NOX5 NAD(P)H oxidase regulates growth and apoptosis in DU 145 prostate cancer cells

Sukhdev S. Brar, Zachary Corbin, Thomas P. Kennedy, Richelle Hemendinger, Lisa Thornton, Bettina Bommarius, Rebecca S. Arnold, A. Richard Whorton, Anne B. Sturrock, Thomas P. Huecksteadt, Mark T. Quinn, Kevin Krenitsky, Kristia G. Ardie, David Lambeth, and John R. Hoidal

NOX5 NAD(P)H oxidase regulates growth and apoptosis in DU 145 prostate cancer cells. Am J Physiol Cell Physiol 285: C353–C369, 2003. First published April 9, 2003; 10.1152/ajpcell.00525.2002.—Reactive oxygen species (ROS) appear to play an important role in regulating growth and survival of prostate cancer. However, the sources for ROS production in prostate cancer cells have not been determined. We report that ROS are generated by intact American Type Culture Collection DU 145 cells and by their membranes through a mechanism blocked by NAD(P)H oxidase inhibitors. ROS are critical for growth in these cells, because NAD(P)H oxidase inhibitors and antioxidants blocked proliferation. Components of the human phagocyte NAD(P)H oxidase, p22\textsuperscript{phox} and gp91\textsuperscript{phox}, as well as the Ca\textsuperscript{2+} concentration-responsive gp91\textsuperscript{phox} homolog NOX5 were demonstrated in DU 145 cells by RT-PCR and sequencing. Although the protein product for p22\textsuperscript{phox} was not detectable, both gp91\textsuperscript{phox} and NOX5 were identified throughout the cell by immunostaining and confocal microscopy and NOX5 immunostaining was enhanced in a perinuclear location, corresponding to enhanced ROS production adjacent to the nuclear membrane imaged by 2',7'-dichlorofluorescein diacetate oxidation. The calcium ionophore ionomycin dramatically stimulated ferricytochrome c reduction in cell media, further supporting the importance of NOX5 for ROS production. Antisense oligonucleotides for NOX5 inhibited ROS production and cell proliferation in DU 145 cells. In contrast, antisense oligonucleotides to p22\textsuperscript{phox} or gp91\textsuperscript{phox} did not impair cell growth. Inhibition of ROS generation with antioxidants or NAD(P)H oxidase inhibitors increased apoptosis in cells. These results indicate that ROS generated by the newly described NOX5 oxidase are essential for prostate cancer growth, possibly by providing trophic intracellular oxidant tone that retards programmed cell death.

AN INCREASING BODY OF EVIDENCE points to a prominent role for reactive oxygen species (ROS) in the pathogenesis of carcinoma of the prostate, which is initiated by oxidative stress (19) and inversely related to dietary consumption of antioxidants such as lycopene (22). Prostate cancer cells produce substantial amounts of ROS (49), in part from stimulation of a nonphagocytic Ca\textsuperscript{2+}-regulated NADPH oxidase (54). ROS produced by prostate cancer cells can activate the p90 ribosomal S6 kinase (p90 RSK) (54) and the redox-regulated transcription factors nuclear factor-\kappaB (NF-\kappaB) and activator protein-1 (AP-1) (50). Antioxidants inhibit cell cycle signaling (48, 62, 64, 65), decrease proliferation of prostate cancer cells in culture (54), reduce growth of prostate cancer xenografts (48), and induce prostate cancer cell apoptosis (26). Thus not only are oxidants important in the origin of prostate cancer, but antioxidants might also be useful in its therapy.

Other tumors likewise generate large amounts of ROS (12, 13, 60). Stable transformation of NIH 3T3 cells with the epidermal growth factor receptor (EGFR), with the HER1-HER2 chimera, or with mistyalted ras enhances cellular ROS production compared with nontransformed control cells (9), and mitogenic signaling through both Ras (30) and Rac (32) is mediated by superoxide (O\textsubscript{2}\textsuperscript{-}). A number of sources have been proposed for the origin of ROS generation in tumor cells, but recent attention has turned to the potential role of a family of membrane-localized NOX oxidases homologous to the gp91\textsuperscript{phox} component of the NAD(P)H oxidase.

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Address for reprint requests and other correspondence: T. P. Kennedy, Carolinas Medical Center, 410 Cannon Research Center, P.O. Box 32861, Charlotte, NC 28232 (E-mail: tkennedy@carolinas.org).

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phagocytic NAD(P)H oxidase (2, 3, 16, 59). A plethora of human tumor types express the various NOX oxidases (12, 16, 34, 55, 59). NOX transfection into normal cells increases ROS production (2, 3, 7, 23, 59), transforms normal fibroblasts (59), and creates cell lines that are tumorigenic (3) and angiogenic (2). Conversely, NOX antisense oligonucleotides inhibit tumor cell proliferation (12). Furthermore, the enhanced ROS production in EGFR-, HER1-HER2- or Ras-transformed NIH 3T3 cells potentiates stress-induced activation of c-Jun NH2-terminal kinase (JNK) and p38 mitogen-activated protein (MAP) kinase and stress-induced JNK and p38 MAP kinase activation is decreased by treatment with antioxidants or with diphenylene iodonium (DPI), an inhibitor of flavoprotein-dependent membrane NAD(P)H oxidases (9).

Because of the importance of ROS in prostate cancer pathogenesis, we have examined the commonly used prostate cancer cell line DU 145 for evidence of a growth-regulatory NAD(P)H oxidase. We report that mRNA coding for the NAD(P)H oxidase components p22phox, gp91phox, and the unique Ca2+-activated gp91phox homolog NOX5 (7, 16) are all expressed in this androgen-independent cell line; however, protein product was present for only gp91phox and NOX5. ROS production is stimulated by increasing intracellular Ca2+ concentration ([Ca2+]i), and ROS generation by both cells and membranes is reduced by the flavoprotein-dependent NAD(P)H oxidase inhibitor DPI. Antisense oligonucleotides for NOX5 but not p22phox or gp91phox inhibit DU 145 cell proliferation in culture and also reduce NOX5 protein expression and ROS generation. NOX5 is additionally expressed in the androgen-responsive LNCaP prostate cancer cell line and in mRNA samples obtained from clinical specimens of prostate cancer. These findings suggest a potential role for this [Ca2+]i-regulated gp91phox homolog oxidase in the pathogenesis or propagation of adenocarcinoma of the prostate.

MATERIALS AND METHODS

Cell culture and materials. DU 145 and LNCaP prostate cancer cells were obtained from American Type Culture Collection (Rockville, MD). Cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and passaged with trypsin-EDTA. Culture media, HEPES, antibiotics (10,000 U penicillin, 10,000 µg streptomycin and 25 µg amphotericin B/ml), and trypsin-EDTA solution were purchased from the GIBCO-BRL division of Life Technologies (Grand Island, NY). FBS and horse serum were purchased from HyClone Laboratories (Logan, UT). The intracellular oxidant-sensitive probe 2′,7′-dichlorofluorescein diacetate (DCFH-DA) was from Molecular Probes (Eugene, OR). Rabbit phospho-specific antibodies for the activated forms of p44/42 (ERK1/2) MAP kinase (phosphorylated at threonine 202/tyrosine 204), p38 MAP kinase (phosphorylated at threonine 180/tyrosine 182), JNK (phosphorylated at threonine 183 and tyrosine 185), and protein kinase B (Akt; phosphorylated at serine 473), and for respective nonphosphorylated proteins, were purchased from Cell Signaling Technology (Beverly, MA). All other materials were purchased from Sigma Chemical (St. Louis, MO), unless specified.

Measurement of proliferation in cell cultures. Anchorage-dependent proliferation of cultured cells seeded into 24-well uncoated plastic plates (Costar) at 50,000 cells per well (except where indicated) was quantitated as previously described (12, 13) with a colorimetric method based on metabolic reduction of the insoluble yellow tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to its insoluble purple formazan by the action of mitochondrial succinyldihydrogenase. For some studies with a final cell density of less than ~40,000 cells per well, direct cell counts were performed on 10 random fields/well of Wright’s-modified Giemsa- and eosin-stained monolayers viewed at a magnification of ×100 with a 1-mm2 ocular grid.

Measurement of ROS generation by intact cells. Intracellular production of ROS by cells was measured with oxidation ofDCFH-DA to 2′,7′-dichlorofluorescein (DCF) (52). Cells (1 × 106) were incubated in the dark for 10 min at 37°C with 50 µmol/l DCFH-DA, harvested, and resuspended in plain medium. Fluorescence was analyzed with a FACStarPLUS flow cytometer (Becton Dickinson, San Jose, CA) with excitation at 488 nm and emission at 530 nm.

To determine where oxidants were produced, DU 145 cells were plated on Lab-Tek chambered coverglass slides (Nalge Nunc International, Naperville, IL) at a density of 20,000 per well, grown to 50–70% confluence and loaded with 10 µmol/l of DCFH-DA for 30 min, the time necessary to obtain a level signal (53). Studies were carried out with a Leica TCS confocal microscope (Leica Microsystems, Heidelberg, Germany) with ×63 lens, numeric aperture 1.4, and pinhole size 50 µm. Microscope parameter values were set for the controls and kept constant thereafter. Signal intensity was recorded with a photomultiplier and converted in a discrete scale ranging from 0 to 248. With the quantification mode of the Leica TCS PowerScan software (Version 1.6.587, Leica Microsystems), a histogram was generated showing the distribution of pixels with different gray-scale intensities.

Measurement of ROS generation by cell membranes. The method of Pagano et al. (43), with centrifugation speeds modified according to Mohazzab-H and Wolin (41), was used to prepare membranes for measurement of ROS. The pellet and supernatant from the 100,000 g centrifugation were saved as representative of mitochondria-free membranes and lactate dehydrogenase-containing soluble elements of cytoplasm, respectively (41). Generation of ROS was measured by superoxide dismutase (SOD)-inhibitable lucigenin chemiluminescence (39, 57) in 500 µl of 50 µmol/l phosphate buffer (pH 7.0) containing 1 mmol/l EDTA, 150 mmol/l sucrose, 5 µmol/l lucigenin, 15 µg of cell membrane protein, 50 µg of cytosolic protein, and 100 µmol/l NADH or NADPH as substrate. Chemiluminescence (in arbitrary light units) was measured with a Turner model 20/20D luminometer (Turner Designs, Sunnyvale, CA) at 30-s intervals for 5 min with and without addition of 300 µl of SOD to determine dependence of light generation on O2 generation. The signal was expressed as the sum of all measurements after subtraction of the buffer blank (57).

Effect of raising intracellular [Ca2+]i on ROS generation. Ferricytochrome c reduction was used to determine whether O2 release into the extracellular medium could be stimulated by increasing intracellular [Ca2+]i. Near-confluent cells (70%) grown on 24-well plates were growth arrested for 48 h in serum-free medium, washed with Dulbecco’s phosphate-buffered saline (DPBS), and incubated with sodium bicarbonate-containing, phenol red-free Hanks’ balanced salt solution (HBSS) to which was added 1.25 mmol/l CaCl2, 160 µmol/l...
ferricytochrome c, and 1,000 U/ml bovine liver catalase (to prevent back-oxidation of ferricytochrome c by H2O2 produced from O2 dismutation). With an ELx800 automated microplate reader (Biotek Instruments, Highland Park, VT), the absorbance of each well was measured at 500 nm initially and at 15, 30, and 60 min after stimulation with 2 μM ionomycin to increase intracellular Ca2+. Monolayers were then washed with DPBS, and cell protein was measured with the bicinchoninic acid protein assay. Results were expressed as nanomoles of ferricytochrome c reduced per milligram of cell protein.

Reverse transcriptase-polymerase chain reaction detection of NAD(P)H oxidase components. To probe for presence of p22phox, gp91phox, and p67phox components of the putative analog of neutrophil NAD(P)H oxidase and the newly described gp91phox NOX homologs (16), semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR) was performed as recently described (12) on confluent cultures of proliferating cells with human gene-specific sense and antisense primers based on sequences published in GenBank: GPDH upstream 5′ ACCCACATGGGAGAGCTGG; GPDH downstream 5′ GCCCAGATTGCC; p22phox upstream 5′ GGACCCGTTGGGGAGACAATCATG; p22phox downstream 5′ GATGGTGCCCTCATGGTGCCGG; gp91phox upstream 5′ TGTTGACACATATCCTTGTGG; gp91phox downstream 5′ AAGGGGCAATACGGCATTCTGAT; NOX1 upstream 5′ CTGTTGTTGTTAACCATTGTTT; NOX1 downstream 5′ ACCAATGGCTTGATCCTCAG; NOX3 upstream 5′ ATGGAACCTCTGGGGTGACGTA; NOX3 downstream 5′GGAGGGGCGACACTCTGCT; NOX4 upstream 5′ TCTCATGTCACCTCTGCAACG; p47phox upstream 5′ ACCAGCCACCACTCACTATGG; p47phox downstream 5′ AGTGCGCTGGTGGAAGGTCT; p47phox downstream 5′ CGAGGAGCTGCTGCAGCTG; p67phox upstream 5′ CTGTTGACCTCTGCT; p67phox downstream 5′ CATGGGAAACTCGGTCAGT.

Except where indicated, amplification was carried out for 30 cycles for GAPDH, 32 cycles for p22phox, 34 cycles for NOX3, and 36 cycles for all other primers at 95°C for 1 min, 58°C for 1 min, and 72°C for 2 min, followed by an extension step at 72°C for 10 min. PCR products from defined bands were purified with QIAquick gel extraction kits (Qiagen, Chatsworth, CA) and sequenced automatically by an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA) with the same respective primers for sequencing as for PCR. RT-PCR was performed on RNA from human leukocytes (PMNs), Caco colon carcinoma cells, and adenovirus-transformed 293 human embryonic kidney cells as positive controls.

Immunoblot for proteins. Immunoblots of cell lysates were performed as detailed previously (12, 13) with phospho-specific antibodies against activated kinases. Membranes were then stripped and immunoblotted again with antibodies against respective nonphosphorylated proteins or β-actin to provide loading controls.

To measure protein expression of p22phox, gp91phox, or NOX5 in various cell compartments, immunoblots were performed on sequential fractions obtained during preparation of cell membranes. Membranes were prepared for immunoblots by harvesting and resuspending the DU 145 cells from six confluent T-75 flasks into ice-cold relaxation buffer (in mmol/l: 100 KCl, 3 NaCl, 3.5 MgCl2, 1.25 EGTA, 10 PIPES, pH 7.3) containing (in mmol/l) 2 sodium orthovanadate, 1 phenylmethylsulfonyl fluoride, and 5 sodium fluoride. Cells were then disrupted by three cycles of sonication for 15 s each at 60 W with a 4710 series Ultrasonic Homogenizer (Cole-Parmer Instruments) Unbroken cells and nuclei were pelleted by centrifugation at 500 g for 10 min at 4°C. The supernatant was carefully layered on top of a discontinuous gradient of 5 ml of 15% (wt/wt) sucrose on 5 ml of 34% (wt/wt) sucrose made up in relaxation buffer and centrifuged at 100,000 g for 40 min at 4°C in an L-80 ultracentrifuge (Beckman, Irvine, CA) with an SW41Ti rotor. The light membrane fractions were collected at the 15–34% sucrose interface and pelleted by ultracentrifugation at 100,000 g for 30 min at 4°C. Membranes were resuspended in electrophoresis boiling buffer, and proteins were immunoblotted with previously described mouse monoclonal antibodies prepared against whole human p22phox (44.1) (14) and gp91phox COOH-terminal peptide (54.1) (14) or rabbit polyclonal antisem (antiserum 8759) prepared against a mixture of unique NOX5 peptides (NH2-YESFKASDPGLRGSKRC-COOH, starting at amino acid 318; NH2-YRHRKRHTCPS-COOH, starting at amino acid 405). Primary antibodies were used at dilutions of 1:1,000.

Cellular localization of NAD(P)H oxidase components with confocal microscopy. Cells were plated on Lab-Tek chambered coverglass slides at a density of 20,000 per well and grown to 50–70% confluence. Cells were fixed for 30 min at room temperature in 2% buffered paraformaldehyde and washed once with DPBS and once with DPBS containing 0.1% saponin and 1% goat serum (DPBS-S-GS). Cells were then incubated for 30 min at room temperature with previously described primary antibodies, diluted in DPBS-S-GS at 5 μg/ml for antibodies against p22phox or gp91phox and 1 μg/ml for NOX5 antisem. After three 10-min washes with DPBS containing 1% goat serum, cells were incubated with fluorescent-conjugated goat anti-mouse (Alexa Fluor 488 goat anti-mouse IgG, Molecular Probes) or goat anti-rabbit (Alexa Fluor 488 goat anti-rabbit IgG, Molecular Probes) secondary antibodies, diluted in DPBS-S-GS at 1 μg/ml, and maintained at room temperature for 45 min in a humidified, darkened, covered chamber to prevent photobleaching from overhead light. Cells were washed again twice in DPBS containing 1% goat serum and then in some experiments incubated in the darkened, humidified chamber with propidium iodide (10 μg/ml in DPBS) for 15 min at room temperature to stain nuclei. After a final washing twice with DPBS-S-GS and once with DPBS, cells were examined under the ×40 objective of the Leica confocal microscope. Sequential images were collected for each sample field and averaged for a composite image of the cells. The images from each individual dye were overlaid to form composite pictures for determining cellular localization of the antigens.

Transfection protocol for antisense oligonucleotides against NAD(P)H oxidase components. To transfet antisense oligonucleotides for NAD(P)H oxidase components, cells were cultured in six-well plates at a density of 20,000 cells per well and grown in RPMI 1640 containing 10% FBS. After 24 h, cells were washed once with DPBS and 800 μl of RPMI 1640 (serum- and antibiotic free) was added to each well. Sense, antisense, and scrambled oligonucleotides or unique phosphorothioate antisense oligonucleotides for p22phox and gp91phox were constructed based on unique sequences chosen from GenBank: scrambled (from p22phox) 5′-CATGGTCATTACCGGCCTTGAGC-3′; p22phox-sense-1 5′-CGTTGTTTCTAGTTGGCAAC-3′; p22phox antisense-1 5′-GCACAAAATCCTACCTGGTG-3′; gp91phox antisense-1 5′-CCTCATTCACTACGCCGAGTT-3′; NOX5 sense-1 5′-GCCTACCTCCCTGAGAGAT-3′; NOX5 antisense-1 5′-AGCTCCTGCAAAGGTATG-3′; NOX5 sense-2 5′-TTC-
Scrambled oligonucleotides were used as controls for gp91<sub>phox</sub> antisense oligonucleotides. Oligonucleotides (0.5–2 μg) and 4.0 μl of Oligofectamine reagent (Invitrogen, Life Technologies) were diluted separately in 100 μl of serum- and antibiotic-free RPMI 1640 and incubated at room temperature for 15 min. Oligonucleotides and Oligofectamine were then mixed and incubated together for another 20 min. This mixture was then added to each well, and cells were incubated at 37°C. After 6 h, the transfection mixture was gently removed and replaced with 2.5 ml of RPMI 1640 containing 10% FBS. Cells were incubated an additional 48 h, fixed with 5% ice-cold buffered formalin, permeabilized with two 30-min treatments of ice-cold methanol, stained with Wright’s modified Giemsa, and counterstained with eosin. Direct cell counts were then performed on five random fields/well at a magnification of ×100 with a 1-mm<sup>2</sup> ocular grid. Confirmation that antisense transfection inhibited protein expression was obtained by immunoblotting performed after transfection reagent was removed and cells were incubated an additional 12 h with complete medium. Finally, to determine whether transfection of antisense oligonucleotides inhibited function, cells were transfected with antisense oligonucleotides for 6 h, recovered 12 h in complete medium containing 10 μM DPI, and imaged by confocal microscopy to determine a cell fluorescence intensity score, as described in Measurement of ROS generation by intact cells.

**DNA cell cycle measurements.** To study the effect of NAD(P)H oxidase inhibition on the DNA cell cycle, cells were grown to confluence in 25-cm<sup>2</sup> plastic flasks and treated with 10% FBS in RPMI 1640, with or without 20 μmol/l DPI. After 12 h cells were trypsinized, washed twice in cold DPBS with
1 mM EDTA and 1% BSA, fixed 30 min in ice-cold 70% ethanol, and stained by incubation for 30 min at 37°C in a 10 mg/ml solution of propidium iodide in DPBS and 1 mg/ml RNase A. DNA cell cycle measurements were made with a FACStarPLUS flow cytometer (Becton Dickinson).

Measurement of apoptosis. Apoptosis was studied by a variety of methods. Nuclear and cellular morphology were studied by simultaneous staining with 5 µg/ml Hoechst 33342 to determine nuclear morphology and total nonspontaneous fluorescence and 1,425 for DPI; median fluorescence was 2,102. Pretreatment of cells for 15 min with DPI (50 µmol/l) reduced DCF oxidation to DCF by 50%. Cytosolic activity of NADPH oxidase was measured by oxidation of DCFH-DA, harvested, and resuspended twice with an equal volume of phenol-chloroform (1:1; Tri saturated, pH 8.0) and treated with 40 µg/ml of RNase A for 1 h at 37°C and 50 µg/ml of proteinase K for 30 min at 37°C. DNA was then precipitated overnight at −20°C with 0.1 volume of 3 M sodium acetate (pH 5.2), 1 volume of isopropanol, and 1 µl of 20 mg/ml glycerol per 600 µl of isopropanol. After microfugation at 14,000 rpm for 15 min and washing with 70% ethanol, the DNA pellet was air dried, resuspended in 20 µl of alkalized H2O2 (DNA hydration solution, Gentra Systems, Minneapolis, MN), and heated at 55°C for 15 min. For each experiment, all the fragmented DNA from two 100-mm culture dishes was electrophoresed on a 2% agarose gel for 4 h at 40 V. Fragmented DNA was stained with ethidium bromide and photographed under UV light.

Electrophoretic mobility shift assays. Nuclear protein was isolated and DNA binding reactions were performed and quantitated as previously detailed (12) with consensus oligonucleotides for the cAMP response element (CRE) (5'-AGAGGTCC-CTGACGTCAGAGCTAG-3' and 3'-TCTCTCACCCAGACTGAGA-5'). Mem, membrane; Cyto, cytosol; Mem+Cyto, membrane + cytosol; Mem+PhenArs, membrane + phenylarsine. Membrane-bound luciferase activity is expressed relative to the untreated control and is presented as the mean ± SE. The significance of differences in luciferase activity was determined by one-way ANOVA. Values are means ± SE of three experiments. *P < 0.001 vs. untreated or NADPH treatment.
incubating the binding reaction with 1 μg of supershifting antibody (Santa Cruz Biotechnology) before electrophoresis.

Human tissues. Five primary tumors and one adjacent normal tissue from patients diagnosed with adenocarcinoma of the prostate were selected from samples residing in the Global Repository at Genomics Collaborative (Cambridge, MA). Staging and grading were established by pathologists at the site of collection and were confirmed by pathologists at Genomics Collaborative. All samples were obtained from human subjects with full informed consent. Tissue samples were procured from surgical specimens and flash frozen in liquid nitrogen. All samples were then stored in liquid nitrogen vapor until RNA was extracted and quality controlled at Genomics Collaborative.

Statistical analysis. Data are expressed as means ± SE for a minimum of four observations, unless otherwise indicated. Differences between two groups were compared with the unpaired Student’s t-test. Two-tailed tests of significance were used. Differences between multiple groups were compared with one-way analysis of variance or repeated-measures two-way analysis of variance for analysis of group, time, and group-time interactions. If significant interactions were found, Tukey’s or Newman-Keuls post hoc multiple-comparisons tests were used.
Fig. 6. DU 145 cells express protein for NOX5 and for the gp91phox, but not p22phox, component of the phagocyte NAD(P)H oxidase: cellular localization of classic phagocyte NAD(P)H oxidase membrane components and NOX5 with confocal microscopy. Cells were fixed and immunostained with primary mouse monoclonal antibodies against human p22phox (14) or gp91phox (14) and secondary fluorescent-conjugated goat anti-mouse antibodies (Alexa Fluor 488 goat anti-mouse IgG; Molecular Probes) or primary polyclonal rabbit antiserum against a mixture of unique NOX5 peptides and secondary fluorescent-conjugated goat anti-rabbit antibodies (Alexa Fluor 488 goat anti-rabbit IgG; Molecular Probes). Immunostained cultures were then examined under a ×40 objective with a Leica TCS confocal microscope (Leica Microsystems). Sequential images were collected for each sample field and averaged for a composite image of the cells. The images from each individual dye were overlaid to form composite pictures for determining cellular localization of the antigens. A: green fluorescent labeling for p22phox was notably absent in DU 145 cells. Nuclei are counterstained red with propidium iodide. Control cultures treated identically except for addition of primary antibodies showed no green fluorescence. B: green fluorescent labeling for gp91phox localized throughout the cytoplasmic compartment, presumably in cytoplasmic membrane structures, but some activity was also found over the nucleus. Control cultures treated identically except for addition of primary antibodies showed no green fluorescence. C: green fluorescent labeling for NOX5 localized throughout DU 145 cells, with perinuclear enhancement. Control cultures treated identically except for addition of primary antibodies showed no green fluorescence. D: immunoblots were performed of cell lysate (lanes 1 and 4), nuclear protein (harvested as for electrophoretic mobility shift assays, lanes 2 and 5), and membranes (lanes 3 and 6) with primary rabbit antiserum (antiserum 8579) to unique NOX5 peptides. In lanes 4–6 primary antiserum was incubated overnight with a mixtures of antigenic blocking peptides (NH$_2$-YESFKASDPLGRGSKRC-COOH, beginning at amino acid 318; NH$_2$-YRHQKRKHTCP-S-COOH, beginning at amino acid 405) before use. The 85-kDa band detected in lysate (lane 1), nuclear protein (lane 2), and membranes (lane 3) was virtually eliminated (lanes 4–6, respectively) by incubation of antiserum with blocking peptides before use.
comparisons tests were applied to locate the sources of differences. Significance was assumed at $P < 0.05$.

**RESULTS**

DU 145 prostate cancer cells produce ROS. To determine whether proliferating DU 145 cells spontaneously generate ROS, we treated cells with DCFH-DA and, with confocal microscopy (Fig. 1) or flow cytometry (Fig. 2), determined whether it was oxidized to DCF, indicating the intracellular production of $\text{H}_2\text{O}_2$ and other oxidants (52). DU 145 cells oxidized DCFH to DCF diffusely throughout the cell, with focally increased cytoplasmic oxidation in a linear reticular pattern that was accentuated in areas bordering the nucleus (Fig. 1, top). Enzymatic potential for ROS production from NADPH was localized to membranes but not cytoplasm (Fig. 3). DU 145 cell membranes readily supported SOD-inhibitable luciferin chemiluminescence (Fig. 3). In contrast, chemiluminescence was not sustained by cytosol and addition of cytosol to the detection system along with membranes did not increase ROS generation compared with membranes alone (Fig. 3). At the luciferin concentration of 5 $\mu$mol/l used to prevent artifactual redox cycling (39, 57), the preferred substrate for ROS generation was NADPH rather than NADH (Fig. 3). This indicates that ROS production is more likely from activity of a membrane-localized NOX oxidase (16), which utilizes NADPH as an electron donor (39, 57), than from cytosolic NADPH:quinone oxidoreductase (NQO), which equivalently utilizes NADH or NADPH (20), or microsomal NQO, which preferentially utilizes NADH (31).

Chemiluminescence was significantly inhibited by addition of SOD (Fig. 3; 300 U/ml), suggesting that light emission is the result of $\text{O}_2^\cdot$ generation. Membrane chemiluminescence was substantially reduced by the flavoprotein inhibitor DPI (Fig. 3; 25 $\mu$mol/l) or the NAD(P)H oxidase inhibitor phenylarsine (Fig. 3; 1 $\mu$mol/l), suggesting that ROS in DU 145 cells are likely derived from a membrane NAD(P)H oxidase. DCFH oxidation was also reduced by DPI (Fig. 1B, 25 $\mu$mol/l; Fig. 2, 50 $\mu$mol/l) but not eliminated, consistent with the probability that other metabolic sources of oxidant generation also exist. Specifically, DPI virtually eliminated (Fig. 1B) the cytoplasmic reticular and perinuclear enhancement of DCF concentration prominent in untreated cells (Fig. 1A).

DU 145 prostate cancer cell growth is reduced by antioxidants and by NAD(P)H oxidase inhibitors. To determine whether ROS produced by DU 145 cells were functionally important for cell proliferation, we treated cells with a variety of ROS scavengers and followed their anchorage-dependent growth for 72 h (Fig. 4A). DU 145 proliferation was significantly reduced by addition of the $\text{H}_2\text{O}_2$ scavenger catalase (3,000 U/ml) or the glutathione peroxidase mimic ebselen (20 $\mu$mol/l) (56) to growth medium (Fig. 4A). SOD (1,000 U/ml), which is restricted to the extracellular compartment, failed to affect growth, but growth was substantially reduced by the cell permeant semiquinone-forming spin trap 4,5-dihydroxy-1,3-benzene disulfonic acid (Tiron; 10 mmol/l), which has been used in biological studies (63) as an intracellular $\text{O}_2^\cdot$ scavenger (25). This suggests that $\text{O}_2^\cdot$ generation is from an

![Fig. 7. Antisense oligonucleotides for NOX5 inhibit DU 145 cell proliferation and ROS generation. A: NOX5 antisense oligonucleotides reduce DU 145 cell growth. DU 145 cells (20,000 cells/well) were seeded into 6-well plastic plates and grown for 24 h. Cells were transfected with 1 of either of 2 pairs of antisense oligonucleotides for NOX5 (1 $\mu$g) with Oligofectamine as detailed in MATERIALS AND METHODS. After 6 h of exposure, transfection solution was replaced with complete medium and cells were grown an additional 48 h. Cells were fixed with formalin, permeabilized with methanol, stained with Wright’s modified Giemsa, and counterstained with eosin. Direct cell counts were made on 5 random fields/well at ×100 power with a 1-mm$^2$ ocular grid. Compared with untreated controls, transfection reagent-treated controls, and cells treated with sense oligonucleotides, treatment with NOX5 antisense oligonucleotides significantly reduced subsequent growth. NOX5–2 antisense oligonucleotides were more effective than NOX5–1 antisense oligonucleotides at suppressing growth. Photomicrographs at bottom are cells treated with NOX5–2 sense or antisense oligonucleotides. *$P < 0.001$ vs. untreated cells, reagent-treated cells, or cells treated with NOX5 sense oligonucleotides. B: NOX5 antisense oligonucleotides suppress expression of NOX5 protein. DU 145 cells (20,000 cells/well) were cultured overnight in 6-well plates in RPMI 1640 with 10% FBS, transfected as in A for 6 h with NOX5 sense or antisense oligonucleotides, and grown in RPMI 1640 with 10% FBS for an additional 12 h. The cells in replicate wells (3 wells each) were then lysed in a pooled manner by scraping the cells of the first well into 250 $\mu$l of lysis buffer and then transferring the lysate sequentially to the next well before scraping. Immunoblots were performed for NOX5 on equal amounts of protein, and the membrane was stripped overnight and then immunoblotted again for β-actin as a loading control. Transfection reagent and NOX5–1 or NOX5–2 sense oligonucleotides did not affect protein expression, but NOX5–1 antisense oligonucleotides moderately inhibited and NOX5–2 antisense oligonucleotides substantially inhibited expression of NOX5 protein, paralleling their potency in A for inhibiting DU 145 cell proliferation. C: ROS generation by DU 145 cells is stimulated by increases in intracellular $\text{Ca}^{2+}$ concentration ([Ca$^{2+}$]). Because NOX5 is the only NOX homolog known to be regulated by [Ca$^{2+}$] (7, 16), this provides additional support for NOX5 as the source of growth-regulatory ROS important in DU 145 cell proliferation. Near-confluent DU 145 cells (70%) grown on 24-well plates were growth arrested for 48 h in serum-free medium, washed with Dulbecco’s phosphate-buffered saline (DPBS), and incubated with sodium bicarbonate-containing, phenol red-free Hank’s balanced salt solution (HBSS) with 160 $\mu$mol/l ferricytochrome c and 1,000 U/ml bovine liver catalase (to prevent back-oxidation of ferrocytochrome c by $\text{H}_2\text{O}_2$ produced from $\text{O}_2^\cdot$ dismutation), with or without 1.25 mmol/l CaCl$_2$ (Ca) or 1,000 U/ml SOD. With a microplate reader, ferricytochrome c reduction was followed by measuring the absorbance of each well at 550 nm measured initially and at 15, 30, and 60 min after stimulation with 2 $\mu$mol/l ionomycin (I) to increase intracellular [Ca$^{2+}$]. Results are expressed as nanomoles of ferricytochrome c reduced per milligram of cell protein. *$P < 0.001$ vs. controls not stimulated with ionomycin or cells stimulated with ionomycin in HBSS; **$P < 0.001$ vs. cells stimulated with ionomycin in the presence of Ca$^{2+}$.
intracellular source. DU 145 proliferation was also reduced in dose-dependent fashion (Fig. 4B) by the flavoprotein inhibitor DPI (1–25 μmol/l), which blocks NAD(P)H oxidases (42). In contrast, DU 145 growth was not affected by treatment of cultures with allopurinol (1 mmol/l) or Nω-nitro-l-arginine (100 μmol/l), inhibitors of the other major cell flavoprotein oxidases, xanthine oxidase and nitric oxide synthase, respectively (data not shown).

DU 145 prostate cancer cells variably express mRNA and protein for membrane components of the phagocyte NAD(P)H oxidase, but they are not functionally important for proliferation. The ability of DPI to inhibit both ROS generation (Figs. 1–3) and proliferation (Fig. 4B) suggested that ROS production in DU 145 cells is from a growth-regulatory NAD(P)H oxidase similar to those recently identified in other nonphagocyte cell lines (16). We therefore sought to determine whether components of the classic phagocyte membrane NAD(P)H oxidase or its reported homologs are expressed in DU 145 prostate cancer cells. When RT-PCR was performed on RNA extracted from proliferating cells stimulated with 10% FBS, DU 145 cells were found to express RNA for the classic phagocyte p22^phox mem-
brane subunit (Fig. 5, lane 2), which was initially detected at 32 cycles. The 252-base pair PCR product obtained was sequenced and found to be identical to bases 221–471 of the reported human mRNA sequence (GenBank accession no. NM000397). Next, using monoclonal primary and fluorescent-conjugated secondary antibodies, we immunostained and viewed DU 145 cells under confocal microscopy to localize the sites of residence for NAD(P)H oxidase protein components expressed within the cell. In neutrophils, p22^phox^ and gp91^phox^ are assembled into a single functional complex primarily associated with cytoplasmic secretory vesicles and specific granules in the resting state and are transferred to the plasma membrane by fusion events in the activated state (4). Given the relatively abundant mRNA (Fig. 5), we therefore expected to see diffuse staining throughout the cell, corresponding to abundant p22^phox^ protein. Instead, we could not detect immunostaining for p22^phox^ in DU 145 cells (Fig. 6A), and in immunoblots of protein extracted from cell membranes there was no prominent band of appropriate size corresponding to p22^phox^ (data not shown).

DU 145 cells express mRNA and protein for the NOX5 NAD(P)H oxidase, which is functionally important for ROS generation and cell proliferation. The disparate protein expression in DU 145 cells of the two membrane components of the classic phagocyte oxidase, combined with evidence for production of ROS, suggested the possibility that another NAD(P)H oxidase might perform a signaling in DU 145 cells. We therefore looked for expression of other NOX homologs in this cell line. No evidence was found in DU 145 cells for NOX1 (Fig. 5, lane 6) or NOX4 (lane 7), and NOX3 was also absent in DU 145 cells (data not shown). However, NOX5 was prominently expressed after 36 cycles as a 239-base pair product that was sequenced and shown identical to bases 859–1097 of the reported GenBank sequence (accession no. AF317889). Therefore looked for NOX5 protein expression in this cell line, using polyclonal rabbit antiserum against specific NH2- and COOH-terminal peptides unique for NOX5. When this antiserum was used to immunostain DU 145 cells, fluorescent staining specific for the anti-rabbit secondary antibody was seen diffusely throughout the cell with perinuclear enhancement (Fig. 6C), similar to the pattern of oxidation seen for DCFH-DA (Fig. 1A). A distinct protein band of the expected 85-kDa size was seen in DU 145 total cell lysate (Fig. 6D, lane 1) and in the 100,000 g membrane fraction (lane 3). Consistent with perinuclear enhancement of staining by confocal microscopy, an 85-kDa protein band was also detectable by immunoblots of nuclear protein prepared as for electrophoretic mobility shift assays (Fig. 6D, lane 2). These bands were abolished in immunoblots of respective cell fractions when antiserum was neutralized with a mixture of both antigenic peptides before primary incubation (Fig. 6D, lanes 4–6), suggesting that the 85-kDa bands in Fig. 6D, lanes 1–3, represent NOX5. When the functional significance of NOX5 for cell proliferation was studied, antiserum but not sense oligonucleotides against NOX5 dramatically reduced DU 145 cell proliferation compared with controls (Fig. 7A). Transfection with NOX5 antisense oligonucleotides also reduced expression of NOX5 protein (Fig. 7B) and decreased intracellular production of ROS, measured by oxidation of DCFH-DA visualized with semiquantitative confocal microscopy (mean cell fluorescence signal intensity = 7.9 ± 1.4 for control cells vs. 3.3 ± 0.3 for cells transfected 12 h earlier with NOX5 antisense oligonucleotides; n = 3; P < 0.05). NOX5 is unique among the NOX homologs in having an NH2-terminal extension of three EF hands conferring an ability to generate O2 in response to cytosolic free [Ca2+] elevations (7, 16). Therefore, we studied the effect of increased intracellular Ca2+ on ROS generation by this cell line. In the presence of Ca2+-containing BHSS, the Ca2+ ionophore ionomycin caused a prompt increase in ferricytochrome c reduction by cultured DU 145 cells (Fig. 7C). This response was inhibited by addition of SOD and was absent when cells were stimulated with ionomycin in Ca2+-free BHSS. Together, these data suggest that NOX5 is a biologically important NOX.
NAD(P)H oxidase regulates growth in prostate cancer cells.

**A** RT-PCR was performed on nearly confluent LNCaP cells. LNCaP cells expressed only NOX1 (lane 6, 36 cycles) and NOX5 (lane 8, 36 cycles). GAPDH is shown in lane 1. PCR products were compared with those from an equal amount of mRNA from human PMNs (lane 2 for p22phox, lane 3 for gp91phox, lane 4 for p47phox, and lane 5 for p67phox), Caco colon carcinoma cells (lane 6 for NOX1 and lane 7 for NOX4), or adenovirus-transformed human embryonic kidney 293 cells (lane 8 for NOX5), respectively, as shown. No NOX3 was found in LNCaP cells. RT-PCR was performed with human gene-specific sense and antisense primers as detailed in the text. B: antioxidants inhibit anchor-

dependent growth of androgen-responsive LNCaP prostate cancer cells. Cells stimulated with 10% FBS were plated at a density of 50,000 cells/well, and antioxidants were added to wells at the indicated concentrations. After 72 h, proliferation was quantitated by assessing the cell number-dependent reduction of the soluble yellow tetrazolium MTT to its insoluble formazan, measured as A540. A control was also performed for DMSO (5 μl/well), used as a vehicle for elselen. Cell growth was inhibited by the H2O2 scavenger catalase (3,000 U/ml), the glutathione peroxidase mimic elselen (20 μmol/l), and the sulfhydryl donor N-acetylcysteine (NAC; 20 mmol/l). The O2 scavenger SOD (1,000 U/ml) reduced proliferation to a much lesser degree than other antioxidant treatments. *P < 0.001 vs. untreated control; †P < 0.001 vs. DMSO control.

Fig. 9. NOX5 is widely expressed in prostatic adenocarcinomas. RT-PCR was performed on RNA samples (Genomics Collaborative, Cambridge, MA) from 5 adenocarcinomas of the prostate (SR10KACT, 3FB4KN3H, STJ7PAFX, TSJC4AWY, and VHVIPNPT) and from 1 specimen of normal prostate (3FB4KA3U) with the same primers used in Figs. 5 and 8. NOX5 was widely expressed in prostatic adenocarcinoma and was also found in normal prostate gland. Bands shown for NOX5 are after 35 cycles, and those shown for GAPDH are after 32 cycles of PCR.

**C** NOX5 oxidase components are also present in the LNCaP prostate cancer cell line and in clinical specimens of prostate cancer. The existence of p22phox, gp91phox, and NOX5 in DU 145 cells prompted us to examine a widely studied androgen-responsive prostate cancer cell line for evidence of NAD(P)H oxidase components. LNCaP cells (Fig. 8A) had no p22phox, gp91phox, p47phox, or p67phox, but NOX5 was strongly expressed and prominent PCR product was also obtained for NOX5. Similar to the results with DU 145 cells, antioxidants and DPI strongly inhibited anchorage-dependent growth of LNCaP cell lines (Fig. 8B), affirming the importance of ROS for prostate cancer proliferation.

To determine how widely NOX5 is expressed in adenocarcinomas of the prostate, we performed RT-PCR on RNA from five clinical prostate cancer specimens and one specimen of normal prostate gland. The results, displayed in Fig. 9, show NOX5 to be widely distributed in both malignant and normal prostate tissue, indicating that it is not a marker of malignant transformation but is more likely a normal component of cell signaling cascades important for malignant proliferation.

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Effect of NAD(P)H oxidase inhibitors on growth signaling cascades, transcription factor DNA binding, and apoptosis. Because of the dramatic growth inhibition caused by NAD(P)H oxidase inhibition or NOX5 antisense oligonucleotides, we hypothesized that ROS from an NAD(P)H oxidase might promote growth signaling by negatively regulating redox-sensitive phosphatases that control MAP kinase activities (8), or by positively enhancing protein kinase C (PKC) activity through oxidative inactivation of the cysteine-rich zinc finger motif in the NH2-terminal regulatory domain that inhibits PKC activity (35). To test these hypotheses, we performed immunoblots on control and DPI-treated DU 145 cell cultures with phosphospecific antibodies for the phosphorylated active enzyme forms of the three classic MAP kinases. DPI did not inhibit constitutive activation of the p42/p44 MAP kinases (ERK1/ERK2), the c-Jun NH2-terminal kinase (JNK), or the p38 stress-activated MAP kinase (p38 MAPK), and DPI also failed to reduce phosphorylation of protein kinase B/Akt (data not shown). This suggested that NAD(P)H oxidase inhibition disrupted DU 145 cell growth by a mechanism distinct from disruption of growth factor-related signaling cascades.

Vitamin E and other antioxidants were previously shown to induce apoptosis in DU 145 prostate cancer cells (26). Recently, caspase-3 activity and apoptosis were induced in NOX1-expressing guinea pig gastric mucosal cells by treatment of cultures with the ROS scavengers N-acetylcysteine or SOD plus catalase or by inhibition of NOX1 oxidase activity with DPI (61). O2 was recently shown to protect cells from apoptotic stimuli (18, 45, 46), and ROS can inhibit and inactivate cysteine-dependent proapoptotic caspases by oxidative modification of the thiol group necessary for protease activity (27). We therefore explored whether ROS from NOX oxidases might inhibit DU 145 cell proliferation by similarly inducing apoptosis in proliferating cells. To remove the trophic effect of serum and better unmask any proapoptotic effect of NAD(P)H oxidase inhibition, we performed experiments similar to those reported in normal gastric mucosal cells (61) and incubated confluent cultures overnight in serum-free medium with or without treatment. Under these conditions DPI and, to a lesser extent, antioxidants induced the apoptotic phenotype in DU 145 prostate cancer cells compared with cells incubated in medium alone (Fig. 10, A–C). DPI-treated cells demonstrated chromatin condensation and fragmentation and showed evidence of fluorescent staining for annexin V, suggesting externalization of phosphatidylserine to the membrane surface (data not shown). Induction of apoptosis was confirmed by evidence of DNA fragmentation in agarose gels (Fig. 10D) and by flow cytometry (Fig. 10E), showing that DPI treatment increased the number of apoptotic aggregates. Finally, DPI treatment also substantially augmented caspase activity. Caspase-3 activity was detectable in untreated control prostate cancer cells after 24 h in serum-free medium (0.26 ± 0.01 μmol·min⁻¹·mg protein⁻¹) but was increased 10-fold in cells treated 24 h with DPI (2.11 ± 0.24 μmol·min⁻¹·mg protein⁻¹; P < 0.001). Thus one important function of an NAD(P)H oxidase in prostate cancer may be oxidant inhibition of caspase activity, thereby retarding cell death.

Others have suggested that disruption of NAD(P)H oxidase function or scavenging of its O2 product may induce apoptosis by inhibiting trophic oxidant activation of the transcription factor NF-κB (61), which up-regulates a number of antiapoptotic proteins in malignancy.

**Fig. 10.** NAD(P)H oxidase inhibition induces apoptosis of DU 145 prostate cancer cells. A and B: nuclear condensation and fragmentation. DU 145 cells were grown to 50% confluence in RPMI 1640 containing 10% FBS, and they were treated with vehicle (A) or 20 μmol/l DPI (B) for 12 h. These cells were then stained with Hoechst 33342 dye. In vehicle control cells (A) the Hoechst 33342 nuclear fluorescence pattern was normal, and no chromatin condensation or nuclear fragmentation was evident at 12 h. In contrast, DU 145 prostate cancer cells treated with DPI (B) showed condensed, fragmented nuclei characteristic of apoptotic cells (some marked with arrows). Micrographs were taken at ×485 magnification. C: apoptosis was quantitated morphologically by the method of Pollack and Ciancio (47), as modified by Hemendinger and Bloom (29). Cells were seeded onto 4-well chamber coverslips, grown to 60% confluence, and treated for 24 h with 20 μmol/l DPI or 10 mM Tiron in serum-free RPMI 1640. Cells were stained by addition of 100 μg/ml Hoechst 33342 (blue fluorescence) and 20 mg/ml propidium iodide (PI; red fluorescence at 37°C in the dark). The double fluorescence was detected by a Leitz microscope with an epifluorescence system and a long-pass filter block for UV excitation (BP 340–380 and long-pass emission LP 425). Dead cells fluoresce red, and live cells fluoresce blue. Apoptotic cells have a characteristic phenotype of condensed, segregated chromatin in intact but shrunken cells. The apoptotic phenotype is easily discriminated from the necrotic phenotype. Necrotic cells are characterized as swollen, having irregular, damaged membranes, being PI positive, and showing minimal condensation of the chromatin with some accumulation near the nuclear membrane. One hundred cells were scored for each sample by a blinded observer (M. Hemendinger) and classified as necrotic, apoptotic, or live. *P < 0.05 vs. untreated; *P < 0.001 vs. untreated. D: DNA ladder formation. DU 145 cells were treated as in C, and cells were harvested and lysed. DNA was extracted as detailed in MATERIALS AND METHODS, treated with RNase A and proteinase K, and subjected to 2% agarose gel electrophoresis for 4 h to detect DNA fragmentation. The fragmented DNA was stained with ethidium bromide and visualized under UV light. Compared with vehicle-treated cells (lane 1), DU 145 prostate cancer cells incubated in serum-free medium 24 h with 20 μmol/l DPI (lane 3) showed marked increase in DNA fragmentation, characteristic of accelerated apoptosis. Results were compared with a positive control treated with 1 μmol/l staurosporine (lane 2). E: flow cytometry analysis of DU 145 cells treated with and without DPI. Near-confluent (70%) monolayers of DU 145 cells were incubated 12 h in the presence or absence of 20 μmol/l DPI. Cells were harvested, ethanol fixed, treated with PI and RNase A, and subjected to DNA cell cycle analysis by flow cytometry. Compared with vehicle-treated cells (top), DU 145 prostate cancer cells incubated with DPI (bottom) demonstrated greater accumulation of apoptotic cell aggregates.
nant cells (6). In DU 145 cells DPI treatment modestly increased DNA binding of NF-κB (data not shown). However, DPI inhibited DNA binding of activating transcription factor (ATF)/CRE binding protein (CREB) family transcription factors (Fig. 11A), which have not been well studied in prostate cancer cell lines but are essential for survival of other malignant cell types such as melanomas (33). Therefore, NAD(P)H oxidase activity may provide an important survival function in prostate cancer cells by influencing multiple pathways retarding apoptosis.

**DISCUSSION**

For several years a growing body of evidence has pointed to a role for a growth-signaling oxidase in the pathogenesis of prostate cancer. However, the metabolic basis for these observations has been unclear. We
now present evidence that signaling ROS may be generated by a growth-regulatory NAD(P)H oxidase similar, but not identical, to that in PMNs. Generation of ROS by whole DU 145 cells and their membranes was reduced by the flavoprotein inhibitor DPI, consistent with an NAD(P)H oxidase as the oxidant source. RT-PCR demonstrated evidence in DU 145 cells for the four main phagocyte NAD(P)H oxidase components, with an NAD(P)H oxidase as the oxidant source. Replicate cultures (n = 3/group) of proliferating DU 145 cells at ~50% confluence were treated with and without 20 μmol/l DPI added to culture medium. After 12 h cells were lysed, nuclear protein was extracted, and EMSAs were performed with 32P-end-labeled consensus oligonucleotides for CRE (5′-AGAGATTGCT-GAGCTCAGAGACTAG-3′ and 3′-TCTCTCTACGACTGACTCTCA-5′) or for OCT1 (5′-TGTCGAATG-CAAATCTAGAA-3′ and 3′-ACAGCTCTAGTTATGCCATTT-5′) as a loading control. Competition experiments were performed with 10× respective unlabeled wild-type oligonucleotide sequences, and supershift experiments were carried out by incubating the binding reaction with 1 μg of supershifting antibody.

In phagocytes, the NAD(P)H oxidase consists of two membrane proteins, gp91phox and p22phox, that bind a flavin adenine nucleotide (FAD) and form a cytochrome with a redox midpoint potential of −245 mV (4). At least two and possibly three cytosolic proteins (p47phox, p67phox, and p40) are also essential, and several other cytosolic components participate, including the small GTPases Rac1 and Rac2. The oxidase is thought to contain all the factors necessary for transporting electrons from the donor substrate NADPH via FAD to generate O2 from molecular O2. The nonphagocytic NAD(P)H oxidase shares some of the components with its phagocyte cousin, but with critical distinctions, including a delayed time course for activation and a lower level of activity (4). Endothelial cells appear to express all the phagocyte oxidase components, including gp91phox, p22phox, p47phox, and p67phox (24). In contrast, vascular smooth muscle cells express p22phox, p47phox, and the unique homologs NOX1 and NOX4 (36). The putative DU 145 NAD(P)H oxidase shares some properties with the phagocytic oxidase, utilizing NADPH to produce O2. However, in the phagocyte, p22phox and gp91phox are colocalized to membranes, whereas their intimate association is required for function (4). In DU 145 cells, gp91phox was identified in the cytoplasmic compartment by confocal microscopy and immunoblot of membranes. In contrast, although mRNA transcript was present, p22phox protein product was not detectable in DU 145 cells. Furthermore, antisense oligonucleotides for p22phox or gp91phox did not inhibit DU 145 prostate cancer cell growth. Because gp91phox is not thought to function without p22phox, it is likely that another oxidase must be responsible for growth-regulatory ROS production in DU 145 cells. NOX5 is a possible candidate. It is strongly expressed in DU 145 cells in a pattern corresponding with the perinuclear enhancement of DCFH-DA oxidation. In addition, although there are limitations to this approach (58), transfection of NOX5 antisense oligonucleotides significantly reduced NOX5 protein expression, DU 145 cell proliferation, and ROS production. Further...
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 thermore, ROS production in DU 145 cells is sensitive to calcium regulation, as previously reported for prostatic carcinoma (54). Of all the reported NOX homologs, only NOX5 has the three NH2-terminal EF hands rendering it capable of generating O2- in response to cytosolic free [Ca2+]i elevations (7, 16). When transfected into cells, NOX homologs generate ROS independently without assistance from the other NAD(P)H oxidase components needed for PMN oxidase function (2, 3, 7, 59). NOX5 is found in prostate cancer cell lines and in a multitude of developing organs in the fetal state (16), but it was previously reported to be absent in normal adult prostate tissue (7, 16). However, we found abundant NOX5 expression by RT-PCR of mRNA from normal prostate gland. Thus expression of the NOX5 oxidase is not a unique marker of the malignant state but may be a normal signaling enzyme important for neoplastic proliferation.

The exact growth signaling pathways for NAD(P)H oxidases are presently uncertain and may be tissue specific. Whereas angiotensin II stimulates NAD(P)H oxidase activity in vascular smooth muscle through stress-activated protein kinase pathways (36), the NAD(P)H oxidase regulates proliferation of human airway smooth muscle (11) and melanomas (12) in part through NF-kB. DPI did not inhibit NF-kB DNA binding in DU 145 cells but did decrease DNA binding to CRE. ATF/CREB family proteins have not been well studied in prostate cancer but act as survival factors in other malignancies (39) and positively regulate important cell cycle events such as expression of cyclins D1 (37) and A (10). How inhibition of intracellular oxidant production from an NAD(P)H oxidase might lead to disruption of DNA binding of ATF/CREB family transcription factors is presently unclear. One candidate pathway in prostate cancer cells is p90 RSK, which plays a prominent role in transcriptional activation (21), is activated by ROS (1), and mediates the growth of prostate cancer tumor spheroids induced by a mitogen-stimulated NADPH oxidase (54). Nonphagocytic NADPH oxidases might also provide a “feed-forward” mechanism to reinforce kinase-based growth signaling pathways by oxidatively inactivating the protein phosphatases that negatively regulate MAP kinase signaling (8). Abnormalities in phosphatase expression or function have been implicated in the etiology of a number of malignancies (44). Alternately, ROS from growth-regulatory NAD(P)H oxidases might stimulate PKC through thiol modification of the zinc finger motif in the PKC NH2-terminal autoregulatory domain (35). However, neither phosphatase inhibition nor PKC activation is consistent with our findings. DPI failed to decrease activities of MAP kinase cascades or inhibit progression through the cell cycle. Therefore, other mechanisms must explain the importance of NAD(P)H oxidase activity for DU 145 cell growth.

In addition to limitless replicative potential, another of the hallmarks of cancer is the ability of tumor cell populations to evade normal mechanisms of apoptosis (28). Several observed mechanisms explain the acquired trait of eluding programmed cell death, including mutations in the p53 tumor suppressor gene (28), augmented expression of NF-kB-linked antiapoptotic proteins (6), and enhanced activity of the phosphatidylinositol 3-kinase (PI3-kinase)-Akt/protein kinase B pathway through mutational loss or inactivation of the PTEN tumor suppressor (15). Oxidants in general have long been thought only to stimulate apoptosis. However, this view has been modified recently (45). Concentrations of H2O2 that trigger apoptosis have been shown to substantially inhibit cellular NAD(P)H oxidase activity, producing a significant decrease in intracellular O2- concentration, reduction of the intracellular milieu and acidification of intracellular pH (17). Scavenging intracellular O2- increases the sensitivity of melanomas to induction of apoptosis by cancer chemotherapeutic agents (46), and inhibiting NAD(P)H oxidase activity with DPI prevents phorbol ester-induced resistance of melanomas to Fas-mediated apoptosis (18). Thus O2- from an NAD(P)H oxidase might provide trophic inhibition of apoptosis through redox activation of NF-kB (11) or oxidative inactivation of PTEN with enhancement of PI-3K activity (38), with interrelationships between the two mechanisms (5, 40, 51). Another potential mechanism by which NAD(P)H oxidases might retard apoptosis is by reversible inhibition of caspases in a fashion similar to that by which O2- reversibly regulates protein tyrosine phosphatase 1B (PTP1B), through modification of cysteine thiol to generate an unstable sulfenic derivative that is S-glutathionylated and can be reactivated by thioltransf erases (8). In serum-deprived DU 145 prostate cancer cells, DPI significantly increased caspase activity, DNA fragmentation, and the fraction of cells developing apoptosis defined by morphological study or cell cycle analysis. Thus O2- from a growth-regulatory NAD(P)H oxidase might exert a cancer-promoting trophic effect by facilitating cellular immortality through resistance to programmed cell death. This association could provide a missing mechanistic link to explain the prominent role of oxidants in prostate cancer biology.

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DISCLOSURES

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