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Slowing Tumorigenic Progression in TRAMP Mice and Prostatic Carcinoma Cell Lines Using Natural Anti-Oxidant from Spinach, NAO—A Comparative Study of Three Anti-Oxidants

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ABSTRACT

The TRAMP model and human prostatic cancer (PCA) cell lines DU145 and PC3 are useful for chemopreventive studies. We compared the efficacy of 3 anti-oxidants [a water-soluble natural anti-oxidant, NAO (200 mg/kg), found in spinach leaves; epigallocatechin-3 gallate, EGCG (200 mg/kg), a major green tea polyphenol; and *N*-acetylcysteine, NAC (125 mg/kg)] plus vehicle in slowing spontaneous tumorigenic progression in TRAMP and wild-type male mice. Sacrifices occurred on weeks 5, 9, and 13. Prostatic histopathology and oxidative-stress blood markers were evaluated. Hyperplasias were ranked by a combination of severity grade and distribution (focal, multifocal, and diffuse). The effectivity of each tested compound in reducing the severity/focalness of hyperplasia varied from lobe to lobe. NAO exerted a significant effect on the dorsal and lateral lobes; NAC, on the anterior and ventral lobes, and EGCG, on the ventral lobe. When the most severe hyperplasia in all 4 lobes of TRAMPs was evaluated, only NAO reduced hyperplasia at weeks 9 and 13. Plasma peroxide levels in TRAMPs were reduced following oral administration of NAO or NAC for 13 weeks; EGCG only slightly reduced these levels. In NAO-treated DU145 and PC3 PCA cells, inhibition of cellular proliferation occurred in a dose-dependent manner, increasing numbers of G1 cells and reducing ROS levels. The anti-oxidative and antiproliferative properties of NAO may explain its efficacy in slowing the spontaneous prostatic carcinogenic process in the TRAMP and its effects in the cell lines.

Keywords. Anti-oxidant; spinach; TRAMP mice; prostate; hyperplasia; cancer.

INTRODUCTION

Studies with many chemical carcinogens have shown that their effects in the initiation, promotion, and progression of carcinogenesis are exerted via the generation of oxygen and other organic, free-radical intermediates (53). In recent years, the naturally occurring polyphenolic anti-oxidants have received increased scrutiny as cancer-preventive agents (62). Polyphenols possess potent anti-oxidant properties, including the scavenging of oxygen radicals, nitric oxide, and lipid radicals (47, 55), and exert profound biochemical and pharmacological effects including anti-oxidation, modulation of carcinogen metabolism, inhibition of cellular proliferation, induction of apoptosis, and cell-cycle arrest (33).

Investigation of the association of fruit and vegetable intake and prostatic cancer risk in men under 65 years of age showed that the consumption of large amounts of vegetables, particularly those in the cruciferous category, was associated with reduced risk of prostate cancer (12). In another study, the lower incidence of prostatic cancer in Asians was linked to the presence of isoflavonoids derived from soy and found at high concentrations in prostatic fluids (42). A recent review of epidemiological studies concluded that reasonable, though mixed, evidence exists for an inverse association between prostatic cancer (PC) risk and consumption of vegetables, including tomatoes, legumes, and beans (10). In other investigations high consumption of foods containing soy resulted in elevated concentrations of phytoestrogens, including genistein (5, 42), in plasma, urine, and prostatic fluid. More recently, soybean phytochemicals, including genistein, inhibited in vivo growth of PC tumors that resulted from the SC injection of PC cells in mice and rats (48, 63, 64). Genistein also inhibited the growth of rat MAT-LyLu and human PC-3 cell lines in vitro (44). Recently investigators demonstrated that dietary administration of genistein (500 mg/kg/day) significantly reduced the percentages of TRAMP (TRansgenic Adenocarcinoma of the Mouse Prostate) males that developed poorly differentiated adenocarcinoma in a dose-dependent manner (40).

Natural anti-oxidant (NAO), a mixture of mainly aromatic polyphenols and flavonoids isolated from spinach leaves, is an effective free-radical scavenger and inhibitor of the lipoxygenase enzyme (24). NAO can easily be used in mice because it is water-soluble and stable at high temperatures and lacks toxicity (LD_{50} of NAO in mice is 1,500 mg/kg). The efficacy

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of NAO as an anti-oxidant in both in vitro and in vivo models (dermal, oral, and IP administration) was shown superior to that of the well-known anti-oxidants vitamin E and butylated hydroxytoluene (BHT) (4, 24). The prophylactic therapeutic efficacy of NAO was demonstrated when tested in endotoxemia and cardiotoxicity models known to be associated with oxidative stress (2, 3, 7, 8, 34, 35). Researchers suggested that the beneficial effect of NAO was related to its anti-oxidative functions, including scavenging of released free radicals, as well as its anti-inflammatory actions (36).

Green tea, a popular beverage consumed worldwide, has been shown to exert chemopreventive effects against tumorigenesis and tumor growth in a wide range of target organs in rodent carcinogenesis models. These effects have been attributed to the biochemical and pharmacological activities of its polyphenolic constituents, most notably epigallocatechin-3-gallate (EGCG) (13, 58, 61). Epidemiological studies, although inconclusive, have suggested a protective effect of tea consumption against some cancers in humans (9, 32). Limited epidemiological studies indicated that men who consume tea regularly may have a lower risk of prostate cancer (26, 31); Japanese and Chinese populations who regularly consume tea, especially green tea, have the lowest incidences of prostatic cancer in the world (18). Oral infusion of a human-achievable dose (equivalent to 6 cups of green tea per day) to the TRAMP model resulted in significant inhibition of development and progression of prostatic cancer and increased survival (25).

N-acetylcysteine (NAC), an analogue and precursor of reduced glutathione, reduced the incidence of neoplastic and preneoplastic lesions induced by several chemical carcinogens in rodents. These lesions included lung adenomas, bladder carcinoma, skin papilloma, colon carcinoma, and mammary adenocarcinoma. Oral administration of NAC (0.025–4 g/kg) revealed anticarcinogenic activity and a dose-related delay in formation of primary tumors (14, 15). Among several anticarcinogenic mechanisms proposed were scavenging of reactive oxygen species (ROS), modulations of DNA repair and metabolism, regulation of cell cycle and apoptosis, and inhibition of invasions and metastasis (16).

In the TRAMP model that closely mimics the progressive form of the human disease, the expression of the SV40 early genes (T and t antigen) is driven by prostate-specific probasin that leads to cell transformation within the prostate (22). This strain develops prostatic cancer without any chemical or hormonal treatment; the prostate-specific lesions are characterized by precursor lesions, analogous to prostatic intraepithelial neoplasia (PIN), and rapidly developing, poorly differentiated carcinoma metastasizing to include lymph nodes and lungs—a sequential process over 12–28 weeks with median survival of 42 weeks (21). According to Matusik and colleagues (37), an advantage of the TRAMP model is that the mice develop relatively quickly tumors that originate in the dorsolateral lobe (human analog) so that therapies for aggressive metastatic cancer can be tested (27). Disadvantages of this model include variable rates of extremely rapid progression among animals and the presence of androgen-independent cells as early as 4 to 12 weeks of age (20).

The objective of this study was to compare the efficacy of three anti-oxidants—NAO, EGCG, and NAC—in slowing the progression of spontaneous tumorigenic progression in the TRAMP prostatic cancer model and suggest a mechanism for this action. Prostatic histopathology and biochemical oxidative-stress parameters in the blood were used to evaulate the observed effects. Prostatic cancer cell lines were used to elucidate the mechanisms by which NAO exerts it anticarcinogenic effects.

METHODS

Test Materials: NAO is a brown powder. It is a water-soluble anti-oxidant composed of a mixture of natural molecules extracted and purified from spinach leaves and containing mainly aromatic polyphenols (4). For its preparation, spinach leaves were homogenized with an equal amount of H_2O (w/w). The supernatant was collected and purified by ultrafiltration using a 3K-pore-size membrane. The filtrated fraction was collected and was used as NAO in this study. NAO solution (40 mg/ml saline) was prepared at the beginning of the study, divided into aliquots of 2 ml, and kept at -20°C until samples were thawed as used. Selection of the gavage dosage of NAO of 200 mg/kg body weight was based on previous experience with treatment of mice in a methylcholantrene model in which the effective oral dose of NAO was 0.1% (1 mg/ml) in drinking water (about 200 mg/kg body weight/day). The NAO dosage of 100–200 mg/kg/day was found to be significantly efficacious and safe in various models. The 100 mg/kg dose level was previously tested in the Tg.AC mouse model for skin cancer and found to be significantly ($p < 0.01$) effective but not toxic (45).

To prepare gavage dosing solutions, the appropriate amount of NAO was dissolved in calcium-free phosphate-buffered saline (PBS) to yield a concentration of 20 mg NAO/ml. The dosing solution was prepared at the beginning of the study, dispensed into brown glass vials cleared with nitrogen, and stored below -20°C until use. NAO was administered by oral gavage at a dosing volume of 5 ml/kg, yielding a dose of 200 mg NAO/kg body weight.

EGCG (98% pure) was supplied by Sigma (St. Louis, MO). The EGCG solution (40 mg/ml saline) was aliquotted at the beginning of the study and kept frozen at -20°C. Samples were defrosted each day for usage on that same day. The solution was administered by gavage at a dosage of 200 mg/kg and a volume dosage of 5 ml/kg; these amounts were based on published data of EGCG oral gavage treatment of mice in a TNF-alpha model in which the effective doses were 100 and 500 mg/kg BW/day (61).

NAC (Sigma Chemical Co, St. Louis, MO) was administered by oral gavage at a dose of 125 mg/kg reported to be effective in mice (1, 14, 15). The maximum feasible dose for NAC was 125 mg/kg/day in mice. Our preliminary testing indicated that higher concentrations of dosing solutions showed low solubility.

Studies Using TRAMP and Wild-Type Mice

Animals: One hundred twenty-eight (128) male TRAMP mice from a C57BL/6 background and 128 male wild-type C57BL/6 littermate mice were generated inhouse in the ILS TRAMP breeding colony. For mating, 1 or 2 female mice were placed in the cage of one male. Females were observed carefully, and, when pregnancy became obvious, nesting material was provided. The female and her litter were disturbed

as little as possible for approximately 10 days after birth. At 4 weeks of age, pups were weaned and sexed and tail snips from all healthy offspring used for genotyping and identifying the heterozygous TRAMP transgene carriers and wild-types.

The animals were housed in the ILS AAALAC-accredited Specific Pathogen-Free (SPF) facility and handled according to the guidelines provided in the NIH *Guide for the Care and Use of Laboratory Animals* (54). Mice were individually housed in polycarbonate cages containing heat-treated absorbent hardwood bedding (BetaChips, Northeastern Products Corp, Warrensburg, NY). The cage size, 476 cm^2 and 13 cm in height, was adequate to house up to 5 mice at the upper weight category (>25 g). All animals were transferred to clean cages with fresh bedding at least once a week. The temperature and humidity of the room were monitored continuously by hygrothermagraph. The lights were maintained on a 12-hour light/dark cycle. Because these animals were produced inhouse, there was no true "quarantine" period; however, the animals were acclimated in the study room prior to the study initiation. Prior to treatment, the animals were observed daily for any signs of illness or death, and only the clinically healthy animals were included in the study. The mice were approximately 7 weeks old at the start of treatment. Pelleted NTP-2000 (Zeigler Bros, Gardeners, PA) chow was provided ad libitum to all mice after weaning and until termination. Durham city tap water in water bottles with stainless steel sipper tubes was provided ad libitum. There were no contaminants in the feed or water that potentially could have impacted this study.

Study Design: Group designations, dose levels, and numbers of animals are presented in Table 1.

Clinical Examination: Animals were observed for mortality/moribundity twice daily during the week and once daily on weekends and holidays. Body weights of all animals were recorded at 6 weeks of age, 1 week prior to treatment, prior to the treatment on day 1, and weekly thereafter. Clinical observations were made at 6 weeks of age, 1 week prior to treatment, prior to treatment on day 1, and weekly thereafter. Clinical observations consisted of a thorough examination including the eyes and all orificies.

Food consumption was measured for 3 days before the first day of treatment and twice a week thereafter until the study termination.

Tissue Preparation and Histologic Examination: Two scheduled interim sacrifices of 5 mice per genotype per group were carried out at weeks 5 and 9 of the study; the terminal necropsy of all remaining mice occurred at week 13. For each animal, prior to necropsy, body weights were taken and blood samples collected from the retro-orbital sinus. Serum was harvested and stored at −70°C.

TABLE 1.—Experimental design—Group designations, dose levels, and numbers of animals.

Group number	Treatment	Number of mice	
		TRAMP	Wild-type
1	Control	32	32
2	NAO, 200 mg/kg	32	32
3	EGCG, 200 mg/kg	32	32
4	NAC, 125 mg/kg	32	32
	Total	128	128

1st interim sacrifice (6 mice/genotype/group): week 5 of treatment.
2nd interim sacrifice (6 mice/genotype/group): week 9 of treatment.
Terminal sacrifice: week 13.

A full necropsy was performed that included examination and removal of all organs. The accessory sex pluck (including prostate, seminal vesicles with coagulating gland/anterior prostate, and urinary bladder) was removed, weighed, placed intact on moistened filter paper, and put into a cassette to prevent curling and maintain anatomic relationships. Tissues were fixed in 10% neutral buffered formalin for 18–24 hours, then placed in 70% ethanol prior to tissue processing.

Trimming, processing, sectioning, and embedment of the sex pluck were performed as previously described (51). The seminal vesicle and coagulating gland were embedded without trimming and sectioned frontally to examine the entire length of the gland. The initial section was hematoxylin-and-eosin (H&E)-stained; 10 unstained sections followed. Subsequently, at 200 mμ deeper into the block, another H&E was followed by 10 unstained sections. This sampling was repeated at 400, 600, and 800 mμ into the block.

Histopathological evaluation using light microscopy was performed on all H&E-stained slides prepared from the prostate of each animal. That not all animals had all lobes examined was taken into consideration when applying the statistical analysis. The classification, quantitation, and progression of proliferative epithelial lesions were scored according to the method described by Suttie et al (50). Briefly, progression of proliferative lesions was divided into 6 grades readily identifiable histologically [grade 1-simple flat lesions; grade 2-papillary or cribriform structures, single-layered or with cell piling; grade 3-papillary or cribriform lesions protruding into lumen; grade 4-lesions occupying significant proportion of lumen; grade 5-lesions filling and expanding lumen, or distinct epithelial mass within lumen; grade 6-poorly differentiated epithelial tissue, local invasion, or distant metastasis (This grade was not seen in this experiment.)]. The grade of the most advanced lesion identified in each lobe was recorded for each animal and the distribution of this lesion grade recorded on a 3-point semiquantitative scale (focal, multifocal, diffuse).

To determine whether the beneficial effect of the 3 antioxidants on various lobes of the prostate is due to a "direct" biologic effect of the compounds on the lesions or an "indirectly" altered lesion development via transgene-expression reduction, the level of transgene expression was evaluated immunohistochemically. The prostates of three TRAMP animals from each treatment group (N = 4) and sacrifice period (N = 3) were selected for immunostaining using mouse anti-SV40 large T antigen (Tag) (BD Pharmingen, San Diego, CA). Samples from wild-type C57Bl/6 mice were also included. Sections were deparaffinized and hydrated to 1× Automation Buffer (Biomeda, Foster City, CA). Endogenous peroxidase was quenched with 3% H_2O_2. Sections were then blocked with 10% normal horse serum (Vector Laboratories, Burlingame, CA) and avidin and biotin (Avidin/Biotin Kit, Vector). The primary antibody, mouse anti-SV40 large Tag, was applied for 1 hour, and sections were then washed with 1× automation buffer. The secondary antibody, horse antimouse (Vector), was then applied at a 1:500 dilution for 30 minutes. Labeling was done with an avidin-biotin complex (ABC Elite Kit, Vector). The complex

was visualized with Liquid DAB (Dako Corporation, Cuperintina, CA). Samples from wild-type C57Bl/6 mice were also included.

Biochemical Analysis of the Plasma: The level of lipid hydroperoxides/H_2O_2 was measured in plasma samples collected from wild-type and TRAMP mice at 2 interim sacrifice intervals and termination of the study. The collected plasma samples were pooled in such a way that every 2 samples were placed in the same group and combined into 1 batch. This combining was done due to the limited amount of plasma (0.1–0.25 ml/animal) that was available for the various tests.

The level of hydroperoxides/H_2O_2 was measured by the FOX method in the presence of xylenol orange (28, 60). Protein concentration was determined in all fractions using bovine serum albumin (BSA) as a standard (6). The activity of superoxide dismutase (SOD) was determined spectrophotometrically in the plasma samples (39) by the ability of SOD to inhibit the reduction of cytochrome *c* in the presence of xanthine and xanthine oxidase. One unit was defined as the amount of enzyme that inhibits the reduction of cytochrome *c* by 50%. Enzyme activity was defined as the amount of activity units per mg protein.

Studies with NAO Using Prostatic Cancer Cell

Cell Lines and Culture: The human PCA cells, DU145 and PC3, were obtained from the American Type Culture Collection (Manassas, VA) and cultured in RPMI-1640 medium (Biological Industries, Inc, Kibbutz Beit Haemek, Israel) supplemented with 10% fetal calf serum, 1% penicillin-streptomycin, and 0.2% amphotericin. The cells were maintained at 37°C with 5% CO_2 in a humid environment.

Cell Viability: The PC3 and DU145 cells were grown to 70% confluence, detached using a solution of 0.1% trypsin, counted, plated $1 \times 10^6/_{10}$ ml dish, and treated with NAO (0.8, 1.6, and 3.2 mg/ml) for 24, 48, and 72 hours. The viable cells were determined using a cytotoxicity detection kit (Roche, Indianapolis, IN, USA).

Proliferation Assay: The effect on cellular proliferation was measured by a modified (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) (Sigma, MO) assay based on the ability of live cells to cleave the tetrazolium ring into a molecule that absorbs at 570 nm in active mitochondria (43). Then, 5×10^3 cells were grown on 96-well microliter plates and incubated with or without NAO (0.8, 1.6, and 3.2 mg/ml). After 72 hours, the medium was changed using 130 ul/well of fresh RPMI-1640 complete media. Next, 20 ul of MTT reagent (5 mg/1 ml PBS) was added for each well, and the cells were further incubated at 37°C for 2 hours. To determine lysis of the cells, 100 ul *N*,*N*-dimethyl formamide solution (50% final concentration of *N*,*N*-dimethyl formamide and 20% of sodium dodecyl sulphate, pH = 4.7) was added to each well for an additional 7 hours; a reading was then obtained on a scanning multiwell spectrophotometer.

DNA Cell Cycle Analysis: The PC3 and DU145 cells were grown to 70% confluence, detached using a solution of 0.1% trypsin, counted, and plated $1 \times 10^6/10$ mm/dish. The cells were treated with NAO (3.2 mg/ml) in RPMI-1640 complete medium for 24 hours, then spun at 300 × g. The pellet was resuspended in 250 ml PBS and 1 ml ethanol for 1 hour at 4°C. After centrifugation at 300 × g for 5 minutes, the pellet was washed twice with PBS, suspended in 400 ul PBS and 400 ul propidium iodide (50 mg/ml final concentration) for 15 minutes, and analyzed by flow cytometry.

Determination of ROS in Cultured Cells: The level of ROS in the cells was assayed as previously described (46). Nonfluorescent 2′,7′-dichlorofluorescein-diacetate (DCFH-DA) was used for monitoring the ROS with a spectrofluorometer (wavelength 485/535 nm) capable of reading microplates. The PC3 and DU145 cells were grown to 70% confluence, detached using a solution of 0.1% trypsin, washed immediately with PBS, counted, and plated 5×10^4/well/50 μl PBS in 96-well, flat-bottomed tissue-culture plates. NAO, or PBS for the control samples, was added to the wells. A stock solution of DCFH-DA was prepared by dissolving 2.0 mg/ml in ethanol, kept at −20°C wrapped in foil in the dark, and diluted 100 times in PBS. All of the plates were incubated for 1 hour at 37°C and then read with a Tican fluorometer (wavelength 485/530 nm).

Statistical Analysis: Body-and-organ-weight, biochemical, and PCA-cell data were analyzed using the ANOVA method followed by the Dunnett's test. Differences were considered significant when $p < 0.05$ (41). The in vitro results are representative of 3 experiments (N = 5 in each experiment). To evaluate the statistical significance of the differences in histopathological changes between the 3 treatments and the control animals, we used a bootstrapped ranks test (19) that allows simultaneous testing of 3 treatments against the controls. Tests were performed for each sacrifice week, each prostatic lobe, and the whole prostate. The whole-prostate tests used the data from the lobe with the highest grade of hyperplasia for each animal.

To calculate the test statistic for comparing a particular treatment with the control group, we ordered the hyperplasias by focalness (ie, focal, multifocal, diffuse) within the severity grades. As an example, a diffuse grade 3 hyperplasia was considered less severe than a focal grade 4 hyperplasia but more severe than a multifocal grade 3 hyperplasia. The null hypothesis for the test is that the distribution of severities and focalnesses is the same for treated animals as for control animals. The distribution of the test statistics under the null hypothesis was obtained by the bootstrap method. If the maximum test statistic for a lobe was significant ($p = 0.05$ or less), then the next smaller test statistic was also tested for significance (conditional on having rejected the null hypothesis for the maximum statistic), again using the bootstrap method. In this case, the null hypothesis was that the distribution of severities and focalnesses for the remaining 2 treatments was the same as for the control animals.

The test was also performed for each sacrifice week and each lobe using just the severity grade, without ordering by focalness within severity grades. Ignoring focalness reduced the available information, so these tests were not as powerful for detecting treatment effects as those that took the focalness into account.

RESULTS

Body and Organ Weight: Mean group body weights and body weight gains in TRAMP or wild-type mice treated

TABLE 2.—Incidence of hyperplasia in the anterior lobe of the prostate of TRAMP mice.

		Number of animals with hyperplasias†										
		Grade 2		Grade 3				Grade 4			Grade 5	
Sacrifice week	Treatment	Focal	Total	Focal	Multifocal	Diffuse	Total	Focal	Multifocal	Total	Focal	Total
5	Control			3	3		6					
	EGCG			2	4		6					
	NAC	1	1	3	3		6					
	NAO			3	3		6					
9	Control			1	4		5	1		1		
	EGCG			1	5		6					
	NAC				5	1	6					
	NAO			2	4		6					
13	Control			2	9		11	4	4	8	1	1
	EGCG			3	14		17	2	1	3		
	NAC*			4	13		17	2		2		
	NAO			1	10	1	12	7	1	8		

†To calculate the test statistic for comparing a treatment with the control group, we ordered the hyperplasias by distribution within the severity grades, eg, among hyperplasias, a diffuse grade 3 is considered less severe than a focal grade 4, but more severe than a multifocal grade 3.
*Significantly different from control at 0.05 level.

with anti-oxidants were similar to those in TRAMP or wild-type controls. At the 2 interim and terminal necropsies, liver, spleen, kidneys, pituitary, testes, epididymides, and sex plucks including the prostate, seminal vesicles, and urinary bladder were weighed, and mean group absolute and relative organ weights were calculated. At the second interim necropsy, a significant ($p < 0.05$) decrease in absolute and relative spleen weights in TRAMP mice treated with NAC relative to TRAMP controls was found. There were no other significant differences in organ weights of mice treated with anti-oxidants relative to their respective controls (data not presented).

Mortality, Clinical Signs, and Gross Findings: Two wild-type mice exposed to EGCG were humanely euthanized at 11 weeks; 2 wild-type mice exposed to NAO were found dead after 1 week; and 1 TRAMP mouse exposed to NAO was found dead after 4 weeks of study. No clinical observations or findings at necropsy were attributable to treatment with an anti-oxidant of TRAMP or wild-type mice.

Histopathological Findings and Statistical Evaluation: In the prostate of wild-type animals there were no proliferative changes in the epithelium other than occasional focal changes that were associated inflammatory lesions and grade-1 lesions in the anterior lobe of some mice. As reported by Suttie et al (50), these grade-1 lesions probably represented ductular epithelium in the neck of the anterior gland, not a true proliferative lesion.

The incidence and severity (ie, focalness) of the histopathologic findings noted in the TRAMP animals are presented in Tables 2–6. Statistical evaluation revealed several findings.

In the anterior lobe, NAC significantly ($p = 0.02$) reduced hyperplasia severity/focalness at week 13 (Table 2). NAC also significantly ($p = 0.02$) reduced hyperplasia when focalness was ignored.

In the dorsal lobe, NAO significantly ($p < 0.05$) reduced hyperplasia severity/focalness at week 9 and week 13 (Table 3). When focalness was not considered, NAO significantly ($p = 0.02$) reduced severity at 13 weeks. Examples of the lesion occurring at the highest frequency in the control group (grade 3 multifocal) and the NAO group (grade 4 focal) at 13 weeks are shown in Figure 1A and B.

In the lateral lobe, NAO significantly ($p = 0.02$) reduced hyperplasia severity/focalness at week 9 (Table 4). When focalness was ignored, no significant effects were detected.

In the ventral lobe, EGCG and NAC significantly ($p < 0.01$) reduced hyperplasia severity/focalness at week 13 (Table 5), and EGCG significantly ($p = 0.02$) reduced severity/focalness at week 9. No significant effects were detected when focalness was ignored.

TABLE 3.—Incidence of hyperplasia in the dorsal lobe of the prostate of TRAMP mice.

		Number of animals with hyperplasias										
		Grade 3				Grade 4				Grade 5		
Sacrifice week	Treatment	Focal	Multifocal	Diffuse	Total	Focal	Multifocal	Diffuse	Total	Focal	Multifocal	Total
5	Control	1	3	1	5							
	EGCG	2	4		6							
	NAC	4	2		6							
	NAO	3	3		6							
9	Control			6	6							
	EGCG		3		3	3			3			
	NAC		4	1	5			1	1			
	NAO*		5		5	1			1			
13	Control	2		3	5	7	3		10	4	1	5
	EGCG	1	2	1	4	9	4		13	2	1	3
	NAC		3	1	4	10	1		11	2		2
	NAO*		8	4	12	4	2		6	1		1

*Significantly different from control at 0.05 level.

TABLE 4.—Incidence of hyperplasia in the lateral lobe of the prostate of TRAMP mice.

Sacrifice week	Treatment	Number of animals with hyperplasias							
		Grade 2		Grade 3				Grade 4	
		Diffuse	Total	Focal	Multifocal	Diffuse	Total	Focal	Total
5	Control			1	3		4		
	EGCG			2	2	2	6		
	NAC				6		6		
	NAO	1	1	2	3		5		
9	Control				2	3	5		
	EGCG			1	4	1	6		
	NAC				5	1	6		
	NAO*			1	5		6		
13	Control	1	1	3	2	7	12	1	1
	EGCG				10	8	18	1	1
	NAC				6	10	16		
	NAO			2	12	3	17	1	1

*Significantly different from control at 0.05 level.

When the most severe hyperplasia in all 4 lobes was considered, irrespective of the lobe in which it was noted, only the NAO significantly reduced hyperplasia severity/focalness at weeks 9 ($p = 0.03$) and 13 ($p = 0.02$). No significant effects were observed when focalness was ignored (Table 6).

Effect of the Anti-Oxidant Agents on Transgene Expression: No difference in Tag levels was observed in the epithelium of the different prostatic lobes between any groups, as assessed by a scoring system using immunohistochemical staining (data not shown).

Biochemical Analysis of the Plasma

Lipid Hydroperoxides/H_2O_2: The biochemistry results are illustrated in Figure 2A and 2B. The level of lipid hydroperoxides/H_2O_2 measured in blood samples collected from control TRAMP mice on week 13 was significantly ($p < 0.01$) higher by 42% compared to the level measured in samples collected on week 5; this trend was not observed in wild-type animals. The level of lipid hydroperoxides/H_2O_2 in wild-types was similar among all the study intervals. In TRAMP mice, the level of lipid hydroperoxides/H_2O_2 was significantly ($p < 0.05$) reduced compared to vehicle, following oral administration of NAO (15% reduction) or NAC (17% reduction) for 13 weeks. Oral administration of EGCG, however, only slightly reduced the level of lipid hydroperoxides/H_2O_2 by 6%. In wild-type mice, no significant effect was obtained following oral administration of the anti-oxidants NAO, NAC, or EGCG up to 13 weeks.

Superoxide Dismutase: The activity of SOD was measured in plasma samples collected from wild-type and TRAMP mice at 2 interim sacrifice intervals (weeks 5 and 9) and at termination of the study (week 13). No significant effect on SOD activity was obtained in TRAMP or wild-type animals during the entire study period following oral administration of the anti-oxidants NAO, NAC, or EGCG, compared to vehicle controls (data not presented).

Studies of Prostatic Cancer Cell Lines Treated with NAO: NAO significantly ($p < 0.05$) inhibited cellular proliferation of PC3 and DU145 in a dose-dependent manner (Figure 3). For inhibition of PC3 proliferation, the NAO IC-50 (concentration that caused 50% inhibition of proliferation compared to control) was 2.5 mg/ml following 72 hours' incubation. For inhibition of DU145 proliferation the IC-50 of NAO was 2.2 mg/ml following 72 hours' incubation.The cytotoxicity assay demonstrated that NAO exerted no toxic effect on either cell line (data not shown). NAO was shown to affect the cell cycle of PC3 and DU145 causing accumulation of cells in the G1 stage (Figure 4). A positive effect of NAO on reducing the basal ROS level was also obtained in both cell lines as shown in Figure 5.

DISCUSSION

In the absence of satisfactory treatment options for prostatic cancer, chemoprevention could be an effective approach to reduce the incidence of the disease. For a variety of reasons, greater emphasis is being placed on identifying naturally occurring dietary substances as cancer chemopreventive agents than finding new treatments. Prostatic cancer is an excellent candidate for disease chemoprevention because it is typically diagnosed in elderly men; therefore, even a modest delay in neoplastic development achieved through pharmacological or nutritional intervention could result in a substantial reduction in the incidence of the clinically detectable disease.

Our findings indicate the benefits of chemoprevention and are consistent with those in recently published clinical and

TABLE 5.—Incidence of hyperplasia in the ventral lobe of the prostate of TRAMP mice.

Sacrifice week	Treatment	Number of animals with hyperplasias									
		Grade 2				Grade 3				Grade 4	
		Focal	Multifocal	Diffuse	Total	Focal	Multifocal	Diffuse	Total	Focal	Total
5	Control		1	1	2	1	3		4		
	EGCG	1			1	5			5		
	NAC		2		2	2	2		4		
	NAO			2	2	1	3		4		
9	Control						4	1	5		
	EGCG*					5	1		6		
	NAC		2		2		1		1	1	1
	NAO		3		3	1	2		3		
13	Control					1	4	4	9		
	EGCG**		2		2	7	2		9		
	NAC**	1	1		2	10	2	1	13		
	NAO		2	1	3	3	4	2	9		

*Significantly different from control at 0.05 level.
**Significantly different from control at 0.01 level.

TABLE 6.—Incidence of most severe hyperplasia (irrespective of the lobe) in the prostate of TRAMP mice.

		Number of animals with hyperplasias												
		Grade 2		Grade 3				Grade 4				Grade 5		
Sacrifice week	Treatment	Focal	Total	Focal	Multifocal	Diffuse	Total	Focal	Multifocal	Diffuse	Total	Focal	Multifocal	Total
5	Control				5	1	6							
	EGCG			1	3	2	6							
	NAC	1	1		6		6							
	NAO			1	5		6							
9	Control					5	5	1			1			
	EGCG				3		3	3			3			
	NAC				2	2	4	1		1	2			
	NAO*				5		5	1			1			
13	Control					5	5	7	2		9	5	1	6
	EGCG				3	1	4	8	5		13	2	1	3
	NAC			1	3	1	5	11	1		12	2		2
	NAO*				7	3	10	7	2		9	1		1

*Significantly different from control at 0.05 level.

experimental surveys reporting the effectiveness of particular food constituents on the incidence of prostatic cancer (11, 23). For example, the effect of tomato sauce-based pasta dishes on human patients already diagnosed with prostatic cancer, whose cases had been closely followed by physicians, was compared to that on other randomly selected prostatic cancer patients. Results showed that this lycopene-containing diet was effective in reducing DNA oxidative damage in the prostate, as well as the serum levels of prostate-specific antigen (PSA), a marker routinely used to determine the risk for prostatic cancer development. Saturated fats have been associated with advanced prostatic cancer risk (23). The results obtained by Wechter et al (57) in the TRAMP model suggested that metastatic potential or invasiveness of the cancer is considerably diminished by a combination of E-7869 (R-flurnipropen, a nonsteroidal anti-inflammatory drug) and a lower saturated-fat content of the diet.

The statistical test used to analyze histopathological changes in the current study was constructed to allow the simultaneous comparison of 3 different treatments and a control group. We applied this test 15 times (3 sacrifice weeks $\times$ 4 lobes + whole prostate) with 8 of the 15 tests showing significant ($p < 0.05$) results for at least 1 compound. In every case, the direction of the change showed that the anti-oxidant reduced hyperplasia severity/focalness. These were changes primarily in "focalness" rather than severity.

Within each lobe, when a significant treatment effect for both weeks 9 and 13 was noted, the anti-oxidant was the same. This association further strengthens the conclusion that the treatment effects were genuine. On the other hand, the effectivity of each tested compound in reducing the severity/focalness of hyperplasia varied from lobe to lobe; NAO exerted a significant effect in the dorsal and lateral lobes, NAC, in the anterior and ventral lobes, and EGCG, in the ventral lobe (Tables 2–5).

Parallelling these data, background severities varied by lobe. For example, at 13 weeks all control severities in the ventral lobe and 12/14 in the lateral lobe were graded 3. In contrast, 75% (15/20) of control severities in the dorsal lobe were 4 or 5 (Tables 3–5). The anterior lobe was intermediate. This variation suggests that, if a localized effect on prostatic hyperplasia severity is suspected, then separate severities should be obtained for each lobe rather than recording only the most severe lesion at any location for a "whole prostate" analysis. For example, for these data, the most severe "whole prostate" severities for each animal were essentially the same as those of the dorsal prostate (see Tables 2–6). Thus, if a chemical reduces severity only at one or more of the other prostatic lobes, where the background severity

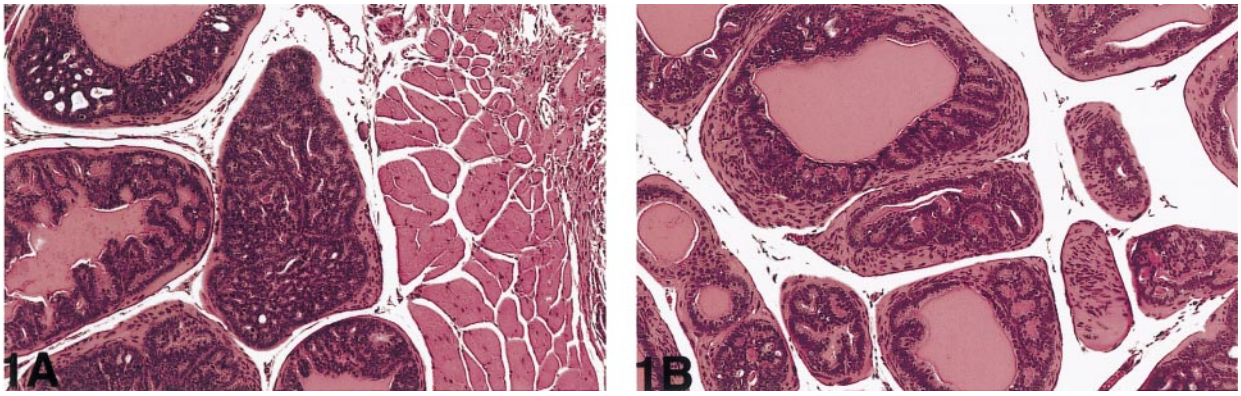
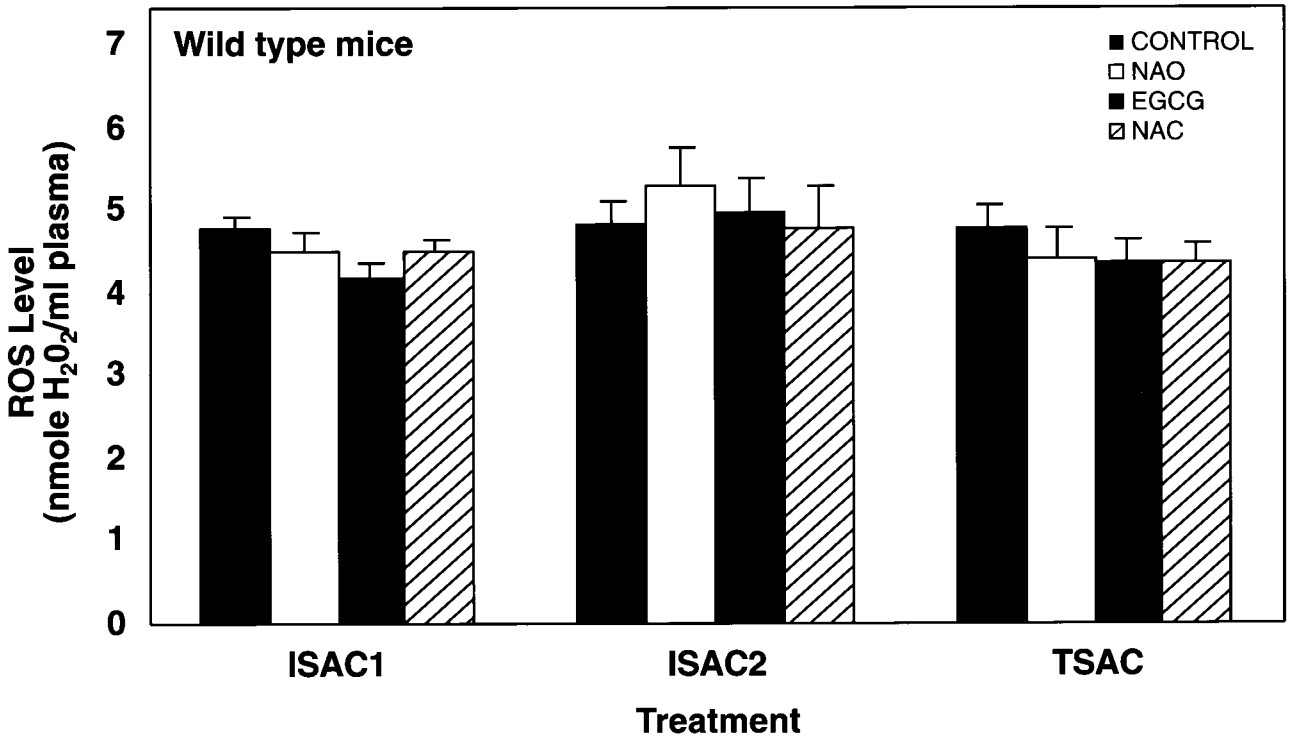
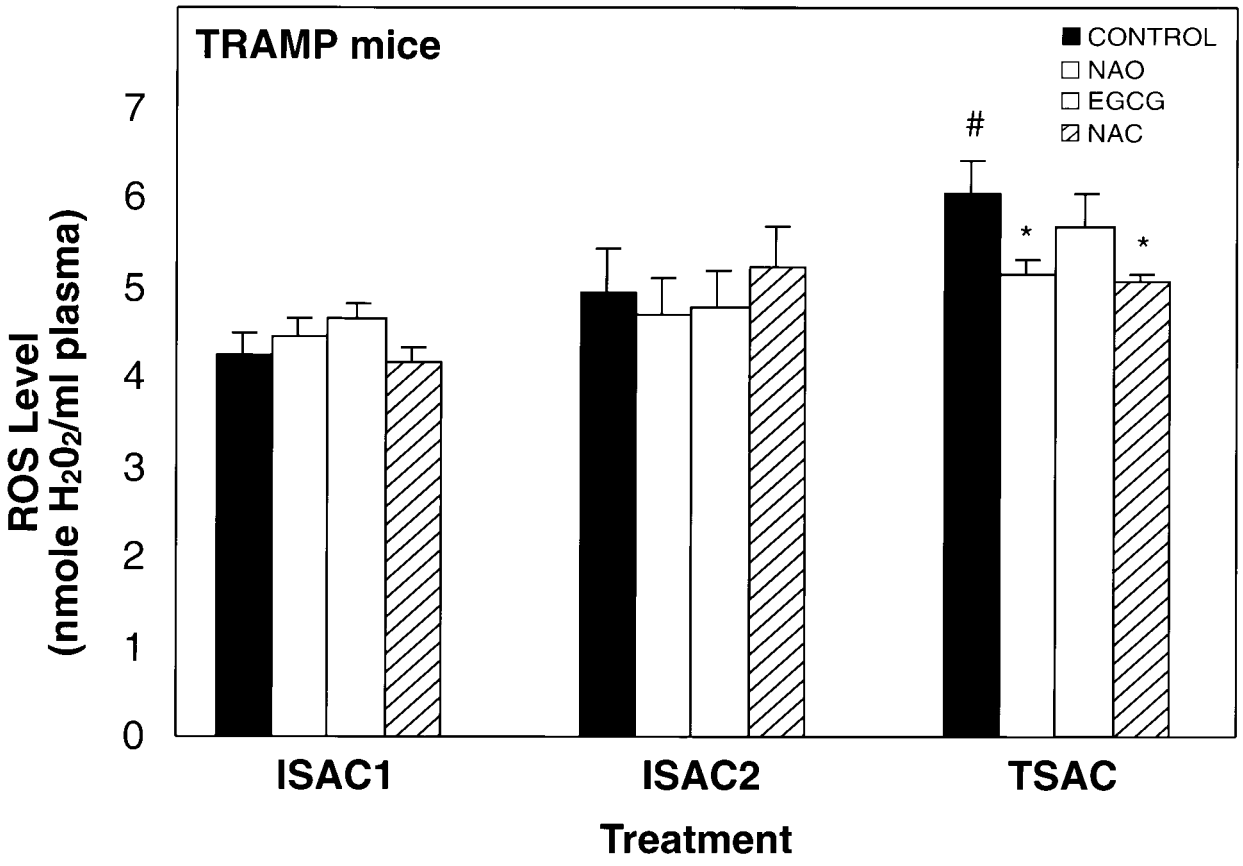


FIGURE 1.—Effect of NAO on proliferative epithelial lesions in dorsal prostate. Photomicrographs showing lesions occurring with highest frequency in TRAMP mice, 13-week terminally sacrificed, control and NAO-treatment groups. A) Control. Grade 4 lesion, focal. Epithelial proliferation occupies acinus. B) NAO. Grade 3 lesion, multifocal. Epithelial proliferation protruding into lumen of acinus.



(A)



(B)

FIGURE 2.—The level of lipid hydroperoxides/H_2O_2 in plasma samples collected from wild-type (A) and TRAMP (B) mice at week 5 (ISAC1), week 9 (ISAC2), and week 13 at terminal sacrifice (TSAC). The "error bars" are SE. N = 6 for interim sacrifice samples and N = 10 for terminal sacrifice samples. #Significantly different compared to control ISAC1 ($p < 0.01$). *Significantly different compared to control TSAC ($p < 0.05$).

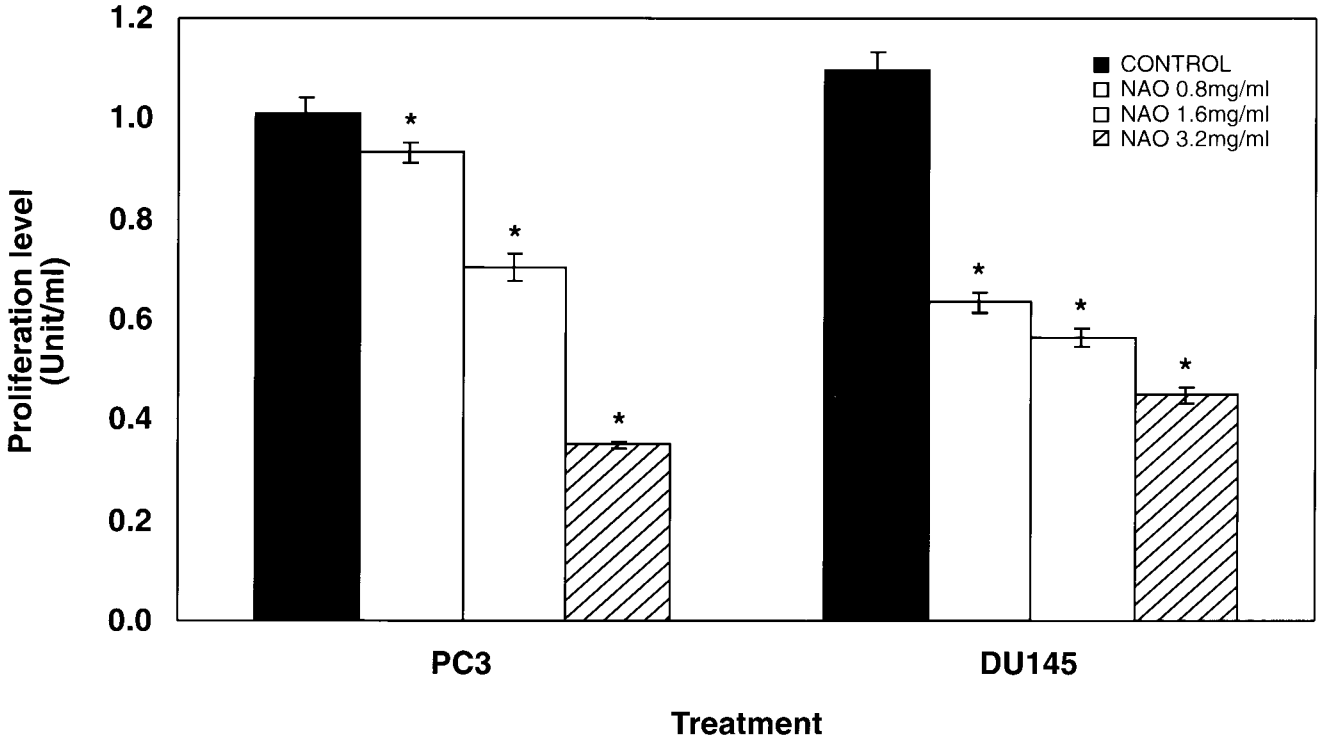


FIGURE 3.—Proliferation of DU145 and PC3 cells following NAO treatment. Cells were incubated with NAO (0.8, 1.6, and 3.2 mg/ml) for 72 hours; viable cells were measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay ($p < 0.05$). The "error bars" are SE. N = 5 wells/per treatment. The results are representative of 3 experiments. *Significantly different compared to control ($p < 0.05$).

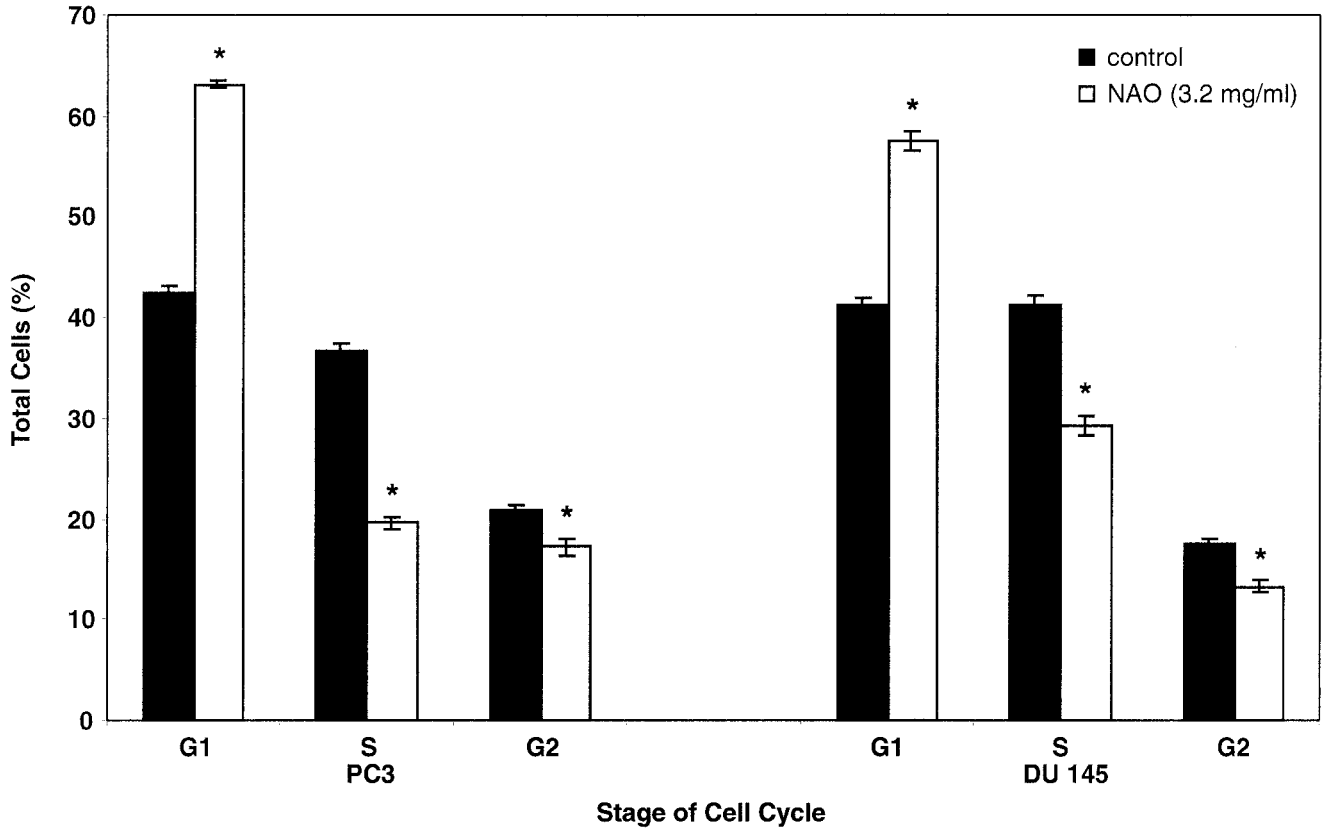


FIGURE 4.—Cell cycle analysis of PC3 and DU-145 cells treated with 3.2 mg/ml NAO for 24 hours and analyzed by flow cytometry. Percentages of cells in G1 and G2 phases were calculated using cell-fit computer software. The "error bars" are SE. N = 5 wells/per treatment. The results are representative of 3 experiments. *Significantly different compared to control ($p < 0.05$).

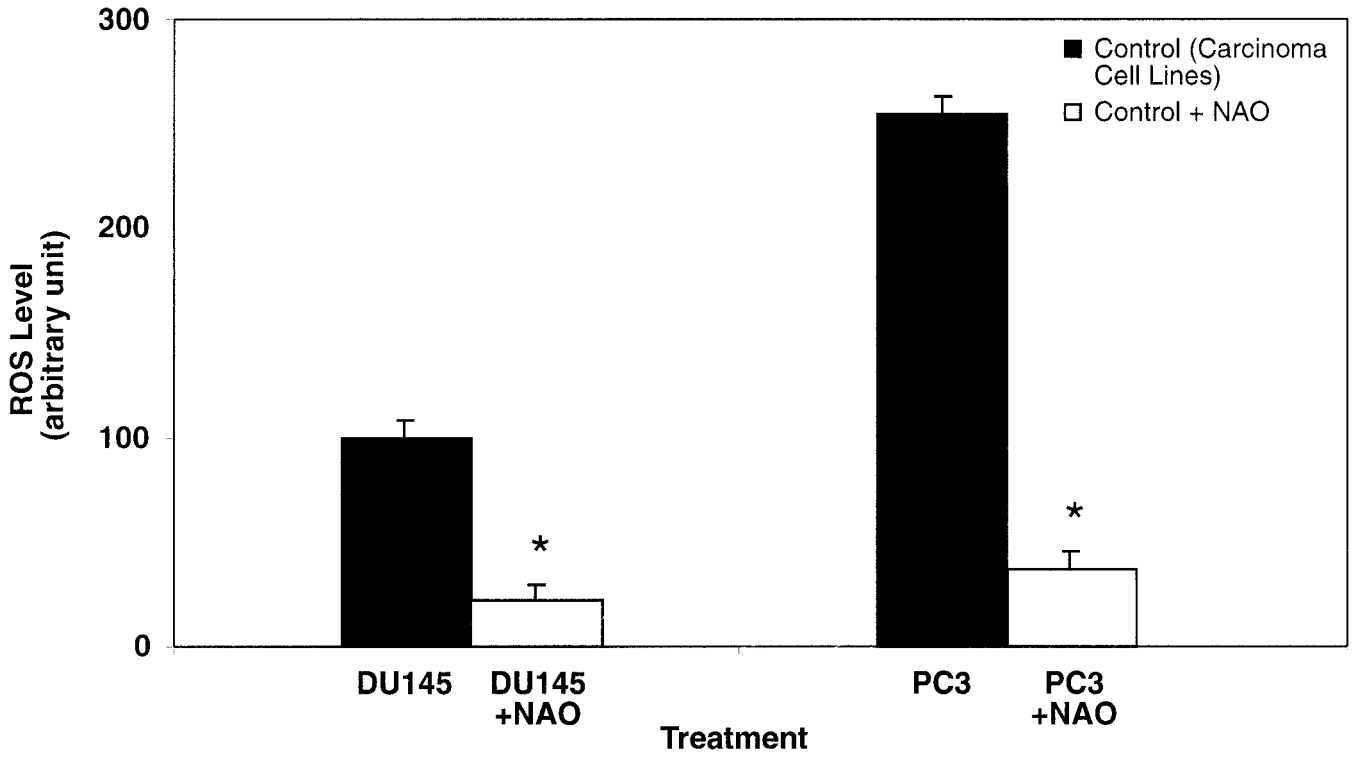


FIGURE 5.—Effect of NAO on ROS levels in human prostatic cancer cell lines. Cells were incubated with 1.6 mg/ml NAO and 2′,7′-dichlorofluorescein diacetate (DCFH-DA) for 1 hour at 37°C; the fluorescent signal was then measured. The "error bars" are SE. N = 5 wells/per treatment. The results are representative of 3 experiments. *Significantly different compared to control ($p < 0.01$).

is lower, then these reductions would likely be undetected by a "whole prostate" analysis. In particular, the significant ($p < 0.05$) reductions in hyperplasia severity in the ventral lobe observed for NAC and EGCG and a similar reduction in the anterior lobe found for NAC at 13 weeks were not detected by a "whole prostate" analysis, because the latter was based primarily on dorsal lobe severities. Not surprisingly, the significant reduction in dorsal prostate severity in the NAO group was also detected in the whole prostate analysis, since the 2 analyses reflected nearly the same data. The reason for the different efficacies exerted by the 3 anti-oxidants tested upon the various lobes is unknown and currently under investigation by the application of in situ immunohistochemical labels for the visualization of the distribution of oxidative stress markers in the prostatic tissue. We suggest that the specific cell type comprising each lobe may react differently to the presence of different anti-oxidants and/or ROS in the blood.

We have shown for the first time that aged TRAMP mice are oxidatively stressed and that, following treatment with NAO or NAC, the level of hydroperoxides/H_2O_2 returned to normal. That reversal may be explained by alteration in the anti-oxidative/oxidative balance in TRAMP mice. The exact molecular pathways that contribute to higher plasma levels of ROS in TRAMP mice are unknown and merit further investigation. The significant reduction of the level of hydroperoxides/H_2O_2 in blood by NAO may contribute to its effectiveness in the reduction of the severity/focalness of prostatic hyperplasia. We emphasize that NAO was effective in reducing hydroperoxides/H_2O_2 in TRAMPs only when oxidative stress occurred, but not basal hydroperoxide/H_2O_2 levels, and not in wild-type animals.

In our study, in contrast to NAO, EGCG failed to reduce hydroperoxide/H_2O_2 levels and apparently was less efficacious in slowing the progression of the prostatic carcinogenesis. Gupta et al (25), however, using the TRAMP model showed that oral administration of green tea in drinking water (500 mg/kg/day) caused a significant inhibition of the development, progression, and metastasis of prostatic cancer to distant organ sites. Furthermore, green tea consumption caused significant apoptosis of prostatic cancer cells, which possibly resulted in reduced dissemination of cancerous cells, thereby causing the inhibition. Oral infusion of green tea polyphenols to TRAMP mice caused significant inhibition of serum IGF-I and restoration of serum IGFBP-3 levels. The findings may suggest the use of IGF-I and IGFBP-3 as serum biomarkers for monitoring prostatic cancer chemoprevention (25). Oral infusion of EGCG is expected to cause a higher systemic exposure compared to oral gavage administration. That may contribute to higher efficacy of EGCG when given in drinking water compared to gavage and may explain the differences in protective effects observed between these previously reported results (25) and our results.

The relatively minor protective effect of EGCG compared to NAO observed in our investigation may be explained to some extent by the pharmacokinetic properties of green tea that has relatively low oral bioavaiability (65), possibly due to slow absorption as well as high metabolic clearance by the

liver (first-pass effect). To elucidate the biological effects of green tea consumption, Kim and colleagues (30) measured plasma and tissue levels of EGCG in rats and mice given a 0.6% green tea polyphenol preparation as the drinking fluid for different periods of time. The EGCG levels became much higher in mice than rats. The results suggested that consumption of EGCG by rodents involved adaptive responses affecting blood and tissue levels of tea catechins with time and that investigation of a similar phenomenon in humans is warranted.

Glycosylation of all aromatic polyphenols isolated from spinach leaves is expected to increase their intestinal absorption and bioavailability and may contribute to beneficial pharmacodynamic effects of NAO compared to other natural flavonoids (4).

In the current study NAC was effective in reducing lipid hydroperoxide/H_2O_2 levels in blood and the severity/focalness of prostatic hyperplasia in the anterior and ventral lobes. NAC has exerted protective effects against a variety of chemical mutagens (16) and induction of preneoplastic and neoplastic lesions in cancer models such as skin, mammary gland, intestine, and lung in rodents. A variety of biomarkers was affected by the oral administration of NAC in rats exposed to cigarette smoke, including protection of the respiratory epithelium from massive histopathological damage, modulation of metabolism, and formation of carcinogen-DNA adducts in several organs. The marked decrease of tumors observed in animals treated with NAC most likely reflects the detoxification of direct-acting mutagens due to its nucleophilic and anti-oxidant properties and less by interfering with the promotion stage of the carcinogenicity process. NAC has appeared to be one of the most promising cancer chemopreventive agents due to its beneficial effects in preclinical studies (15); however, when tested in a 2-year clinical trial (project Euroscan) (56), it was found ineffective on lung and neck cancer.

NAO has previously exhibited dramatic anti-inflammatory properties in lipopolysaccharide(LPS)-induced septic shock in rat and rabbit models (3, 4, 34, 35). Anti-inflammatory compounds have been proven effective in preventing prostatic cancer development in transgenic TRAMP mice (57). Animals treated daily by oral gavage with R-Flurbiprofen demonstrated lower incidences of primary tumors and metastasis. In addition to its anti-inflammatory and anti-oxidant properties, NAO was shown by immunohistochemical staining to inhibit the expression of cyclooxygenase (COX)-2 in various organs (36). The inhibition of prostaglandin production may play an important role in slowing or inhibiting carcinogenesis. Prostaglandins increase numbers of cells and colonies in numerous cancer cell lines (49, 52). Several papers have demonstrated an increase in prostaglandin E2 (PGE2) levels in colorectal carcinoma tumors compared to surrounding normal tissue (38). Researchers have also demonstrated that PGE2 can inhibit apoptosis and upregulate bcl-2 expression in colon cancer cell lines (49).

A dose- and time-dependent inhibition of prostatic cell growth by polyphenols was described for different wine polyphenol anti-oxidants (29). That effect was reported to correlate with antiproliferative action and inhibition of inducible nitric oxide synthase (NOS). Wine polyphenols include phenolic acids such as *p*-coumaric and cinamic and various flavonoids (catechin, epicatechin, and quercetin). The proliferation of LNCaP and PC3 cells was preferentially inhibited by flavonoids, whereas resveratrol was the most potent inhibitor of DU145. All other phenols showed only low interactions. Production of oxygen species after mitogen stimulation and H_2O_2 sensitivity of these cell lines did not correlate with the observed antiproliferative effects and ruled out such a mode of action.

NAO is a mixture of spinach anti-oxidants composed mainly of coumaric acid derivatives and flavonoids. Its antiproliferative effect is more likely related to the flavonoid fraction. The antiproliferative effect of specific isolated NAO fractions is under investigation. The G1-phase cell-cycle arrest of prostatic cancer cells by NAO suggests that this agent may slow the growth of cancerous cells by artificially imposing the cell-cycle checkpoint.

The similarity in the Tag expression in the prostatic epithelium of untreated and antioxidant-treated groups suggests that the mechanism of action of these anti-oxidant agents against TRAMP prostatic cancer is related to a direct anticarcinogenic effect, not through downregulation of Tag.

The reason for the statistically significant decrease in absolute and relative spleen weights noted in the second interim sacrifice in TRAMP mice treated with NAC relative to TRAMP controls is not known. Although the spleen was not checked histologically, NAC is known to protect lymphocytes from potential damage from biologically and chemically toxic compounds (17, 59).

In summary, our studies demonstrated that the effectivity of each of the tested antioxidants in reducing the severity/focalness of hyperplasia varied from lobe to lobe. NAO exerted a significant effect on the dorsal and lateral lobes; NAC, on the anterior and ventral lobes, and EGCG, on the ventral lobe. When the most severe hyperplasia in all 4 lobes of TRAMPs was evaluated, only NAO reduced hyperplasia at weeks 9 and 13 of sacrifice. We demonstrated that our method for scoring the progression of proliferative epithelial lesions of the prostate (50) allowed consistent quantification and comparison of the beneficial effect of chemical and dietary interventions in the TRAMP model. NAO reduced peroxide levels in TRAMP plasma and PCA cell lines, where inhibition of cellular proliferation and increased proportions of G1-arrest cells also occurred. The anti-oxidative and antiproliferative properties of NAO may explain, at least in part, its effectiveness, greater than that of NAC or EGCG, in slowing the spontaneous prostatic carcinogenic process in the TRAMP and its effects in the 2 cell lines. Our findings propose the protective benefits and efficacy of using natural anti-oxidants, particularly NAO, in reducing the risk or slowing the progression of prostatic cancer without any apparent safety concerns.

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