer cells line, log-phase cells were grown on 8 chamber slides, incubated with 10 mmol/L of dihydroethidium in warm HBSS, and were visualized by using an inverted fluorescence microscope (Leica RX-DA) using Cooke software (magnification, ×10) according to Yu et al. (13). For superoxide quantification, cells were treated with the above dye (dihydroethidium) and were scanned for the 60 min in the microplate reader at λex = 535 ± 20 and λem = 620 ± 20 nm.

Where indicated, freshly frozen tissue sections from prostatectomy specimens (collected under an Institutional Review Board–approved protocol) were used. The tissue sections were thawed to room temperature in the dark, and 200 μL of dihydroethidium in HBSS was added to immerse the tissue. After incubation of 20 min at 37°C, slices were washed once with the PBS and pictures were taken immediately by using the fluorescence microscope (14). Four randomly selected regions of each sample were analyzed for each time point. Parallel sections adjoining the selected samples were stained by 4-4hydroxynonenal to determine pathologic stage of the tissue.

**Measurement of proliferation and viability in cell culture.** Log-phase cells were seeded into the 96-well plate (10,000 cells) and were allowed for the attachment overnight followed by the treatment with diphenyliodonium and NAC for 48 h. Cellular proliferation was quantified with colorimetric methods based on the metabolic reduction of the soluble yellow MTT dye to its insoluble formazan (15). For viability assay, all the cell lines were seeded in a 24-well plate (2 × 10⁴) and were treated with diphenyliodonium and NAC for 48 h, followed by the addition of 0.4% crystal violet in 0.2 mol/L citrate buffer for 30 min (16). Dyes were solubilized with ethanol, and the readings were taken at 595 nm.

**Reverse transcription-PCR.** Total RNA was extracted using RNEasy mini kit (Qiagen). One microgram of RNA was used to prepare cDNA using iScript second strand cDNA synthesis kit (Bio-Rad). One microgram of synthesized cDNA was used to amplify Nox1, Nox2, Nox3, Nox4, and Nox5, p22phox, or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA using human gene—specific, primer primers described in Vaquero et al. (17).

**Measurement of clonogenic potential.** For foci formation assays, cells were treated with diphenyliodonium or NAC for 48 h before trypsinization, and randomly thousand cells were plated in 100-mm dishes and were allowed to form the foci. Foci were counted after 2 wk after fixation with glutaraldehyde (6% v/v) and stained with crystal violet (0.5% w/v; ref. 18). Experiments were performed in triplicates and repeated at least twice.

To measure anchorage-independent growth in soft agar, assay was performed according to Rizzino (19). After 14 d, pictures of the colonies were taken and colonies bigger than 0.2 mm were counted in the representative fields. Colony formation assays were always measured in quadruplicate.

**Cell cycle analysis.** Subconfluent cultures were treated with either diphenyliodonium or NAC for 48 h, harvested, washed with the cold PBS, and were kept overnight with the staining solution (Rnase, 20 μg/mL; propidium iodide, 50 μg/mL) at 4°C. Cell cycle distribution was then determined using a fluorescence-activated cell sorting (FACS) instrument (Beckman Coulter FC500) in the FACS Core Facility of the University of Colorado Health Sciences System at Denver. ModFit LT 3.0 (Verity House Software) cell cycle analysis software was used to determine the percentage of cells in the different phase of the cell cycle.

**Cell motility and migration assays.** For the persistence migratory directionality assay, confluent cultures (80%–90% in serum-free medium) were used for the in vitro scratch wound–healing assay (20). For migration assays, transwell chamber were used (21).

**Matrigel invasion assay.** The in vitro invasion assays were carried out in BD BioCoat Matrigel chambers (Transwell; Corning) according to Repesh et al. (22).

**Matrix metalloproteinase—Zymography.** Cells in semiconfluent cultures (~80% confluent) were placed into serum-free medium, treated with either diphenyliodonium or NAC, cultured for an additional 36 h, and conditioned medium were concentrated (Microcon YM-10 centrifugal filter Millipore) and separated on 7% SDS polyacrylamide gel containing 0.1% (w/v) gelatin under nonreducing conditions. Zymogram for matrix metalloproteinase (MMP)-9 and MMP-2 were performed according to Bernhard and Muschel (23). Pure human MMP-9 and MMP-2 (50 ng) protein were used as a positive control.

**Measurement of mitochondrial transmembrane potential.** The change in mitochondrial transmembrane potential (ΔΨm) induced by diphenyliodonium and NAC in prostate cancer cell lines was (a) observed microscopically (Leica RX-DA) and (b) flow cytometrically by using appropriate fluorescent probes such as JC-1 (24). Cells (1 × 10⁵) plated in 8 chamber slides were treated with either diphenyliodonium or NAC for 8 h, labeled with JC-1 (2.5 μg/mL) for 30 min at 37°C, followed by washing twice with PBS, and pictures were taken at ×63 magnification. For FACS study, treated cells were labeled with 2 μmol/L of JC-1 for 15 min at 37°C, washed with PBS, and analyzed on a flow cytometer using 488-nm excitation with 530- and 585-nm band pass emission filters. Change in color from red to green were quantified and analyzed. CCCP-treated cells (10 μmol/L) were taken as a positive control.

**Western blot analysis.** Where indicated, cells were treated with either diphenyliodonium or NAC for predetermined periods, cells were lysed in radioimmunoprecipitation assay buffer, separated on SDS-PAGE, and proteins bands were transferred to a polyvinylidene difluoride membrane followed by incubation with appropriate antibodies. The binding of antibodies was detected with an enhanced chemiluminescence substrate.

**Results**

Prostate cancer cells generate high levels of ROS, and the generation of ROS increases with aggressiveness of the cells. High levels of ROS (H₂O₂ and superoxide) were produced constitutively in all three different adherent human prostate cell lines tested here (PC3 and Du145 (androgen independent), and LNCaP (androgen dependent)) compared with normal cell lines (Table 1). Both the androgen-independent cell lines produce more H₂O₂ than the androgen-dependent cell line, LNCaP. Similar results of ROS production were also visualized in immunofluorescence experiments (Fig. 1A) with these cell lines. PC3 cells produced H₂O₂ with the higher rate than the DU145 and LNCaP. Similarly, results presented in Fig. 1C show that rate of production of H₂O₂ were greatest in the PC3 cells compared with other cells.

In view of the increased H₂O₂ in cancer cells, we also examined prostate cancer cell lines for superoxide production (Fig. 1B and D). Increased superoxide production was observed in all three cancer cell lines, with the rate [arbitrary fluorescence units (AFU)/min] of the production of superoxide being highest in PC3 cells (Table 1).
We also observed increased ROS generation in frozen sections from prostate tumor tissues. Increased dihydroethidium fluorescence was observed in tumor tissue sections as compared to normal tissue, indicating a higher level of superoxide in the cancer cells in vivo as well. Figure 1E shows a representative image of normal and tumor tissue dihydroethidium fluorescence.

**Increased ROS generation in prostate cancer cells are contributed by Nox system.** To identify the source of ROS generation, we evaluated the effects of various agents (an antioxidant, NAC; mitochondrial complex I inhibitor, rotenone; or mitochondrial complex III inhibitor, antimycin; or Nox inhibitor, diphenyliodonium) on H$_2$O$_2$ levels in prostate cancer cells. Results presented (Fig. 2A) show that diphenyliodonium selectively inhibited ROS generation, whereas rotenone and antimycin had no effect.

Results presented in Fig. 2B show expression of various Nox isoforms in prostate cancer cells. Nox4 expression was found in DU145, PC3, and LNCaP lineage cell lines, whereas it is absent in normal cell line (WPMY1). Nox2 expression was observed primarily in DU145 cells and PC3 and LNCaP cells but not in LNCaP-derived (C4 and C42B) cell lines or normal cell line. Nox5 was absent in WPMY1 but expressed in cancer cell lines. p22$^{	ext{phox}}$ was present only in DU145 and WPMY1. Specifically, Nox1 and Nox3 were not detected in any cell line tested. These results show expression of various isoforms of Nox in prostate cancer cells but not in normal cells.

Taken together, these results show expression of Nox isoforms in prostate cancer cells, and show that extramitochondrial Nox system contributed to increased ROS generation in prostate cancer cells.

**Inhibition of Nox and neutralization of ROS have distinct effects on cell growth and proliferation.** Results of cell proliferation determined show that diphenyliodonium significantly inhibited cell proliferation (in concentration dependent manner; Supplementary Data), whereas NAC was less effective in inhibiting cell proliferation (Fig. 2C). Similar results were observed in viability assay with the crystal violet (Fig. 2D). These finding indicated that inherent oxidative stress present in the prostate cancer cell lines are, in part, responsible for proliferation and survival of the cells. Interestingly, agents that inhibited ROS generation (diphenyliodonium) were more effective in blocking cell growth compared with antioxidant (NAC) that neutralized ROS, suggesting that inhibition of ROS generation rather than its neutralization might be a better strategy in inhibiting growth and proliferation of prostate cancer cells.

**ROS generation is critical for foci formation and anchorage-independent growth of prostate cancer cells.** Formation of foci was completely blocked by diphenyliodonium, but NAC had no effect in PC3 cells and only had a mild inhibitory effect in DU145 and LNCaP cells (Fig. 3A). Similarly, diphenyliodonium also inhibited the anchorage-independent growth of all three cell lines tested (Fig. 3B). Again, NAC had no significant effect in PC3 cells, but in LNCaP cells, NAC reduced the size and number of colonies, whereas in DU145, it reduced only the number of the colonies (Fig. 3B). These results suggested that increased ROS generation through extramitochondrial source is necessary for the malignant phenotype of these cancer cells.

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**Table 1. Rate of generation of free radicals in prostate cancer cell lines as detected by using specific fluorescent probes described in Materials and Methods**

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Rate of production of H$_2$O$_2$ (AFU/min)</th>
<th>Rate of production of O$_2$ (AFU/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WPMY1</td>
<td>3.6 ± 0.29</td>
<td>1.6 ± 0.16</td>
</tr>
<tr>
<td>RWPE1</td>
<td>3.9 ± 0.32</td>
<td>0.6 ± 0.11</td>
</tr>
<tr>
<td>Primary epithelial</td>
<td>3.9 ± 0.41</td>
<td>0.4 ± 0.09</td>
</tr>
<tr>
<td>LNCaP</td>
<td>5.4 ± 0.40$^a$</td>
<td>2.1 ± 0.01</td>
</tr>
<tr>
<td>DU145</td>
<td>5.9 ± 0.64$^b$</td>
<td>3.4 ± 0.71$^a$</td>
</tr>
<tr>
<td>PC3</td>
<td>9.9 ± 1.16$^c$</td>
<td>3.9 ± 0.19$^c$</td>
</tr>
</tbody>
</table>

$^aP < 0.05$.

$^bP < 0.01$.

$^cP < 0.001$ (Student’s $t$ test) versus control group (RWPE1).

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**Figure 1.** Prostate cancer cells generate high levels of ROS. A, cells were labeled with the 10 $\mu$mol/L of CM-H$_2$DCFDA for 15 min, and intracellular generation of H$_2$O$_2$ were analyzed by fluorescence microscopy (Leica RX-DA) using FITC filter. B, cells were labeled with the 10 $\mu$mol/L of dihydroethidium for 30 min, staining was visualized, and photographs were immediately taken. C and D, quantitative analysis of generation of H$_2$O$_2$ and superoxide, respectively in log-phase growing human prostate cancer cell lines as described in Materials and Methods. A, DU145; B, LNCaP; E, frozen sections of human prostate cancer tissue were brought to room temperature and, in the dark, were covered with 200 $\mu$L of 10 $\mu$mol/L of dihydroethidium in Hank’s buffer, and dihydroethidium staining was visualized and photographed by fluorescence microscopy using CY3 filter. A, U., arbitrary units.
Inhibition of extramitochondrial ROS generation induces cell cycle arrest in prostate cancer cell lines. We observed that treatment of PC3 cells with diphenyliodonium resulted in an irreversible cell cycle arrest in G2-M phase of the cell cycle (Fig. 4A and B), whereas treatment with NAC has no significant effect. Similar results were observed in LNCaP and DU145 (data not shown). Cell cycle arrest induced by diphenyliodonium was associated with a significant decrease in protein levels of cyclin B1 (Fig. 4C). These results suggested that extramitochondrial source of ROS generator, Nox, is important for prostate cancer cell cycle progression especially in G2-M phase.

Nox inhibitor decreased cell migration as measured by scratch and transwell migration assays. Treatment of PC3 cells with the diphenyliodonium reduced the cell motility as determined in scratch assay, whereas NAC treatment has no effect (Fig. 5A). Similarly, results from the transwell migration assays also show that diphenyliodonium inhibited cell migration, whereas NAC had no significant effect (Fig. 5B). These results show the critical role of NAD(P)H-dependent ROS generation in cell migration.

ROS generation is essential for invasive phenotype of prostate cancer. We also tested the effect of diphenyliodonium and NAC on cell invasion using Matrigel-coated transwell chambers. Treatment of diphenyliodonium, compared with NAC and untreated control, strongly inhibited cell invasion (Fig. 5C). These results show that ROS generation from Nox system is essential for the invasive phenotype of the prostate cancer and suggested that any inhibition of this system could affect the invasion/metastasis of this cancer. MMPs are a family of enzymes whose function primarily relates to degradation of extracellular matrix proteins and is necessary for cell invasion (25). As can be seen in Fig. 5D, diphenyliodonium decreased MMP-9 activity in PC3 cells, whereas we did not find any MMP-9 activity in LNCaP cell line and very low MMP-9 activity in DU145 (data not shown). However, treatment of NAC has very little effect in the MMP-9 activity (Fig. 5D). We did not find any MMP-2 activity in any of the cell lines tested here. These results suggested that PC3 cells require ROS generation for MMP-9 activity and maintenance of invasive phenotype.

Inhibition of ROS generation resulted in suppression of growth regulatory proteins and decreased mitochondrial potential. Previous studies suggested a possibility that ROS, from Nox system, might promote growth-signaling cascade by negatively regulating redox-sensitive phosphatase that control the MAPK activities (26). To test this hypothesis, we evaluated the effects of diphenyliodonium and NAC on levels of phosphorylated (active enzyme forms) p42/p44 MAPK (ERK1/ERK2) and the p38 stress-activated MAPK (p38 MAPK). Our results indicated that diphenyliodonium treatment decreased the levels of active (phospho-ERK1/ERK2 and p38) MAPK in all the three cell lines but had no effect on total ERK1/ERK2 and p38 stress-activated MAPK (p38 MAPK). We did not observe any inhibition with NAC. We also observed that treatment with diphenyliodonium also inhibited phosphorylation of AKT in all the cell lines. Moreover, diphenyliodonium also decreased the levels of total AKT in the case of LNCaP and DU145 (Fig. 6A). Taken together, these results indicated that ROS from the extramitochondrial source play an important role in modulating activities of MAPK and protein kinase B in prostate cancer cells.
To get a better insight into the mechanism of diphenyliodonium-induced cytotoxicity, we measured the change in mitochondrial potential after the diphenyliodonium and NAC treatments. Results (Fig. 6B and C) show that diphenyliodonium treatment caused loss of mitochondrial membrane potential, suggesting a plausible mechanism for cell death after inhibition of Nox system by diphenyliodonium.

Discussion

ROS and the oxidative stress have long been associated with the tumor progression. It has been suggested that elimination of excessive ROS by chemical or antioxidants may decrease the metastasis of various types of cancer and has opened up new areas of research for the cell biologist (27). There is a growing body of evidence suggesting a role for oxidative stress in the pathogenesis of prostate cancer. Prostate may be particularly vulnerable to oxidative stress because androgen activity may alter the pro-oxidant–antioxidant balance of prostate cells. Physiologic levels of androgens increase mitochondrial activity and oxidative stress in androgen-responsive LNCaP prostate carcinoma cells (28). Oxidative stress can also be exacerbated by prostatitis. Acute and chronic inflammatory cells generate superoxide, hydrogen peroxide, and other ROS (29). Indeed, recent studies indicate that oxidative stress is higher within the epithelium of prostate cancer patients than men without the disease. In recent years, several antioxidant trials have been conducted against prostate cancer, but we will have to wait for conclusive evidence either in favor or against the usefulness of such therapies (reviewed in ref. 30).

In the present studies, the role of inherent oxidative stress in prostate cancer progression has been characterized in vitro in three different cell lines, PC3, DU145, and LNCaP. We show for the first time that an extramitochondrial ROS-generating system, Nox, is responsible for inherent ROS generation in prostate cancer cells. We also show for the first time that Nox system is critical for maintenance of phenotypic characteristics of the cancer cells, in that inhibition of Nox resulted in inhibition of growth and proliferation, decrease in clonogenic activity, migration, as well as cell invasion and cell cycle arrest in G2-M phase of the cell cycle. We first characterized the basal level of ROS generation in all the three cell lines, and results indicated that PC3 cell lines generate more free radicals than the DU145 and LNCaP (Fig. 1), and rate of H2O2 and superoxide production (Table 1) is also highest in the case of PC3. These results indicated that within these three cell lines, ROS generation was directly proportional to aggressive phenotype. We also observed higher superoxide levels in tumor tissue compared with normal tissue (Fig. 1E).

To further identify the source of ROS generation, we used a general antioxidant, NAC; mitochondrial complex I inhibitor, rotenone; or mitochondrial complex III inhibitor, antimycin; or Nox inhibitor, diphenyliodonium. Results presented (Fig. 2A) show that diphenyliodonium selectively inhibited ROS generation, whereas rotenone and antimycin had no effect. These findings indicated Nox system as the major source of ROS generation in prostate cancer cells. Nox and Nox family as a source of ROS in cancer is an exciting finding, particularly in prostate cancer. The NAD(P)H-dependent reduction of molecular oxygen to generate superoxide anion (O2·−), which is dismutated to form peroxide (H2O2) in the cells, is primarily responsible for phagocyte respiratory burst (31). The phagocyte respiratory burst–associated Nox consists of gp91phox and p67phox (plasma membrane–bound catalytic protein subunit) and p22phox (cytochrome b558). The active assembly complex also includes p67phox and p47phox (two cytosolic protein components), and small GTPase Rac (31, 32).

![Figure 3](image-url). ROS generation is critical for the malignant phenotype of cancer cells. A, cells were analyzed for the foci formation during the course of 2 wk in the absence of or presence of either diphenyliodonium (DPI) or NAC. B, similar to A, all the cell lines were also analyzed for the anchorage-independent growth in soft agar in the presence of above inhibitors. Experiments were performed twice in triplicate. ■, control; □, diphenyliodonium; △, NAC.
During the last decade and a half, several studies documented NAD(P)H-dependent ROS generation not only in phagocytes but also in several other nonphagocyte cells (33–37). Although investigators presumed enzymatic structure to be similar to phagocyte enzyme complex, absence of gp91phox in nonphagocytic cells critically limited such presumptions. Recently, homologues of gp91phox have been identified and named as Nox (for Nox) proteins in nonphagocytic cells (38, 39), providing an explanation for nonphagocytic cell Nox activity. To date, Nox family consists of five members (Nox1–5). These oxidases are believed to play a role in a variety of signaling events, including cell growth, cell survival, and death; however, the exact functional role of these oxidases has largely remained unexplored. Ectopic expression of Nox1 in prostate cancer cells enhances growth, tumorigenicity, and angiogenicity (40), whereas down-regulation of Nox5 causes growth arrest and apoptosis (5).

A recent study showed that castration resulted in dramatic increases of three ROS-generating Nox including Nox1, Nox2, and Nox4 (41). Our studies for the first time identified expression of various Nox isoforms in prostate cancer cells (Fig. 2B). Taken together, these data show that ROS generation in different cancer cells may be contributed by different Nox isoforms.

We also evaluated the effects of diphenyliodonium, a selective Nox inhibitor on the proliferation/viability of the cells; our results show that diphenyliodonium not only inhibit the production of ROS (Fig. 2A) but also inhibited the proliferation, viability, foci formation, and anchorage-independent growth of the cells (Figs. 2C, D, and 3). Furthermore, we showed that inhibition of ROS generation by diphenyliodonium caused cell cycle arrest. Control of the cell cycle progression in cancer cells is considered to be a potentially effective strategy for the control of tumor growth as the molecular analyses of the human cancers have revealed that cell cycle regulators are frequently mutated in most common malignancies (42, 43). Our in vitro data (Fig. 4) indicated that treatment of both androgen-sensitive and androgen-insensitive cells with diphenyliodonium resulted in significant G2–M arrest, which suggested that one of the mechanisms by which the diphenyliodonium inhibit the proliferation of the cells is inhibition of cell cycle progression. We also observed a significant decrease in the cyclin B. Taken together, these results suggest that ROS-mediated cell cycle progression might result from an increased cyclin B activity. ROS have been shown to play a role in the MAPK activation, which plays essential roles in cancer cells, and any inhibition of these kinases may cause the reduction of growth of the cells (44). Moreover, recent studies (12) suggested that p38α can negatively regulate the tumor growth in response to oncogene-induced ROS. To this end, our results show for the first time that diphenyliodonium treatment reduced the activity of phospho-ERK and p38 MAPK (Fig. 6A). Thus, inhibition of extramitochondrial ROS generation seems to modulate p38 and ERK-MAPK activities and might impair the proliferation and anchorage-independent growth of the prostate cancer cell lines. Besides MAPK, other PI3-kinase targets that are involved in the antiapoptotic signaling pathway, such as AKT/PKB, are also activated by ROS. Our results show that inhibition of ROS caused the loss of expression of AKT in prostate cancer cells (Fig. 6A). These results suggest that diphenyliodonium blocks the ROS production by the prostate cancer cell lines, thus inhibiting the AKT expression. Because AKT is associated with cell survival by countering mitochondrial apoptotic signals, we evaluated the effects of diphenyliodonium on mitochondrial membrane potential. Results of these studies (Fig. 6B and C) show loss of mitochondrial membrane potential in diphenyliodonium-treated cells. These results offer one possible regulatory mechanism of cell survival modulated by ROS generation.
In addition to regulating tumor growth and survival, ROS also participate in those mechanisms that are associated with the tumor metastasis, such as migration and invasion (45). Oxidative stress may augment tumor invasion and metastasis by increasing the rate of the cell migration (46). We made a novel observation that the cell lines with higher ROS are more migratory and invasive than the cell lines with low ROS. Diphenyliodonium treatment was able to inhibit the migration and invasion (Fig. 5A, B, and C).

These results suggested requirement of ROS production in the metastatic phenotype of prostate cancer cells. A separate mechanism by which the oxidative stress may induce tumor cell migration and invasion is the up-regulation of the MMPs. Increased MMPs such as MMP-2 or MMP-9 correlates with the increased cell invasion and metastasis (47). Our results showed a strong MMP-9 activity in PC3 cells with no activity or low activity in LNCaP cells and DU145 cells in basal state. Moreover, inhibition of MMP-9 activity by the Nox inhibitor blocked cell migration and invasion (Fig. 5A).
ROS generation by diphenyliodonium inhibited the MMP-9 activity, whereas neutralization of ROS by NAC had no effect on the activity (Fig. 5D). These results show that active ROS generation rather than ROS accumulation might be more important in prostate cancer cell migration and invasion. These findings provide a possible mechanism of high invasive and aggressive nature of PC3 cells compared with LNCaP and DU145 cells.

Our finding of expression of various Nox isoforms in prostate cancer cell lines and a cross-talk between the endogenous ROS generation by Nox system and the tumorigenic potential suggest that this pathway might play a critical role in tumor modulation. Our observations of expression of different combinations of Nox2, Nox4, Nox5, and p22phox, but not Nox1 or Nox3 in various prostate cancer cell lines, suggest multiple parallel pathways of generation of ROS in prostate cancer cells. It is important to point out that association of p22phox (a membranous components of Nox) with gp91phox (homologous of Nox enzymes) is required for their function except for Nox5 (48, 49). Thus, coexpression p22phox is essential for ROS production by all Nox isoforms except Nox5. Our additional results with Nox5 siRNA, suggest a significant role for Nox 5 in ROS generation and proliferation of the prostate cancer cell lines (data not shown). However, involvement of Nox2 and Nox4 in ROS generation in the case of prostate cancer cannot be neglected. Based on these discussions, we speculate that increased Nox (either coexpression of Nox isoforms 2 and 4 with p22phox or overexpression of Nox5 alone in the absence of p22phox) expression–driven ROS generation may modulate tumor phenotypes by modulating various signaling cascades Fig. 6D.

Taken together, the results of our current studies show that inherent oxidative stress present in prostate cancer cells is responsible for many phenotypic characteristics of these cells, such as uncontrolled cell cycle, clonogenicity, invasion, and metastasis. In addition, increased ROS generation may have fundamental role in the initiation, maintenance, and promotion of prostate cancer phenotype. These results also indicate that ROS production rather than accumulation plays an important role in prostate cancer cell phenotypic behavior. Based on these observations, it is tempting to speculate that treatment aimed at reducing ROS production rather than ROS neutralization might offer an effective means against prostate cancer in particular and
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