Chemoprevention of esophageal cancer with black raspberries, their component anthocyanins, and a major anthocyanin metabolite, protocatechuic acid

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Abstract

Diets containing either freeze-dried black raspberries (BRB) or their polyphenolic anthocyanins (AC) have been shown to inhibit the development of *N*-nitrosomethylbenzylamine (NMBA)-induced esophageal cancer in rats. The present study was conducted to determine if PCA, a major microbial metabolite of BRB AC, also prevents NMBA-induced esophageal cancer in rats. F344 rats were injected with NMBA three times a week (wk) for five weeks (wks) and then fed control or experimental diets containing 6.1% BRB, an AC-rich fraction derived from BRB, or PCA. Animals were exsanguinated at wks 15, 25, and 35 to quantify the development of preneoplastic lesions and tumors in the esophagus, and to relate this to the expression of inflammatory biomarkers. At wks 15 and 25, all experimental diets were equally effective in reducing NMBA-induced esophageal tumorigenesis, as well as in reducing the expression of Pentraxin-3 (PTX3), a cytokine produced by peripheral blood mononuclear cells in response to IL-1β and TNF-α. All experimental diets were also active at reducing tumorigenesis at wk 35; however, the BRB diet was significantly more effective than the AC and PCA diets. Further, all experimental diets inhibited inflammation in the esophagus via reducing biomarker (COX-2, iNOS, p-NF-κB, sEH) and cytokine (PTX3) expression. Overall, our data suggest that BRB, their component AC and PCA inhibit NMBA-induced esophageal tumorigenesis, at least in part, by their inhibitory effects on genes associated with inflammation.

Keywords

esophagus; cancer; rodent; black raspberry; anthocyanin; protocatechuic acid; chemoprevention

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INTRODUCTION

Esophageal cancer is the third most common gastrointestinal cancer and sixth most common cancer worldwide. There are two types of esophageal cancer, squamous cell carcinoma (SCC) and adenocarcinoma, and SCC accounts for 90% of the disease worldwide (1, 2). The incidence of esophageal SCC is highly variable throughout the world with more than one-half of all cases occurring in China. The occurrence of the disease in males exceeds that in females by a factor of 3- to-4. Risk factors associated with the etiology of esophageal SCC include tobacco and alcohol use, consumption of foods contaminated with mold, vitamin and mineral deficiencies, temperature hot beverages and food, inadequate intake of vegetables and fruit, and infection with human papilloma virus (HPV) (3-8). Nitrosamine carcinogens and *N*-nitroso precursors present in foodstuffs and produced in the acidic environment of the stomach are also thought to contribute to the disease (9). Esophageal SCC likely develops through a progressive sequence from hyperplasia >mild, moderate and severe dysplasia> carcinoma *in situ*> SCC. Because esophageal cancers are generally detected in the late stages of development, the five-year survival rate for SCC remains a dismal 15-20% (10).

Life style changes such as avoidance of tobacco, alcohol and moldy foods are likely to be effective in reducing the incidence of esophageal SCC. Chemoprevention also has potential for reducing the risk for development of the disease. Support for this comes from epidemiological studies which have observed protective effects of naturally-occurring fruits and vegetables on the risk for esophageal SCC (11, 12). In that regard, preclinical studies in our laboratory have demonstrated inhibitory effects of different berry types on the development of *N*-nitrosomethylbenzylamine (NMBA)-induced esophageal tumors in rats (13-15) a model of human esophageal SCC. A recent phase II clinical trial by Chen, et al. (16) demonstrated an ~ 80% reduction in histologic grade of mildly dysplastic lesions of the esophagus of Chinese patients who ingested a total of 60g (30g, 2×/day) of freeze-dried strawberries daily in a slurry of water for six months.

The use of black raspberries (BRB) as a chemoprevention agent has gained interest and 6 human trials have been completed to date to assess the efficacy of BRB formulations for cancer prevention (17). High concentrations of chemopreventive compounds such as the anthocyanins, ellagic acid, quercetin, and β-sitosterol have been identified in BRB (14, 18). BRB and their component anthocyanins (AC) have the ability to inhibit cell proliferation, inflammation and angiogenesis and to stimulate apoptosis, cell differentiation and cell adhesion (15). They do this by protectively modulating the expression levels of multiple genes and proteins in signaling pathways associated with various cellular functions including P13K/Akt/mTOR, AP-1, MAPK, Erk1/2, and p38 (cell proliferation), COX-2, iNOS, NF-κB, CD45, IL-1β, IL-12, IL-10 (inflammation), Muc-2, and various keratin genes (differentiation), VEGF, HIF-1α and CD34 (angiogenesis) and Bcl-2, Bax and caspase 3/7 (apoptosis) (15, 19-26). BRB also re-activate suppressor genes that have been silenced in tumors by hypermethylation (27, 28).

The absorption and bioavailability of BRB constituents including the AC is a fundamental aspect of their physiological role in disease prevention. Recent studies have demonstrated that the uptake of orally administered BRB AC into blood is less than 1% of the administered dose (29, 30). The majority of AC enter the colon where they are metabolized by colonic bacteria into smaller, and more bioavailable phenolic acids such as protocatechuic acid (PCA) (31, 32). PCA is known to function as an antioxidant and an anti-diabetic agent (33). In addition, it is effective as a chemopreventive against colon, bladder and liver cancer in rodents (34-36) and has anti-proliferative and pro-apoptotic capabilities (37, 38).

Our laboratory has used the Fischer-344 (F-344) rat model for studies of the etiology, biology and chemoprevention of esophageal SCC for several decades. Esophageal tumors (mainly papillomas) are induced by subcutaneous (s.c.) injection of rats with the carcinogen, NMBA (9). Repeated s.c. injections of NMBA into rats dependably and reproducibly induce esophageal tumor formation within 15-to-26 weeks (wks). Preneoplastic changes closely resemble changes observed in human esophageal SCC including hyperplasia and mild, moderate and severe dysplasia. In the present study, we evaluated the relative ability of whole BRB, their component AC and PCA to prevent the development of esophageal cancer in F-344 rats. The actions of these agents were quantified in terms of their effects on the prevalence of preneoplastic lesions, tumor multiplicity and burden, and on the expression of the inflammatory markers COX-2, iNOS, NF-κB and soluble epoxide hydrolase (sEH). The effects on sEH expression was examined because this enzyme converts the anti-inflammatory epoxyeicosatrienoic acids (EET) into vincinal diols which are rapidly excreted (39). sEH, therefore, is pro-inflammatory and there is interest in developing inhibitory agents for this enzyme. The effects of BRB, AC and PCA on the expression of pentraxin-3 (PTX3), a cytokine and anti-angiogenic factor (40), was also examined because of the reported silencing of this gene in human esophageal SCC (41).

MATERIALS AND METHODS

Black raspberry powder

Freeze-dried black raspberry (*Rubus occidentalis*) powder was purchased from Decker Farms, Inc. (Hillsboro, OR) and from BerriProducts, Inc. (Corvallis, OR) and stored at 4°C in vacuum-sealed plastic bags at the Medical College of Wisconsin (MCW). About 100 g of each lot of powder from both vendors was shipped to Covance Laboratories (Madison, WI) for quantification of specific minerals, phenolic acids, vitamins, phytosterols, carotenoids, fungicides, pesticides, and herbicides as described before (14). The content of the three major AC in each lot of BRB powder; i.e., cyanidin-3-*O*-glucoside, cyanidin-3-*O*-rutinoside and cyanidin-3-*O*-xylosylrutinoside, was determined in the laboratory of Dr. Stephen Hecht via high-performance liquid chromatography (HPLC). A portion of the powder was shipped from MCW to Dr. Hecht's laboratory to prepare the AC-enriched fraction, and the remaining powder was used in the carcinogenesis bioassay conducted at MCW.

Preparation of the AC-enriched fraction

Extraction of freeze-dried BRB powder—A filter-bag, made from untreated canvas (Harris Machinery and Canvas Warehouse, Minneapolis, MN) was placed inside a high density polyethylene (HDPE) bucket. BRB powder (2.0 kg) and 0.1N HCl (8 L) were added to the bag and mixed briefly to ensure homogeneity, then the mixture was stirred for 30 minutes (mins). The bag containing the BRB/HCl slurry was then transferred to the reservoir of a modified fruit press. The bag was sealed by rolling its top down to the surface of the mixture. Wooden blocks were placed on top and then, over ~45 mins, pressure was slowly applied to force the BRB extract through the canvas filter-bag and drain it into a HDPE bucket. The filter bag containing the extracted BRBs and residual HCl was then transferred to a clean bucket, and the extraction process was repeated two additional times using 6 L 0.1 N HCl each time.

Enrichment of AC—SP-710 Polystyrenic adsorbent resin (Itochu Chemical, White Plains, NY) was conditioned overnight in 1.1 bed volumes (BV) 200 proof ethanol (Decon Laboratories, King of Prussia, PA) and then washed with de-ionized water immediately prior to use. The extract from above was added to the resin and the mixture was stirred for 1 h. The resin was collected by filtering the mixture through polyester fabric netting (JoAnn Fabrics, Hudson, OH). After filtration, the resin was stirred with the following for 15 mins each: 2×2 BV H2O, 1.25 BV H20, 0.25 M pH 7 potassium phosphate buffer, and three times with 2 BV H2O. The resin was kept in the fabric throughout the washes, allowing for rapid drainage of each wash and transfer to the next. After the final wash, the resin, still in the polyester filter, was partially dried for ~25 mins under N2 using a HDPE bucket with 25 ¼" holes in the bottom and an N2 line inserted in the top. After removing most of the water, the AC were desorbed from the resin using 3×3 L of 200 proof ethanol. Each ethanol wash was stirred in the resin for 30 mins before collection into a HDPE bucket. This was accomplished by draining through the polyester filter fabric by gravity for ~5 mins followed by 5-10 seconds (s) of N2 pressure using the same drying assembly described above. To remove any stray resin beads, the ethanol desorbate was filtered one final time through 4 layers of the polyester filter fabric, before being collected in polyethylene jugs and stored at −20°C.

Solvent removal—Most of the ethanol was first removed using a Büchi R-220 preparatory scale rotary evaporator set to a temperature of 22°C, a vacuum of approximately 15 torr and a speed of approximately 70 rpm. This stage was considered complete when there was a marked decrease in the rate of evaporation. In the second stage, the water-rich solutions were combined and rotary evaporated at the minimum temperature necessary to yield a drip-rate of 2-3 droplets/second without heating above 40°C. Rotary evaporation was stopped when the extract had reached the consistency of syrup, ~60% solid by weight. The extract was then transferred to plastic jugs and stored at −20°C before overnight shipment under dry-ice to the MCW. Aliquots were removed for AC determination by HPLC and H2O content determination by lyophilization.

Analysis of AC in BRB extract—Total AC were determined by HPLC, as cyanidin-3-O-glucoside equivalents. Extract and standard solutions of cyanidin-3-O-glucoside

(Extrasynthese, Genay Cedex, France) were prepared in 5% aqueous formic acid. The standard solution was further diluted with 0.1N HCl, the absorbance was measured at 510 nm using a Beckman DU-7400 spectrophotometer for standardization.

HPLC was performed using a Luna C18(2) 5μ 250 × 4.6 mm column (Phenomenex, Torrance, CA), with detection at 515 nm using a SPD-10A UV-Vis detector (Shimadzu, Columbia, IL). Solvent A was 30.5% methanol in H2O with 0.1% phosphoric acid and solvent B was 100% methanol. Elution was isocratic in 100% A for 0-20 mins, then switched to 100% B in 0.5 mins and held for 5 mins, before returning to 100% A in 0.5 mins and re-equilibrating for 10 mins. The flow rate was 0.9 mL/mins.

Chemicals

NMBA was purchased from Ash Stevens (Detroit, MI) and was found to be >98% pure by HPLC. Protocatechuic acid ethyl ester (PCA, 97% pure) was purchased from Sigma-Aldrich (St. Louis, MO).

Diet Preparation

Diets were prepared using a Hobart mixer. BRB powder, the AC enriched fraction, or PCA was weighed and added to American Institute of Nutrition-76A (AIN-76A) synthetic diet (Dyets, Inc., Bethlehem, PA) at the proper concentration and allowed to mix for 20 mins. Diets were evaluated for content of BRB, AC and PCA via HPLC to ensure homogeneity.

Animals

Male F-344 rats, 3-5 wks old, were purchased from Harlan Sprague-Dawley (Indianapolis, IN). Rats were housed two animals per cage under standard conditions ($20 \pm 2°C$, $50 \pm 10\%$ relative humidity, 12-hour light/dark cycles). AIN-76A diet and water were available *ad libitum*. Hygienic conditions were maintained by twice-weekly cage changes. Food intake and body weights were taken weekly over the course of the study. Animals were kept according to the recommendations of the American Association of Laboratory Animal Care.

Chemoprevention bioassay

Rats were randomly assigned to five separate groups and placed on AIN-76A diet for one week (wk) to acclimatize to the facility. They were then given s.c. injections with 0.2 ml of either 20% DMSO in water (vehicle control) or 20% DMSO in water + NMBA (0.35 mg/kg b.w.; carcinogen control) three times per wk for five wks. Following the injections, rats were placed on experimental diets as follows (Table 1): AIN-76A diet + DMSO in water (Group 1), AIN-76A diet + NMBA (Group 2), 6.1% BRB powder in AIN-76A diet + NMBA (Group 3), 3.8 μmol AC/g AIN-76A diet + NMBA (Group 4), 500 ppm PCA in AIN-76A diet + NMBA (Group 5). A 6.1% BRB diet was prepared in order for the AC content in Group 3 to be equivalent to that in Group 4. At wks 15 and 25, nine rats from each group were euthanized, each esophagus was opened longitudinally, and tumors counted, mapped and sized. Lesions greater than 0.5 mm in a single dimension were counted as tumors. Tumor volume was calculated using the length × width × height × ð/6 formula (expressed as mm^3). Tumor burden was calculated by summing the tumor volume for each esophagus. The esophagi were cut in half; one-half was snap frozen in liquid nitrogen for extraction of DNA,

RNA and protein, and the other half was fixed for 24 hours (hrs) in 10% neutral buffered formalin and then stored in PBS for subsequent histopathologic evaluation. At wk 35, all remaining rats were terminated, esophageal tumors quantified, and tissues processed using the same protocol.

Histological analysis

Formalin-fixed esophageal tissue was paraffin embedded, cut, stained by H&E, and evaluated by routine histopathology. Areas of normal tissue, hyperplasia, low- and high-grade dysplasia were scored and quantified based on their occurrence within each esophagus as described by Kresty, *et al.* 2001 (14).

qPCR analysis

RNA from each esophagus was extracted using a DNA/RNA kit (Qiagen, Valencia, CA). RNA was then standardized and converted to cDNA using Superscript III reverse transcriptase (Life Technologies, Brown Deer, WI). COX-2, iNOS, NF-κB, sEH mRNA expression was quantified using exon specific primers in a SYBR green based qPCR assay, using GAPDH as an internal control (Life Technologies). These levels were statistically analyzed using the $(2^{-\triangle \triangle Ct})$ method (42). The qPCR was carried out using an Applied Biosciences Step One Plus real-time PCR system (Life Technologies). The conditions were 2 mins 95°C denaturation, 30 cycles of 94°C for 30s, 58°C for 30 s, and 72°C for 30 s. Final extension was completed at 72°C for five mins. See Supplementary table 1 for the respective primer sequences.

Measurement of PTX3 in plasma

Whole blood was collected in heparin coated tubes (BD Biosciences, San Jose, CA), centrifuged at $3,000 \times$ g for 10 mins, and plasma collected and stored at -80°C until analyzed. 100 μL of plasma from each rat was used to quantify PTX3 concentrations using an enzyme-linked immunosorbent assay (ELISA) (Cusabio, Carlsbad, CA). **Pyrosequencing** DNA was extracted from snap frozen esophageal epithelium (Qiagen) and standardized to 500 ng. It was then bisulfite converted using an EZ DNA Methylation Kit (Zymo Research, Orange, CA). A region within the PTX3 gene in each esophagus was amplified via PCR and the product was sequenced using a pyrosequencer (Qiagen). Gene methylation of PTX3 was quantified using PyroQ-CpG software (Qiagen).

Immunoblotting

Esophageal tissues were disrupted by sonication and solubilized in modified RIPA buffer (50 mM Tris-HCl, pH 7.3, 150 mM NaCl, 0.25% (v/v) sodium deoxycholate, 1.0% (v/v) NP-40, 0.1% (v/v) SDS and 1 mM EDTA) supplemented with Protease Inhibitor Cocktail Set III (EMD Biosciences, San Diego, CA) and 10 mM orthovanadate, 40 mM glycerophosphate, and 20 mM sodium fluoride as phosphatase inhibitors. Lysates were centrifuged (10,000 rpm, 10 mins, 4°C) and the supernatant collected. Protein concentrations were measured using a DC™ protein assay kit (Bio-Rad, Berkeley, CA) and standardized to 2 μg/μL. A total of 50 μg of protein was resolved on precasted SDS-PAGE gels. Blots were prepared using preset transfer paper and run on the Trans-Blot® Turbo™ Transfer System (Bio-Rad,

Berkeley, CA). Blots were blocked in 5% BSA for 30 mins and then incubated with primary antibody to PTX3, iNOS, sEH (Santa Cruz Biotechnology, Berkeley, CA), COX-2 (Thermo Fisher Scientific, Waltham, MA), NF-κB or p-NF-κB (Cell Signaling Technology, Beverly, MA). A secondary antibody labeled with horse radish peroxidase (GE Healthcare, Pittsburgh, PA) was used in conjunction with an ECL detection kit (GE Healthcare, Pittsburgh, PA) to detect the presence of proteins. Western blot analysis was performed on esophagi from 3 animals per diet group (n=3). Densitometric analysis of relative protein abundance compared to β-actin was determined using ImageLab 4.0.1 software (Bio-Rad, Berkeley, CA).

Statistical Analysis

Body weight, food consumption, tumor multiplicity and burden, histopathological analysis and immunohistochemical staining data, qPCR analysis, plasma PTX3 levels, and western blot image density analysis were compared using ANOVA via Prism 5 (GraphPad). A P value < 0.05 was considered to be statistically significant. The post-hoc test used was Tukey's test in which all treatment groups were compared to the AIN-76A diet + NMBA-injection group.

RESULTS

General observations

There were no significant differences in animal body weights or food consumption throughout the course of the study ($P > 0.05$; data not shown). Esophageal tumors were examined under a light microscope and all were found to have the histological features of squamous cell papillomas. No tumors were seen in any DMSO-injected animals at any time point, and no invasive carcinomas were identified in the stroma or muscle tissue of any NMBA-treated esophagi at any time point. This was not unexpected because NMBA-treated rats are typically euthanized before carcinomas develop due to occlusion of the lumen of the esophagus by the expanding papillomas. BRB, AC, and PCA diets were well tolerated and did not produce any gross or histological abnormalities in the esophagus, liver, intestinal tract, kidneys or spleen of any of the treated rats.

Effects of diets on NMBA-induced preneoplastic lesions and tumors

Effects on NMBA-induced preneoplastic lesions—The inhibition of preneoplastic esophageal lesions by the administered diets is summarized in Table 2. At wk 15, when compared to NMBA control rats, the esophagi of rats fed diets supplemented with BRB, AC, or PCA had reduced areas of hyperplasia ($P < 0.05$). Only two animals had high-grade dysplastic lesions at wk 15. At wk 25, the BRB, AC, and PCA diets reduced the occurrence of high-grade dysplasia by 60.0, 70.4, and 69.7%, respectively ($P < 0.05$) when compared to NMBA control rats, but no differences were observed in the proportion of normal epithelium, hyperplasia, or low-grade dysplasia. At wk 35, there was a higher proportion of normal epithelial tissue as well as a lower proportion of high-grade dysplasia in esophagi from the BRB, AC and PCA-treated groups than in NMBA controls ($P < 0.05$). These results suggest that the three dietary treatments delayed the development of preneoplastic lesions

including high grade dysplasia, and likely the subsequent conversion of high-grade dysplastic lesions to papillomas.

Inhibition of tumorigenesis by treatment diets—No tumors were observed in NMBA-treated rats at wk 15. The effects of the BRB, AC and PCA diets on tumor multiplicity and tumor burden at wks 25 and 35 are shown in Figs. 1A-D. All three experimental diets were about equally effective in reducing tumor multiplicity and burden at wk 25 (P< 0.05) as shown in Figs. 1A and 1B respectively. At wk 35, all three experimental diets reduced tumor multiplicity and burden as shown in Figs. 1C and 1D respectively (P< 0.05), but the BRB treatment was significantly more effective than either the AC or PCA treatments (P< 0.05). No significant differences in tumor size were observed across all NMBA-treated groups at any time point.

Inflammatory marker expression in NMBA-treated rats

Previous studies have shown that BRB and their component AC reduce the mRNA and protein expression levels of the inflammatory markers, COX-2, iNOS, and NF-κB in NMBA-treated rat esophagus (15, 21). Based on these observations, we determined if PCA might exhibit similar down-regulatory effects on these markers. In addition, the effects of all three experimental diets on the expression of the pro-inflammatory enzyme, sEH, was determined.

mRNA expression—Relative mRNA expression levels of the above-mentioned inflammatory markers in the esophagi of all animals in the study are summarized in Figs. 2A-D. At wks 15 and 35 all treatment groups decreased the expression levels of sEH mRNA compared to the NMBA control rats (P< 0.05) (Fig. 2A), while no significant change was observed at wk 25 (P> 0.05). COX-2 (Fig. 2B) and iNOS (Fig. 2C) were not affected by any of the diets at wks 15 and 25 (P> 0.05). At wk 35, all three diets reduced COX-2 (Fig. 2B) mRNA expression levels (P<0.05), while the BRB and PCA diets, but not the AC diet, decreased the total iNOS mRNA levels (P< 0.05) (Fig. 2C). No change in the expression of NF-κB mRNA was found with any of the dietary treatments at any time point (P> 0.05) (Fig. 2D). These results suggest that BRB, AC and PCA are more likely to influence the expression levels of the inflammatory markers at a later stage of esophageal carcinogenesis, and that these agents vary in their ability to influence the expression levels of specific inflammatory markers.

Western blot—Protein levels of the inflammatory markers were determined by Western blot only at wk 35 (Figs. 3A-C) because the esophageal tissues at wks 15 and 25 were insufficient for analysis. For each marker, esophagi from 3 rats per diet group were analyzed and the band image densities quantified and compared to the β-actin band density. COX-2 protein expression was significantly reduced by the BRB and AC diets but not the PCA diet (P< 0.05) (Fig. 3B). Expression of sEH protein (Fig. 3B) was reduced by all three experimental diets (P<0.05) and iNOS protein expression was reduced by the BRB and PCA diets (P< 0.05) (Fig. 3B), but not by the AC diet (P> 0.05). These data correlated positively with mRNA expression results. Naïve NF-κB expression was not altered by any of the diets (P> 0.05) (Fig. 3C); however, p-NF-κB expression was reduced by all three diets (P< 0.05)

(Fig. 3C). Collectively, these data suggest that BRB, AC, and PCA all reduce inflammation in the esophagus and, potentially, by differential effects on individual biomarkers.

PTX3 expression—We presumed that PTX3 could be a new anti-inflammatory marker for esophageal tumors in rats because its expression was shown to be highly down-regulated in human ESCC cell lines and tissue through hypermethylation of the promoter region (41). In addition, PTX3 has been shown to elicit anti-carcinogenic effects via its ability to prevent neutrophil migration into tissue sites during acute lung injury (43), as well as anti-angiogenic effects (40, 44).

Plasma PTX3 levels—No differences in plasma PTX3 levels were observed in any diet group at wk 15 (P> 0.05) (Fig. 4A). At wk 25, the plasma PTX3 level in PCA-fed rats was significantly higher than in rats treated with NMBA only (P< 0.05) (Fig. 4A). At wk 35, BRB-, AC- and PCA-fed rats all had higher plasma PTX3 levels when compared to NMBA-treated rats on control diet (P< 0.05) (Fig. 4A).

Western blot analysis—A representative blot depicts the relative levels of PTX3 expression of the five experimental groups (Fig. 4B). PTX3 protein expression was significantly upregulated by all three experimental diets to levels similar to the vehicle control based on image density analysis (P< 0.05) (Fig. 4C).

PTX3 methylation—Extracted esophageal DNA was analyzed for the relative percentage of DNA methylation at wks 15, 25, and 35 (Fig. 4D). No significant differences were seen in relative DNA methylation across all diet groups and time points (P> 0.05). These results suggest that the mechanism for downregulation of PTX3 expression in NMBA-induced rat esophageal papillomas is not through hypermethylation of the PTX3 gene as is the case in human esophageal SCC (41).

DISCUSSION

We reported that synthetic AIN-76A diet containing either 5% or 10% BRB was effective at reducing NMBA-induced esophageal carcinogenesis in rats (14). Continued research demonstrated that the four AC in BRB are nearly as effective as whole BRB in reducing esophageal carcinogenesis (15). Results from the present study confirm the chemopreventive activity of whole BRB and BRB AC against NMBA-induced rat esophageal carcinogenesis and also demonstrate the ability of PCA, a major metabolite of BRB AC, to reduce esophageal carcinogenesis. A pharmacokinetic study in humans indicated that about 70% of the administered AC in BRB are converted to PCA in the human gut (45, 46). Therefore, for comparative purposes, we fed rats an amount of PCA (500 ppm in the diet) equivalent to about 70% of the anthocyanin content in the AC-enriched diet. The observation that PCA was effective at this dose suggests that it may be responsible for at least some of the chemopreventive activity of whole BRB and BRB AC. Further studies are underway to confirm this observation including a metabolism study to determine the extent of PCA production from BRB AC by microbiota in the rat intestine. If confirmed, then PCA would appear to be worthy of additional evaluation as a chemopreventive agent for the esophagus and potentially other organs. When compared to whole BRB and the AC fraction, PCA has

several advantages for chemoprevention in that it delivers a constant dose, is commercially available at low cost, and it is more readily bioavailable than the cyanidin-type AC found in BRB (47).

Consistent with their ability to inhibit NMBA-induced tumors in the rat esophagus, the BRB, AC and PCA diets were all effective at reducing premalignant lesions. When compared to the NMBA control group, all three diets appeared to cause a delay in the formation of preneoplastic lesions in NMBA-treated esophagus as well as the progression of these lesions to papillomas. The delay in formation of preneoplastic lesions was observed as early as 15 wks after the first injection of NMBA (post-initiation). The BRB and AC results confirm earlier data (15, 20), and lend credence to the concept of using these agents in human clinical trials involving patients with endoscopically-identified preneoplastic esophageal lesions as was done with strawberry powder in Chinese patients by Chen et al. (16). With additional experimentation, PCA may also prove to be a viable candidate for these trials.

The present study also confirms earlier data demonstrating the inhibitory effects of whole BRB and their component AC on inflammatory biomarkers and extends these activities to PCA. The AC repeatedly decreased expression of the inflammatory markers; COX-2, activated NF-κB, PTX3 and sEH at levels similar to whole BRB powder. PCA appeared to be active in reducing inflammatory biomarkers but less so than the BRB. The relative efficacy of these three agents to reduce esophageal tumorigenesis and preneoplastic lesions, therefore, appears to parallel their relative inhibitory effects on the inflammatory markers. The enhanced ability of BRB to reduce the inflammatory markers may be due to an additive effect of other compounds in BRB such as ellagic, ferulic and chlorogenic acids and quercetin, or to their fiber content. For example, the fiber fraction of fruits has been shown to select for, and enhance the production of, specific bacterial types in the intestine that exhibit anti-inflammatory effects (48). In that regard, we have shown that the residue (non-alcohol soluble) or fiber fraction of BRB has chemopreventive activity for NMBA-treated rat esophagus (15).

Recent work has also indicated that the PTX3 promoter is hypermethylated in human ESCC cell lines and esophageal tumor tissue (41). This was not observed in NMBA-treated rat esophagus in the present study. PTX3 has both anti-angiogenic and anti-tumorigenic activity in human prostate cancer cell lines (40), and steroid-hormone regulated tumors S115 (mouse mammary tumor cells) (49). Interestingly, PCA was the most effective treatment at inducing PTX3 expression in the plasma at the 25 wk time point (PTX3 plasma level = 0.47 pg/ml compared to 0.23 pg/ml in the NMBA-control), but this was reduced to the same level as in BRB- and AC-treated rats at 35 wks. The increased level at 25 wks may account for the decrease in high grade dysplasia seen at 25 wks in the PCA treated group. As PTX3 has been shown to alter immune cell migration via inhibiting P-selectin mediated rolling adhesion (43), this altered cytokine expression may lead to changes in esophagus immune cell trafficking.

Minor differences observed in the effectiveness of the AC fraction and PCA to reduce tumorigenesis and inflammatory biomarkers in the present study may be a result of the

kinetics of anthocyanin and PCA metabolism and bioavailability. Following oral administration, PCA can be absorbed in the stomach and small intestine as indicated by its recovery in the plasma within 2.9 mins and reaching a peak by 5 mins, a time period too short for the anthocyanins to have reached the colon (50). Orally administration of PCA would increase its exposure to absorptive sites in the GI tract allowing for higher amounts to be absorbed into the circulation. This would allow for a longer exposure time of PCA to tissues in animals fed PCA when compared to the PCA produced from AC by the enteric microbiota.

In summary, results of the present study support the notion that while the AC in BRB are important for their chemopreventive activity, PCA, a major metabolite of BRB AC, is also effective in inhibiting tumorigenesis and inflammatory signaling. Whole BRB appear to be more effective than either AC or PCA in reducing NMBA-induced esophageal tumorigenesis which undoubtedly reflects their entire content of potential chemopreventive agents. The effectiveness of PCA as a chemopreventive agent in the present study is interesting however, because PCA is available commercially at a reasonable cost and is more easily synthesized and stable than the AC. PCA appears to be a viable candidate for additional mechanistic studies in preclinical rodent models and, potentially, for human clinical trials of cancer prevention in the esophagus and other organs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

References

1. Stoner GD, Rustgi AK. Biology of esophageal squamous cell carcinoma. Gastrointest Cancers Biol Diagn Ther. 1995; 8:141–6.

2. Beer DG, Stoner GD. Clinical models of chemoprevention for the esophagus. Hematol Oncol Clin North Am. 1998; 12:1055–77. [PubMed: 9888021]

3. Tuyns AJ. Recherches concernant les facteurs etiologiques du cancer de l'oesophage dans l'ouest de la France. Bull Cancer. 1980; 67:1174–85.

4. Li MH, Cheng SJ. Carcinoma of the esophagus and gastric cardia. In: Huang GJ, Kai WY, editorsEtiology of carcinoma of the esophagus. Springer-Verlag; New York: 1984. 25–51.

5. Li MH, Cheng SJ. Occurence of nitroso compounds in fungi-contaminated foods: a review. Nutr. Cancer. 1986; 8:63–9. [PubMed: 3520493]

6. Togawa K, Jaskiewicz K, Takahashi H, Meltzer SJ, Rustgi AK. Human papillomavirus DNA sequences in esophageal squamous cell carcinoma. Gastroenterology. 1994:128–36. [PubMed: 7912679]

7. Hecht SS, Stoner GD. Lung and esophageal carcinogenesis. In: Aisner J, Arriagada R, Green MR, Martini N, Perry MC, editorsComprehensive textbook of thoracic oncology. Williams and Wilkins; Philidelphia: 1996. 25–50.

8. Ribeiro J,U, Posner MC, Safalte-Ribeiro AV, Reynolds JC. Risk factors for squamous cell carcinoma of the esophagus. Br J Surg. 1996; 83:1174–85. [PubMed: 8983603]

9. Stoner GD, Gupta A. Etiology and chemoprevention of esophageal squamous cell carcinoma. Carcinogenesis. 2001; 22:1737–46. [PubMed: 11698334]

10. Rice TW, Adelstein DJ, Zuccaro G, Falk GW, Goldblum JR. Advances in treatment of esophageal carcinoma. Gastroenterologist. 1997; 5:278–94. [PubMed: 9436004]

11. Rijken PJ, Timmer WG, van de Kooji AJ, Van Benschop IM, Wiseman SA, Meijers M, et al. Effect of vegetable and carotenoid consumption on aberrant crypt multiplicity, a surrogate end-point marker for colorectal cancer in azoxymethane-induced rats. Carcinogenesis. 1999; 20:2267–72. [PubMed: 10590218]

12. Foschi R, Pelucchi C, Dal Maso L, Rossi M, Levi F, Talamini R, et al. Citrus fruit and cancer risk in a network of case-control studies. Cancer Causes Control. 2010; 21:237–42. [PubMed: 19856118]

13. Carlton PS, Kresty LA, Siglin JC, Morse MA, Lu J, Morgan C, et al. Inhibition of N-nitrosomethylbenzylamine-induced tumorigenesis in the rat esophagus by dietary freeze-dried strawberries. Carcinogenesis. 2001; 22:441–6. [PubMed: 11238184]

14. Kresty LA, Morse MA, Morgan C, Carlton PS, Lu J, Gupta A, et al. Chemoprevention of esophageal tumorigenesis by dietary administration of lyophilized black raspberries. Cancer Res. 2001; 61:6112–9. [PubMed: 11507061]

15. Wang LS, Hecht SS, Carmella SG, Yu N, Larue B, Henry C, et al. Anthocyanins in black raspberries prevent esophageal tumors in rats. Cancer Prev Res. 2009; 2:84–93.

16. Chen T, Yan F, Qian J, Guo M, Zhang H, Tang X, et al. Randomized phase II trial of lyophilized strawberries in patients with dysplastic precancerous lesions of the esophagus. Cancer Prev Res. 2012; 5:41–50.

17. Stoner G, Wang LS. Chemoprevention of esophageal squamous cell carcinoma with berries. In: Pezzuto JM, Suh N, editorsNatural products in cancer prevention and therapy. Springer; Berlin: 2013. 1–20.

18. Stoner GD. Foodstuffs for preventing cancer: The preclinical and clinical development of berries. Cancer Prev Res. 2009; 2:187–94.

19. Huang C, Huang Y, Li J, Hu W, Aziz R, Tang MS, et al. Inhibition of benzo(a)pyrene diol-epoxide-induced transactivation of activated protein 1 and nuclear factor κB by black raspberry extracts. Cancer Res. 2002; 62:6857–63. [PubMed: 12460899]

20. Chen T, Hwang H, Rose ME, Nines RG, Stoner GD. Chemopreventive properties of black raspberries in N-nitrosomethylbenzylamine-induced rat esophageal tumorigenesis: down-regulation of cyclooxygenase-2, inducible nitric oxide synthase, and c-Jun. Cancer Res. 2006; 66:2853–9. [PubMed: 16510608]

21. Chen T, Rose ME, Hwang H, Nines RG, Stoner GD. Black raspberries inhibit N-nitrosomethylbenzylamine (NMBA)-induced angiogenesis in rat esophagus parallel to the suppression of COX-2 and iNOS. Carcinogenesis. 2006; 27:2301–7. [PubMed: 16777990]

22. Hecht SS, Huang C, Stoner GD, Li J, Kenney PM, Sturla SJ, et al. Identification of cyanidin glycosides as constituents of freeze-dried black raspberries which inhibit anti-benzo[a]pyrene-7,8-diol-9,10-epoxide induced NFκB and AP-1 activity. Carcinogenesis. 2006; 27:1617–26. [PubMed: 16522666]

23. Stoner GD, Dombkowski AA, Reen RK, Cukovic D, Salagrama S, Wang LS, et al. Carcinogen-altered genes in rat esophagus positively modulated to normal levels of expression by both black raspberries and phenylethyl isothiocyanate. Cancer Res. 2008; 68:6460–7. [PubMed: 18676871]

24. Zikri NN, Riedl KM, Wang LS, Lechner JF, Schwartz SJ, Stoner GD. Black raspberry components inhibit proliferation, induce apoptosis, and modulate gene expression in rat esophageal epithelial cells. Nutr. Cancer. 2009; 61:816–26. [PubMed: 20155622]

25. Bi X, Fang W, Wang LS, Stoner GD, Yang W. Black raspberries inhibit intestinal tumorigenesis in Apc1638+/− and Muc2−/− mouse models of colorectal cancer. Cancer Prev Res. 2010; 3:1443–50.

26. Montrose DC, Horelik NA, Madigan JP, Stoner GD, Wang LS, Bruno RS, et al. Anti-inflammatory effects of freeze-dried black raspberry powder in ulcerative colitis. Carcinogenesis. 2011; 32:343–50. [PubMed: 21098643]

27. Wang LS, Dombkowski AA, Seguin C, Rocha C, Cukovic D, Mukundan A, et al. Mechanistic basis for the chemopreventive effects of black raspberries at a late stage of rat esophageal carcinogenesis. Mol Carcinog. 2011; 50:291–300. [PubMed: 21465577]

28. Wang LS, Kuo CT, Stoner K, Yearsley M, Oshima K, Yu J, et al. Dietary black raspberries modulate DNA methylation in dextran sodium sulfate (DSS)-induced ulcerative colitis. Carcinogenesis. 2013 Epub ahead of print.

29. Passamonti S, Vrhovsek U, Vanzo A, Mattivi F. The stomach as a site for anthocyanins absorption from food. FEBS Lett. 2003; 544:210–3. [PubMed: 12782318]

30. Stoner GD, Sardo C, Apseloff G, Mullet D, Wargo W, Pound V, et al. Pharmacokinetics of anthocyanins and ellagic acid in healthy volunteers fed freeze-dried black raspberries daily for 7 days. J Clin Pharmacol. 2005; 45:1153–64. [PubMed: 16172180]

31. Keppler K, Humpf HU. Metabolism of anthcyanins and their phenolic degradation products by the intestinal microflora. Bioorg Med Chem. 2005; 13:5195–205. [PubMed: 15963727]

32. Hidalgo M, Martin-Santamaria S, Recio I, Sanchez-Moreno C, de Pascual-Teresea B, Rimbach G, et al. Potential anti-inflammatory, anti-adhesive, anti/estrogenic, and angiotensin-converting enzyme inhibitory activities of anthcyanins and their gut metabolites. Genes Nutr. 2012; 7:295–306. [PubMed: 22218934]

33. Lin CY, Huang CY, Yin MC. Anticoagulatory, antiinflammatory, and antioxidative effects of protocatechuic acid in diabetic mice. J Agric Food Chem. 2009; 57:6661–7. [PubMed: 19722571]

34. Tanaka T, Kojima T, Kawamori T, Yoshimi N, Mori H. Chemoprevention of diethylnitrosamine-induced heptaocarcinogenesis by a simple phenolic acid protocatechuic acid in rats. Cancer Res. 1993; 53:2775–9. [PubMed: 8504418]

35. Tanaka T, Kojima T, Suzui M, Mori H. Chemoprevention of colon carcinogenesis by the natural product of a simple phenolic compound protocatechuic acid: suppressing effects on tumor development and biomarkers expression of colon tumorigenesis. Cancer Res. 1993; 53:3908–13. [PubMed: 8358716]

36. Mori H, Tanaka T, Sugie S, Yoshimi N, Kawamori T, Hirose Y, et al. Chemoprevention by naturally occurring and synthetic agents in oral, liver, and large bowel carcinogenesis. J Cell Biochem Suppl. 1997; 27:35–41. [PubMed: 9591191]

37. Anter J, Romero-Jiménez M, Fernández-Bedmar Z, Villatoro-Pulido M, Analla M, Alonso-Moraga A, et al. Antigenotoxicity, cytotoxicity, and apoptosis induction by apigenin, bisabolol, and protocatechuic acid. Med Food. 2011; 14:276–83.

38. Suzuki R, Kohno H, Sugie S, Tanaka T. Dietary protocatechuic acid during the progression phase exerts chemopreventive effects on chemically induced tongue carcinogenesis. Asian Pac J Cancer Prev. 2003; 4:319–26. [PubMed: 14728590]

39. Moghaddam MF, Grant DF, Cheek JM, Greene JF, Williamson KC, Hammock BD. Bioactivation of leukotoxins to their toxic diols by epoxide hydrolase. Nat Med. 1997; 3:562–6. [PubMed: 9142128]

40. Ronca R, Alessi P, Coltrini D, Di Salle E, Giacomini A, Leali D, et al. Long pentraxin-3 as an epithelial-stromal fibroblast growth factor-targeting inhibitor in prostate cancer. J Pathol. 2013; 230:228–38. [PubMed: 23424081]

41. Wang JX, He YL, Zhu ST, Yang S, Zhang ST. Aberrant methylation of the 3q25 tumor suppressor gene PTX3 in human esophageal squamous cell carcinoma. World J Gastroenterol. 2011; 17:4225–30. [PubMed: 22072855]

42. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001; 25:402–8. [PubMed: 11846609]

43. Deban L, Russo RC, Sironi M, Moalli F, Scanziani M, Zambelli V, et al. Regulation of leukocyte recruitment by the long pentraxin PTX3. Nat Immunol. 2010; 11:328–34. [PubMed: 20208538]

44. Alessi P, Leali D, Camozzi M, Cantelmo A, Albini A, Presta M. Anti-FGF2 approaches as a strategy to compensate resistane to anti-VEGF therapy: pentraxin-3 as a novel antiangiogenic FGF2-antagonist. Eur Cytokine Netw. 2009; 20:225–34. [PubMed: 20167562]

45. Aura AM, Martin-Lopez P, O'Leary KA, Williamson G, Oksman-Caldentey KM, Poutanen K, et al. In vitro metabolism of anthocyanins by human gut microflora. Eur J Nutr. 2005; 44:133–42. [PubMed: 15309431]

46. Vitaglione P, Donnarumma G, Napolitano A, Galvano F, Gallo A, Scalfi L, et al. Protocatechuic acid is the major human metabolite of cyanidin-glucosides. J Nutr. 2007; 137:2043–8. [PubMed: 17709440]

47. Wang D, Zou T, Yang Y, Yan X, Ling W. Cyanidin-3-O-β-glucoside with the aid of its metabolite protocatechuic acid, reduces monocyte infiltration in apolipoprotein E-deficient mice. Biochem Pharmacol. 2011; 82:713–9. [PubMed: 21569764]

48. Tuohy KM, Conterno L, Gasperotti M, Viola R. Up-regulating the human intestinal microbiome using whole plant foods, polyphenols, and/or fiber. J Agric Food Chem. 2012; 60:8776–82. [PubMed: 22607578]

49. Leali D, Alessi P, Coltrini D, Ronca R, Corsini M, Nardo G, et al. Long pentraxin-e inhibits FGF8b-dependent angiogenesis and growth of steroid hormone-regulated tumors. Mol Cancer Ther. 2011; 10:1600–10. [PubMed: 21764903]

50. Chen W, Wang D, Wang LS, Bei D, Wang J, See WA, et al. Pharmacokinetics of protocatechuic acid in mouse and its quantification in human plasma using LC-tandem mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci. 2012; 908:39–44.

# Week 25

Week 35
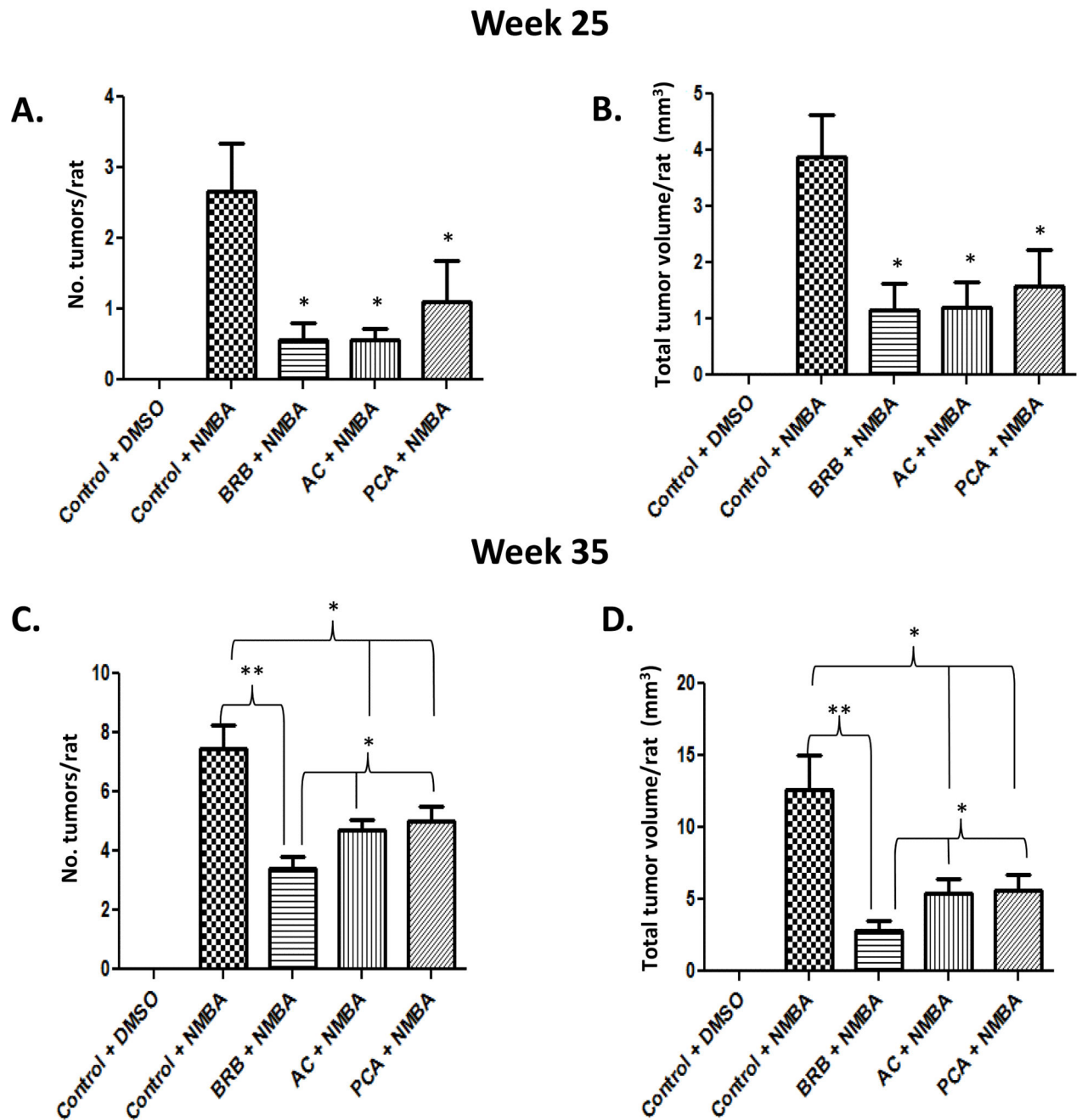
Figure 1.
Effects of dietary BRB, AC, and PCA on tumor response and burden in NMBA-treated rat esophagus. Fig. 1A shows the number of tumors/rat was significantly reduced by all treatment groups relative to the NMBA control at wk 25 (P< 0.05). Fig. 1B illustrates that this reduction in tumor multiplicity correlates with a reduction in tumor burden in the treatment groups compared to the NMBA control at wk 25 (P< 0.05). Fig. 1C reveals that tumor multiplicity was also reduced by all treatment groups at wk 35, however the BRB group had significantly lower tumors/rat compared to the AC and PCA groups (P< 0.05).

Fig. 1D shows that total tumor burden/rat was also significantly reduced by all treatment groups compared to the NMBA control at wk 35 (P< 0.05), and that the BRB group significantly reduced tumor burden when compared to the AC and PCA groups. *Columns,* mean (n= 9 for wk 25, n= 30 for wk 35), *bars,* SD. * and ** indicates results were significantly lower (P< 0.05, 0.01 respectively) than rats treated with NMBA and fed control diet or a diet containing AC or PCA.
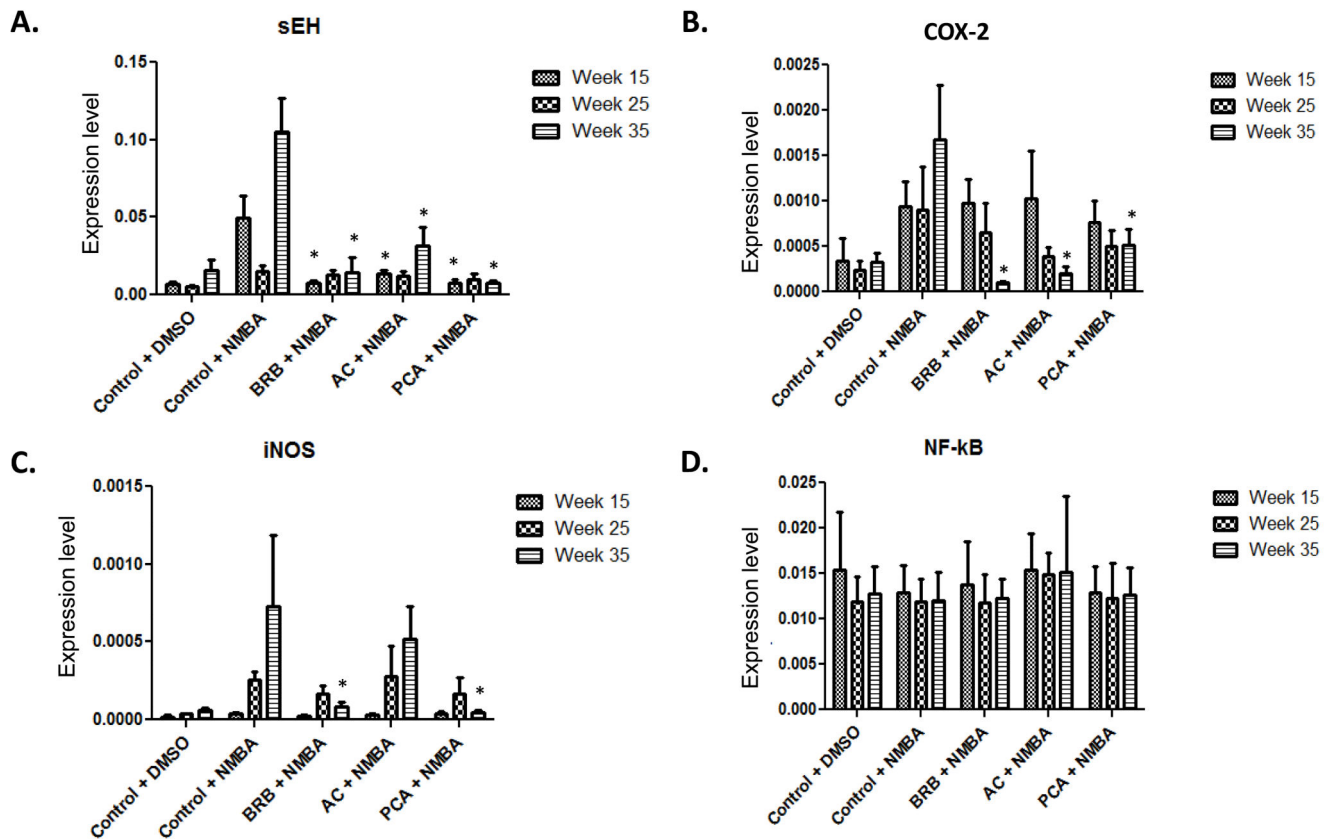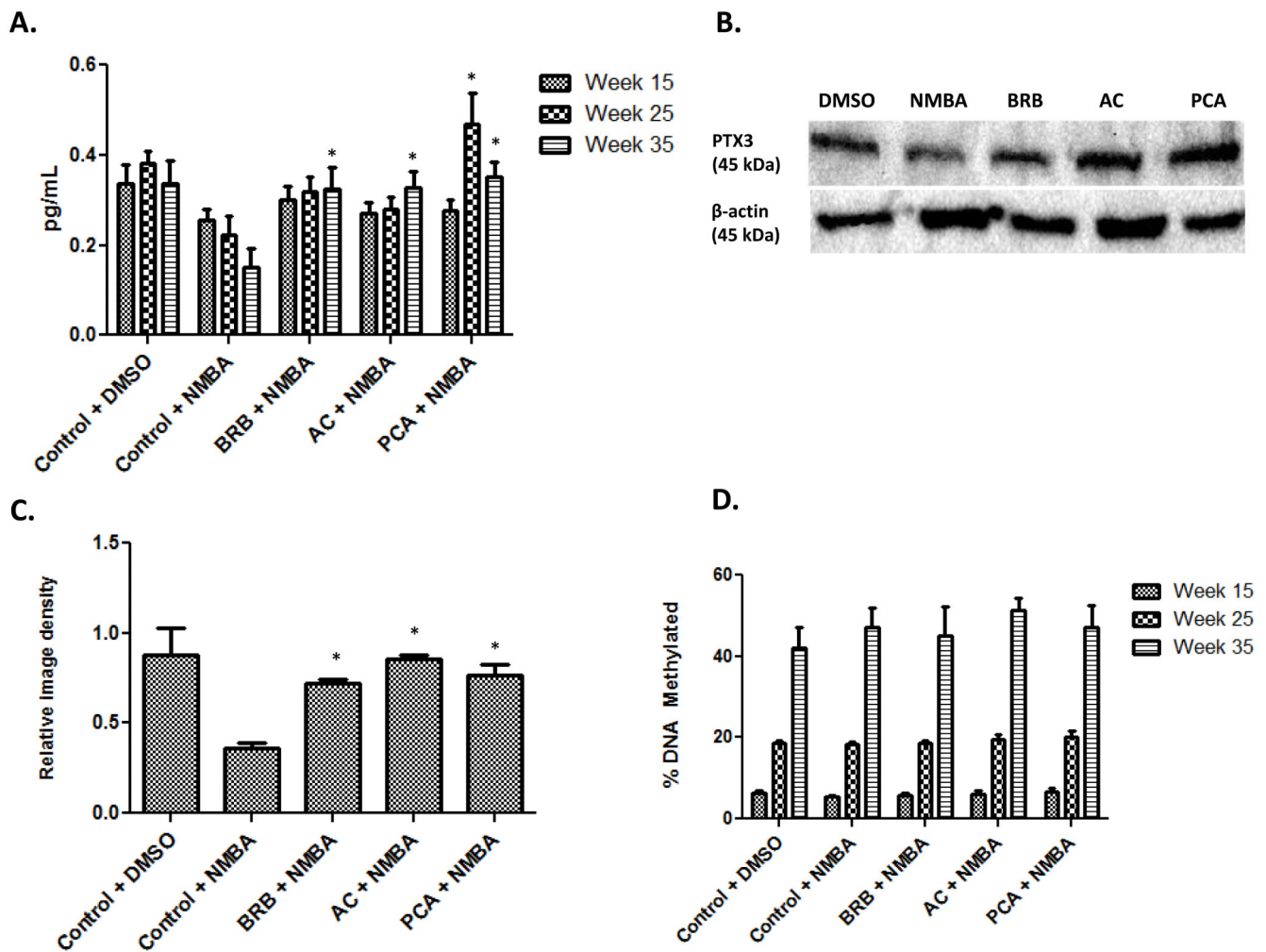
Figure 2.
Effects of dietary BRB, AC, and PCA on mRNA expression of inflammatory biomarkers using GAPDH as an internal control. Fig. 2A illustrates that sEH mRNA expression was significantly reduced in all treatment groups at wks 15 and 35 (P< 0.05). Depicted in Fig. 2B, COX-2 mRNA expression was significantly reduced at wk 35 for all groups (P< 0.05), while Fig. 2C shows only the BRB and PCA diets reduced iNOS mRNA expression significantly at wk 35 (P< 0.05). Fig. 2D reveals no significant change in NF-κB mRNA expression at any time point. *Columns,* mean (n= 9 for wks 15 and 25, n= 10 for wk 35), *bars,* SD. *, significantly lower (P< 0.05) than rats treated with NMBA and fed control diet.
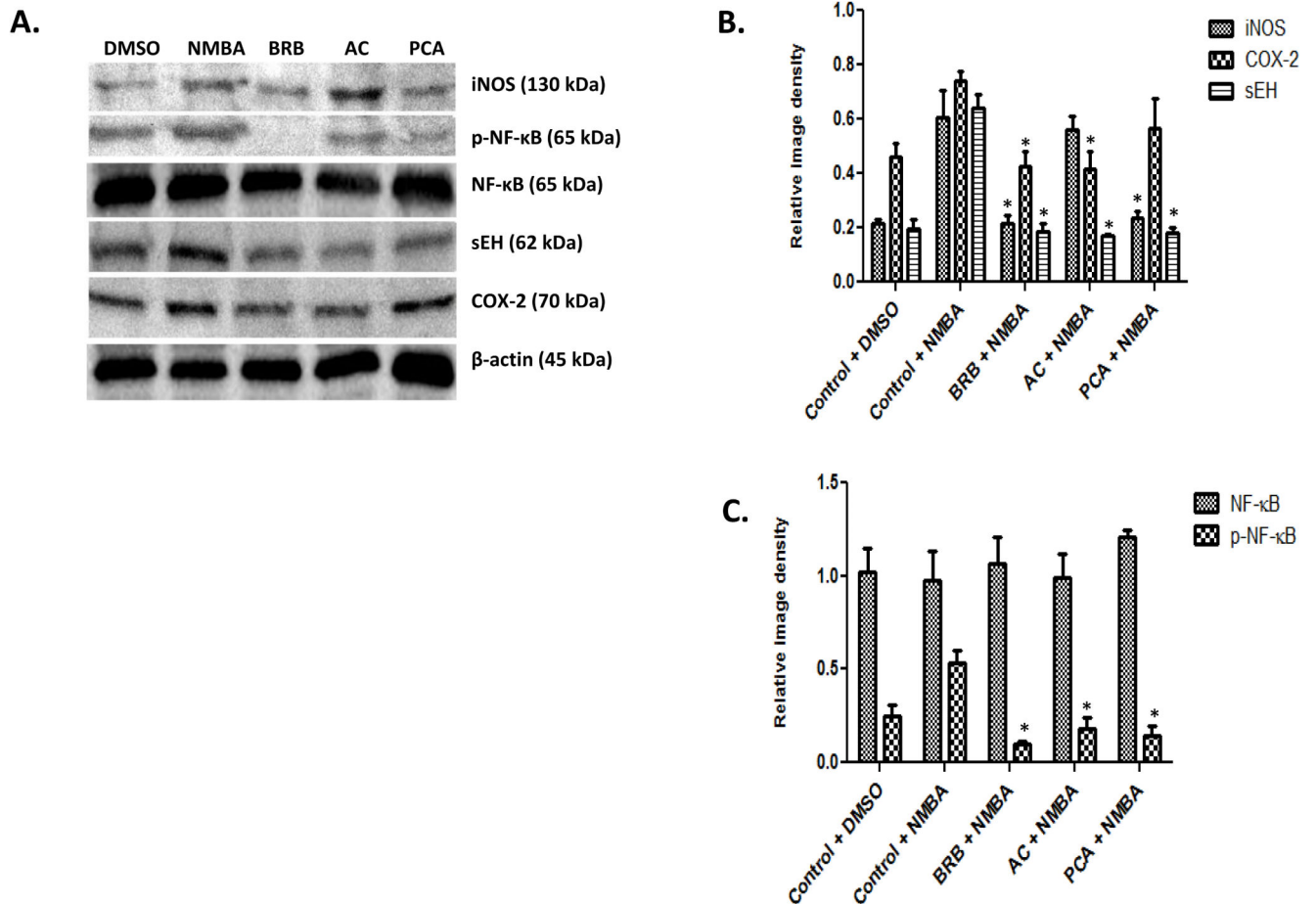
Figure 3.
Effects of dietary BRB, AC, and PCA on protein expression of inflammatory markers in the esophagus via immunoblot at wk 35. Fig. 3A depicts representative blots for all the inflammatory markers measured. Fig. 3B indicates that sEH protein expression was reduced by the BRB, AC, and PCA diets as well (P< 0.05). COX-2 expression was significantly reduced by the BRB and AC diets in the esophagus (P< 0.05), while iNOS expression was reduced by the BRB and PCA diets (P< 0.05). Fig. 3C shows that naive NF-κB expression was unchanged, while p-NF-κB expression was significantly reduced by the BRB, AC, and PCA diets. *Columns,* mean (n=3), *bars,* SD. *, significantly lower (P< 0.05) than rats treated with NMBA and fed control diet.

Figure 4.
Effects of dietary intake of BRB, AC, and PCA on PTX3 expression in the plasma and esophagus shown via ELISA and immunoblotting. Fig. 4A shows that the PCA group has significantly higher PTX3 expression at wk 25, while all three experimental diets significantly upregulated PTX3 expression globally in the plasma at wk 35 (n=9 at wks 15 and 25, n=10 at wk 35). Fig. 4B depicts a representative blot for PTX3 at wk 35 in the esophagus, showing increased expression of PTX3 in the BRB, AC, and PCA dietary groups. Fig. 4C illustrates increased PTX3 expression in the esophagus at wk 35 quantified through image density analysis. This correlated with plasma PTX3 levels at wk 35 (n=3). Fig. 4D indicates no difference in PTX3 promoter methylation at any time point (n=9). *Columns* represent mean, *bars* show SD. *, significantly higher (P< 0.05) than rats treated with NMBA and fed control diet.

Table 1

Experimental diet overview

Group	No. of rats (wk 15, 25, 35)	Treatment	[a]Diet	Concentration of active compound (μmol/g)	Addition to diet (% by weight)
1	9, 9, 30	[b]DMSO	AIN-76A	N/A	N/A
2	9, 9, 30	[c]NMBA	AIN-76A	N/A	N/A
3	9, 9, 30	NMBA	AIN-76A + BRB	3.8	6.1
4	9, 9, 30	NMBA	AIN-76A + AC	3.8	1.6
5	9, 9, 30	NMBA	AIN-76A + PCA	3.24	0.05

Abbreviations: Black berry raspberry powder (BRB), Anthocyanins (AC)

[a]Diets fed to animals following DMSO/NMBA injection

[b]DMSO is vehicle for NMBA (20% DMSO/Water solution)

[c]NMBA administered by s.c injection (.35 mg/kg) in volume of 0.2 mL vehicle

Table 2

Progression of preneoplastic lesions

Group	NMBA (0.35 mg/kg)	Diet administered	% Normal Epithelium or Preneoplastic Esophageal Lesions (±SE)			
			Normal	Epithelial hyperplasia	Low-grade dysplasia	High-grade dysplasia
			Week 15			
1	-	AIN-76A	98.5^a (3.1)	1.5^a (3.1)	0	0
2	+	AIN-76A	53.2 (21.6)	39.4 (16.2)	7.1 (9.2)	0.3 (0.2)
3	+	6.1% BRB	96.2^a (4.2)	3.2^a (3.5)	0.6 (1.7)	0
4	+	3.8 μmol AC/g	92.7^a (10.9)	6.9^a (10.6)	0.4 (1.3)	0
5	+	500 ppm PCA	86.9^a (12.7)	10.2^a (10.4)	2.5 (3.3)	0.4 (0.1)
			Week 25			
1	-	AIN-76A	86.2^a (1.2)	13.8^a (1.2)	0	0
2	+	AIN-76A	51.4 (3.2)	20.7 (2.0)	13.4 (0.4)	14.5 (0.5)
3	+	6.1% BRB	52.9 (2.9)	25.3 (1.4)	16.0 (1.6)	5.8^a (0.8)
4	+	3.8 μmol AC/g	56.7 (1.3)	25.1 (0.7)	13.9 (0.5)	4.3^a (0.4)
5	+	500 ppm PCA	57.7 (2.0)	26.7 (1.1)	11.2 (0.8)	4.4^a (0.5)
			Week 35			
1	-	AIN-76A	93.6^a (0.8)	6.2^a (0.5)	4.7^a (0.5)	0
2	+	AIN-76A	31.6 (0.5)	22.4 (0.7)	21.3 (0.6)	24.7 (0.5)
3	+	6.1% BRB	51.3^a (1.1)	22.4 (0.5)	15.2^a (0.8)	11.1^a (0.5)
4	+	3.8 μmol AC/g	53.4^a (1.2)	21.0 (0.7)	16.0^a (0.6)	9.6^a (0.7)
5	+	500 ppm PCA	50.5^a (1.4)	25.3 (0.7)	16.6^a (0.8)	7.7^a (0.4)

BRB, AC, & PCA diets mixed with AIN-76A

[a] Statistically significant relative to NMBA controls (Group 2) (P< 0.05)