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Chemopreventive Action of Anthocyanin-rich Black Soybean Fraction in *APC*$^{Min/+}$ Intestinal Polyposis Model

ORIGINAL ARTICLE

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Background: Anthocyanins have been shown to inhibit cancer cell growth by suppressing oxidative stress and inflammatory responses. The purpose of this study was to investigate the effects of an anthocyanin-rich extract (AE) from black soybean coat on intestinal carcinogenesis.
Methods: *Apc*$^{Min/+}$ mice were fed a diet of 0.2% or 0.5% AE for 7 weeks. We analyzed the number of intestinal tumors, oxidative stress and inflammatory markers associated with β-catenin and cytosolic phospholipase A_2 (cPLA$_2$) signals. The number of intestinal tumors, and cellular expression of β-catenin were determined.
Results: The number of intestinal tumors was significantly lower in mice fed a 0.5% AE diet compared to those of the other groups. Cytosolic β-catenin expression was significantly decreased in the AE supplemented groups compared to that of the control animals. In addition, mucosa expression of cyclooxygenase-2 and cPLA$_2$ were also significantly decreased in the 0.5% AE group, by 32% and 62%, respectively, compared to the control group.
Conclusions: These results suggest that dietary AE reduced the development of intestinal tumors, possibly through the ability to suppress oxidative stresses, decreasing inflammatory responses mediated by β-catenin associated signals.
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Key Words: Anthocyanins, *APC*$^{Min/+}$, Black soybean, Inflammation, Intestinal tumors

INTRODUCTION

Environmental factors, including a western style diet rich in animal fat and protein, increase the risk of colon, breast and prostate cancers. Colorectal cancer was the fourth highest cause of cancer deaths worldwide in 2012.[1] Although plant food-based diets are known to reduce the risk of developing colorectal cancer,[2,3] the cause-effect evidence is not strong enough to show a negative association. This may be due to complex interactions between individual plant food components to exert biological responses. In addition, most of the efficacy evaluation studies used an in vitro cell culture system, which does not accurately reflect complex biological systems.

Soybeans are one of the most frequently studied legumes for their biological activities. Black soybeans have been widely used as materials in oriental medicine. A distinctive difference between yellow and black soybeans is their anthocyanin contents, which are responsible for the dark-blue colors exhibited by the plants. Anthocyanins are especially abundant in the epidermis palisade layer of the black soybean coat,[4] synthesized via the phenylpropanoid pathway.[5] The most abundant anthocyanins in plant foods are cyanidin-3-glucoside, delphinidin-3-glucoside and melvidin-3-glucoside, all of which are known to possess strong antioxidant and anticarcinogenic activity.[4] It is reported that

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administration of bilberry anthocyanins decreased tumor proliferation in colorectal cancer patients.[6] Previous studies also has been reported that anti-carcinogenesis effects of anthocyanins from several plant foods such as sweet potato, grapes, tart cherry and berries in colon cancer animal models.[7-11] However, few mechanistic explanations on the inhibitory effects of anthocyanins on tumor growth in vivo have been provided.

We previously demonstrated that anthocyanidins and anthocyanin-rich extract (AE) from black soybean coat exerted anti-inflammatory and anti-proliferative effects in colon cancer cells.[12] The biological properties of anthocyanins are due to their peculiar chemical structure, as they are excellent scavengers with reactivity towards reactive oxygen species (ROS).[13] One of the best described cellular effects of ROS is the oxidative modification of fatty acids within membrane phospholipids, altering membrane fluidity, protein structure and cell signaling.[14] ROS also activate nuclear transcription factors such as AP-1 and NF-κB, which in turn induce the expression of inflammatory target proteins, including cytosolic phospholipase A_2 ($cPLA_2$).[15] $cPLA_2$ hydrolyzes the *sn*-2 ester bond in phospholipids, releasing free fatty acids and lysophospholipids.[16] Increased $cPLA_2$ activity gives rise to a number of biologically active mediators, such as arachidonic acid (AA)-derived metabolites, including prostaglandins and lysophosphatidylcholine. For this reason, $cPLA_2$ has an important role in the modulation of membrane stability, as well as in the formation of inflammatory prostaglandins.

Apc$^{Min/+}$ mice exhibit a germ-line nonsense mutation at codon 850 of the adenomatous polyposis coli (*APC*) gene, causing the spontaneous development of multiple polyps in the small and large intestines at the age of 10 to 12 weeks.[17] Mutations in the *APC* tumor suppressor gene occur very early in cancer, and are found in a majority of sporadic colorectal tumors, as well as in familial adenomatous polyposis (FAP), a hereditary form of intestinal cancer.[18] The main tumor suppressing activity of the APC protein is due to its ability to down regulate cellular β-catenin.[19] Cytosolic β-catenin can be translocated into the nucleus, where it interacts with the transcriptional factor Tcf/Lef to stimulate transcription of inflammatory response genes, including cyclooxygenase-2 (COX-2). Enhanced levels of β-catenin and several inflammatory mediators are favored factors for the development of colorectal cancer.[20] Previous studies have also indicated that ROS-induced activation of nuclear transcription factors increase the expression of COX-2. COX-2 converts AA to prostaglandin E_2 (PGE_2), mediating inflammatory responses in colonic carcinogenesis.[21,22] AA is liberated from membrane phospholipids by the action of $cPLA_2$. Since the expression of $cPLA_2$ is shown to be up-regulated by ROS,[23] decreasing the oxidative stress by intake of anthocyanins may reduce the synthesis of PGE_2, suppressing colon carcinogenesis.

In the present study, we investigated the inhibitory activity and the potential underlying mechanism of action of an AE from black soybean coat against intestinal tumorigenesis in *Apc*$^{Min/+}$ mice.

MATERIALS AND METHODS

1. Animals

The mice were bred in Sookmyung Women's University, from inbred mice originally purchased from the Jackson Laboratory (Bar Harbor, ME, USA). The *Apc*$^{Min/+}$ pedigree was maintained by mating *Apc*$^{+/+}$ females with *Apc*$^{Min/+}$ males, and procedures to secure inbreeding were followed. The *Apc*$^{Min/+}$ mice were identified by allele- specific PCR on DNA isolated from the tail. All animals were housed in wire cages (3 mice/cage) with free access to drinking water and basal diet CE-2 (CLEA Japan Inc., Tokyo, Japan), under controlled conditions of humidity (50% ± 10%), lighting (12 hours light/dark cycle), and temperature (23°C ± 2°C), which were maintained until termination of the study.

2. Preparation of anthocyanin-rich extract from black soybean seed coat

AE was prepared as previously described.[6] Briefly, the seed coats of black soybean were peeled manually after heating at 105°C for 2 hours. The separated seed coats were extracted three times with 1,000 mL of 1% HCl-40% CH_3OH at 4°C for 24 hours. The combined extracts were filtered and concentrated at 30°C in vacuo. The main anthocyanin contents in the seed coats of the black soybeans were determined by measurement of the HPLC peak area, monitored at 530 nm. Cyanidin-3-glucoside, delphinidin-3-glucoside, and petunidin-3-glucoside content were determined to be 0.38, 0.16, and 0.05 mg/g black soybean seed coat, respectively.

3. Study design

A total of 30 *Apc*$^{Min/+}$ male mice (5 weeks old) were used in this study. The *Apc*$^{Min/+}$ mice were randomly assigned to three groups (ten mice per group) following a 1 week acclimatization period. The control group of *Apc*$^{Min/+}$ mice were fed the AIN-93G control diet, and 2 groups of *Apc*$^{Min/+}$ mice were fed AE diets at different concentrations (0.2% and 0.5%, respectively). Diets were prepared by replacing sucrose in the AIN-93G diet with powdered AE. All mice were fed these diets for 7 weeks. Food intake were measured every 2nd day after diet is replaced with fresh diet. Body weight

is measured every other day. At the end of the experimental period, the mice were sacrificed, and the intestines and colons were harvested. After scoring of tumors, the small intestinal and colonic mucosa was scraped with blades and kept at −80°C to use for mRNA and protein analysis. Blood was also obtained for biochemical analysis. The experiment was approved by the Animal Care and Use Committee of the Department of Food and Nutrition of Sookmyung Women's University (Seoul, Korea).

4. Tumor scoring

All organs were removed at sacrifice, and the small and large intestines were cut open along the longitudinal axis. The small intestine and colon were then spread flat on a microscope slide, and the number of tumors was counted under an inverse light microscope at a magnification of 1 × 10, while blind to the dietary treatment.

5. Measurement of cytosolic phospholipase A_2 activity

$cPLA_2$ activity was determined in the cytosolic faction using a $cPLA_2$ assay kit (Cayman Chemical, Ann Arbor, MI, USA). Arachidonoyl thio-PC (1-*O*-hexadecyl-2-deoxy-2-thio-*R*-(arachidonoyl)-*sn*-glyceral-3-phosphoryl-choline) was used as a synthetic substrate to detect PLA_2 activity. Hydrolysis of the arachidonoyl thioester bond at the *sn*-2 position by PLA_2 releases free thiol, which is detected by Ellman's reagent. Activity was reported as nanomol per minute per milligram of cytosolic protein.

6. RNA extraction and semi-quantitative reverse transcription-PCR

Total RNA was isolated from mouse tissues using Trizol (Invitrogen, Carlsbad, CA, USA), following the manufacturer's recommendations. Single-stranded cDNAs were generated from total RNA using the superscript first-strand synthesis system (Invitrogen). An aliquot of 100 ng of RNA was used for reverse transcription, and subsequent amplification was carried out using 1 μL of cDNA in a PCR at a final volume of 20 μL with an MJ Research PTC-0150 MiniCycler (MJ Research Inc., Waltham, MA, USA), using HotStarTaq (Intron Biotechnology Inc, Seongnam, Korea). PCR oligonucleotide sequences for intestinal mucosa used were as follows: COX-2 (forward 5′-TGTATCCCCCCACAGTCAAAG ACAC-3′, reverse 5′-GTGCTCCCGAAGCCAGATGG-3′), $cPLA_2$ (forward 5′-GT GTCTGGGCAGTGCCTTT-3′, reverse 5′-GTTGAAAATGGCGA TTCGGG-3′), β-actin (forward 5′-TGTGAGGTGGGAATGGGTCAG-3′, reverse 5′-TTTGATGTCACGCACGATTTCC-3′). The conditions of PCR for the target genes were as follows: COX-2 gene: 30 cycles at 94°C for 1 minute, 62°C for 1 minute, and 72°C for 2 minutes; $cPLA_2$ gene: 30 cycles at 94°C for 1 minute, 60°C for 1 minute, and 72°C for 2 minutes. The termination cycle included a prolonged extension at 72°C for 5 minutes. PCR products were confirmed by 1.6% agarose gel electrophoresis, and visualized by UV transillumination (Bio-Rad Laboratories Inc., Hercules, CA, USA). All signals were normalized to the mRNA levels of the housekeeping gene β-actin, and expressed as a ratio.

7. Biochemistry of intestinal mucosa

Because the subcellular localization of β-catenin is considered to be an important determinant in cancer, β-catenin was analyzed separately from cytosolic and membranous fractions of adenoma and normal-appearing mucosa. Small intestinal and colonic mucosa homogenates were centrifuged for 10 minutes at 200 *g* to remove intact cells. Cleared supernatants were centrifuged further for 10 minutes at 1,000 *g*. Supernatants were used for cytosolic and membranous fraction isolations, and pellets for nuclear fraction isolations. Ice-cold homogenization buffer was added to the supernatants to make a final volume of 5 mL. These mixtures were centrifuged for 60 minutes at 100,000 *g*. Newly formed supernatants were collected and used as the cytosolic fractions of the mucosal epithelial cells. The pellets were resuspended in 5 mL of ice-cold homogenization buffer containing 2 mL/L Triton X-100, incubated for 30 minutes and centrifuged for 60 minutes at 100,000 *g*. The resulting supernatants were used as the membranous fractions of the mucosal epithelial cells. The pellets formed in the first step of the procedure containing the nuclear fractions were resuspended in 1.8 mL of ice-cold homogenization buffer containing 2 mL/L Triton X-100, incubated for 20 minutes and centrifuged for 10 minutes at 15,000 *g*. The resulting supernatants were used as the nuclear fractions of the mucosal epithelial cells. All steps were performed at 4°C. All fractions were concentrated using Amicon Ultra-4 Centrifugal Filter Devices (Millipore, Bedford, MA, USA), and the protein concentrations of the resulting samples were measured using a Bio-Rad protein assay reagent (Bio-Rad Laboratories Inc.).

8. Western blot analysis

For the immunoblotting analysis of β-catenin, 30 μg of the sample proteins were electrophoresed through 7.5% SDS PAGE and transferred to polyvinylidenedifluoride membranes (Amersham, Arlington Heights, IL, USA). The transferred membrane was blocked using 2% skim milk to inhibit non-specific proteins, and primary antibodies were added. β-catenin (BD Transduction, San Diego, CA, USA), β-actin, and β-tubulin (Cell Signaling, Beverly, MA, USA) were used as the primary antibodies. Protein detection

was carried out using secondary anti-mouse HPR antibodies (Zymed Laboratories, South San Francisco, CA, USA). Each protein band was then confirmed and quantified using an enhanced chemiluminescence system (Amersham). Blots were scanned and analyzed using a multiple image analyzer and the Quantity One program (Bio-Rad Laboratories Inc.).

9. Measurement of serum prostaglandin E_2 level

The PGE_2 levels in the serum were measured using commercially available ELISA kits (R&D, Ann Arbor, MI, USA). This assay is based on the competitive binding technique, in which the PGE_2 present in a sample competes with a fixed amount of horseradish peroxidase labeled PGE_2 for the sites on a mouse monoclonal antibody. The absorbance was determined at 450 nm, with the wavelength correction set at 540 nm.

10. Statistical analysis

For the results of all experimental analyses, means and standard errors in each group were calculated using an SAS program (SAS Institute, Cary, NC, USA). To determine the statistical significance of differences between the control and AE-treated groups, one-way analysis of variance was performed, followed by the Student's *t*-test. *P*-values less than 0.05 were considered to be statistically significant.

RESULTS

1. Effect of dietary anthocyanin-rich extract on food intake, body weight, intestinal length, liver weight and tumor formation

As indicated in Table 1, there was no significant differences in the body weight gain between the groups. Further, there were no considerable changes in liver weight and intestinal length (Table 1). The food intake was measured every 2nd day after replacing the feed with fresh diet. The average food consumption for 2 days were as follows: control group, 7.84 ± 0.47 g; 0.2% AE group, 8.00 ± 0.51 g; 0.5% AE group, 8.10 ± 0.55 g and there is no significant difference. The effect of dietary AE from black soybean coats on tumor formation was assessed at week 7 after supplying the different experimental diets. Results showed that the average number of tumors were 41.3 ± 8.14, 34.7 ± 14.51, and 28.3 ± 11.50 per mouse fed either control diet, 0.2% AE diet or 0.5% AE diet, respectively (Fig. 1). Although we did not differentially counted the tumor numbers from small and large intestine seperatly, more than 90% of the tumors were located in the small intestine. We could find only 2 to 3 polyps from large intestine. The reduction observed with the 0.2% anthocyanin extract diet was not statistically significant; however, mice fed the 0.5% AE diet had a significant decrease in the number of intestinal tumors ($P < 0.05$; Fig. 1).

Table 1. Effects of dietary AE on body weight, intestinal length and liver weight in $APC^{Min/+}$ mice

Group	Body weight (g)	Intestinal length (cm)	Liver weight (g)
Control	30.64 ± 1.04[a]	39.31 ± 1.11[a]	9.34 ± 0.31[a]
0.2% AE	33.11 ± 1.13	38.73 ± 1.18	9.02 ± 0.19
0.5% AE	26.30 ± 1.72	38.74 ± 1.14	9.09 ± 0.16

Values are presented as mean ± SE. The experimental mice were fed 0.2% or 0.5% anthocyanin-rich extract (AE) supplemented diets for 7 weeks. Statistical significance of the differences was evaluated by *t*-test. [a]NS, not significant.

2. Effect of dietary anthocyanin-rich extract on intestinal β-catenin expression

Cytosolic and nucleic β-catenin protein expression levels were determined, revealing that the cytosolic β-catenin expression was significantly decreased in animals fed either 0.2% AE ($P < 0.01$) or 0.5% AE ($P < 0.001$; Fig. 2A), compared to the control. However, nucleic β-catenin protein level was not significantly different between the groups (Fig. 2B).

3. Effect of dietary anthocyanin-rich extract on intestinal cytosolic phospholipase A_2 activity and mRNA expression

The effects of an AE supplemented diet on $cPLA_2$ activity and $cPLA_2$ mRNA expression in the intestinal mucosa were determined.

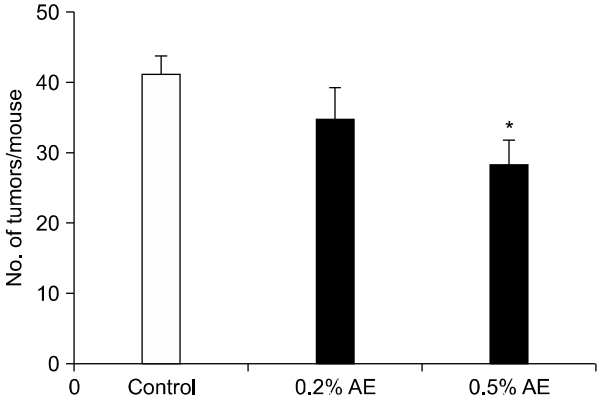

Figure 1. Effect of dietary anthocyanin-rich extract (AE) on tumor formation in $APC^{Min/+}$ mice. The mice were fed 0.2% or 0.5% AE supplemented diets for 7 weeks. After 7 weeks, the number of tumors was scored. Each bar represents the mean ± SE; n = 10 mice for each group. Statistical significance of the differences was evaluated by *t*-test (*$P < 0.05$). NS, not significant.

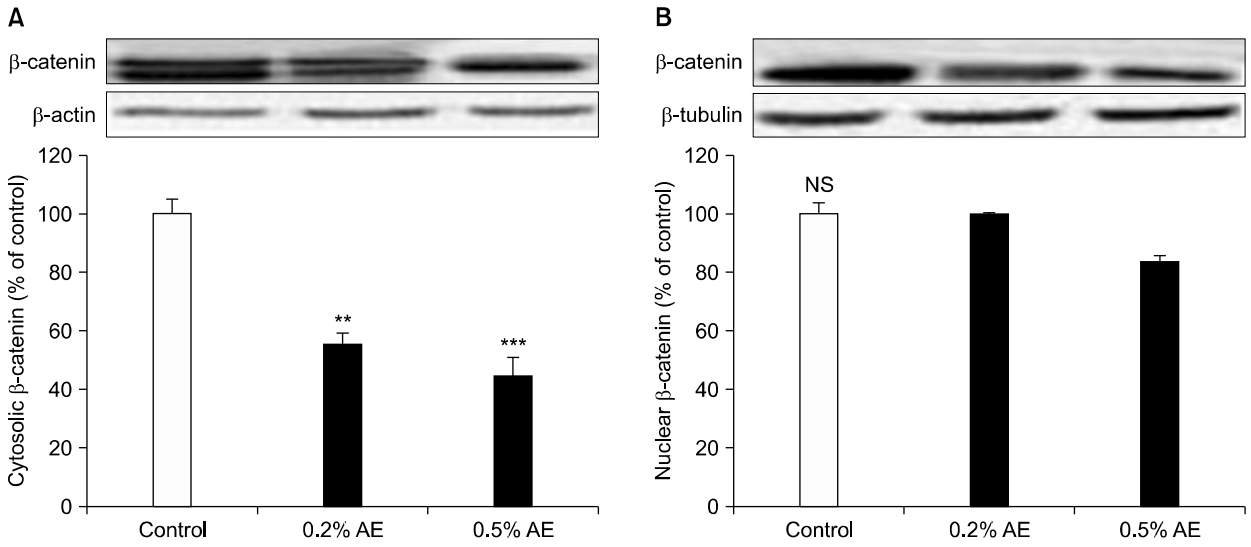

Figure 2. Effect of dietary anthocyanin-rich extract (AE) on (A) cytosolic β-catenin protein and (B) nuclear β-catenin protein level in $Apc^{Min/+}$ mice. The mice were fed 0.2% or 0.5% AE supplemented diets for 7 weeks. After 7 weeks, (A) cytosolic β-catenin protein and (B) nuclear β-catenin protein level in the intestines were measured. The white bars indicate non-supplemented diets, while the black bars represents diets supplemented with AE. Each bar represents the mean ± SE; n = 10 mice for each group. The experiments are repeated 3 times. Statistical significance of differences was evaluated by *t*-test (**$P < 0.01$, ***$P < 0.001$). NS, not significant.

Results showed that $cPLA_2$ activity was decreased in both the 0.2% and 0.5% AE supplemented group; however, the results were not statistically significant (Fig. 3A). In contrast to activity, the $cPLA_2$ mRNA expression was significantly reduced in the 0.5% AE-fed group by 62% compared to that of the control group ($P < 0.05$, Fig. 3B).

4. Effect of dietary anthocyanin-rich extract on intestinal COX-2 mRNA expression

COX-2 transforms membrane AA to cause a pro-inflammatory response. Figure 4A shows that the diet of 0.5% AE significantly decreased the expression of intestinal COX-2 mRNA by 32% ($P < 0.05$) compared with the control diet.

5. Effect of dietary anthocyanin-rich extract on serum prostaglandin E_2 and nitric oxide

COX-2 generates PGE_2 and production of PGE_2 is related to various pathogenic processes, and to the stability of the cell membrane. The results of this study showed that serum PGE_2 levels were not statistically significant in AE supplemented groups (Fig. 4B).

DISCUSSION

We previously reported that anthocyanins or anthocyanin-rich black soybeans inhibited colon cancer cell growth and the formation of precancerous aberrant crypt foci (ACF) by suppressing inflammatory response.[12] In this study, we further investigated the AE of black soybean coat on colon tumor formation in $APC^{Min/+}$ mice.

The results of this study suggest that the dietary administration of AE in this study significantly decreased the cytosolic β-catenin level, with no significant difference in nuclear β-catenin expression. Although β-catenin nuclear translocation has been used as a marker for colon cancer in many studies, the relationship between cytosolic and nuclear β-catenin accumulation in intestinal tumor development has not been clearly defined. In a β-catenin expression pattern study, most colorectal tumors demonstrated increased β-catenin in the cytosolic fraction, while nuclear β-catenin expression was found in only 64% of primary colorectal tumors and 21% of corresponding liver metastases,[24] suggesting intestinal tumor formation and growth are correlated more with the cytosolic levels of β-catenin than with the nuclear levels. It was also previously suggested that nuclear accumulation of β-catenin is the very first event of tumor development, followed by a substantial increase of β-catenin in the entire cytosol.[25] Another study showed that the nuclear β-catenin level was significantly correlated with the number of adenomas at the early stage of tumor development, while the cytosolic, but not nuclear or membranous β-catenin, was strong correlated with adenoma growth at the later developmental stages in $Apc^{Min/+}$ mice.[19] In this regard, it should be noted that cytoplasmic expression of β-catenin may be the most frequent abnormality in human ACF, adenomas, and carcinomas development.[26] There

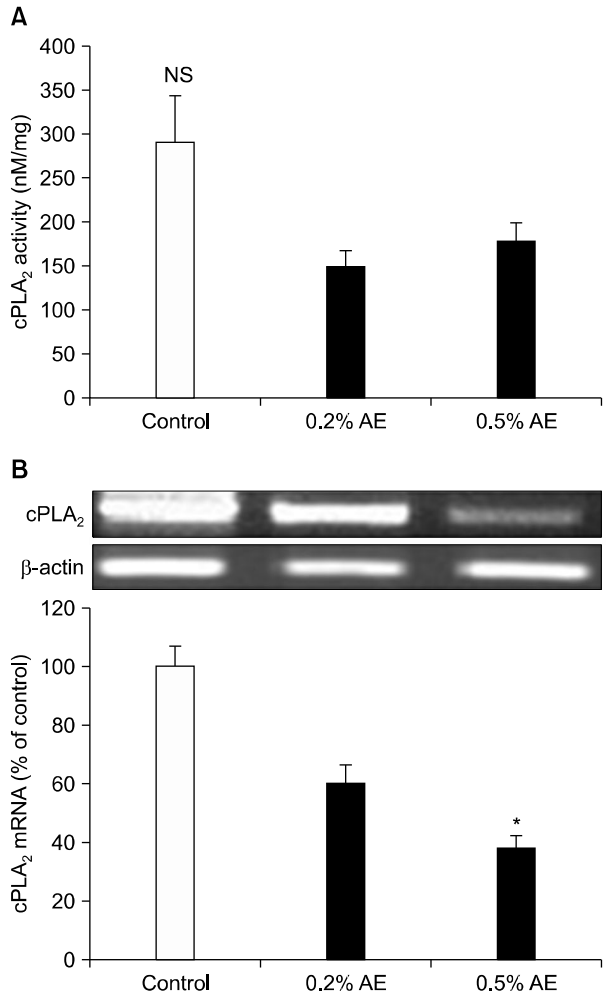

Figure 3. Effect of dietary anthocyanin-rich extract (AE) on (A) cytosolic phospholipase A2 (cPLA$_2$) activity and (B) cPLA$_2$ mRNA expression in *Apc*$^{Min/+}$ mice. The mice were fed 0.2% or 0.5% AE supplemented diets for 7 weeks. After 7 weeks, (A) cPLA$_2$ activity and (B) cPLA$_2$ mRNA expression in the intestinal mucosa were measured. Each bar represents the mean ± SE; n = 10 mice for each group. The experiments are repeated 3 times. Statistical significance of the differences was evaluated by *t*-test (*$P < 0.05$). NS, not significant.

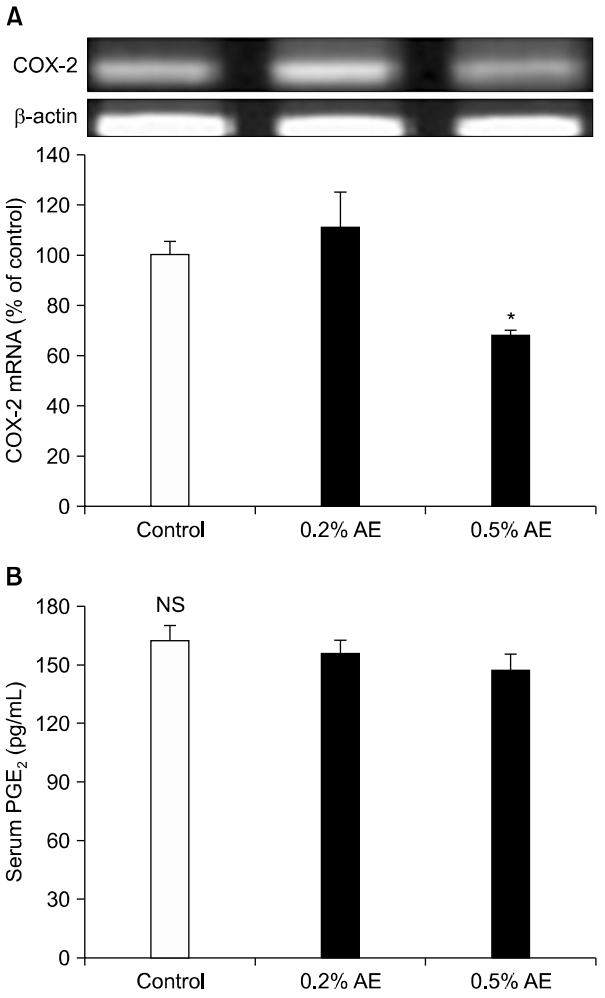

Figure 4. Effect of dietary anthocyanin-rich extract (AE) on (A) intestinal COX-2 and (B) serum levels of prostaglandin E$_2$ (PGE$_2$) in *Apc*$^{Min/+}$ mice. The mice were fed 0.2% or 0.5% AE supplemented diets for 7 weeks. After 7 weeks, (A) intestinal COX-2 and (B) serum PGE2 level were measured. The white bars represent non-supplemented diets, and black bars indicate diets supplemented with AE. Each bar represents the mean ± SE; n = 10 mice for each group. The experiments are repeated 3 times. Statistical significance of the differences was evaluated by *t*-test (*$P < 0.05$). NS, not significant.

are few studies that show that the direct effects of anthocyanins on the activation of β-catenin. Two previous studies showed that anthocyanins from black raspberry and fruits of *Vitis coignetiae* Pulliat effectively suppressed β-catenin expression in colon cancer cells and uterine cervical cancer cells, respectively.[27,28] Here, we found that the AE diet specifically down regulated the cytosolic β-catenin level, which may play a critical role in the intestinal tumor development.

cPLA$_2$ is an enzyme which releases AA from cell membrane phospholipids, which is an upstream event in the eicosanoid synthesis pathway involving COX-2.[29] COX-2 overexpression is also observed in human colorectal adenocarcinomas and adenomas.[30] In this study, although the 0.5% AE diet significantly decreased cPLA$_2$ mRNA expression, the cPLA$_2$ activity was not changed.

Meanwhile COX2 mRNA expression in the intestinal mucosa was effectively decreased by 0.5% AE diet compared to the control diet (Fig. 4A). It is reported that AEs inhibited COX-2 mRNA expression in colon cancer model.[10] These results indicate that the inhibitory effects of an AE diet on colon tumor formation may be partially attributed by the inhibition of COX-2. Anthocyanins were also shown to regulate various cellular signaling pathways involving the PLA$_2$, PI3K/Akt and NF-κB pathways in several pathological conditions.[31] Cai et al.[8] showed that expression of

Akt was reduced in *Apc*$^{Min/+}$ mice received red grape pomace extract. These data suggest that the protective effects of anthocyanins on cancer are not due to the antioxidant activity alone, but also to the direct blockage of signaling pathways. In this study, a tendency towards lower serum concentrations of PGE_2, a metabolite of COX-2, was observed by AE supplementation. We assume that higher amounts of and/or longer periods of AE supplementation may cause a significant reduction in the serum PGE2 concentration.

The highest content of anthocyanin in AE was cyanidin-3-glycoside (C3G), followed by delphinidin-3-glucoside and petunidin-3-glucoside. In a pharmacokinetic study, C57BL/6J mice received an oral administration of 500 mg C3G/kg body weight and gastrointestinal (GI) mucosa contained 10^{-4} to 10^{-3} M C3G which is similar to effective concentrations exerting anti-proliferative effects against cancer cells in vitro.[32] The authors showed that these levels were higher than those seen in any other of the tissues. Moreover, the elimination rate constants and half-lives in mucosa were 0.39 h^{-1} and 1.8 h, respectively. These data indicated that C3G stay in the tissue long enough to exert biological effects. In our previous study, cyanidin and delphinidin exerted significant growth inhibitory effects at $\geq$1 μM concentrations in human colon cancer cells.[12] These data indicated that the anti-proliferative effects of these phenolics would be in part responsible for the anticancer activity of AE diet.

Supplementation at levels of 0.2% to 0.5% crude anthocyanin extract corresponds to 2 to 5 g crude anthocyanins per day assuming we consume about 1 kg of food material a day. These levels of supplementation will reach around 0.1 mM to 1 mM in GI mucosa of a 70 kg person since it has been suggested that GI mucosa contained 0.1 mM to 1 mM of cyaniding-3-glucoside when 500 mg/kg body weight of cyanidin-3-glucoside was administered.[32,33] However, these concentrations might not be accomplishable by consuming black soybeans by diet especially because 100% of anthocyanins are present in soybean coat which is often removed before consumption. Therefore, the use of black soybean coat anthocyanin may be more appropriate as a dietary supplement rather than the use of whole beans. Since we did not analyze all of the constituents present in black soybean coat extract, other bioactive constituents may be present. Black soybean coat also contains several phenolic compounds, isoflavones, and tannins. Since it was reported that above constituents also possess anti-carcinogenic activities against colon cancer,[34-45] anthocyanins might not be the only group of compounds suppressing tumor formation. However, we used the extraction method specifically extract crude anthocyanins from black

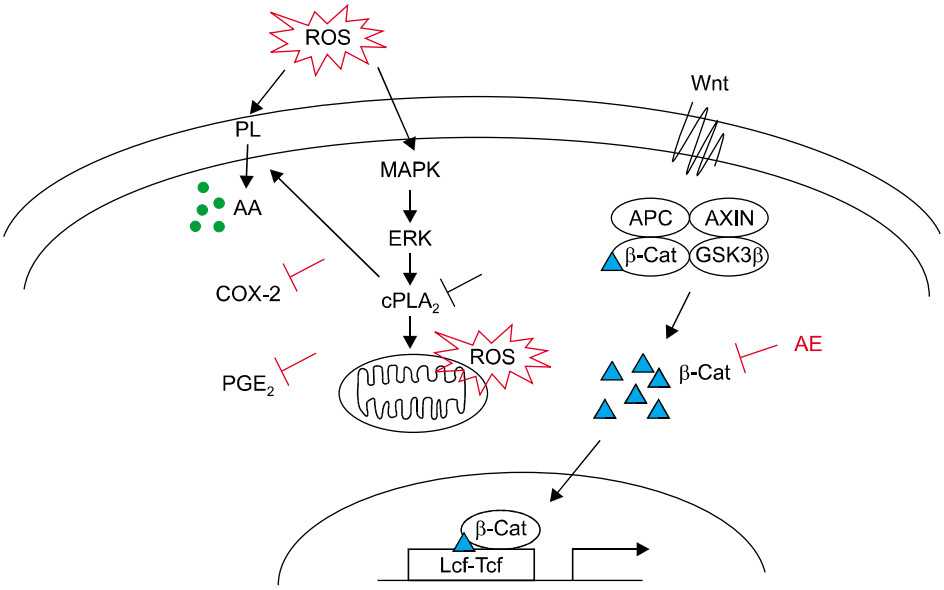

Figure 5. The hypothetical scheme of the mechanisms by which an anthocyanin-rich extract (AE) diet inhibits tumor formation in *Apc*$^{Min/+}$ mice. The adenomatous polyposis coli (APC) mutation in *Apc*$^{Min/+}$ mice leads to accumulation of cytosolic and nuclear β-Cat which leads to Wnt-specific target gene translation. Reactive oxygen species (ROS) activate AA release from membrane, leading to increase of inflammatory mediators such as COX-2 and prostaglandin E_2 (PGE_2) which accelerate tumorigenesis. AE diet effectively inhibits cytosolic β-Cat accumulation and suppresses ROS-induced COX-2 and PGE_2 expressions. β-Cat, β-catenin; AA, arachidonic acid; AXIN, Axis inhibition protein; COX-2, cyclooxygenase-2; $cPLA_2$, cytosolic phospholipase A_2; ERK, extracellular signal-regulated kinase; GSK3β, glycogen synthase kinase 3β; MAPK, mitogen-activated protein kinase; PL, phospholipid.

soybean coat[46] and we assumed major constituents in the extract are anthocyanins. A study reported by Xu and Chang[47] indicated that seed coat of black soybean contains less than 10% of phenolic acids and flavonoids including isoflavones compared to dehulled beans. Also, 100% of anthocyanins are present in seed coat. Presuming these phytochemicals possess similar potential to exert anti-carcinogenic activities, anthocyanins might be major anti-carcinogenic compounds in seed coat especially in this crude anthocyanin extract. To elucidate the contribution of each soybean coat components regarding the anticancer activities, a further study will be needed.

In conclusion, dietary AE possesses chemopreventive action in intestinal carcinogenesis by reducing the cytosolic levels of β-catenin, and by suppressing inflammatory responses (Fig. 5). These results may have implications for dietary intervention in human FAP and colorectal cancer. Further studies are required to provide explanations for the precise mechanisms of action.

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CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

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