Research Article

Cancer Prevention Research

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 (BRBs) or their polyphenolic anthocyanins (ACs) have been shown to inhibit the development of *N*-nitrosomethylbenzylamine (NMBA)–induced esophageal cancer in rats. The present study was conducted to determine whether PCA, a major microbial metabolite of black raspberry (BRB) ACs, also prevents NMBA-induced esophageal cancer in rats. F344 rats were injected with NMBA three times a week for 5 weeks and then fed control or experimental diets containing 6.1% BRBs, an anthocyanin (AC)-enriched fraction derived from BRBs, or protocatechuic acid (PCA). Animals were exsanguinated at weeks 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 weeks 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 interleukin (IL)-1β and TNF-α. All experimental diets were also active at reducing tumorigenesis at week 35; however, the BRB diet was significantly more effective than the AC and PCA diets. Furthermore, all experimental diets inhibited inflammation in the esophagus via reducing biomarker (COX-2, iNOS, p-NF-κB, and sEH) and cytokine (PTX3) expression. Overall, our data suggest that BRBs, their component ACs, and PCA inhibit NMBA-induced esophageal tumorigenesis, at least in part, by their inhibitory effects on genes associated with inflammation. *Cancer Prev Res; 7(6); 574–84. ©2014 AACR.*

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 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, high temperature beverages and food, inadequate intake of vegetables and fruit, and infection with human papilloma virus (HPV; refs. 3–8). Nitrosamine carcinogens present in foodstuffs as well as *N*-Nitroso precursors, which can undergo nitrosation in the acidic environment of the stomach, are also thought to contribute to disease burden (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 5-year survival rate for SCC remains a dismal 15% to 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 epidemiologic studies that 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 and colleagues (16) demonstrated an approximately 80% reduction in histologic grade of mildly

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dysplastic lesions of the esophagus of Chinese patients who ingested a total of 60 g (30 g, 2×/day) of freeze-dried strawberries daily in a slurry of water for 6 months.

The use of black raspberries (BRBs) as a chemoprevention agent has gained interest, and six human trials have been completed to date to assess the efficacy of black raspberry (BRB) formulations for cancer prevention (17). High concentrations of chemopreventive compounds such as the anthocyanins (ACs), ellagic acid, quercetin, and β-sitosterol have been identified in BRB (14, 18). BRBs and their component ACs 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, and 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; refs. 15, 19–26). BRBs also re-activate suppressor genes that have been silenced in tumors by hypermethylation (27, 28).

The absorption and bioavailability of BRB constituents including the ACs is a fundamental aspect of their physiologic role in disease prevention. Recent studies have demonstrated that the uptake of orally administered BRB ACs into blood is less than 1% of the administered dose (29, 30). The majority of ACs enter the colon where they are metabolized by colonic bacteria into smaller, and more bioavailable phenolic acids such as protocatechuic acid (PCA; refs. 31, 32). PCA is known to function as an antioxidant and an antidiabetic agent (33). In addition, it is effective as a chemopreventive against colon, bladder, and liver cancer in rodents (34–36) and has antiproliferative and proapoptotic 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 injection of rats with the carcinogen, NMBA (9). Repeated subcutaneous injections of NMBA into rats dependably and reproducibly induce esophageal tumor formation within 15 to 26 weeks. 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 BRBs, their component ACs, 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 were 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 of interest in developing inhibitory agents for this enzyme. The effects of BRBs, ACs, and PCA on the expression of pentraxin-3 (PTX3), a cytokine and antiangiogenic factor (40), were also examined because of the reported silencing of this gene in human esophageal SCC (41).

Materials and Methods

BRB powder

Freeze-dried BRBs (*Rubus occidentalis*) powder was purchased from Decker Farms, Inc. and from BerriProducts, LLC. 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 for quantification of specific minerals, phenolic acis, vitamins, phytosterols, carotenoids, fungicides, pesticides, and herbicides as described previously (14). The content of the three major ACs 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 a high-performance liquid chromatography (HPLC) method described below. A portion of the powder was shipped from MCW to Dr. Hecht's laboratory at the University of Minnesota Cancer Center to prepare the anthocyanin (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), was placed inside a high-density polyethylene (HDPE) bucket. BRB powder (2.0 kg) and 0.1 N HCl (8 L) were added to the bag and mixed briefly to ensure homogeneity and then the mixture was stirred for 30 minutes. 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 approximately 45 minutes, 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 Chemicals America Inc.) was conditioned overnight in 1.1 bed volumes (BV) 200 proof ethanol (Decon Laboratories) and then washed with deionized water immediately before use. The extract from above was added to the resin and the mixture was stirred for 1 hour. The resin was collected by filtering the mixture through polyester fabric netting (Jo-Ann Stores, LLC.). After filtration, the resin was stirred with the following for 15 minutes each: twice with 2 BV H_2O, once with 1.25 BV 0.25 mol/L pH 7.0 aqueous phosphate buffer, and three times with 2 BV H_2O. 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 approximately 25 minutes under N_2 using

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an HDPE bucket with 25, $^1/_4$-inch holes in the bottom and an N_2 line inserted in the top. After removing most of the water, the ACs were desorbed from the resin using 3×3 L, of 200 proof ethanol. Each ethanol wash was stirred in the resin for 30 minutes before collection into a HDPE bucket. This was accomplished by draining through the polyester filter fabric by gravity for approximately 5 minutes followed by 5 to 10 seconds of N_2 pressure using the same drying assembly described above. To remove any stray resin beads, the ethanol desorbate was filtered one final time through four layers of the polyester filter fabric, before being collected in polyethylene jugs and stored at −20°C.

Solvent removal. Solvent removal was performed in two stages using a Büchi R-220 preparatory scale rotary evaporator set at a vacuum of approximately 15 torr and a rotation speed of 70 rpm. First, the majority of the ethanol was removed at a temperature of 22°C, in batches of 15 L. After observing a marked decrease in the rate of evaporation, the ethanol extract was removed and stored at −20°C, and the process was repeated until all of the ethanol rich extract was converted to a water rich semi-concentrate. In the second stage, the semi-concentrates were combined and evaporated at the minimum temperature required to yield a drip-rate of 2 to 3 droplets/second, without heating above 40°C. Rotary evaporation was stopped when the extract had reached the consistency of syrup, or approximately 60% solid by weight. The extract was then transferred to plastic jugs and stored at −20°C before overnight shipment under dry ice to MCW. Aliquots were removed for AC determination by HPLC and H2O content determination by lyophilization.

Analysis of AC in BRB extract. Total ACs were determined by HPLC, as cyanidin-3-O-glucoside equivalents. Extract and standard solutions of cyanidin-3-O-glucoside (Extrasynthese) were prepared in 5% aqueous formic acid. The standard solution was further diluted with 0.1 N HCL.

HPLC was performed using a Luna C18(2) 5 μm 250 × 4.6 mm column (Phenomenex), with detection at 515 nm using a SPD-10A UV-Vis detector (Shimadzu). Solvent A was 30.5% methanol in H_2O with 0.1% phosphoric acid and solvent B was 100% methanol. Elution was isocratic in 100% A for 0 to 20 minutes, then switched to 100% B in 0.5 minutes and held for 5 minutes, before returning to 100% A in 0.5 minutes and reequilibrating for 10 minutes. The flow rate was 0.9 mL/min.

Chemicals

NMBA was purchased from Ash Stevens and was found to be >98% pure by HPLC. Protocatechuic acid (PCA, 97% pure) was purchased from Sigma-Aldrich.

Diet preparation

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

Animals

Male F-344 rats, 3-to 5-weeks old, were purchased from Harlan Sprague-Dawley. Rats were housed two animals per cage under standard conditions (20°C ± 2°C, 50% ± 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 1 week to acclimatize to the facility. They were then given subcutaneous injections with 0.2 mL of either 20% dimethyl sulfoxide (DMSO) in water (vehicle control) or 20% DMSO in water + NMBA (0.35 mg/kg body weight; carcinogen control) three times per week for 5 weeks. 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 ACs/g AIN-76A diet + NMBA (group 4), and 500 ppm PCA in AIN-76A diet + NMBA (group 5). A 6.1% BRB diet was prepared for the AC content in group 3 to be equivalent to that in group 4. At weeks 15 and 25, 9 rats from each group were

Table 1. Experimental diet overview

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

[a]Diets fed to animals following DMSO/NMBA injection.
[b]DMSO is vehicle for NMBA (20% DMSO/water solution).
[c]NMBA administered by subcutaneous injection (0.35 mg/kg) in volume of 0.2 mL vehicle.

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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 in 10% neutral buffered formalin and then stored in PBS for subsequent histopathologic evaluation. At week 35, all remaining rats were terminated, esophageal tumors quantified, and tissues processed using the same protocol.

Histologic analysis

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

Quantitative PCR analysis

RNA from each esophagus was extracted using a DNA/RNA kit (Qiagen). RNA was then standardized and converted to cDNA using SuperScript III Reverse Transcriptase (Life Technologies). *COX-2*, *iNOS*, *NF-κB*, and *sEH* mRNA expression was quantified using exon-specific primers in a SYBR green–based quantitative PCR (qPCR) assay, using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control (Life Technologies). These levels were statistically analyzed using the $2^{-\Delta\Delta C_t}$ method (42). The qPCR was carried out using an Applied Biosciences StepOnePlus Real-Time PCR System (Life Technologies). The conditions were 2 minutes 95°C denaturation, 30 cycles of 94°C for 30 seconds, 58°C for 30 seconds, and 72°C for 30 seconds. Final extension was completed at 72°C for 5 minutes. See Supplementary Table S1 for the respective primer sequences.

Measurement of PTX3 in plasma

Whole blood was collected in heparin-coated tubes (BD Biosciences), centrifuged at 3,000 ×*g* for 10 minutes, and plasma was collected and stored at −80°C until analyzed. Of note, 100 μL of plasma from each rat was used to quantify PTX3 concentrations using ELISA (CUSABIO).

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). 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 mmol/L Tris-HCl, pH 7.3, 150 mmol/L NaCl, 0.25% (v/v) sodium deoxycholate, 1.0% (v/v) NP-40, 0.1% (v/v) SDS, and 1 mmol/L EDTA) supplemented with Protease Inhibitor Cocktail Set III (EMD Biosciences) and 10 mmol/L orthovanadate, 40 mmol/L glycerophosphate, and 20 mmol/L sodium fluoride as phosphatase inhibitors. Lysates were centrifuged (10,000 rpm, 10 minutes, 4°C) and the supernatant was collected. Protein concentrations were measured using a DC Protein Assay kit (Bio-Rad) 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). Blots were blocked in 5% bovine serum albumin (BSA) for 30 minutes and then incubated with primary antibody to PTX3, iNOS, sEH (Santa Cruz Biotechnology), COX-2 (Thermo Fisher Scientific), NF-κB, or p-NF-κB (Cell Signaling Technology). A secondary antibody labeled with horseradish peroxidase (GE Healthcare) was used in conjunction with an ECL detection kit (GE Healthcare) 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 with β-actin was determined using ImageLab 4.0.1 software (Bio-Rad).

Statistical analysis

Body weight, food consumption, tumor multiplicity and burden, histopathologic 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 the Tukey test in which all treatment groups were compared with 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 ($P > 0.05$; unpublished data). Esophageal tumors were examined under a light microscope and all were found to have the histologic 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 histologic 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 week 15, when

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compared with NMBA control rats, the esophagi of rats fed diets supplemented with BRBs, ACs, or PCA had reduced areas of hyperplasia ($P < 0.05$). Only two animals had high-grade dysplastic lesions at week 15. At week 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 with NMBA control rats, but no differences were observed in the proportion of normal epithelium, hyperplasia, or low-grade dysplasia. At week 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 week 15. The effects of the BRBs, ACs, and PCA diets on tumor multiplicity and tumor burden at weeks 25 and 35 are shown in Fig. 1A–D. All three experimental diets were about equally effective in reducing tumor multiplicity and burden at week 25 ($P < 0.05$) as shown in Fig. 1A and B, respectively. At week 35, all three experimental diets reduced tumor multiplicity and burden as shown in Fig. 1C and D, 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 BRBs and their component ACs reduce the mRNA and protein expression levels of the inflammatory markers, COX-2, iNOS, and NF-κB, in NMBA-treated rat esophagus (15, 21). On the basis of these observations, we determined if PCA might exhibit similar downregulatory effects on these markers. In addition, the effects of all three experimental diets on the expression of the proinflammatory enzyme, sEH, was determined.

mRNA expression. Relative mRNA expression levels of the abovementioned inflammatory markers in the esophagi of all animals in the study are summarized in Fig. 2A–D. At weeks 15 and 35, all treatment groups decreased the expression levels of *sEH* mRNA compared with the NMBA control rats ($P < 0.05$; Fig. 2A), whereas no significant change was observed at week 25 ($P > 0.05$). *COX-2* (Fig. 2B) and *iNOS* (Fig. 2C) were not affected by any of the diets at weeks 15 and 25 ($P > 0.05$). At week 35, all three diets reduced *COX-2* (Fig. 2B) mRNA expression levels ($P < 0.05$), whereas 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

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 ACs/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 ACs/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 ACs/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)

NOTE: BRB, AC, and PCA diets were mixed with AIN-76A.
[a]Statistically significant relative to NMBA controls (group 2; $P < 0.05$).

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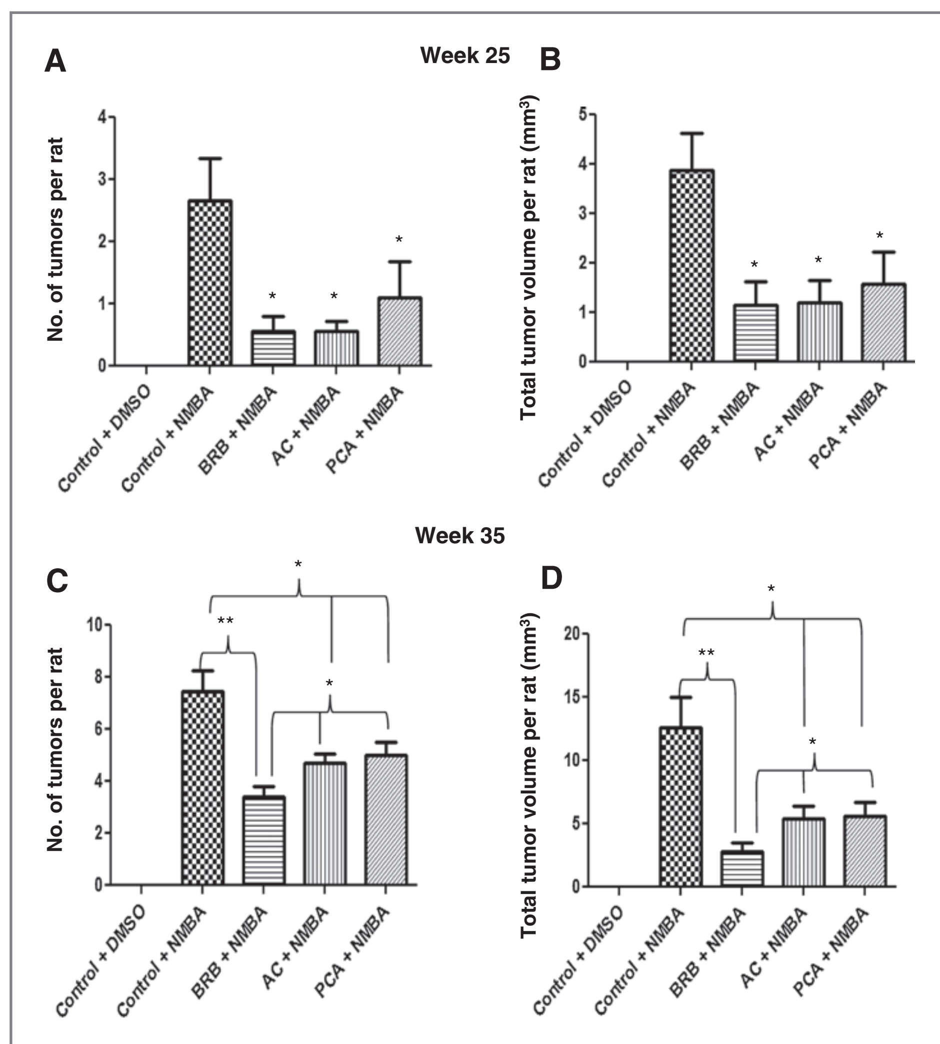

Figure 1. Effects of dietary BRBs, ACs, and PCA on tumor response and burden in NMBA-treated rat esophagus. A, the number of tumors per rat was significantly reduced by all treatment groups relative to the NMBA control at week 25 ($P < 0.05$). B, this reduction in tumor multiplicity correlates with a reduction in tumor burden in the treatment groups compared with the NMBA control at week 25 ($P < 0.05$). C, tumor multiplicity was also reduced by all treatment groups at week 35; however, the BRB group had significantly lower tumors per rat compared with the AC and PCA groups ($P < 0.05$). D, total tumor burden per rat was also significantly reduced by all treatment groups compared with the NMBA control at week 35 ($P < 0.05$), and the BRB group significantly reduced tumor burden when compared with the AC and PCA groups. Columns, mean ($n = 9$ for week 25, $n = 30$ for week 35); bars, SD. The asterisks (*, $P < 0.05$; **, $P < 0.01$) indicate that results were significantly lower than rats treated with NMBA and fed control diet or a diet containing ACs or PCA.

that these agents vary in their ability to influence the expression levels of specific inflammatory markers.

Western blot analysis. Protein levels of the inflammatory markers were determined by Western blot analysis only at week 35 (Fig. 3A–C) because the esophageal tissues at weeks 15 and 25 were insufficient for analysis. For each marker, esophagi from 3 rats per diet group were analyzed and the band image densities were quantified and compared with 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 BRBs, ACs, and PCA all reduce inflammation in the esophagus and, potentially, by differential effects on individual targets.

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 downregulated in human esophageal SCC cell lines and tissue through hypermethylation of the promoter region (41). In addition, PTX3 has been shown to elicit anticarcinogenic effects via its ability to prevent neutrophil migration into tissue sites during acute lung injury (43), as well as antiangiogenic effects (40, 44).

Plasma PTX3 levels. No differences in plasma PTX3 levels were observed in any diet group at week 15 ($P > 0.05$; Fig. 4A). At week 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 week 35, BRB, AC, and PCA-fed rats all had higher plasma PTX3 levels when compared with 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

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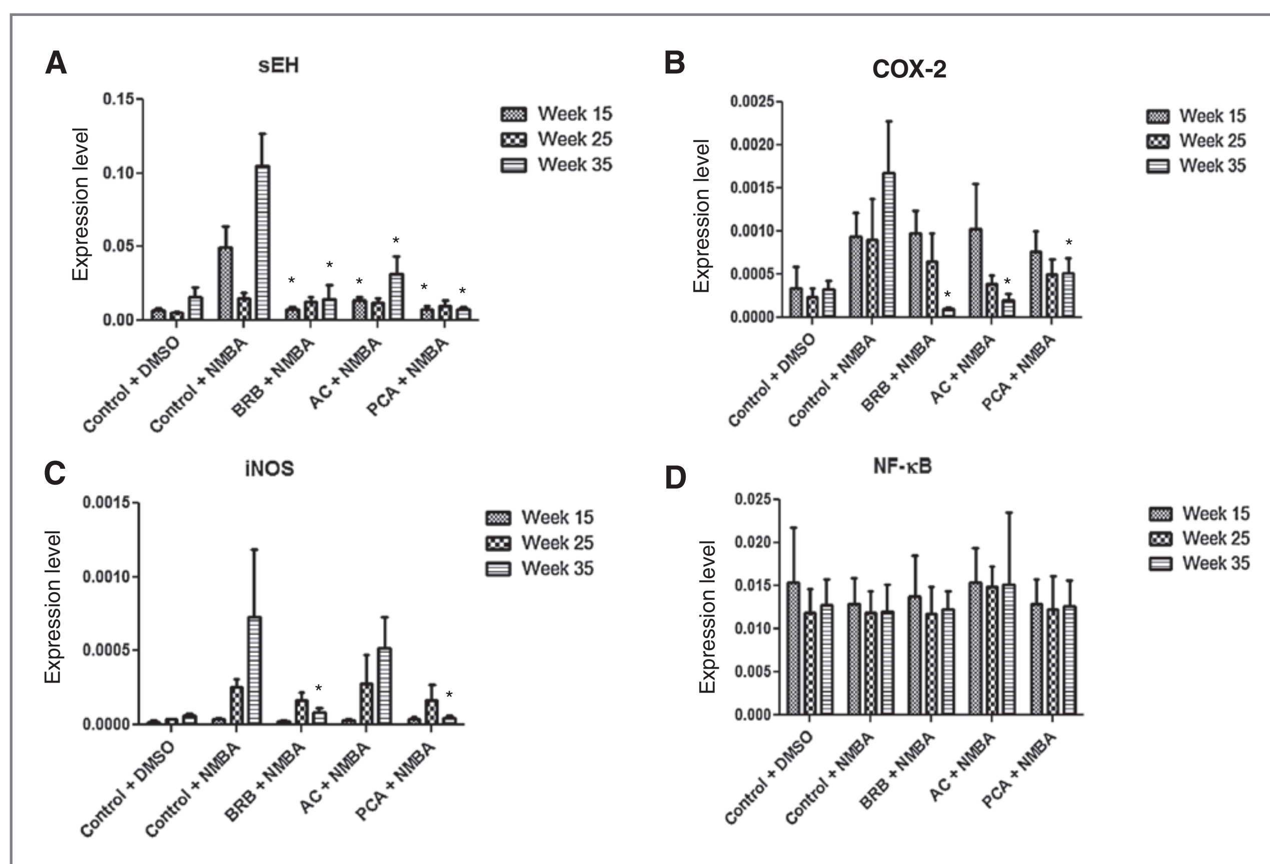

Figure 2. Effects of dietary BRBs, ACs, and PCA on mRNA expression of inflammatory biomarkers using GAPDH as an internal control. A, the sEH mRNA expression was significantly reduced in all treatment groups at weeks 15 and 35 ($P < 0.05$). B, COX-2 mRNA expression was significantly reduced at week 35 for all groups ($P < 0.05$). C, only the BRB and PCA diets reduced iNOS mRNA expression significantly at week 35 ($P < 0.05$). D, no significant change in NF-κB mRNA expression at any time point is revealed. Columns, mean ($n = 9$ for weeks 15 and 25, $n = 10$ for week 35); bars, SD. Asterisk (*, $P < 0.05$) indicates significantly lower results than rats treated with NMBA and fed control diet.

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 weeks 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% BRBs was effective at reducing NMBA-induced esophageal carcinogenesis in rats (14). Continued research demonstrated that the four ACs in BRBs are nearly as effective as whole BRBs in reducing esophageal carcinogenesis (15). Results from the present study confirm the chemopreventive activity of whole BRBs and BRB ACs against NMBA-induced rat esophageal carcinogenesis and also demonstrate the ability of PCA, a major metabolite of BRB ACs, to reduce esophageal carcinogenesis. A pharmacokinetic study in humans indicated that about 70% of the administered ACs in BRBs 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 AC 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 BRBs and BRB ACs. Further studies are underway to confirm this observation including a metabolism study to determine the extent of PCA production from BRB ACs by microbiota in the rat intestine. If confirmed, then PCA would seem to be worthy of additional evaluation as a chemopreventive agent for the esophagus and potentially other organs. When compared with whole BRBs and the AC-enriched 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 ACs found in BRBs (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

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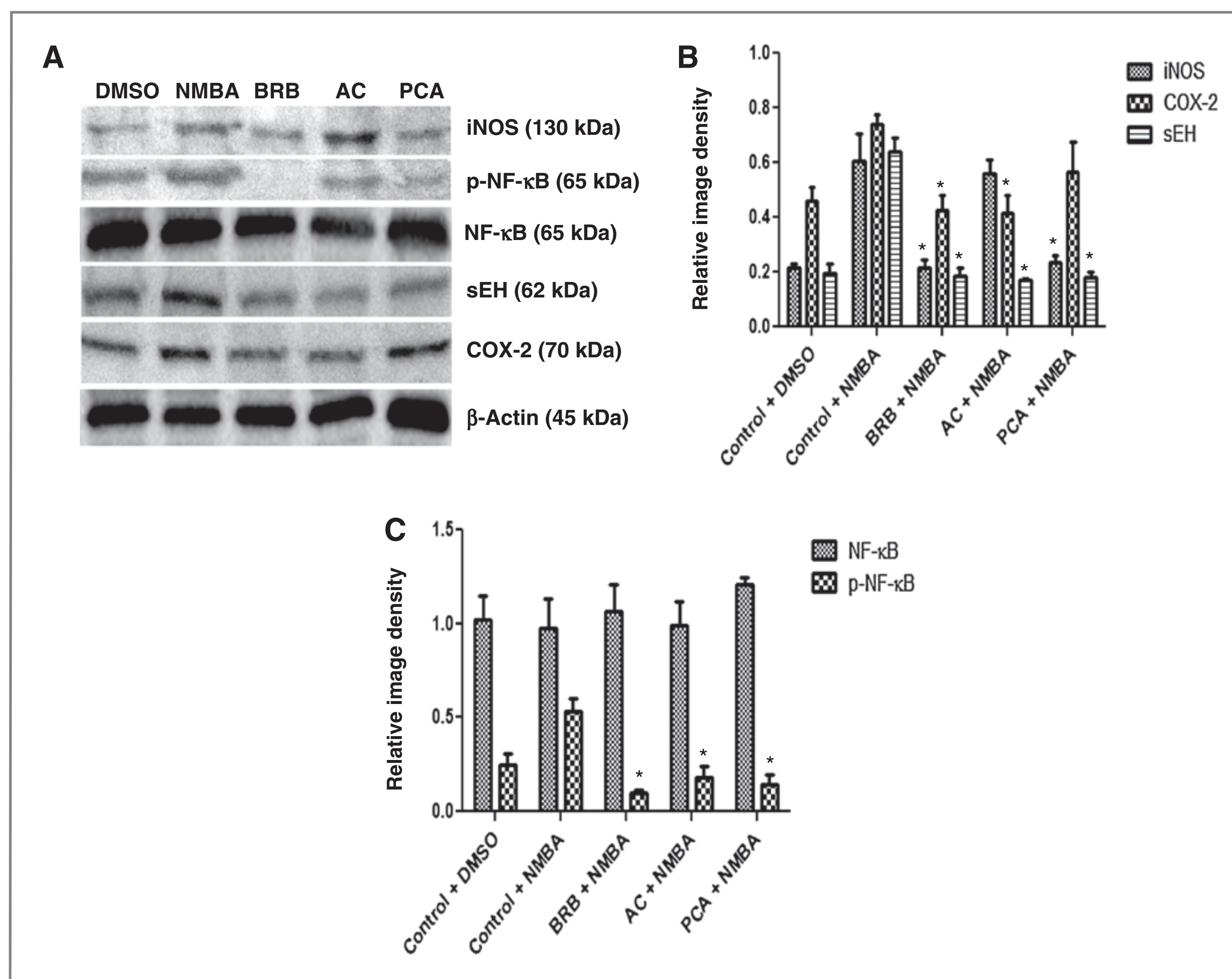

Figure 3. Effects of dietary BRBs, ACs, and PCA on protein expression of inflammatory markers in the esophagus via immunoblot at week 35. A, representative blots for all the inflammatory markers measured. B, sEH protein expression was reduced by the BRB, AC, and PCA diets as well ($P < 0.05$). COX-2 expression was significantly reduced by the BRB and AC diets in the esophagus ($P < 0.05$), whereas iNOS expression was reduced by the BRB and PCA diets ($P < 0.05$). C, naive NF-κB expression was unchanged, whereas p-NF-κB expression was significantly reduced by the BRB, AC, and PCA diets. Columns, mean ($n = 3$); bars, SD. Asterisk (*, $P < 0.05$) indicates significantly lower results than rats treated with NMBA and fed control diet.

compared with the NMBA control group, all three diets seemed 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 weeks after the first injection of NMBA (postinitiation). The BRB and AC results confirm the 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 and colleagues (16). With additional experimentation, PCA may also prove to be a viable candidate for these trials.

The present study also confirms the earlier data demonstrating the inhibitory effects of whole BRBs and their component ACs on inflammatory biomarkers and extends these activities to PCA. The ACs repeatedly decreased expression of the inflammatory markers, COX-2, and activated NF-κB, PTX3, and sEH at levels similar to whole BRB powder. PCA seemed to be active in reducing inflammatory biomarkers but less so than the BRBs. The relative efficacy of these three agents to reduce esophageal tumorigenesis and preneoplastic lesions, therefore, seems to parallel their relative inhibitory effects on the inflammatory markers. The enhanced ability of BRBs to reduce the inflammatory markers may be due to an additive effect of other compounds in BRBs such as ellagic, ferulic, and chlorogenic acids and quercetin, or 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 esophageal SCC cell lines and

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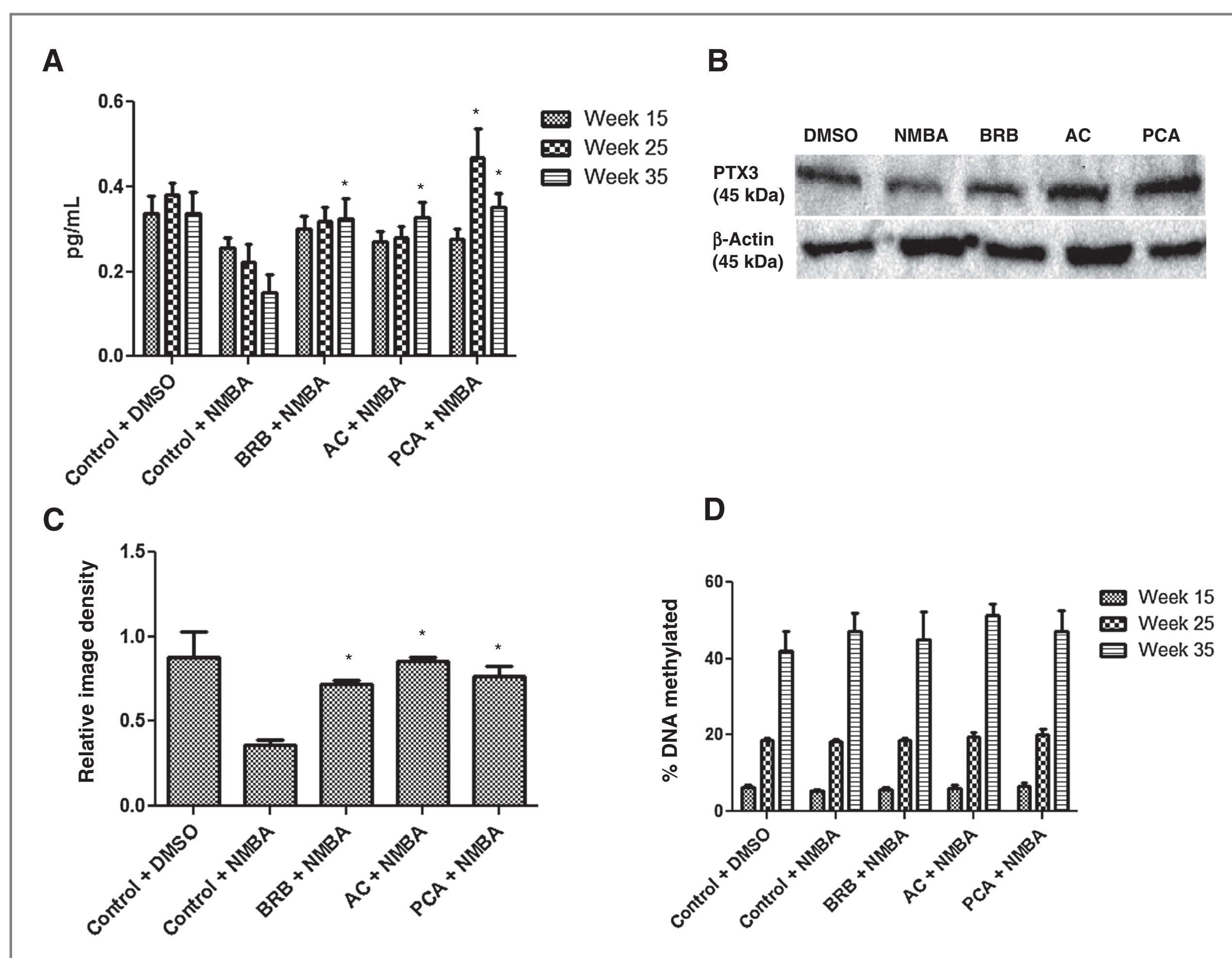

Figure 4. Effects of dietary intake of BRBs, ACs, and PCA on PTX3 expression in the plasma and esophagus shown via ELISA and immunoblotting. A, the PCA group has significantly higher PTX3 expression at week 25, whereas all three experimental diets significantly upregulated PTX3 expression globally in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

tumor tissue (41). This was not observed in NMBA-treated rat esophagus in the present study. PTX3 has both anti-angiogenic and antitumorigenic activity in human prostate cancer cell lines (40), and steroid hormone–regulated tumors S115 (mouse mammary tumor cells; ref. 49). Interestingly, PCA was the most effective treatment at inducing PTX3 expression in the plasma at the 25 week time point (PTX3 plasma level = 0.47 pg/mL compared with 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 weeks. The increased level at 25 weeks may account for the decrease in high-grade dysplasia seen at 25 weeks 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 immune cell trafficking in the esophagus.

Minor differences observed in the effectiveness of the AC-enriched fraction and PCA to reduce tumorigenesis and inflammatory biomarkers in the present study may be a result of the kinetics of AC 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 minutes and reaching a peak by 5 minutes, a time period too short for the ACs to have reached the colon (50). Oral administration of PCA would increase its exposure to absorptive sites in the gastrointestinal 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 with the PCA produced from ACs by the enteric microbiota.

In summary, results of the present study support the notion that although the ACs in BRBs are important for their chemopreventive activity, PCA, a major metabolite of BRB ACs, is also effective in inhibiting tumorigenesis and inflammatory signaling. Whole BRBs seem to be more effective than either ACs or PCA in reducing NMBA-induced

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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 and, because PCA is available commercially at a reasonable cost and is more easily synthesized and stable than BRB ACs, PCA seems 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.

Disclosure of Potential Conflicts of Interest

N.P. Zimmerman is employed as the Vice President of research and development in Agro BioSciences Inc. No potential conflicts of interest were disclosed by the other authors.

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