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Androgenic Regulation of Oxidative Stress in the Rat Prostate

Involvement of NAD(P)H Oxidases and Antioxidant Defense Machinery during Prostatic Involution and Regrowth

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Little is known about the roles of androgens in the regulation of redox state in the prostate, a cellular process believed to profoundly influence normal and aberrant prostate functions. We demonstrate that castration induced discrete oxidative stress (OS) in the acinar epithelium of rat ventral prostate (VP), as evident from marked increases in 8-hydroxy-2′-deoxyguanosine and 4-hydroxynonenal protein adducts in the regressing epithelium. Testosterone replacement partially reduced OS in VP epithelia of castrates, but the level remained higher than in intact rats. Quantification of steady-state mRNA levels of 14 genes involved in the anabolism and catabolism of reactive oxygen species (ROS) showed that castration resulted in dramatic increases of three ROS-generating NAD(P)H oxidases (Noxs) including Nox1, gp91^{phox}, and Nox4, significant reductions of key ROS-detoxifying enzymes (superoxide dismutase 2, glutathione peroxidase 1, thioredoxin, and peroxiredoxin 5), and unchanged levels of catalase, glutathione reductase, γ-glutamyl transpeptidase, and glutathione synthetase. Testosterone replacement in castrated rats partially reduced expression of Noxs but restored expression of superoxide dismutase 2, glutathione peroxidase 1, thioredoxin, and peroxiredoxin 5 to complete normalcy and induced a compensatory increase in expression of catalase, glutathione reductase, γ-glutamyl transpeptidase, and glutathione synthetase in the regenerating VP. Expression of superoxide dismutase 1, glutathione S-transferase-π, and glucose-6-phosphate dehydrogenase was unaffected by castration and testosterone replacement. These findings indicate androgen-deprivation induces OS in the rat VP through elevation of ROS anabolism and diminution of antioxidant detoxification. Androgen replacement partially reduces OS in rat VP to precastration levels. Expression of Noxs remained high amid a broad-based recovery of antioxidant defense mechanism(s). These data might have implications on the use of androgen blockade for prostate cancer prevention and androgen therapy for andropause treatment in elderly men. ***(Am J Pathol 2003, 163:2513–2522)***

Persistent generation of reactive oxygen species (ROS) such as superoxide, hydrogen peroxide (H_2O_2), and hydroxyl radicals is an inevitable consequence of mitochondrial respiration in aerobic organisms. It is now known that low levels of ROS are required in the regulation of a broad range of normal cellular responses, including proliferation and cell survival[1–3] via oxidative modifications of redox-sensitive transcription factors (eg, AP-1, nuclear factor-κB, and HIF-1α) and intermediate signaling molecules (eg, protein kinase C, ERK, and JNK).[1,4] In contrast, induction of high levels of ROS subjects the cell to a state of oxidative stress (OS), which may damage cellular DNA, proteins, and lipids and result in cell-cycle arrest, cellular senescence, and cell death.[1] Chronic OS has been implicated in neoplastic transformation[5] and promotion of tumorigenesis.[6] Apropos to the development of diseases in the prostate, increased oxidative damage to cellular macromolecules has been observed with aging[7] and the development of malignancy.[8,9]

The degree of OS in a cell is dependent on the balance between ROS anabolism and catabolism (Figure 1). Although mitochondrial respiration is a major source of ROS, superoxide is also produced by a family of membrane-bound enzymes known as NAD(P)H oxidases (Noxs). Human Nox1, gp91^{phox} (the catalytic subunit of Nox2), Nox3, Nox4, Nox5, and rat Nox1, gp91^{phox}, and Nox4 have been cloned.[5,10] Different Noxs are expressed in different cell types, including phagocytes and nonphagocytic cells.[11,12] Little is known about expression of Noxs and their functions in the prostate. Ectopic

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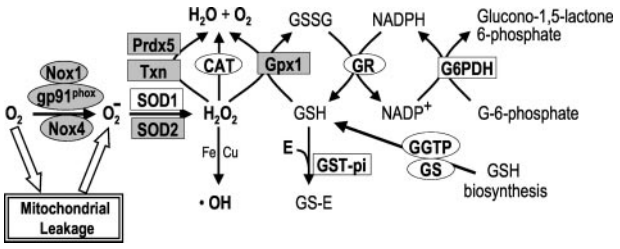

Figure 1. Generation of ROS by NAD(P)H oxidases (Noxs) and mitochondrial leakage and the antioxidant defense machinery against damage by ROS. Superoxide anions (O_2^-) are produced by a family of Noxs including Nox1, $gp91^{phox}$, and Nox4. In addition, mitochondria are believed to be a major source of ROS, which are persistently generated as a consequence of leakage from the mitochondrial respiration. The first line of defense against damage by ROS includes the cytosolic SOD1 and mitochondrial SOD2 converting superoxide to H_2O_2. Because H_2O_2 reacts with iron or copper ions (via Fenton reaction) to produce hydroxyl radicals (•OH) that are highly damaging to cellular macromolecules, Gpx1, catalase (CAT), and Prdx5 are involved in removing H_2O_2 in cells. Additionally, glutathione *S*-transferases such as GST-π are directly responsible for elimination of electrophilic oxidants (E) at the expense of glutathione (GSH). The second line of cellular antioxidant defense includes GR, G6PDH, GGTP, and GS that are involved in the regeneration of intracellular reducing powers such as GSH and NAD(P)H. Txn directly detoxifies ROS and also serves as an electron donor to other antioxidant enzymes including Prdx5. **Gray oval boxes** (Nox1, $gp91^{phox}$, and Nox4): genes demonstrating elevated mRNA levels in the VPs of castrated rats followed by partial reduction in transcript levels in VPs of testosterone-treated rats (see Figure 3A). **Gray rectangles** (SOD2, Gpx1, Txn, and Prdx5): genes showing diminished levels in VPs of castrated animal *versus* control and testosterone replacement group (see Figure 3B). **Open oval boxes** (catalase, GR, GGTP, and GS): genes demonstrating no changes in mRNA levels in rat VP after castration but elevated levels after testosterone replacement (see Figure 3C). **Open rectangles** (SOD1, GST-π, and G6PDH): genes showing no changes in transcript level after castration or after subsequent testosterone replacement (see Figure 3D). Other abbreviations: Fe, iron ions; Cu, copper ions; GSSG, glutathione disulfide; GS-E, glutathione-electrophile conjugates; G-6-phosphate, glucose-6-phosphate.

expression of Nox1 in prostate cancer cells enhances growth, tumorigenicity, and angiogenicity,[6] whereas down-regulation of Nox5 causes growth arrest and apoptosis.[12] No information is available on Nox expression in the prostate gland *in vivo*.

To cope with OS, cells are equipped with multiple antioxidant defense mechanisms.[13] The first line of defense includes two superoxide dismutases, the cytosolic superoxide dismutase (SOD)1 and mitochondrial SOD2, which convert superoxide to H_2O_2. Because accumulation of H_2O_2 leads to the production of hydroxyl radicals that are highly damaging to cellular macromolecules, all cells are equipped with multiple enzymatic pathways for its removal. Among them are three major pathways involving glutathione peroxidase 1 (Gpx1), catalase, and peroxiredoxin 5 (Prdx5). Additionally, glutathione *S*-transferases (GSTs) are directly responsible for the elimination of electrophilic oxidants at the expense of glutathione (GSH). The second line of cellular defense against antioxidants involves generation of intracellular reducing power, such as the reduced form of glutathione and NAD(P)H, within the cell.[14] Enzymes such as glutathione reductase (GR), glucose-6-phosphate dehydrogenase (G6PDH), γ-glutamyl transpeptidase (GGTP), and glutathione synthetase (GS) play major interactive roles in the replenishment of cellular reducing power. Other antioxidant molecules include thioredoxin (Txn), a protein thiol that directly detoxifies ROS and serves as an electron donor to other antioxidant enzymes, including Prdx5.[15]

Androgens are required for maintaining the homeostasis of cell proliferation and apoptosis in the prostate gland. Castration-induced androgen ablation results in regression of the prostate with a rapid rate of decline in fresh weight, RNA, DNA, and protein content mainly via apoptosis of epithelial cells, cell shrinkage, reduced protein synthesis, and secretion.[16–19] Androgen replacement to castrated animals stimulates regeneration of the prostatic epithelium and other cellular and structural components.[19] Although prostatic involution and regrowth have long been regarded as direct responses to circulating androgen levels, recent reports suggest that some cellular events are mediated by indirect processes, such as hypoxia[17,20] or alterations in the redox state.[21] Furthermore, as men age, the incidence of prostatic diseases rises dramatically.[22] Related issues such as the risk associated with androgen replacement therapy for andropause treatment[23,24] and the risk/benefit ratio of the use of Finasteride for prevention of prostate cancer[25] continue to grow in magnitude in response to demographic changes. All in all, these unresolved issues related to the prostate and prostate diseases have prompted us to hypothesize that androgen may play a significant role in regulating the cellular redox state, ie, the balance in ROS levels, in the prostate, which in turn influences cell growth, apoptosis, and disease development in the gland.

Using immunohistological detection of the specific OS biomarkers,[26] 8-hydroxy-2′-deoxyguanosine (8-OHdG) and 4-hydroxynonenal protein adducts (4-HNE), we localized a marked elevation in oxidative damages to epithelial cells of the rat ventral prostate (VP) after castration and a partial recovery after androgen replacement. These findings support the hypothesis that ROS levels may mediate involution and regeneration of the prostatic epithelium of the rat. Alternatively, changes in ROS levels in the rat prostate after castration and testosterone replacement may simply be part of the cellular events associated with prostatic involution and regeneration. Nevertheless, quantitative polymerase chain reaction (PCR) analyses revealed marked changes in expression levels of Noxs and antioxidant defense enzymes, which may explain the drastic alterations in ROS levels in rat VP after castration and testosterone replacement. These findings provide direct evidence that androgens regulate ROS balance in the prostate, which is now known to have broad ramifications on prostatic aging and disease development in the gland.

Materials and Methods

Castration and Androgen Replacement in Animals

Male NBL rats (5 to 6 weeks old) were purchased from Charles River Laboratories (Wilmington, MA). Rats were housed at the university's animal facility on a 12-hour light/12-hour dark cycle and allowed access to food and water *ad libitum*. At 11 to 12 weeks of age, rats weighing 280 to 300 g were surgically castrated via the scrotal

route under light isoflurane (Abbott Laboratories, North Chicago, IL) anesthesia. Castrated rats ($n = 5$) were sacrificed 3 days after castration. For the androgen replacement study, one group of rats ($n = 5$) that had been castrated 7 days previously before they were implanted subcutaneously at the subscapular region with two hormone-filled capsules, each 2 cm long, that were made of Silastic tubing (1.0-mm inner diameter × 2.2-mm outer diameter; Dow-Corning Corp., Midland, MI) and packed with testosterone (Sigma Chemical Co., St. Louis, MO).[27] Each tube contained ~14.4 ± 2.1 mg of testosterone. Castrated rats were treated with testosterone for 7 additional days before sacrifice. Sham-operated age-matched intact control rats ($n = 5$) were used as controls. VPs were carefully excised from the prostate-urethra-bladder complex and cut in half. One half was formalin-fixed and paraffin-embedded for histological and immunohistochemical studies. The other half was snap-frozen in liquid nitrogen and kept at −70°C until RNA isolation. Protocols of animal usage were approved previously by the University of Massachusetts Medical School Animal Care and Usage Committee.

RNA Isolation and cDNA Synthesis via Reverse Transcription

Total RNA was isolated using TRI reagent (Sigma) according to the manufacturer's protocol. RNA integrity was checked on denatured agarose gels. Two μg of total RNA was reverse-transcribed using the SuperScript II RNase H^- reverse transcriptase system (Invitrogen, Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. After reverse transcription, the resulting cDNA was brought up to a volume of 40 μl with nuclease-free water (Ambion, Austin, TX).

Real-Time Quantitative PCR

Quantitative real-time PCR was performed with the iCycler IQ real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA) as previously described.[28] Before the real-time PCR was performed, cDNA obtained from the VPs of intact control rats, 3-day castrated rats, and 7-day castrated rats treated with testosterone replacement for 7 days were further diluted 10-fold with nuclease-free water. The diluted cDNA (2.5 μl) was amplified in a 25-μl reaction mix containing 1× iQ SYBR Green Supermix (Bio-Rad Laboratories) and 150 nmol/L of each primer. After a 15-minute *Taq* activation step at 95°C (hot start), reactions were subjected to 40 cycles of 30 seconds of denaturation at 94°C, 30 seconds of annealing at 60°C, and 30 seconds of extension at 72°C. Primers were purchased from MWG Biotech (High Point, NC). Intron-spanning primer pairs were designed to minimize primer dimerization and to generate an amplicon between 150 and 350 bp. The sequences of oligonucleotide primers used in this study are listed in Table 1. Optical data were collected during the annealing step of each cycle. Amplification specificity was verified by performing melting-curve analysis by melting PCR products for 3 minutes at 90°C and then lowering the temperature to 55°C in 0.2°C increments, with 1 second per increment. Optical data were collected throughout the duration of the temperature drop, with a dramatic increase in fluorescence seen when the strands reannealed. Immediately after completion of melting-curve analysis, products were checked with agarose gel electrophoresis. Relative expression of the real-time reverse transcriptase-PCR products was determined by the $\Delta\Delta C_t$ method. This method calculates relative expression using the equation: fold induction $= 2^{-[\Delta\Delta Ct]}$, where C_t = the threshold cycle, ie, the cycle number at which the sample's relative fluorescence rises above the background fluorescence, and $\Delta\Delta C_t = [C_t$ gene of interest (unknown sample) $- C_t$ housekeeping gene (unknown sample)] $- [C_t$ gene of interest (calibrator sample) $- C_t$ housekeeping gene (calibrator sample)]. One of the control samples was chosen as the calibrator sample and used in each PCR. Each sample was run in triplicate, and the mean C_t was used in the $\Delta\Delta C_t$ equation. The relative abundance of the mRNAs of interest in the VPs of castrated rats and castrates with testosterone replacement was calculated by arbitrarily assigning the abundance in the VPs of untreated intact controls a value of 1. RLP19 was chosen for normalization because this gene showed consistent expression relative to other housekeeping genes (GAPDH and β-actin) among the three treatment groups in our initial experiments.

Statistical Analyses

Statistical significance of differences in expression level between treatment groups was determined using Systat software (Student version 6.0.1; SPSS, Chicago, IL) to perform one-way analysis of variance with Tukey post hoc analyses. A P value of <0.05 was taken as a statistically significant difference between the two groups.

Immunohistochemical Analysis of OS Biomarkers

Mouse monoclonal antibodies to 8-OHdG (N45.1) and 4-hydroxynonenal protein adducts (HNEJ-2) were obtained from the Japan Institute for the Control of Aging (Fukuroi, Shizuoka, Japan). Immunohistochemical staining was performed with the Vestastain Elite ABC kit (Vector Laboratories, Burlingame, CA). The sections (5 μm) were dewaxed and then rehydrated in graded alcohols and distilled water. After deparaffinization, sections for 8-OHdG staining were antigen-retrieved by autoclaving at 121°C for 15 minutes in a 10% zinc sulfate solution and then treated with 2 mol/L of HCl at 37°C for 30 minutes;[29] no antigen retrieval was needed for immunostaining of 4-HNE protein adducts. Sections were then incubated with 10% normal horse serum for 30 minutes at 37°C before overnight incubation with the primary antibody to 8-OHdG (1:40) and 4-HNE protein adducts (1:40) at 4°C. The slides were then incubated with the biotinylated anti-mouse IgG at a dilution of 1:200 for 30 minutes at 37°C followed by quenching of endogenous peroxidase activity by 3% H_2O_2 in methanol for 30 minutes at room temperature. The rationale for treating sections with H_2O_2

Table 1. Sequences of Oligonucleotide Primers for Quantative Real-Time RT-PCR

Gene name	Primer sequences (5′ to 3′)*	GenBank accession no.
NAD(P)H oxidase 1 (Nox1)	F: TTTTATCGCTCCCGGCAGAA R: CAGTCCCCTGCTGCTCGAAT	NM_053524
gp91^{phox}, also termed Nox2	F: CCAGTGTGTCGGAATCTCCT R: ATGTGCAATGGTGTGAATGG	NM_023965
NAD(P)H oxidase 4 (Nox4)	F: ACAACTGTTCCGGGCCTGAC R: TCAACAAGCCACCCGAAACA	NM_053524
Superoxide dismutase 2 (SOD2)	F: AGCTGCACCACAGCAAGCAC R: TCCACCACCCTTAGGGCTCA	NM_017051
Glutathione peroxidase 1 (Gpx1)	F: CGGTTTCCCGTGCAATCAGT R: ACACCGGGGACCAAATGATG	NM_030826
Thioredoxin (Txn)	F: CTCTGCCACGTGGTGTGGAC R: GAAGGTCGGCATGCATTTGA	NM_053800
Peroxiredoxin 5 (Prdx5)	F: CTATGGCCCCGATCAAGGTG R: GTGCTCCCTTGGCCTTCAGA	NM_053610
Catalase	F: CGACCGAGGGATTCCAGATG R: ATCCGGGTCTTCCTGTGCAA	NM_012520
Glutathione reductase (GR)	F: AGCCCACAGCGGAAGTCAAC R: CAATGTAACCGGCACCCACA	NM_053906
γ-Glutamyl transpeptidase (GGTP)	F: CGTATCGTGGAGGCCTTTCG R: TAGGCGGTTGGGTGAGTGGT	NM_053840
Glutathione synthetase (GS)	F: CACCAGCTGGGGAAGCATCT R: GGTGAGGGGAAGAGCGTGAA	NM_012962
Superoxide dismutase1 (SOD1)	F: GCGGTGAACCAGTTGTGGTG R: AGCCACATTGCCCAGGTCTC	NM_017050
Glutathione *S*-transferase-π (GST-π)	F: CCTGCCTGGGCATCTGAAAC R: GCACTGAGGCGAGCCACATA	X02904
Glucose-6-phosphate dehydrogenase (G6PDH)	F: AGCTGGTCATCCGTGTGCAG R: TGCATTTGGCTCCCACAGAA	NM_017006
Ribosomal protein L19 (RPL19)	F: GCATATGGGCATAGGGAAGA R: CCATGAGAATCCGCTTGTTT	NM_031103
β-Actin	F: GGCATCCTGACCCTGAAGTA R: GGGGTGTTGAAGGTCTCAAA	NM_031144
Glyceraldehyde-3-phosphate-dehydrogenase (GAPDH)	F: AACTCCCATTCCTCCACCTT R: GAGGGCCTCTCTCTTGCTCT	M17701

*F, forward primer; R, reverse primer.

after primary antibody incubation was to ensure that all of the detected oxidative damages were not because of oxidation by H_2O_2 in the quenching step. The immunostaining was developed by peroxidase-conjugated avidin-biotin complexes and diaminobenzidine. For controls, the primary antibodies were preincubated with either excess antigens or 1.5 mg/ml 8-OHdG (Sigma) or were replaced with the corresponding normal isotype sera (Zymed, S. San Francisco, CA). Human seminal vesicle was used as a positive control tissue.[8]

Results

Castration Induced OS and Its Partial Attenuation by Testosterone Replacement

OS in prostate was demonstrated by immunohistochemical detection of 8-OHdG and 4-HNE protein adducts, both of which are biomarkers for OS.[26]

8-OHdG Immunostaining

Positive staining was found exclusively in nuclei of the prostate tissues in the present study. In the acinar region of VPs from the intact control group (Figure 2A), positive nuclear staining was found in glandular epithelial cells but was not often found in periacinar smooth muscle and interstitial stromal cells. Negatively stained nuclei were occasionally seen interspersed with positive nuclei in the glandular epithelium (Figure 2A and insets). In the ductal region (Figure 2D), positive nuclear staining of 8-OHdG was observed in both glandular epithelial cells and periductal smooth muscle cells. The intensity of 8-OHdG staining was conspicuously higher in the ductal region than in acinar region. Three days after castration, the immunoreactivity of 8-OHdG was markedly increased in glandular epithelial cells with no noticeable increase in periacinar smooth muscle cells and interstitial stromal cells such as fibroblasts, inflammatory cells, and endothelial cells (Figure 2B). Interestingly, strong 8-OHdG staining often was detected in the condensed nuclei of apoptotic cells in the glandular epithelium. In the ductal region of castrated animals (Figure 2E), strong immunoreactivity of 8-OHdG was found in both epithelial cells and periductal smooth muscle cells. In castrated animals with testosterone replacement, most acinar epithelia exhibited a general decline in 8-OHdG staining, with foci of strongly stained nuclei often localized adjacent to weakly or negatively stained nuclei within the same epithelium (Figure 2C and inset). Periacinar smooth cells and stromal cells showed a weak or negative signal for 8-OHdG

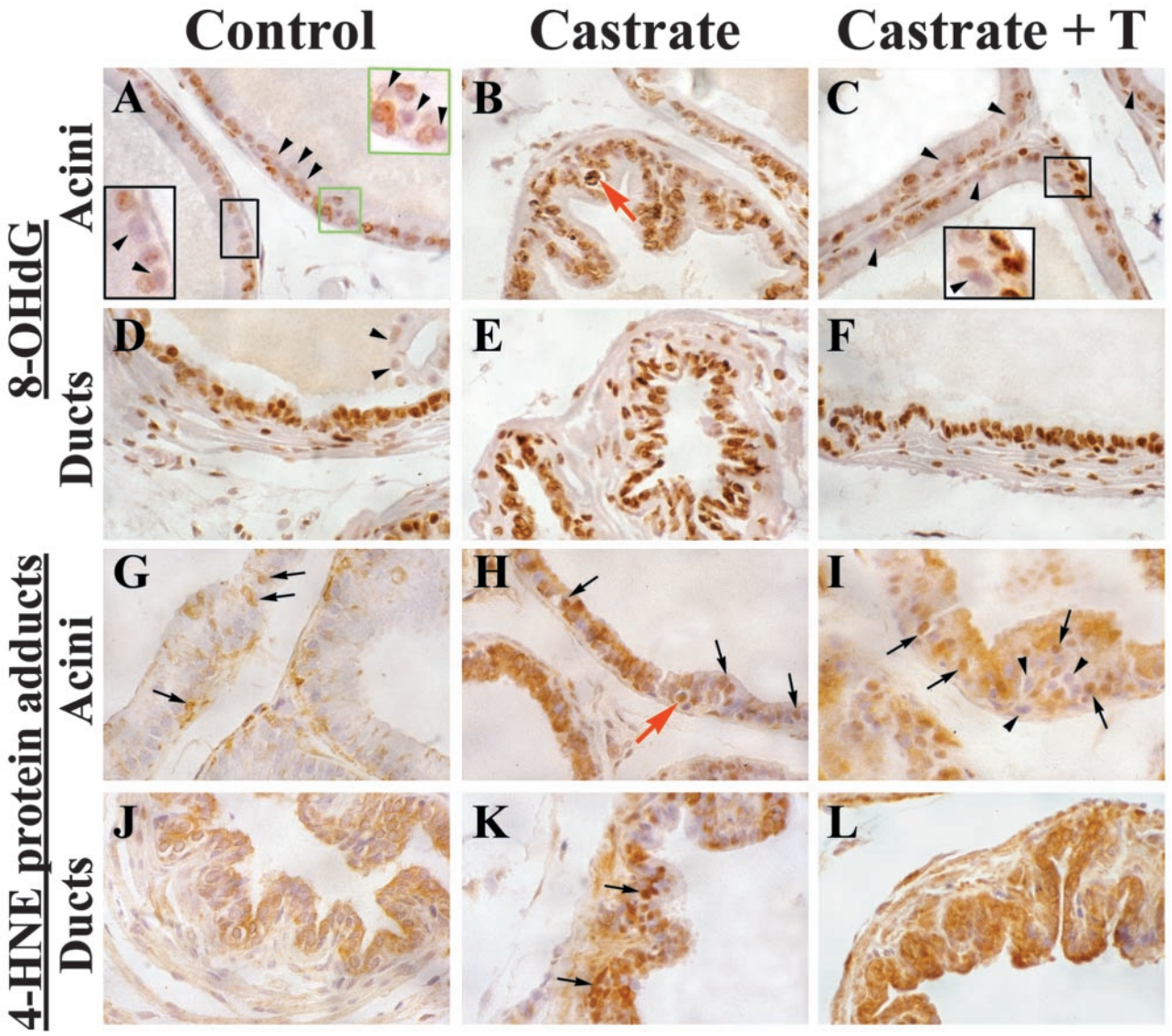

Figure 2. Immunohistochemical analysis for OS biomarkers, 8-OHdG and 4-HNE protein adducts in the VP from sham-operated control, castrated, and testosterone-replaced animals. Representative acinar and ductal areas from VP sections immunostained for 8-OHdG are shown in **A–C** and **D–F**, respectively, whereas representative images demonstrate the localization of 4-HNE protein adducts in acini (**G–I**) and ducts (**J–L**). **A:** Negatively or weakly stained nuclei (**arrowheads**) were often found interspersed with positive nuclei in the acinar epithelium of control VPs. The **insets** at the **top right** and the **bottom left** demonstrate high magnification of areas outlined by **green** and **black lines**, respectively. **B:** There was an increase in 8-OHdG immunostaining in most nuclei of acinar epithelium after castration. Note the strong staining in the nucleus of an apoptotic cell (**red arrow**). **C:** Reappearance of negatively or weakly stained nuclei (**arrowheads**) often localized adjacent to positive nuclei within the same epithelial tubule after testosterone replacement. **Insets**: High magnification of an area demonstrating the presence of a negative nucleus (**arrowhead**) mingled with positive nuclei in acinar epithelium. **D–F:** Both epithelial and stromal nuclei of ducts showed strong staining compared with that of the acini of respective animal groups. Note in **D**, the negative or weakly stained nuclei in a fragment of columnar epithelium (**arrows**) that was likely sloughed off from distal acini. **G:** Acinar epithelium of control VP exhibited sporadic and faint nuclear staining (**arrows**) with minimal cytoplasmic staining. **H:** There was a marked increase in immunostaining of 4-HNE protein adducts in cytoplasm and nuclei (**arrows**) of acinar epithelium after castration. Apoptotic cells also show strong staining in the epithelium (**red arrow**). **I:** Testosterone replacement only partially reversed the staining of 4-HNE protein adducts. Note the patchy and diffuse cytoplasmic staining in epithelium. Negative (**arrowheads**) and positive (**arrows**) nuclei were often found intermingled within the same epithelial region. **J:** 4-HNE protein adducts were uniformly localized in ductal epithelium (mainly in cytoplasm) of control VPs. **K:** Nuclear staining of 4-HNE protein adducts (**arrows**) was markedly increased in ducts after castration. **L:** Strong staining was seen in 4-HNE in ductal epithelium (cytoplasm and nuclei) and stroma after androgen replacement. Original magnifications: ×187.5 (**A–L**); ×750 (**insets**).

immunoreactivity. In the ductal region (Figure 2F), the immunostaining of 8-OHdG remained strong in the nuclei of epithelial cells and periductal smooth muscle cells. As a positive control tissue, human seminal vesicle exhibited intense nuclear staining in the pseudostratified columnar epithelium (data not shown), as previously documented.[8] A signal was absent or undetectable in tissue samples with 8-OHdG antibody preabsorbed with excessive amounts of 8-OHdG (data not shown), demonstrating the specificity of the antibody.

4-HNE Protein Adducts

In the intact control group, acinar epithelium exhibited weak or no immunostaining of 4-HNE protein adducts, which if present were localized in the cytoplasm and

nuclei of epithelial cells (Figure 2G). In ducts, immunostaining of 4-HNE-modified proteins was diffuse and localized mainly in the cytoplasm of epithelial cells (Figure 2J). The smooth muscle cells and stromal cells in both acinar and ductal regions showed weak or no staining. Three days after castration, the immunoreactivity of 4-HNE protein adducts was distinctly increased in nuclei and cytoplasm of cells in both glandular and ductal epithelium (Figure 2, H and K, respectively). The staining was focally distributed within epithelial tubules with foci of strongly stained regions often localized adjacent to weakly or negatively stained areas. Most important, 4-HNE protein adducts were detected in epithelial cells that had undergone apoptosis. In the acinar region, there was no noticeable increase in the staining of 4-HNE protein adducts in smooth muscle cells and stromal cells, including interstitial fibroblasts, inflammatory cells, and endothelial cells (Figure 2H). However, increased 4-HNE protein adducts were found in smooth muscle cells and stroma of prostatic ducts (Figure 2K). In castrated animals treated with testosterone for 7 days, the amount of 4-HNE protein adducts was significantly reduced, and they presented a patchy distribution in the glandular epithelium with diffuse and heterogeneous cytoplasmic staining (Figure 2I). Discretely positive- and negative-stained nuclei were found mingled together within the same epithelial region. Periacinar smooth muscle and stromal cells showed weak or no staining for 4-HNE-modified proteins. In the ductal region (Figure 2L), however, 4-HNE protein adducts were uniformly distributed in the epithelium and in periductal smooth muscle cells, with diffuse staining found in nuclei and cytoplasm. As a positive control tissue, human seminal vesicle exhibited intense cytoplasmic staining in the pseudostratified columnar epithelium (data not shown), as previously reported.[8] The signal was absent or undetectable in tissue samples with primary antibody replaced by normal isotype antiserum (data not shown), demonstrating the specificity of the antibody.

Induction of NAD(P)H Oxidase Expression by Castration and Its Partial Reversal by Androgen Replacement

Quantitative real-time reverse transcriptase-PCR was used to measure the relative abundance of steady-state mRNA levels of rat gp91^{phox} (the catalytic subunit of Nox2) and two other members of the family, Nox1 and Nox4 (Figure 3A). Relative mRNA levels of Nox1, gp91^{phox}, and Nox4 in the VPs of castrated rats were twofold ($P < 0.05$), eightfold ($P < 0.01$), and 10-fold ($P < 0.001$) higher than levels found in VPs of intact control rats, respectively. After testosterone replacement in castrated rats, transcript levels of gp91^{phox} and Nox4 were significantly decreased ($P < 0.05$ and 0.001, respectively) as compared with levels in castrated rats but were still elevated by twofold ($P < 0.05$) and 2.5-fold ($P < 0.01$) over levels found in intact rats, respectively. However, in the castrates with testosterone replacement, Nox1 exhibited only a trend of reduction in expression

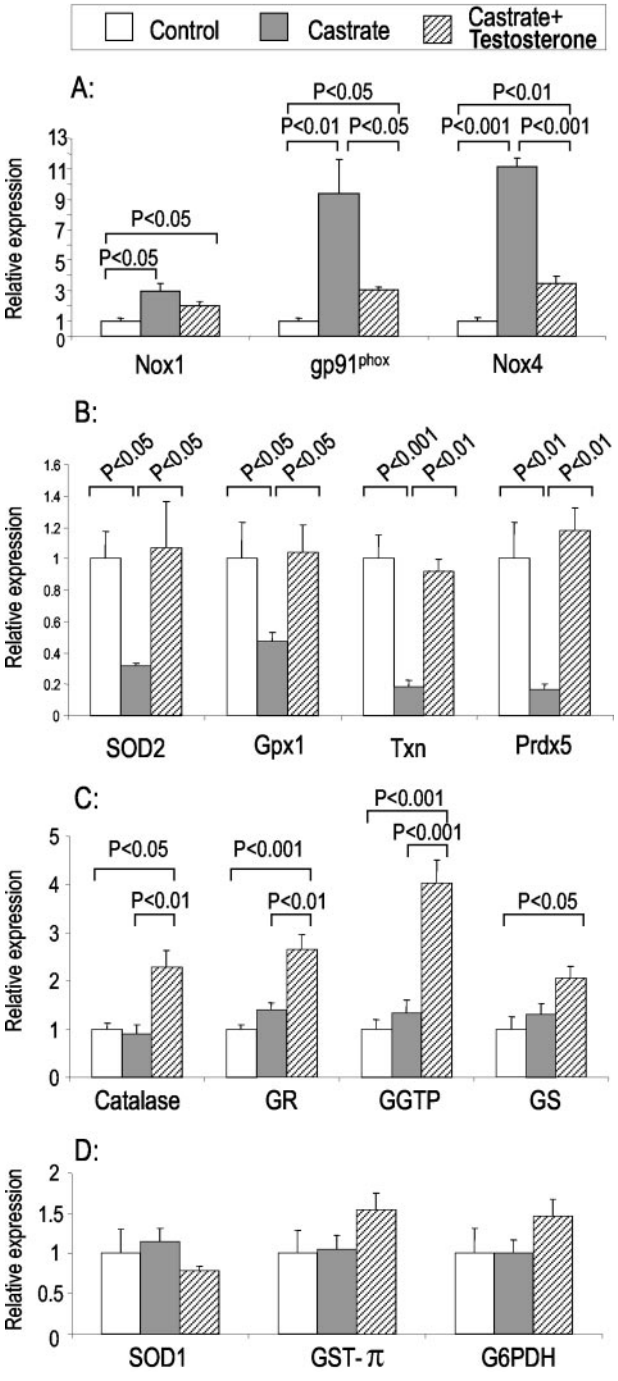

Figure 3. Quantitative real-time PCR analyses of mRNA abundance of major ROS-generating and ROS-detoxification enzymes and selected ROS scavengers in the VPs of control, castrated, and testosterone-replaced castrated animals. **A:** Genes demonstrating elevated mRNA levels in the VPs of castrated rats followed by partial reduction in transcript levels in VPs of testosterone-treated rats. **A** includes Nox1, gp91^{phox}, and Nox4. **B:** Genes showing diminished mRNA levels in VPs of castrated animal *versus* control and testosterone replacement group. **B** includes SOD2, Gpx1, Txn, and Prdx5. **C:** Genes demonstrating no changes in mRNA levels in rat VP after castration but elevated levels after testosterone replacement. **C** includes catalase, GR, GGTP, and GS. **D** includes genes showing no changes in mRNA levels in VPs after castration or after subsequent testosterone replacement. **D** includes SOD1, GST-π, and G6PDH. Levels of expression in control animals were set to 1. Each column represents the average value from five animals and error bars represent SEM.

($P > 0.05$) as compared with values in castrates but remained onefold elevated ($P < 0.05$) over levels in intact controls.

Alterations of Expression of Antioxidant Defense Genes during Castration and Testosterone Replacement

Transcript levels of a total of 11 genes critically involved in the intracellular antioxidant defense or ROS scavenging were quantified in the rat VP after castration and administration of testosterone to castrated rats. Based on their patterns of response to castration and testosterone replacement, the genes were grouped into three clusters. The first cluster of genes (Figure 3B) was regarded as androgen-inducible genes whose expression was decreased by castration and restored to complete normalcy on subsequent testosterone replacement. Castration caused a significant reduction in relative mRNA abundance of SOD2 (threefold, $P < 0.05$), Gpx1 (twofold, $P < 0.05$), Txn (sixfold, $P < 0.001$), and Prdx5 (sixfold, $P < 0.01$) in the VPs. On testosterone replacement, mRNA levels of genes in this cluster were restored to the levels in intact rats. The second cluster of genes (Figure 3C) demonstrated a more complex relationship with *in vivo* androgen status. Relative mRNA abundance of catalase, GR, GGTP, and GS were insensitive to castration-induced androgen deprivation but were markedly up-regulated by testosterone replacement to ~220% ($P < 0.05$), 260% ($P < 0.001$), 400% ($P < 0.001$), and 200% ($P < 0.05$), respectively, of levels in intact controls. Finally, expression of the third cluster (Figure 3D) of genes, including SOD1, GST-π, and G6PDH, appeared to be insensitive to castration and testosterone replacement.

Discussion

The present study provided the first evidence that androgen regulates redox status *in vivo* in the rat VP. This corroborates findings from a single previously published *in vitro* study[30] that reported induction of OS in a prostate cancer cell line by androgen. Castration clearly induced an elevated pro-oxidant state in the regressing VP, particularly in the epithelium, as revealed by marked increases in the number of epithelial cells exhibiting signs of oxidative DNA damages (nuclear 8-OHdG positivity), and lipid peroxidation-induced protein damages (4-HNE protein adducts). Testosterone replacement in castrated rats was only partially effective in reducing OS levels in the VP of the castrates. The extent of oxidative damage in VPs of testosterone-treated castrates was still higher than that of intact rats.

To elucidate the mechanism(s) underlying androgenic regulation of redox state in rat VP, we focused on the metabolic pathways responsible for ROS anabolism and catabolism. Specifically, quantitative real-time PCR was used to measure transcript levels of three NAD(P)H oxidases (superoxide-generating enzymes), a broad spectrum of key antioxidant enzymes, and key ROS scavengers. Steady-state mRNA levels of all three NAD(P)H oxidases, Nox1, gp91^{phox} (Nox2), and Nox4, were significantly up-regulated in the rat VP after castration. Concomitantly, expression of several key ROS detoxification enzymes/scavengers, SOD2, Gpx1, Txn, and Prdx5 declined whereas transcripts levels of catalase, GR, GGTP, and GS remained unchanged in the VPs of castrated rats. Because Noxs are responsible for generating superoxide and SOD2 responsible for its removal, whereas Gpx1, Txn, and Prdx5 are intimately linked to the removal of H_2O_2, alterations in the expression of these genes, collectively, are expected to result in accumulation of ROS, leading to a higher pro-oxidant state in the VPs of castrated rats. In contrast, testosterone replacement in castrated rats caused a complete restoration of expression of SOD2, Gpx1, Txn, and Prdx5 mRNA to levels found in intact rats. However, expression of the Nox genes, although much reduced when compared with levels in castrates, were still significantly higher than those found in intact rats. Interestingly, although levels of mRNA expression of catalase, GR, GGTP, and GS were not changed by castration, they were significantly elevated when androgen was replaced in castrated rats. Collectively, these changes in expression of ROS anabolic and catabolic enzymes and ROS scavengers may explain why evidence of OS continued to exist in the VPs of testosterone-treated castrates. Our findings are in general agreement with recent findings demonstrating altered expression of antioxidant enzymes and specific genes related to ROS scavenging in rat VP after castration.[21,31]

A large body of evidence has demonstrated that ROS evokes apoptosis[1] and proliferation,[2] two extremely contrasting cellular events. In culture, proliferating cells exhibit a broad spectrum of responses to graded levels of oxidants.[3] A very low level of H_2O_2 (3 to 15 μmol/L) causes a significant mitogenic response. Higher levels of H_2O_2 (120 to 400 μmol/L) results in a growth-arrested state, whereas a further increase of H_2O_2 to 1 mmol/L triggers apoptosis. However, the pro-apoptotic or pro-proliferation role of ROS in the prostate gland has yet to be demonstrated. In this study, we observed high levels of OS in the regressing VP and moderate levels of OS in the regenerating gland. Extrapolated from cell culture data, these findings thus support the hypothesis that different levels of ROS may contribute to cell death, as well as to cell growth, *in vivo*. Specifically, 8-OHdG and 4-HNE protein adducts were found to be highly expressed in the regressing VP epithelia, with the frequent association of these markers with apoptotic cells, suggesting a possible causal relationship between OS and apoptosis. OS-mediated apoptosis has been demonstrated in a variety of biological systems, including brain-derived neurotrophic factor- or zinc-induced neuronal cell death,[32,33] anti-cancer drug-induced apoptosis,[34] and hyperoxia- or vanadium-induced pulmonary apoptosis.[35,36] On the other hand, it has been shown that ROS can elicit a mitogenic response via redox-sensitive transcription factors, such as AP-1 and nuclear factor-κB,[37] as well as through other signaling mediators such as protein kinase C[4] and MAP kinases.[38] Previous reports have revealed that low concentrations of oxidants are

effective in stimulating the growth of fibroblasts in cultures.[2,3] Treatment of hepatoma cells with low concentrations of ROS *in vitro* induces proliferation through the phosphatidylinositol 3-kinase/Akt pathway.[39] After the testosterone-treatment of castrated rats, we observed evidence of continued elevation of OS at moderate levels in the regenerating VP, suggesting a plausible role for low levels of ROS in the regulation of cell proliferation, differentiation, and/or tissue remodeling in the gland.

In situ detection of oxidative damages by the immunohistochemical approach allows examination of OS at the cellular level. Oxidative damages, as revealed by the intensity of immunopositive 8OHdG and 4-HNE protein adducts, were always higher in the ducts of the VP than in the acini. Castration and androgen replacement had minimal effects on the levels of OS markers in the ducts but distinctly regulated those in the acini. These findings indicate differential regulation of OS in ducts *versus* that in acini by circulating androgen. Previous studies have reported that prostatic ducts are markedly different from acini with regard to morphology, hormonal responsiveness, proliferation, apoptosis, stromal/epithelial cell ratio, and composition of the extracellular matrix.[40–42] Thus, constitutively high levels of OS found in the ductal regions may explain the reported high susceptibility of tumor development from prostatic ducts in hormone-treated Noble rats.[43,44] It is currently unknown why ductal epithelia express such a high level of OS constitutively. One possible explanation may be related to previously reported retrograde transport of genotoxic substances or pathogens from the urethra to prostatic ducts, a mechanism implicated in prostatitis of the human gland.[45]

NAD(P)H oxidases are directly involved in the production of superoxide, a first step in the oxidant anabolism pathway.[46] The exact mechanisms regulating expression of these genes in nonphagocytic cells are presently unknown and may be cell- or tissue-specific. It has been demonstrated that protein kinase C mediates the regulation of gene expression for NAD(P)H oxidases by angiotensin II in vascular smooth muscle cells[47] and by zinc in neuronal cells.[48] Serum, platelet-derived growth factor, and prostaglandin $F_{2\alpha}$ cause alterations in gene expression of NAD(P)H oxidases in vascular smooth muscle cells.[11,49] Our results clearly demonstrate that expression of NAD(P)H oxidases in rat VP is dependent on the androgen status of the animal. We had examined the promoter sequences (at ~1 to 2 kb upstream of the transcription initiation site) of rat and human NAD(P)H oxidases *in silico* (unpublished observations) and did not find any androgen-responsive elements. A previous study failed to show regulation of superoxide production by testosterone in macrophages.[50] Taken together, these findings suggest that androgenic regulation of NAD(P)H oxidases may be indirect. Expression of these enzymes may be secondary to physiological changes that occur during castration-induced involution and androgen-induced regeneration of the rat VP. The sharp increase in the expression of $gp91^{phox}$ in the VPs of castrated rats raises the possibility that invasion of the immune cell may contribute to the elevated OS status in these glands. It has been documented[51] that migrating macrophages phagocytosing apoptotic bodies appeared in rat VPs 2 days after castration. Leukocyte infiltration has been reported in the prostates of castrated mice[52] and in localized prostate cancer after androgen ablation therapy in humans.[53] However, we did not observe any noticeable increase in the number of lymphocytes or neutrophils in the VP of castrated rats. These discrepancies may be a result of species- and/or disease state-governed variations. Alternatively, it is possible that all three NAD(P)H oxidases are also expressed by epithelial cells. Future investigations involving immunolocalization of the enzymes or transcript analyses in microdissected samples are needed to answer this question. Evidence is emerging to suggest that different Nox enzymes are regulated differentially and have different biological outcomes in a cell or a tissue. For example, ectopic expression of Nox1 in fibroblasts induces proliferation and transformation[5] but that expression of Nox4 induces senescence.[54] In human prostate cancer cells, overexpression of Nox1 increases tumorigenicity[6] and anti-sense inhibition of Nox5 suppresses proliferation and triggers apoptosis,[12] whereas down-regulation of $gp91^{phox}$ does not have any impacts on cell growth and survival/apoptosis.[12] In vascular smooth muscle cells, Nox1 and Nox4 exhibited opposing responses to the same inducing agents.[11] In the present study, androgenic regulation of $gp91^{phox}$ and Nox4 stand in contrast to that of Nox1, suggesting that they may be regulated by dissimilar mechanisms and may play nonredundant roles in the control of ROS anabolism in the rat VP.

We could classify the antioxidant defense enzymes and ROS scavengers that we studied in the rat VP into three categories (Figure 3) according to their responsiveness to androgen withdrawal and resupplementation. The first group of VP genes includes SOD2, Gpx1, Txn, and Prdx5. These genes are most likely regulated by androgen directly; their expression declines on androgen withdrawal and rebounds to precastration levels on testosterone replacement. SOD2, also termed MnSOD, catalyzes the dismutation of superoxide into H_2O_2 in the mitochondria, which are considered the main site of ROS production. Gpx1 and Prdx5 convert H_2O_2 into H_2O, thus preventing accumulation of the peroxide and its metabolic activation to hydroxyl radicals. Txn plays an essential role in eliminating ROS by acting as an electron donor to a number of antioxidant enzymes, including those in the peroxiredoxin family.[15] The second set of VP genes, including catalase, GR, GGTP, and GS, are insensitive to androgen withdrawal but are significantly up-regulated on testosterone replacement in castrated animals. Intriguingly, this set of genes, except for catalase, are more distantly related to the removal of ROS but closely associated with replenishment of GSH.[14] Specifically, GR is involved in the regeneration of GSH from its disulfide (GSSG) in GSH salvage whereas GGTP and GS play a critical role in *de novo* GSH synthesis. Taken together, our data suggest that replenishment of GSH is an important cellular event during androgen-induced prostatic regeneration. Our data agree with a previous finding demonstrating that androgen-treatment induces GGTP gene expression and enzyme activity in a human prostate cancer cell line.[55] The last set of genes, including SOD1 (also termed CuZn SOD), GST-π, and G6PDH, showed no re-

sponse to changes in the androgenic status of the rats. Although studies in the literature have implicated their action of these genes in the maintenance of redox homeostasis, they may not participate directly in the androgen-regulated prostatic involution and regeneration. Our data clearly demonstrate that androgens regulate the expression of mitochondrial SOD2 but not the cytosolic SOD1. This finding suggests that elevated ROS levels expressed in the prostates of castrated rats may, in part, be derived from the mitochondria. Future studies involving localization of key pro- and/or anti-OS mediators both cellularly and subcellularly in the rat prostate are necessary to identify the cell type(s) and the organelle(s) exhibiting most redox changes in response to androgen manipulations.

Recently, vascular endothelial cells were shown to play an important role in mediating the castration-induced regression in the prostate.[17,20] Degeneration and constriction of the vascular system in the prostate were found to be early events after castration, leading to reduced oxygen tension in prostate epithelial cells and activation of hypoxia- and stress-signaling pathways. Similarly, oxygen deprivation alone and combined oxygen and glucose deprivation induce ROS production in microvasculature[56] and neurons,[57] respectively. It is therefore logical to speculate that castration induces a hypoxic environment in the regressing prostate that leads directly or indirectly to elevation of OS in this gland. In a positive-feedback manner, increased OS in prostatic epithelium may, in turn, inflict additional oxidative damages to the vasculature and produce a vicious cycle of hypoxia- and OS-driven events that lead to a rapid involution of rat VP after castration.

In summary, our data suggest that normal physiological levels of androgen maintain a homeostasis between cellular pro-oxidant and antioxidant contents, as well as a balance between cell death and proliferation in the prostate. When normal androgenic status is disrupted, such as under the condition of castration-induced deprivation, OS is induced in the prostate via up-regulation of Nox-dependent ROS anabolism and down-regulation of a number of key antioxidant enzymes/ROS scavengers. The elevated OS state may play a critical role in mediating epithelial apoptosis and involution. When androgen is replaced, epithelial OS levels decline but fail to return to normalcy within a 7-day period, because of primarily high residual levels of Nox expression, although most antioxidant enzymes/ROS scavengers have returned to normalcy and exhibit an overcompensatory rebound to replenish cellular antioxidants and reducing power. This mild state of OS may contribute to cell proliferation, differentiation, and tissue remodeling in the regenerating VP. Collectively, this study provides the first mechanistic evidence that fills a significant data gap regarding the interrelationship between androgen status and redox homeostasis in the prostate. From a broader perspective, understanding androgenic regulation of OS in the prostate is of prime importance because both aging of the gland[7] and pathogenesis of prostatic diseases, such as benign prostatic hyperplasia, prostate cancer, and prostatitis, are believed to be caused by oxidative damages of cellular macromolecules in the human prostate.[6,8,9,30] Considering the fact that the use of androgen replacement therapy will be increased in our aging male population,[23,24] issues surrounding its suspected risk in promoting prostate cancer should be evaluated in light of the present findings. It is possible that long-term use of testosterone may elevate prostatic ROS anabolism and raise the likelihood of tumor initiation and promotion. Furthermore, our data may explain to some degree the results from the Finasteride Prostate Cancer Prevention Trial,[25] which demonstrated that androgen-blockade at the cellular level lowers prostate cancer risk but increases the prevalence of high-grade cancers. It is possible that the protective effect of androgen-blockade through Finasteride is mediated via reduction in cell proliferation, while the promotional effects are caused by androgen deprivation-induced chronic oxidative damages at the cellular/tissue level. Therefore, future studies focusing on the androgenic regulation on ROS production and elimination may result in better preventative or therapeutic modalities in controlling prostate diseases.

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References

1. Martindale JL, Holbrook NJ: Cellular response to oxidative stress: signaling for suicide and survival. J Cell Physiol 2002, 192:1–15
2. Burdon RH: Superoxide and hydrogen peroxide in relation to mammalian cell proliferation. Free Radic Biol Med 1995, 18:775–794
3. Davies KJ: The broad spectrum of responses to oxidants in proliferating cells: a new paradigm for oxidative stress. IUBMB Life 1999, 48:41–47
4. Gopalakrishna R, Jaken S: Protein kinase C signaling and oxidative stress. Free Radic Biol Med 2000, 28:1349–1361
5. Suh YA, Arnold RS, Lassegue B, Shi J, Xu X, Sorescu D, Chung AB, Griendling KK, Lambeth JD: Cell transformation by the superoxide-generating oxidase Mox1. Nature 1999, 401:79–82
6. Arbiser JL, Petros J, Klafter R, Govindajaran B, McLaughlin ER, Brown LF, Cohen C, Moses M, Kilroy S, Arnold RS, Lambeth JD: Reactive oxygen generated by Nox1 triggers the angiogenic switch. Proc Natl Acad Sci USA 2002, 99:715–720
7. Ghatak S, Ho SM: Age-related changes in the activities of antioxidant enzymes and lipid peroxidation status in ventral and dorsolateral prostate lobes of noble rats. Biochem Biophys Res Commun 1996, 222:362–367
8. Oberley TD, Zhong W, Szweda LI, Oberley LW: Localization of antioxidant enzymes and oxidative damage products in normal and malignant prostate epithelium. Prostate 2000, 44:144–155
9. Bostwick DG, Alexander EE, Singh R, Shan A, Qian J, Santella RM, Oberley LW, Yan T, Zhong W, Jiang X, Oberley TD: Antioxidant enzyme expression and reactive oxygen species damage in prostatic intraepithelial neoplasia and cancer. Cancer 2000, 89:123–134
10. Cheng G, Cao Z, Xu X, van Meir EG, Lambeth JD: Homologs of gp91phox: cloning and tissue expression of Nox3, Nox4, and Nox5. Gene 2001, 269:131–140
11. Lassegue B, Sorescu D, Szocs K, Yin Q, Akers M, Zhang Y, Grant SL, Lambeth JD, Griendling KK: Novel gp91(phox) homologues in vascular smooth muscle cells: Nox1 mediates angiotensin II-induced superoxide formation and redox-sensitive signaling pathways. Circ Res 2001, 88:888–894
12. Brar SS, Corbin Z, Kennedy TP, Hemendinger R, Thornton L, Bommarius B, Arnold RS, Whorton AR, Sturrock AB, Huecksteadt TP, Quinn MT, Krenitsky K, Ardie KG, Lambeth JD, Hoidal JR: NOX5 NAD(P)H oxidase regulates growth and apoptosis in DU 145 prostate cancer cells. Am J Physiol 2003, 285:C353–C369

13. Mates JM, Perez-Gomez C, Nunez DCI: Antioxidant enzymes and human diseases. Clin Biochem 1999, 32:595–603
14. Dickinson DA, Forman HJ: Glutathione in defense and signaling: lessons from a small thiol. Ann NY Acad Sci 2002, 973:488–504
15. Chae HZ, Kim HJ, Kang SW, Rhee SG: Characterization of three isoforms of mammalian peroxiredoxin that reduce peroxides in the presence of thioredoxin. Diabetes Res Clin Pract 1999, 45:101–112
16. Isaacs JT: Antagonistic effect of androgen on prostatic cell death. Prostate 1984, 5:545–557
17. Buttyan R, Shabsigh A, Perlman H, Colombel M: Regulation of apoptosis in the prostate gland by androgenic steroids. Trends Endocrinol Metab 1999, 10:47–54
18. Lee C: Physiology of castration-induced regression in rat prostate. Prog Clin Biol Res 1981, 75A:145–159
19. English HF, Drago JR, Santen RJ: Cellular response to androgen depletion and repletion in the rat ventral prostate: autoradiography and morphometric analysis. Prostate 1985, 7:41–51
20. Shabsigh A, Ghafar MA, de la Taille A, Burchardt M, Kaplan SA, Anastasiadis AG, Buttyan R: Biomarker analysis demonstrates a hypoxic environment in the castrated rat ventral prostate gland. J Cell Biochem 2001, 81:437–444
21. Pang ST, Dillner K, Wu X, Pousette A, Norstedt G, Flores-Morales A: Gene expression profiling of androgen deficiency predicts a pathway of prostate apoptosis that involves genes related to oxidative stress. Endocrinology 2002, 143:4897–4906
22. Schulman C, Lunenfeld B: The ageing male. World J Urol 2002, 20:4–10
23. Stas SN, Anastasiadis AG, Fisch H, Benson MC, Shabsigh R: Urologic aspects of andropause. Urology 2003, 61:261–266
24. de Lignieres B: Transdermal dihydrotestosterone treatment of 'andropause.' Ann Med 1993, 25:235–241
25. Thompson IM, Goodman PJ, Tangen CM, Lucia MS, Miller GJ, Ford LG, Lieber MM, Cespedes RD, Atkins JN, Lippman SM, Carlin SM, Ryan A, Szczepanek CM, Crowley JJ, Coltman Jr CA: The influence of finasteride on the development of prostate cancer. N Engl J Med 2003, 349:215–224
26. Kohen R, Nyska A: Oxidation of biological systems: oxidative stress phenomena, antioxidants, redox reactions, and methods for their quantification. Toxicol Pathol 2002, 30:620–650
27. Leav I, Ho SM, Ofner P, Merk FB, Kwan PW, Damassa D: Biochemical alterations in sex hormone-induced hyperplasia and dysplasia of the dorsolateral prostates of Noble rats. J Natl Cancer Inst 1988, 80:1045–1053
28. Thompson CJ, Tam NN, Joyce JM, Leav I, Ho SM: Gene expression profiling of testosterone and estradiol-17 beta-induced prostatic dysplasia in Noble rats and response to the antiestrogen ICI 182, 780. Endocrinology 2002, 143:2093–2105
29. Nakae D, Akai H, Kishida H, Kusuoka O, Tsutsumi M, Konishi Y: Age and organ dependent spontaneous generation of nuclear 8-hydroxydeoxyguanosine in male Fischer 344 rats. Lab Invest 2000, 80:249–261
30. Ripple MO, Henry WF, Rago RP, Wilding G: Prooxidant-antioxidant shift induced by androgen treatment of human prostate carcinoma cells. J Natl Cancer Inst 1997, 89:40–48
31. Morrissey C, Buser A, Scolaro J, O'Sullivan J, Moquin A, Tenniswood M: Changes in hormone sensitivity in the ventral prostate of aging Sprague-Dawley rats. J Androl 2002, 23:341–351
32. Hwang JJ, Choi SY, Koh JY: The role of NADPH oxidase, neuronal nitric oxide synthase and poly(ADP ribose) polymerase in oxidative neuronal death induced in cortical cultures by brain-derived neurotrophic factor and neurotrophin-4/5. J Neurochem 2002, 82:894–902
33. Kim YH, Koh JY: The role of NADPH oxidase and neuronal nitric oxide synthase in zinc-induced poly(ADP-ribose) polymerase activation and cell death in cortical culture. Exp Neurol 2002, 177:407–418
34. Mizutani H, Tada-Oikawa S, Hiraku Y, Oikawa S, Kojima M, Kawanishi S: Mechanism of apoptosis induced by a new topoisomerase inhibitor through the generation of hydrogen peroxide. J Biol Chem 2002, 277:30684–30689
35. Wang L, Medan D, Mercer R, Overmiller D, Leornard S, Castranova V, Shi X, Ding M, Huang C, Rojanasakul Y: Vanadium-induced apoptosis and pulmonary inflammation in mice: role of reactive oxygen species. J Cell Physiol 2003, 195:99–107
36. Zhang X, Shan P, Sasidhar M, Chupp GL, Flavell RA, Choi AM, Lee PJ: Reactive oxygen species and extracellular signal-regulated kinase 1/2 mitogen-activated protein kinase mediate hyperoxia-induced cell death in lung epithelium. Am J Respir Cell Mol Biol 2003, 28:305–315
37. Hsu TC, Young MR, Cmarik J, Colburn NH: Activator protein 1 (AP-1)- and nuclear factor kappaB (NF-kappaB)-dependent transcriptional events in carcinogenesis. Free Radic Biol Med 2000, 28:1338–1348
38. Gupta A, Rosenberger SF, Bowden GT: Increased ROS levels contribute to elevated transcription factor and MAP kinase activities in malignantly progressed mouse keratinocyte cell lines. Carcinogenesis 1999, 20:2063–2073
39. Shi DY, Deng YR, Liu SL, Zhang YD, Wei L: Redox stress regulates cell proliferation and apoptosis of human hepatoma through Akt protein phosphorylation. FEBS Lett 2003, 542:60–64
40. Ilio KY, Nemeth JA, Sensibar JA, Lang S, Lee C: Prostatic ductal system in rats: changes in regional distribution of extracellular matrix proteins during castration-induced regression. Prostate 2000, 43:3–10
41. Lee C, Sensibar JA, Dudek SM, Hiipakka RA, Liao ST: Prostatic ductal system in rats: regional variation in morphological and functional activities. Biol Reprod 1990, 43:1079–1086
42. Nemeth JA, Lee C: Prostatic ductal system in rats: regional variation in stromal organization. Prostate 1996, 28:124–128
43. Bosland MC, Ford H, Horton L: Induction at high incidence of ductal prostate adenocarcinomas in NBL/Cr and Sprague-Dawley Hsd:SD rats treated with a combination of testosterone and estradiol-17 beta or diethylstilbestrol. Carcinogenesis 1995, 16:1311–1317
44. Tam NN, Chung SS, Lee DT, Wong YC: Aberrant expression of hepatocyte growth factor and its receptor, c-Met, during sex hormone-induced prostatic carcinogenesis in the Noble rat. Carcinogenesis 2000, 21:2183–2191
45. Kirby RS, Lowe D, Bultitude MI, Shuttleworth KE: Intra-prostatic urinary reflux: an aetiological factor in abacterial prostatitis. Br J Urol 1982, 54:729–731
46. Vignais PV: The superoxide-generating NADPH oxidase: structural aspects and activation mechanism. Cell Mol Life Sci 2002, 59:1428–1459
47. Mollnau H, Wendt M, Szocs K, Lassegue B, Schulz E, Oelze M, Li H, Bodenschatz M, August M, Kleschyov AL, Tsilimingas N, Walter U, Forstermann U, Meinertz T, Griendling K, Munzel T: Effects of angiotensin II infusion on the expression and function of NAD(P)H oxidase and components of nitric oxide/cGMP signaling. Circ Res 2002, 90:E58–E65
48. Noh KM, Koh JY: Induction and activation by zinc of NADPH oxidase in cultured cortical neurons and astrocytes. J Neurosci 2000, 20:RC111:1–5
49. Katsuyama M, Fan C, Yabe-Nishimura C: NADPH oxidase is involved in prostaglandin F2alpha-induced hypertrophy of vascular smooth muscle cells: induction of NOX1 by PGF2alpha. J Biol Chem 2002, 277:13438–13442
50. Mohan PF, Jacobson MS: Inhibition of macrophage superoxide generation by dehydroepiandrosterone. Am J Med Sci 1993, 306:10–15
51. Kerr JFR, Winterford CM, Harmon BV: Morphological criteria for identifying apoptosis. Cell Biology. A Laboratory Handbook, vol 1. Edited by JE Celis. San Diego, Academic Press, 1994, pp 319–329
52. Sinha AA, Bentley MD, Pomroy Jr FE, Jamuar MP: Freeze-fracture, ultrastructural and autoradiographic analysis of the ventral prostate glands in castrated mice. Cell Tissue Res 1981, 215:547–561
53. Mercader M, Bodner BK, Moser MT, Kwon PS, Park ES, Manecke RG, Ellis TM, Wojcik EM, Yang D, Flanigan RC, Waters WB, Kast WM, Kwon ED: T cell infiltration of the prostate induced by androgen withdrawal in patients with prostate cancer. Proc Natl Acad Sci USA 2001, 98:14565–14570
54. Geiszt M, Kopp JB, Varnai P, Leto TL: Identification of renox, an NAD(P)H oxidase in kidney. Proc Natl Acad Sci USA 2000, 97:8010–8014
55. Ripple MO, Pickhardt PA, Wilding G: Alteration in gamma-glutamyl transpeptidase activity and messenger RNA of human prostate carcinoma cells by androgen. Cancer Res 1997, 57:2428–2433
56. Steiner DR, Gonzalez NC, Wood JG: Interaction between reactive oxygen species and nitric oxide in the microvascular response to systemic hypoxia. J Appl Physiol 2002, 93:1411–1418
57. Almeida A, Delgado-Esteban M, Bolanos JP, Medina JM: Oxygen and glucose deprivation induces mitochondrial dysfunction and oxidative stress in neurones but not in astrocytes in primary culture. J Neurochem 2002, 81:207–217