Effects of Dietary Saw Palmetto on the Prostate of Transgenic Adenocarcinoma of the Mouse Prostate Model (TRAMP)

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BACKGROUND. Several of the proposed mechanisms for the actions of the liposterolic extract of saw palmetto (SPE) are exerted on known risk factors for prostate cancer (CaP). This study investigated whether SPE could prevent the progression of CaP in a transgenic adenocarcinoma of the mouse prostate (TRAMP) model.
METHODS. Two different doses of SPE designed to deliver 50 mg/kg/day SPE and 300 mg/kg/day SPE were administered in a custom diet to TRAMP mice for 12 or 24 weeks. Body and organ weights were used to evaluate toxicity, and radioimmunoassay was used to measure plasma and tissue androgen levels to monitor effects of SPE on 5α reductase activity. Prostate tissues were evaluated histologically to determine the effect of treatment on tumor grade, cell proliferation, and apoptosis.
RESULTS. Treatment with 300 mg/kg/day SPE from 4 to 24 weeks of age significantly reduced the concentration of 5α-dihydrotestosterone (DHT) in the prostate and resulted in a significant increase in apoptosis and significant decrease in pathological tumor grade and frank tumor incidence.
CONCLUSIONS. Dietary supplementation with SPE may be effective in controlling CaP tumorigenesis. SPE suppression of prostatic DHT levels lends support to the hypothesis that inhibition of the enzyme 5α-reductase is a mechanism of action of this substance. *Prostate 67: 661–673, 2007.* © 2007 Wiley-Liss, Inc.

KEY WORDS: *Serenoa repens*; **prostate cancer; 5α-reductase; apoptosis; Ki67**

INTRODUCTION

The limited options for the treatment of prostate cancer (CaP) have prompted the need for chemopreventive strategies. CaP is the most common malignancy among men and is second to lung cancer in death rate [1]. It is typically a slow-growing disease diagnosed in men with a median age of 72 years [2]. Because of the unique profile of this disease, non-toxic, phytotherapeutic interventions that cause even a modest delay in its progression could result in a substantial reduction in its prevalence. Here, we evaluated the chemopreventive potential of the popular self-prescribed herbal preparation saw palmetto extract (SPE) on the transgenic adenocarcinoma of the mouse prostate (TRAMP) model.

Numerous mechanisms are implicated in CaP pathogenesis. The androgen dependency of CaP has led to intense focus on the role of androgen metabolism as a risk factor. 5α-reductase is the enzyme that converts testosterone (T) to dihydrotestosterone (DHT), the

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most biologically active androgen. While the 5α-reductase-inhibitor finasteride prevents or delays the appearance of CaP, this benefit is confounded by sexual side effects and the increased risk of high-grade CaP [3]. Chronic or recurrent inflammation in men with a history of clinical prostatitis, sexually transmitted infections, and/or circulating markers of inflammation is also thought to be associated with an increased relative risk for the development of CaP [4,5], and frequent use of nonsteroidal anti-inflammatory drugs is linked to a reduced risk of CaP occurrence [6]. Epidemiological and molecular biological studies also implicate dysregulation of the insulin-like growth factor (IGF) system in prostate tumorigenesis. Specifically, high circulating levels of IGF-I increase CaP risk in men, and expression of IGF-I in prostate basal epithelial cells in transgenic mice produces prostate neoplasia that is similar to human prostate intraepithelial neoplasm (PIN) and adenocarcinoma [7–9].

SPE, derived from the berry of the American palm tree (*Serenoa repens)*, is the most widely used, self-prescribed treatment for urinary symptoms caused by benign prostate hyperplasia (BPH) [10,11]. SPE is also one of the most commonly self-prescribed phytochemicals used in conjunction with conventional medical treatments to promote prostate health and prevent the progression of existing CaP [12]. In a national survey conducted in 2002, over 2 million men reported the use of SPE [13]. Despite the prevalence of its use, no studies have been performed to evaluate whether SPE is effective at preventing the in vivo development or progression of CaP. Several in vitro studies show that SPE inhibits the growth of CaP cell lines in culture, suggesting that it may possess chemopreventive potential [14–16]. The lipophilic extract consists primarily of free fatty acids, notably oleic, lauric, and myristic acids. It also contains small amounts of phytosterols, fatty alcohols, flavonoids, and polyprenoids [17]. The action of SPE appears to be specific for prostate tissue because radioactivity from oral SPE supplemented with ^{14}C-labeled oleic and lauric acids is selectively concentrated in the rat prostate, and SPE selectively and specifically induces apoptosis in human primary epithelial and stromal cells of prostate origin, but not other target organs [18,19]. The effects of SPE appear to be mediated by several potential mechanisms, including anti-androgenic actions through inhibition of 5α-reductase, competition with DHT for binding to the AR, inhibition of fibroblast growth factor-induced prostate proliferation, and inhibition of the pro-inflammatory enzymes cyclooxygenase-2 (COX-2) and lipoxygenase (LOX) [11,14,15,20]. In addition, we recently reported that SPE suppresses growth and induces apoptosis in vitro by inhibiting IGF-I signaling [16]. Because SPE is a complex phytochemical mixture, complementary pleiotropic actions may act in concert to inhibit growth and malignant transformation of the prostate.

Several studies have established the utility of TRAMP mice for dietary chemoprevention studies [21–23]. In this model of CaP, expression of the SV40 early genes (T and t antigen, Tag) are driven by the prostate-specific probasin promoter, which leads to cell transformation within the prostate [24]. Elevated serum IGF-I concentrations correlate with CaP progression in the TRAMP mouse [25]. In the present study, we randomly assigned TRAMP mice to cohorts that would receive one of two doses of SPE between 4 and 12 or 4 and 24 weeks of age. These two time points were chosen because 12 weeks represents the period of prostate intraepithelial neoplasia and preneoplastic development in the prostate of TRAMP mice, but before development of adenocarcinoma; 24 weeks represents the age at which TRAMP mice develop differentiated prostate adenocarcinoma [26]. We found that at 24 weeks, SPE inhibited 5α reductase in vivo, decreased tumor grade, and increased prostate epithelial cell apoptosis, suggesting that SPE may delay the progression of CaP.

MATERIALS AND METHODS

In Vitro Cell Proliferation Analysis

TRAMP C2 cells, isolated originally from primary TRAMP mouse prostate tumors (American Type Culture Collection, Manassas, VA), were cultured in a defined medium composed of phenol red-free, high glucose, with L-glutamine and without sodium pyruvate Dulbecco's modified Eagles' medium (DMEM) (Gibco, Carlsbad, CA) supplemented with 5% fetal bovine serum and 5% Nu-Serum IV (BD Biosciences, San Jose, CA). Also added to the medium was 5 μg/ml insulin (Sigma, St. Louis, MO), 25 μg/ml Penicillin-Streptomycin (Gibco), 10^{-8} M DHT (Steraloids, Newport, RI) in 100% ethanol, and 2.5 μg/ml fungizone (Gibco). Cells were maintained in a tissue culture incubator at 37°C in humidified air containing 5% CO_2.

To test the effects of SPE on TRAMP C2 cell viability, cells were plated in 6-well flasks at a density of 2.25×10^5 cells/well, cultured in complete medium to approximately 80% confluency, then starved overnight in phenol red-free and serum-free DMEM. After the starvation period, cells were treated with SPE for 24 hr. SPE was dissolved in ethanol to give a concentrated stock solution and centrifuged at 12,000*g* for 5 min to remove insoluble debris. Aliquots of stock solution and/or ethanol were added to serum-free DMEM at 37°C to give the final desired concentrations of 0–200 mg/ml SPE and a final ethanol concentration under

0.5%. The solutions were vigorously vortexed for 3 min, re-warmed to 37°C, and added to the cells. Cell proliferation was determined 24 hr later by measuring the bioreduction of MTS using the CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI) as per manufacturer's instructions.

Mice

TRAMP mice, heterozygous for the probasin-Tag transgene, were bred in our colony at the Oregon Health and Science University. Transgenic females (originally obtained from the MMHCC® of the National Cancer Institute-Frederick (http://emice.nci.nih.gov/emice) were bred with nontransgenic C57BL/6 males. After weaning at 3 weeks of age, the gender of the offspring was determined, males were separated from females, and a tail biopsy was collected from each mouse. Tail DNA was extracted and amplified using the REDExtract-N-AMP™ Tissue PCR Kit according to manufacturer's instructions (Sigma) and transgene incorporation determined by PCR as described by Greenberg et al. [24]. All mice were maintained in a climate-controlled environment with a 12-hr light/12-hr dark cycle, and diet and water supplied ad libitum.

SPE Administration

Control mice were fed AIN-76A (Research Diets, Inc., New Brunswick, NJ), a semipurified diet containing no detectable phyotoestrogens. Treated mice were fed AIN-76A supplemented with one of two doses of SPE-purified extract (SPE, Sabalselect™ (batch 28140/1, Indena® s.a., Milan, Italy). Doses of 0.5 and 3.0 g SPE/kg diet were designed to deliver 50 and 300 mg SPE per kg body weight per day, respectively. These doses were calculated by allometry to approximate a normal human therapeutic dose used for BPH (320 mg/day) and one sixfold greater. Diets were stored at −20°C. Four groups of mice consisting of 15–20 mice per group were studied: non-transgenic controls, TRAMP controls, TRAMP mice given 50 mg/kg/day SPE, and TRAMP mice given 300 mg/kg/day SPE. Food consumption was monitored on a weekly basis. Treatments began at 4 weeks of age and continued until the mice were killed at either 12 or 24 weeks of age. Throughout the study, each mouse was assessed daily for symptoms of toxicity and weighed weekly. All procedures involving animals were conducted in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of Oregon Health and Science University.

Preparation and Analysis of Tissues

Animals were killed with isoflurane asphyxiation. At necropsy, internal organs of the animals were exposed by an incision in the abdominal cavity. Internal organs and urogenital tract (UGT) were visually examined for gross tumors, metastases, and other abnormalities. Gross tumors were documented and recorded. The kidneys, testes, spleen, and UGT (including the bladder, seminal vesicles, and prostate) were removed and weighed. The dorsolateral (DLP), ventral prostates (VP), anterior prostates (AP) were then rapidly dissected and weighed. Samples of DLPs and VPs were frozen on dry ice and stored at −80°C for Western blot analysis and hormone measurements. The remaining DLP and VP tissue was fixed overnight in Z-fix (Anatech Ltd., Battle Creek, MI). Fixed tissues were embedded in paraffin, and 5-μm sections mounted on microscope slides.

Pathologic Analysis

Slides were stained with hematoxylin and eosin (H&E). Slides were then reviewed by two scientists without knowledge of the age, genotype, or treatment of the mice. Each DLP lobe was graded as normal, PIN, well-differentiated adenocarcinoma (WD), moderately differentiated adenocarcinoma (MD), and poorly differentiated adenocarcinoma (PD) using a scale that has been established for TRAMP mice [26]. The percentage of total area corresponding to each pathological stage was recorded; 100% of the pathology was accounted for in each sample. The scores were averaged and expressed as mean ± SEM.

Immunohistochemistry

Paraffin embedded tissues were dewaxed in xylenes and rehydrated through graded alcohols. Antigen retrieval was performed by incubating tissues with Citra solution (BioGenex, San Ramon, CA), pH 6 at 90°C followed by 10 min of steam. Tissues were washed in Tris-buffered saline (TBS) for 5 min then treated with H_2O_2 (25% in methanol) for 10 min to inhibit endogenous peroxidases. Following washing with phosphate buffered saline (PBS), tissues were blocked with 1% bovine serum albumin (BSA) in PBS, then treated with polyclonal anti-Ki67 (Novocastra Laboratories Ltd., Newcastle upon Tyne, UK) at a dilution of 1:400 in PBS 1 hr. Following washing with PBS, tissues were incubated with EnvisionSystem Anti Rabbit solution (Dako, Carpinteria, CA) for 30 min, washed with PBS, incubated with diaminobenzene chromagen (Dako) for 10 min, and washed with water. Slides were counterstained with hematoxylin, dehydrated, and coverslipped. Tissue from mouse small intestine was

similarly processed and used as a positive control. As a negative control, normal rabbit IgG (Vector Laboratories, Burlingame, CA) was used instead of the primary antibody (data not shown). Digital images of stained tissues were recorded at 250× magnification on a Zeiss Axioplan 2 imaging microscope and analyzed using Axiovision software. The density of Ki67-positive cells (detected at 400× magnification) was calculated by dividing the total number of positive cells by the total area of cells in each DLP tissue slice.

Apoptosis Determination

Apoptotic nuclei in paraffin-embedded tissue sections were detected by Fragment End Labeling (FragEL^{TM}, EMD Biosciences, Inc., La Jolla, CA) using manufacturer's instructions. The number of DAB-positive nuclei were counted at 400× magnification from five random 0.5-mm^2 areas from each slide.

Western Blot Analysis

DLPs from TRAMP mice were homogenized in ice-cold lysis buffer (25 mM HEPES, 5 mM EDTA, 5 mM EGTA, 100 mM NaCl, 100 mM sodium pyrophosphate, 25 mM NaF, 1 mM sodium vanadate, and 1% Triton X, pH 7.5) at a ratio of 60 μl lysis buffer per mg of tissue. The lysate was sonicated on ice for 10 sec and centrifuged at 10,000*g* for 10 min at 4°C. The supernatant was collected and protein content determined using the DC Protein Assay (Bio-Rad Laboratories, Hercules, CA) according to manufacturer's instructions. After addition of 50 mM dithiothreitol and 0.005% bromphenol blue, lysates were heated at 95°C for 5 min. Protein samples (30 μg) were resolved under denaturing/reducing conditions on pre-cast 4–12% Bis-Tris gels (Invitrogen, Carlsbad, CA) and transferred to nitrocellulose membranes. Blots were blocked 1 hr in blocking buffer [5% nonfat dry milk dissolved in 10 mM Tris-HCl (pH 7.4), 200 mM NaCl, and 0.1% Tween 20 (TBS-T)], then incubated overnight in TBS-T containing anti-phospho-Akt antibodies (1:750, Cell Signaling Technologies, Beverly, MA) and 5% BSA. Blots were subsequently rinsed in TBS-T, then incubated 1 hr in blocking buffer containing goat-anti-rabbit-horseradish peroxidase (HRP) secondary antibody (1:5,000, Santa Cruz Biotechnology, Santa Cruz, CA). After washing, the immune complexes were detected by enhanced chemiluminescence (ECL) using Supersignal Ultra chemiluminescent substrate (Pierce Biotechnology, Rockford, IL). Signals were detected using the UVP Bioimaging System and LabWorks 4.0 Image Acquisition Software and signal intensity quantified with Bio-Rad Molecular Analyst software. Blots were stripped (Re-Blot plus; Chemicon, Temecula, CA), blocked in blocking buffer and re-probed with polyclonal anti-Akt antibodies (Cell Signaling Technologies). Blots were rinsed and incubated in goat-anti-rabbit-HRP secondary antibody (Santa Cruz Biotechnology). After washing, immunoreactive bands were visualized as described above.

Steroid Determinations

VPs were weighed individually, homogenized in 0.5 ml of distilled water and extracted with 7 ml of freshly redistilled diethyl ether. The ether was evaporated at 37°C and the residue concentrated into the bottom of the extraction tube with two 1 ml ether washes. The concentrated extracts were stored in 500 μl of redistilled ethanol until they were fractionated by liquid chromatography on LH-20 columns to isolate T and DHT. The solvent system used was hexane: benzene:methanol (85:15:5 vol/vol/vol) and the concentrations of T and DHT in the appropriate elution fractions were estimated by radioimmunoassay as previously described [27]. Quadruplicate water blanks and radioactive recovery standards (^{3}H-T and ^{14}C-DHT) were included to correct for background steroids and recovery, respectively. Quality control samples of gonadectomized sheep serum spiked with known quantities of T and DHT were run in each assay to evaluate intra- and inter-assay coefficients of variation. The serum samples were similarly extracted with ether, subjected to chromatography, and assayed for T and DHT using the same radioimmunoassay as for the tissue samples. The respective mean percentages of recovery, water blanks, and intraassay coefficients of variability were: 69%, 9.4 pg, and 8.5% for DHT and 76%, 3.8 pg, and 10.5% for T.

Fatty Acid Analysis

Diet. Fatty acid content of mouse chow and SPE was determined as previously described [28]. Briefly, total lipids were extracted from ground mouse chow and SPE with chloroform-methanol (2:1) containing 65 μg/ml butylated hydroxytoluene (BHT). Fatty acids were liberated by incubation of dried extracts with ethanolic KOH. After addition of water, acidification, and hexane extraction, fatty acid methyl esters were prepared by heating the dried extracts with methanolic BF_3. Fatty acid methyl esters were separated on a 30-m Supelco SP 2330 fused silica capillary column (column temperature 191°C) attached to a Perkin-Elmer Sigma 3B gas chromatograph equipped with an HP 85 computer/3390A integrator. Results are reported as percent of total fatty acids, by weight.

Plasma and tissue. Lipids were extracted from the DLP using previously described methodology [29].

Briefly, tissues were homogenized in ethyl acetate/ethanol (1:1) containing 40 μg of internal standard (diheptadecanoic acid phosphatidylcholine (di-17:0 PC). The mixture was centrifuged for 5 min at 3,000*g* and the supernatant saved. The extraction was repeated twice and the three supernatants combined and dried under nitrogen. The dried extracts were dissolved in 50 μl of methylene chloride and transesterified with METH-PREP II (Alltech, Deerfield, IL) according to manufacturer's instructions and stored at −20 °C until analysis.

Lipids were extracted from mouse plasma as previously described [30]. Briefly, plasma (50 μl) and di-17:0 PC (40 μg) were mixed with 950 μl of PBS, pH 7.4, 1 ml of BHT (100 μg/ml), and 2 ml CH_2Cl_2. After vortex mixing for 3 min, the mixture was centrifuged for 3 min at 1,800*g*. The lower layer was collected and passed through a Pasteur pipette containing anhydrous Na_2SO_4, then dried under nitrogen. The lipid extract was dissolved in 50 μl CH_2Cl_2, then transesterified as described above. The methylated fatty acids were further purified by an additional hexane extraction (2 ml) after the dried reaction was mixed with 1.0 ml PBS and 1.0 ml methanol. The sample was mixed vigorously for 3 min and centrifuged for 3 min at 1,800*g*. The hexane upper phase was collected and dried under nitrogen. The residue was dissolved in 50 μl iso-octane and stored at −20°C until analysis.

Fatty acid methyl esters were separated on a Trace GC 2000 in combination with a DSQ mass spectrometer (ThermoElectron Corporation). Samples were injected using an AS2000 autosampler and a split/splitless injector. The column was a DB5-MS capillary column (30 m, 0.25-mm id, 0.25-μm film thickness). Helium was the carrier gas at a flow rate of 1 ml/min. The injection port and transfer line were maintained at 225 and 300°C, respectively. A 1-μl sample was injected at a split ratio of 1:50 at an initial oven temperature of 50°C and then increased at 15°C/min to 120°C. The temperature was then increased at 3.5°C/min to a final temperature of 300°C and held for 20 min. The mass spectrometer was operated at a source temperature of 250°C in full scan positive electron impact mode at a scan rate of 3,000 scans/sec from *m/z* of 50 to 400. Fatty acid methyl esters were identified by co-chromatography with standards and by comparison of the mass spectrum with the standard spectra in the NIST library. The instrument was controlled and data analyzed using the Xcalibur data system software (ThermoElectron Corporation).

Statistical Analysis

Statistical comparisons of body weights between control and treatment groups were analyzed by two-way ANOVA for repeated measures after log transformation of the data to normalize the variances. All other comparisons, except apoptosis index, were performed with one-way ANOVA. If a significant difference was found, planned contrasts were utilized to compare control and treatment groups, using the Fishers' least significant difference (LSD) test. The statistical program GB-Stat Ver. 7.0 (Dynamic Microsystems, Inc., Silver Spring, MD) was used for these analyses. Differences were considered statistically significant at a level of $P < 0.05$.

RESULTS

SPE Suppresses Tramp C-2 Cell Proliferation In Vitro

We have previously shown that SPE inhibits proliferation of AR-negative human P69 cells, with an IC_{50} of 140 μg/ml [16]. In order to test whether SPE suppresses the viability of cells derived from TRAMP prostate tumors, MTS assays were used to determine the effects of 24-hr SPE treatment on TRAMP-C2 cells. The IC_{50} for inhibition of TRAMP-C2 cell proliferation at 24 hr was approximately 100 μg/ml (Fig. 1), the same as the IC_{50} previously reported for androgen-sensitive LNCaP cells [31]. In vitro cell proliferation measurements correlate with cell counts ($r^2 = 0.9507$, $P < 0.05$; data not shown), which confirms that SPE does not interfere with the MTS colorometric assay.

SPE Reduces Prostatic Concentration of DHT In Vivo

SPE has been shown to inhibit the activity of 5α reductase in vitro [32] and in vivo [33]. Therefore, in

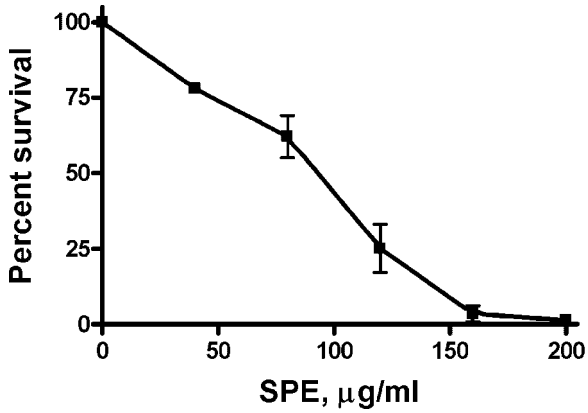

Fig. 1. Effect of SPE on Tramp C-2 cell viability. Cells were plated in 6-well flasks, at a density of 4×10^5 cells/well, cultured in complete medium to 80% confluency, and then treated with varying doses of SPE for 24 hr. Cell viability was determined by the MTS assay, as described in "Materials and Methods." Data are expressed as the percentage of cells in the control treatment at 24 hr. Results are mean ± SE, n = 3.

order to provide evidence that SPE formulated in the diet is biologically active in the prostate, we monitored tissue levels of DHT, which depend on the active metabolism of T by 5α-reductase. We also measured plasma concentrations of T and DHT from TRAMP mice maintained on control, low, and high dose SPE. The concentration of DHT was 40 to 50-fold higher than T in the VP. High-dose SPE decreased levels of DHT in the VP by 31% from 38.9 ± 4.7 pg/ml in TRAMP control mice to 26.9 ± 1.8 pg/ml in TRAMP mice treated with 300 mg/kg/day SPE ($P < 0.05$, Fig. 2a). Low-dose SPE did not significantly alter prostate DHT levels. Neither dose of SPE significantly affected circulating androgen levels or prostate T levels (Figs. 2b–d).

Dietary SPE Is Not Toxic

Changes in body weight and changes in organ weight were used as criteria to evaluate potential SPE toxicity. Weekly body weights between 4 and 24 weeks were not affected by SPE treatment (Fig. 3a). The weights of testes, kidney, and spleen relative to body weights for TRAMP mice at 12 weeks (data not shown) and 24 weeks (Fig. 3b) were not significantly different between treatment groups. These results establish that the concentrations of SPE used in our study are not toxic in mice.

Mice Received Desired Doses of SPE

According to the United States Pharmacopoeia [34], SPE is commonly standardized/characterized by the presence of fatty acids, phytosterols, and long-chain alcohols. We determined the fatty acid content of SPE, control mouse chow, and SPE-supplemented mouse chow to confirm that SPE was stably incorporated into the diet. The fatty acid composition of SPE (batch 28140/1) consisted of 2.0% capric acid, 28.7% lauric acid, 11.7% myristic acid, 9.9% palmitic acid, 2.2% stearic acid, 36.7% oleic acid, 6.9% linoleic acid, and 0.6% linolenic acid. SPE and SPE-supplemented diets contained three short chain fatty acids that were not detected in the control diet (AIN76A): capric, lauric, and myristic acids.

Using values for weekly food consumption, animal weights, and the concentration of SPE in the formulated diets, we calculated that short-term SPE treatment animals on the low-dose diet received an average of 57 ± 3 mg SPE/kg/day and animals on the high-dose diet received an average of 349 ± 14 mg SPE/kg/day. For long-term treatment, animals on the low-dose diet received an average of 47 ± 3 mg SPE/kg/day and animals on the high-dose diet received an average of 289 ± 15 mg SPE/kg/day (Fig. 4). Variations in dosages between short- and long-term cohorts occurred because mice in all treatment groups consumed less

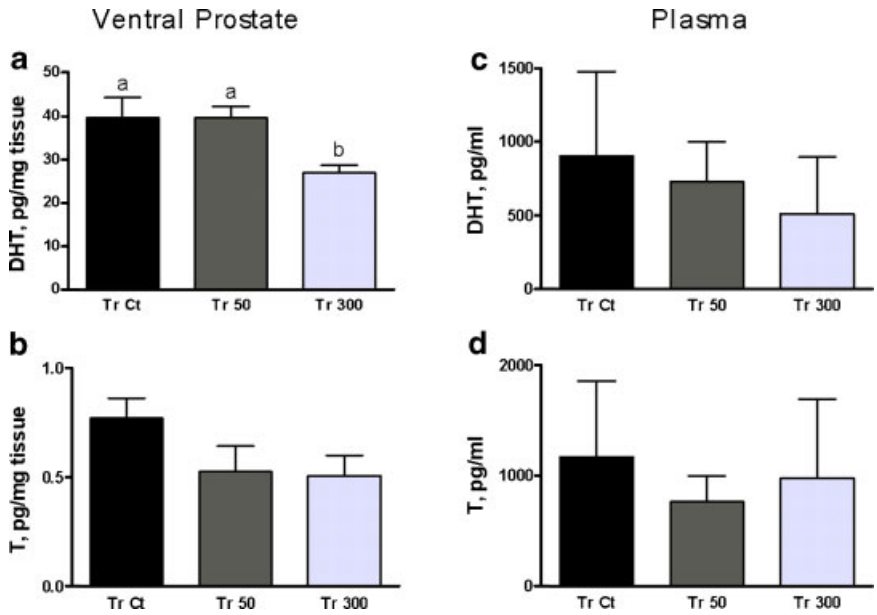

Fig. 2. Effect of SPE on Prostate (a,b) and Plasma (c,d) Androgens. Between 4 and 24 weeks of age, wild-type controls and TRAMP males were fed AIN-76A containing no SPE (Wt Ct, n = 18; Tr Ct, n = 15) and TRAMP males fed AIN-76A supplemented with SPE to provide average doses of 50 mg/kg/day or 300 mg/kg/day (Tr 50, n = 13;Tr 300, n = 18). Plasma and ventral prostate concentrations of testosterone (T) and dihydrotestosterone (DHT) were measured by RIA. Results are mean ± SE. One-way ANOVA followed by Bartlett's test for equal variances revealed a significant difference between DHT levels between mice treated with control diet and mice treated with 300 mg/kg/day SPE, $P < 0.05$. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

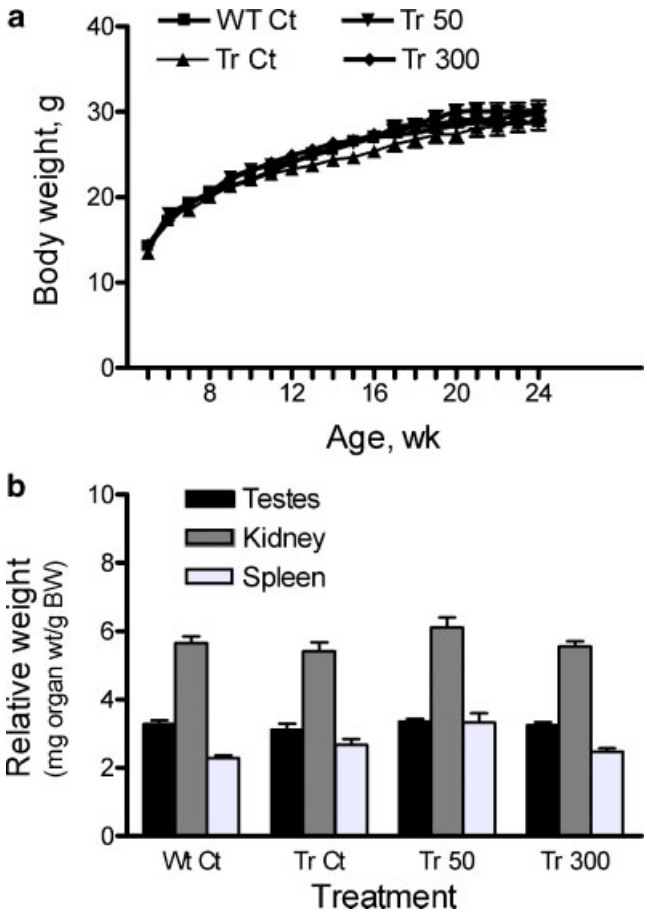

Fig. 3. Effect of dietary SPE on body (**a**) and organ (**b**) weights. TRAMP mice were treated as described in Figure 2. Results are mean ± SE. Two-way ANOVA revealed no significant difference in body weights and one-way ANOVA revealed no significant difference in organ weights between control and SPE-treated TRAMP mice. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

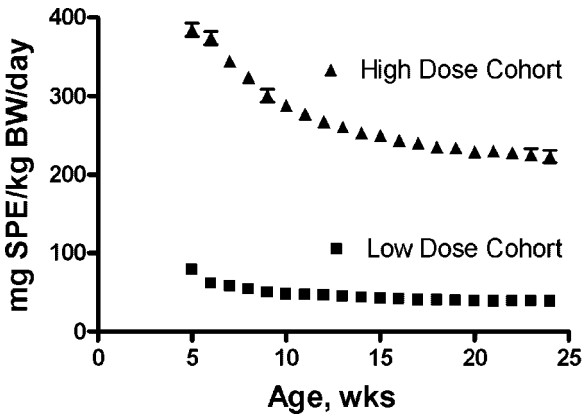

Fig. 4. Average daily SPE doses. Using values for weekly food consumption, animal weights, and the concentration of SPE in the formulated diets, the average daily dose of SPE was calculated for TRAMP mice between the ages of 4 and 24 weeks. Results are mean ± SE.

food per kg of body weight with aging. No unique fatty acid profile was detected in the plasma or prostate of SPE-treated mice compared to controls, and we were not able to detect the unique short chain fatty acids (i.e., capric, lauric, and myristic acid) that were present only in the SPE-formulated diet.

SPE Does Not Affect Relative UGTor DLP Weights

Having demonstrated the ability of SPE to suppress proliferation of TRAMP-derived CaP cells in vitro, we tested the effects of SPE on TRAMP mice in vivo, using relative UGT and DLP weights as indirect measures of tumor volume [35]. Short-term SPE treatment had no effect on relative UGT (Fig. 5a) or DLP (Fig. 5b) weights in 12-week-old TRAMP mice. Likewise, long-term treatment with SPE did not alter relative UGT

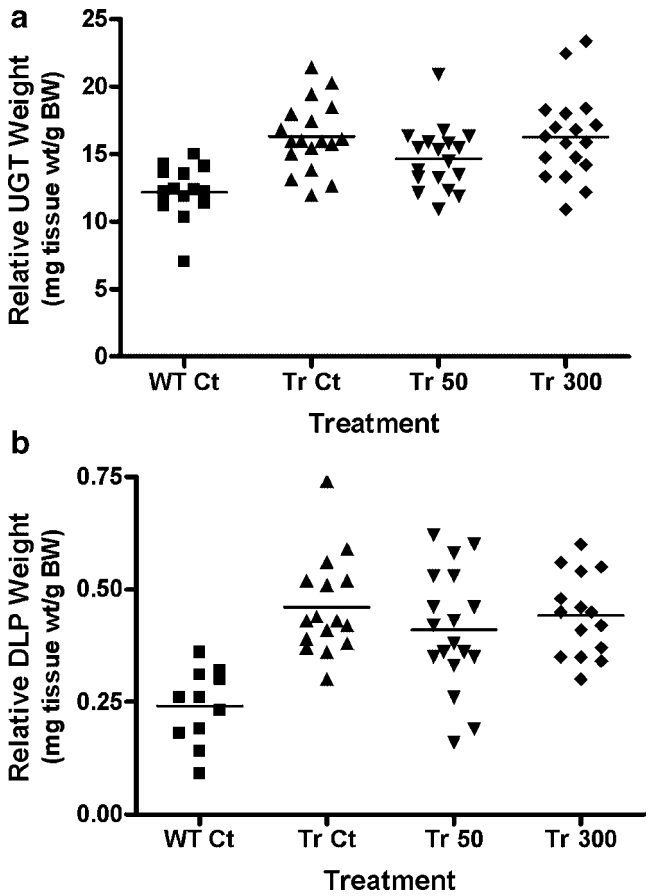

Fig. 5. Effect of short-term SPE treatment on relative UGT (**a**) or DLP (**b**) weights. Between 4 and 12 weeks of age, wild-type controls and TRAMP males were fed AIN-76A containing 0 mg/kg day SPE (Wt Ct, n = 15; Tr Ct, n = 18) or TRAMP males were fed AIN-76A supplemented with 50 mg/kg day or 300 mg/kg day SPE (Tr 50, n = 18; Tr 300, n = 18). At necropsy, body, UGT, and DLP weights were recorded. The horizontal line represents the mean weight. One-way ANOVA revealed no significant difference in UGTor DLP weights between control or SPE-treated TRAMP mice.

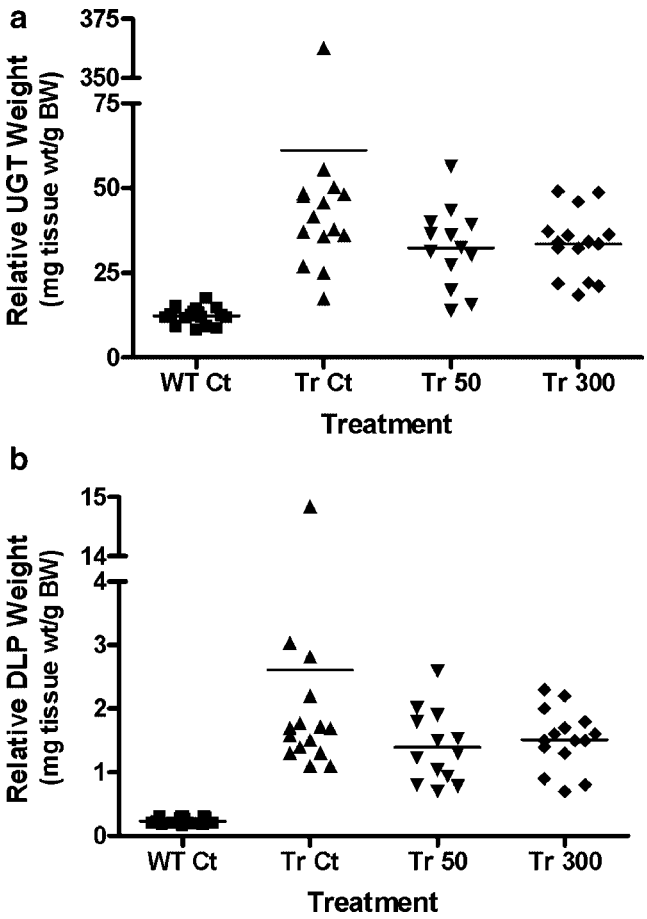

Fig. 6. Effect of long-term SPE treatment on relative UGT (**a**) or DLP (**b**) weights. Mice were treated as described in the legend to Figure 2. At necropsy, body, UGT, and DLP weights were recorded. The horizontal line represents the mean weight. One-way ANOVA revealed no significant difference in UGT or DLP weights between control or SPE-treated TRAMP mice.

(Fig. 6a) and DLP (Fig. 6b) weights in 24-week-old TRAMP mice.

SPE Decreases Tumor Grade and Gross Tumor Incidence After 24 Weeks of Treatment

SPE decreased the incidence of gross, spherical UGT tumors in 24-week-old TRAMP mice. Frank tumors were observed in 4/15 of 24-week-old control TRAMP mice, 1/13 of TRAMP mice treated with 50 mg/kg/day SPE, and 2/18 of TRAMP mice treated with 300 mg/kg/day SPE (Fig. 7a). Histological assessment of DLP tissues revealed that SPE treatment significantly shifted tumor pathology to less advanced grades in all mice in the cohort. The incidence of PIN lesions increased from $21.6 \pm 3.6\%$ in control TRAMP mice to $36.4 \pm 6.7\%$ in mice treated with 50 mg/kg/day SPE ($P < 0.05$ for treatments vs. control) and $38.3 \pm 5.5\%$ in mice treated

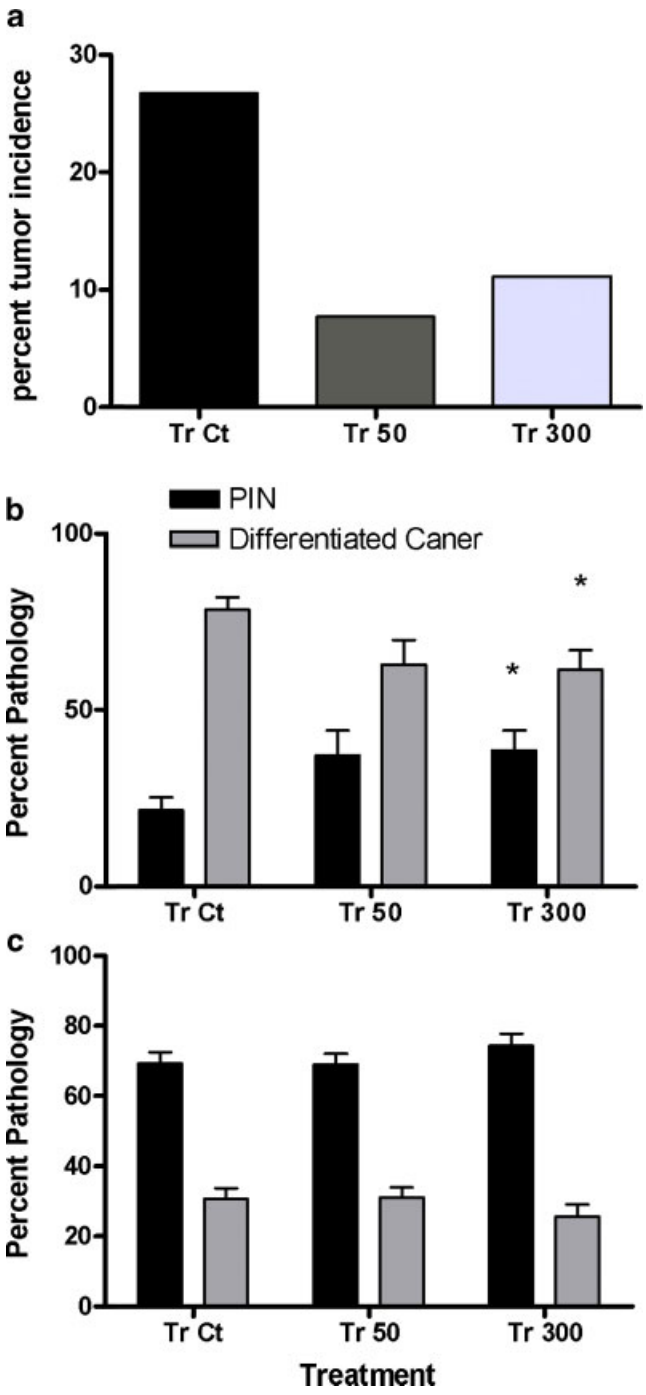

Fig. 7. Effects of SPE on tumor incidence and pathologic cancer grade. **a**: The number of frank prostate tumors observed at necropsy and (**b**) (%) PIN and (%) differentiated tumors were recorded from 24-week-old TRAMP mice treated as described in the legend to Figure 2. Results are mean ± SE. One-way ANOVA followed by Fishers' LSD revealed that there is a significant difference in the percent PIN and percent differentiated cancer between mice treated with control diet and mice treated with 300 mg/kg/day SPE, $P < 0.05$. **c**: The effects of SPE treatment on histopathology from 12-week-old TRAMP mice treated with SPE as described in the legend to Figure 5. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

with 300 mg/kg/day SPE ($P < 0.05$ for high-dose SPE treatment vs. control). The incidence of differentiated tumors decreased from $78.4 \pm 3.6\%$ in TRAMP control mice to $62.8 \pm 7.0\%$ and $61.4 \pm 5.6\%$ in mice treated with 50 and 300 mg/kg/day SPE, respectively ($P < 0.05$ for treatments vs. control, Fig. 7b). Because frank tumors were not observed in 12-week-old TRAMP mice and short-term SPE treatment had no effect on the histopathology of DLPs from these cohorts (Fig. 7c), further analysis was focused on long-term SPE treatments.

SPE Has No Effect on Cell Proliferation In Vivo

Tumor growth and development may be due to dysregulated cellular proliferation. We investigated the effect of SPE on cellular proliferation in the DLPs of TRAMP mice by immunohistochemically staining for Ki-67, a nuclear antigen expressed in all active stages of the cell cycle except at rest [36]. While our previous in vitro findings suggested that SPE inhibits epithelial proliferation [16], there was no difference in the number of Ki67-positive cells per square millimeter in the DLPs from control vs SPE-treated 24-week old mice (Fig. 8).

SPE Increases Apoptosis in the DLPs of 24-Week Old TRAMP Mice

We have previously reported that SPE induces apoptosis in the P69 prostate epithelial cancer cell line as shown by cleavage of the enzyme poly(ADP-ribose)polymerase (PARP) [16]. Consistent with these findings, the number of apoptotic nuclei (detected by fragment end labeling) in paraffin-embedded tissue sections from the DLPs of 24-week old SPE-treated mice was significantly increased when compared to TRAMP control mice (Fig. 9). The number of apoptotic cells per square millimeter increased from 41.7 ± 16 in TRAMP controls to 93.1 ± 16.4 in mice treated with 50 mg/kg/day SPE ($P < 0.001$) and 79.6 ± 17.5 in mice treated with 300 mg/kg/day SPE ($P < 0.05$).

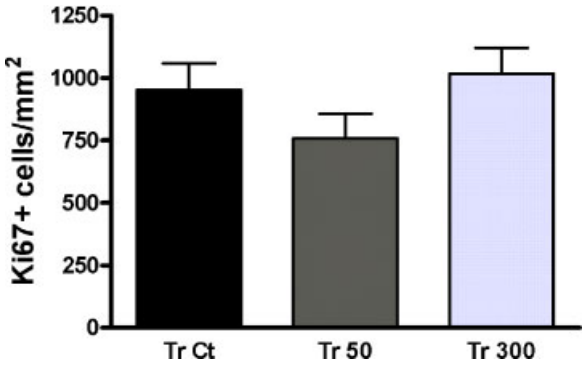

Fig. 8. Effect of SPE on cellular proliferation in DLP tissues from 24-week-old TRAMP mice. The number of Ki67-positive nuclei in paraffin-embedded tissue sections were counted at 400× magnification from five random 500,000 m^2 areas from TRAMP mice treated as described in the legend to Figure 2. Results are mean ± SE. One-way ANOVA revealed no significant difference in Ki67 density between TRAMP mice treated with control versus SPE-formulated diets. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

SPE Has No Effect on AKT Phosphorylation in the DLPs of 24-Week-Old TRAMP Mice

While we previously reported that SPE suppresses growth and induces apoptosis in vitro by inhibiting insulin-like-growth factor-I (IGF-I) signaling [16], Western blot analysis did not reveal a significant difference in the phosphorylation/activation of Akt in DLPs from control or SPE-treated TRAMP mice (data not shown).

DISCUSSION

CaP is an invasive and frequently diagnosed malignancy for which there are limited treatment options. However, because of its long latency period and because it is typically a disease of elderly men, CaP is thought to be an excellent candidate for chemoprevention. Here, we show for the first time that oral administration with SPE increases apoptosis, and reduces tumor grade and frank tumor incidence in TRAMP mice. Although SPE treatment produced only modest effects, dietary supplementation with SPE could possibly be effective in controlling tumorigenesis by slightly delaying the onset and progression of CaP.

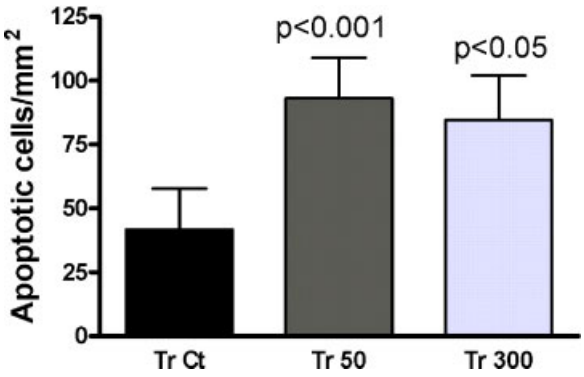

Fig. 9. Effect of SPE on apoptosis in DLP tissues from 24-week-old TRAMP mice. Apoptotic nuclei in paraffin-embedded tissue sections were detected by Fragment End Labeling as described in "Materials and Methods." The number of positive nuclei were counted at 400× magnification from seven random areas from TRAMP mice treated as described in the legend to Figure 2. Results are mean ± SE. One-way ANOVA followed by Fishers' LSD test revealed that compared with TRAMP control mice, there is a significant difference in the number of apoptotic nuclei in mice treated with 50 mg/kg/day SPE and mice treated with 300 mg/kg/day SPE ($P < 0.01$). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Ample evidence suggests that androgens influence the development of CaP. Before the onset of androgen refractoriness, CaP cells require androgen not only for growth, but also to prevent apoptosis [37]. The most potent primary nuclear androgen responsible for the maintenance of epithelial function is DHT. By inhibiting DHT synthesis, 5α-reductase inhibitors decrease the androgen drive to malignant prostate cells [38]. Our results suggest that dietary SPE functions as an in vivo inhibitor of 5α-reductase. High-dose SPE treatment significantly decreased levels of DHT in the VP but not plasma of TRAMP mice. This is consistent with human studies showing that SPE decreases DHT levels in prostate tissue but not in serum [33,39]. While the 5α-reductase inhibitor finasteride prevents or delays the appearance of CaP, its possible benefits are confounded by sexual side effects and the increased risk of high-grade CaP [3]. SPE may, therefore, offer a therapeutic advantage over finasteride because SPE is a less potent 5-α reductase inhibitor [32,33].

Many of the molecular changes identified in prostate tumorigenesis are involved in apoptosis regulation, and therapeutic strategies targeted at inducing apoptosis could be effective in controlling CaP [37]. The increase in apoptosis in the DLPs of TRAMP mice is in accordance with our previous observations which demonstrated that SPE induced epithelial prostate cell apoptosis in vitro [16]. SPE has also been shown to increase molecular markers involved in the apoptotic process in BPH patients who received 160 mg SPE orally twice daily for a 3-month period [40]. Taken together, this data suggests that an active factor in SPE may inhibit prostate tumors by increasing epithelial cell apoptosis. Antiandrogenic activities of SPE can only partially explain its effects on apoptosis because 5α-reductase was inhibited with only high-dose SPE, while apoptosis was induced at both doses. The specific apoptotic signaling pathway induced by SPE is yet to be determined. We have previously shown that SPE has activating effects on the enzyme SAPK/JNK, which has been mechanistically implicated in the initiation of apoptosis in many cell types [16]. Further experimental work will address whether SPE induces SAPK/JNK phosphorylation in TRAMP mice.

The IGF axis is particularly important for prostate growth and development and changes in IGF signaling have important implications in malignant growth of CaP [41]. During the course of cancer progression, TRAMP mice exhibit increased levels of IGF-I and IGF-I signaling, including increased levels of phosphorylated Akt [21,42]. While our previous studies suggest that SPE at doses of 100–150 μg/ml inhibit epithelial cell proliferation and IGF-induced activation of Akt in vitro by exerting direct effects on specific components of the IGF-I signaling pathway, SPE had no effect on epithelial proliferation or the activation/phosphorylation of Akt in DLPs from 24-week-old TRAMP mice. The doses of SPE administered to TRAMP mice were calculated by allometry to approximate a normal human therapeutic dose of 320 mg/day and one sixfold higher. Pharmacokinetic studies in humans show that a single 640-mg dose of SPE leads to peak plasma levels of 2.6 μg/ml [43]. Therefore, although SPE is thought to selectively concentrate in the prostate [18], the concentration of SPE may not have been high enough to affect IGF signaling in vivo, or it is possible that continuous dietary administration of SPE leads to lower plasma concentrations of active principles than SPE administered in a bolus dose. Different batches of SPE were used for our in vivo and in vitro studies. It is also possible that the proportional content of non-standardized components could vary between batches of SPE, thus influencing the activity of the product. Finally, SPE-induced inhibition of IGF-I signaling may not have been sufficient to abrogate simultaneous activation/phosphorylation of Akt from other multiple signal tranduction pathways. Our results demonstrate why caution must be exercised when attributing the in vitro actions of a phytochemical to effects in a spontaneous autochthonous animal model of cancer.

The TRAMP model is an ideal system for investigating chemopreventative potential because mice develop early PIN lesions of the prostate, which gradually progress to invasive CaP, a process that closely mimics human disease [24]. Numerous investigators have used the TRAMP system to study chemoprevention strategies with natural products; however, limited responses to intervention may occur due to the strong oncogenic pressure exerted by SV40 Tag-induced disturbances in p53 and Rb-regulated cell functions. The upregulation of sex steroid receptors and multiple growth signaling pathways support the concept that multiple dysregulation contributes to carcinogenesis in TRAMP mice [44]. While SPE administration increased apoptosis, decreased the incidence of frank prostate tumors, and decreased histological grade of prostate tumors in TRAMP mice, it had no effect on relative UGT weights or epithelial cell proliferation. Other agents that show promise in cancer chemoprevention have been shown to exhibit limited chemopreventive responses in TRAMP mice. Retinoic acid slows prostate tumor cell proliferation, induces apoptosis, and shifts pathology toward lower grades, yet confers no protection against tumor incidence and does not influence prostate weights in TRAMP mice [23]. Dietary rye bran increases epithelial cell apoptosis but does not affect epithelial cell proliferation or prostate lobe weights in TRAMP mice, and is less effective in the TRAMP model than other models [45]. Flaxseed treatment which decreases cellular proliferation and increases apoptosis

in TRAMP mice after 20 weeks of treatment, significantly decreases urogenital/tumor weight at the end of 30 weeks, but not at earlier time points [46]. It may, therefore, be necessary to treat TRAMP mice for a longer time period in order to observe effects of SPE on UGT weight.

SPE is a complex mixture composed of saturated and unsaturated fatty acids in both the free and esterified forms, with a high content of lauric acid, myristic acid, palmitic acid, and oleic acid. It also contains small amounts of the phytosterols sitosterol, campesterol, and stigmasterol as well as flavonoids, carotenoids, polyprenoids, and saccharides [17]. As such, it is likely that its components exert pleiotropic and synergistic effects. The fatty acid components of SPE have been suggested as the active elements of the extract [20,31,47]. The inhibitory activity of SPE on 5α-reductase type 1 and type 2 has been attributed to the flavonoids quercitrin and kaempferol and free fatty acids, pariticularly oleic, linolenic, lauric, and myristic acids [17,48]. In our studies, the control, low-, and high-dose diets were calorically equivalent, containing 5, 4.95, and 4.70% corn oil, respectively. The only fatty acids detected in SPE but not corn oil were capric, lauric, and myristic acids. However, we were unable to detect these three unique short chain fatty acid components of the SPE-supplemented diets in the plasma or prostates of SPE-treated mice, perhaps because short chain fatty acids are rapidly metabolized in vivo [49]. Phytosterols alone are also most likely not responsible for the unique effects of SPE because corn oil is a richer source of sitosterol, campesterol, and stigmasterol than SPE [50]. Most likely, the activity of SPE is not due to a single lipid component, but rather to a mixture of free fatty acids and sterols which modulate the lipid environment in the prostate as well as non-lipid components such as flavonoids and polyprenoids which have been shown to exhibit anticancer and antiproliferative capabilities [17].

SPE is currently used by over 2 million men in the United States and is widely used in Europe for the treatment of BPH [13]. The efficacy of SPE in BPH treatment is controversial. While a recent double-blind trial suggests that SPE does not improve symptoms or objective measures of BPH [51], several randomized trials have reported small improvements in the severity of symptoms, quality of life score, and urinary flow rates in BPH patients [52]. Our results suggest that SPE may inhibit the progression of CaP. Further studies are ongoing to elucidate active components of SPE, determine whether SPE improves the survival of TRAMP mice, and to evaluate whether COX-2 inhibition may contribute to the mechanism by which SPE acts as a chemopreventive agent against CaP.

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