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Systemic metabolite changes in wild-type C57BL/6 mice fed black raspberries

Pan Pan¹, Chad W. Skaer¹, Hsin-Tzu Wang¹, Michael A. Kreiser¹, Steven M. Stirdivant², Kiyoko Oshima³, Yi-Wen Huang⁴, Matthew R. Young⁵, and Li-Shu Wang¹

¹Division of Hematology and Oncology, Department of Medicine, Medical College of Wisconsin, Milwaukee, WI, USA

²Clinical Research and Development, Metabolon, Inc., Durham, NC, USA

³Department of Pathology, Medical College of Wisconsin, Milwaukee, WI, USA

⁴Department of Obstetrics and Gynecology, Medical College of Wisconsin, Milwaukee, WI, USA

⁵Division of Cancer Prevention, National Cancer Institute, Rockville, MD, USA

Abstract

Introduction—Freeze-dried black raspberries (BRBs) elicit chemopreventive effects against colorectal cancer in humans and in rodents. The study objective was to investigate potential BRB-caused metabolite changes using wild-type (WT) C57BL/6 mice.

Methods and results—WT mice were fed either control diet or control diet supplemented with 5% BRBs for 8 weeks. A non-targeted metabolomic analysis was conducted on colonic mucosa, liver, and fecal specimens collected from both diet groups. BRBs significantly changed the levels of 41 colonic mucosa metabolites, 40 liver metabolites and 34 fecal metabolites compared to control diet-fed mice. BRBs reduced 34 lipid metabolites in colonic mucosa and increased levels of amino acids in liver. One metabolite, 3-[3-(sulfoxy) phenyl] propanoic acid, might be a useful biomarker of BRB consumption. In addition, BRB powder was found to contain 30-fold higher levels of linolenate compared to control diets. Consistently, multiple omega-3 polyunsaturated fatty acids (ω -3 PUFAs), including stearidonate, docosapentaenoate (ω -3 DPA), eicosapentaenoate (EPA) and docosahexaenoate (DHA), were significantly elevated in livers of BRB-fed mice.

Conclusion—The data from the current study suggest that BRBs produce systemic metabolite changes in multiple tissue matrices, supporting our hypothesis that BRBs may serve as both a chemopreventive agent and a beneficial dietary supplement.

Keywords

Cancer prevention; Dietary intervention; Edible berries; DHA; Metabolome

Corresponding author: Li-Shu Wang, Division of Hematology and Oncology, Department of Medicine, Medical College of Wisconsin, Rm C4930, 8701 Watertown Plank Rd, Milwaukee, WI, 53226, USA. liswang@mcw.edu, Phone: 414-955-2827, Fax: 414-955-6059.

Conflict of interest

No potential conflicts of interest were disclosed.

Introduction

Cancer is one of the leading causes of morbidity and mortality worldwide (1). An estimated 14 million new cases were diagnosed and 8.2 million deaths were attributed to cancer globally in 2012 (1). In the United States, it has been estimated that there would be approximate 1,685,210 new cases of cancer and 595,690 cancer-related deaths in 2016 (2). Of these, an estimated 134,490 new cases and 49,190 deaths would be attributable to colorectal cancer (2). The leading risk factors for all cancers include high body mass index, low fruit and vegetable intake, lack of physical activity, alcohol use, and tobacco use (1). A reported association between increased risk of colorectal cancer and an “unhealthy” diet, such as diets high in red meat and processed food (3–5), has led to the hypothesis that certain “healthy” diets might prevent colorectal cancer or have beneficial effects to colorectal cancer patients (6). Indeed, many studies have observed protective effects of certain foods such as berries (7), beans (8), broccoli (9) and tea (10) against the development of colorectal cancer.

We previously reported that an average of four-week dietary administration of 60 g of BRBs daily, which is equivalent to a rodent diet of 7% BRBs, resulted in a significant reduction in cell proliferation and angiogenesis, and an increase in cellular apoptosis in colorectal tumors for a group of 28 colorectal cancer patients (11). Additionally, in a nine-month phase Ib study of 14 patients with familial adenomatous polyposis, BRB treatment significantly decreased the tumor burden of rectal polyps (12). In mouse models of colorectal cancer ($Apc^{Min/+}$, $Apc^{1638+/-}$ and $Muc2^{-/-}$), feeding of BRBs significantly inhibited intestinal tumor development and cancer cell proliferation (13, 14).

Metabolomics is a comprehensive analysis of metabolites in biological samples, and provides sensitive and quantitative information on metabolic changes in the body. It is a promising tool to identify systemic metabolite changes caused by specific dietary constituents. We previously demonstrated that BRB-caused metabolite changes in 28 colorectal cancer patients (15) and $Apc^{Min/+}$ mice (14). Therefore, the present study aimed to investigate the BRB-dependent metabolite changes in cancer-free WT C57BL/6 mice using metabolomic profiling. Dietary BRB administration resulted in significant alterations in 41 metabolites in colonic mucosa, 40 metabolites in liver and 34 metabolites in feces compared to specimens from control diet-fed mice. Multiple amino acids were markedly increased in liver and feces, likely due to BRB intake. Additionally, BRBs significantly decreased lipid metabolites in colonic mucosa, which may have been due to an overall reduction in fatty acid synthesis. Furthermore, ω -3 PUFAs, including stearidonate, EPA, ω -3 DPA and DHA, were significantly elevated in liver by BRBs. Our results suggest that BRBs may be a dietary source of ω -3 PUFAs that are potentially chemopreventive.

Materials and Methods

Animals and BRBs

All protocols followed the institutional guidelines for animal care set by the Medical College of Wisconsin Animal Care and Use Committee. WT C57BL/6 mice aged 3–4 weeks were purchased from the Jackson Laboratory (Bar Harbor, ME) and integrated into the protocol

one week following arrival. WT mice were fed either the American Institute of Nutrition synthetic diet 76A (AIN-76A; Dyets Inc., Bethlehem, PA) as the control diet (n=7) or AIN-76A diet supplemented with 5% BRB powder (w/w) (n=10) for a duration of eight weeks. BRB powder was purchased from Decker Farms, Inc. (Hillsboro, OR) and from Berri Products LLC (Corvallis, OR), stored at 4°C in vacuum-sealed plastic bags, and prepared via methods detailed by Montrose and colleagues (16). Mice were euthanized by CO₂ asphyxiation, and colonic mucosa, liver and fecal specimens of the animals were collected for metabolic profiling.

Metabolomic profiling

Mass spectrometer platforms, sample extraction and preparation, instrumentation, and data handling were executed by Metabolon, Inc. (Research Triangle Park, NC) (17). The key components are summarized as follows: sample preparation was performed with an automated MicroLab STAR® system (Reno, NV). Recovery standards were incorporated prior to extraction for QC purposes. Homogenized liver, mucosa, and fecal samples were extracted using 5µL, 5µL, and 25µL of methanol per mg tissue, respectively. Extracts were then divided into three fractions for non-targeted metabolic profiling and randomized for analysis. Each sample was vacuum-dried to remove organic solvent. Sample characterization was achieved using three independent platforms: ultra-high-performance-liquid chromatography/tandem mass spectrometry (UHPLC-MS/MS) in the negative ion mode, UHPLC-MS/MS in the positive ion mode, and gas chromatography-mass spectrometry (GC-MS) after sialylation. Chemical entities were identified by comparison to a metabolomic library of purified standards based on chromatographic properties and mass spectra.

Fatty acid profiling

Gas chromatography, sample extraction and preparation, instrumentation, and data handling were performed by Covance Inc. (Madison, WI). In summary, an internal standard, C11:0 triglyceride (triundecanoin), was added prior to the lipid extraction. The lipid was extracted, saponified with 0.5N methanolic sodium hydroxide, and methylated with 14% BF₃-methanol. The resultant methyl esters were extracted with heptane, analyzed by gas chromatography. Both internal and external standards are used in the calculation. The results are calculated by first generating a linear regression curve for each analyte using the ratio of analyte to internal standard peak areas versus the known concentrations in the 5 point methyl ester working standards. The concentration of each analyte in the sample is derived from this curve, accounting for the sample weight and amount of internal standard added.

Statistical analysis

Mass spectrum peaks were quantified using area-under-the-curve. Raw area counts for each metabolite in each sample were normalized to correct for variation resulting from instrument inter-day tuning differences by the median value for each run-day, therefore, setting the medians to 1.0 for each run. This preserved variation between samples but allowed metabolites of widely different raw peak areas to be compared on a similar graphical scale. Missing values were imputed with the observed minimum, for each individual metabolite, after normalization. Welsh's two sample t-tests, which assume unequal variance, were employed in R version 2.14.2 (18), to identify statistically significant metabolite differences

between mice fed a BRB diet and control diet. Statistical analyses were performed on natural log-transformed data to mitigate the effect of any potential outliers in the data as described in the previous studies (14, 15). A p-value ≤ 0.05 was considered statistically significant.

Results and Discussion

WT mice were fed either AIN-76A control diet (n=7) or control diet supplemented with 5% BRB powder (w/w) (n=10) for 8 weeks, and colonic mucosa, liver and fecal specimens were collected. A MS-based non-targeted metabolomic analysis was conducted and more than 400 metabolites were annotated in each specimen. Of these, levels of 41 metabolites in colonic mucosa (Supplementary Table 1), 40 metabolites in liver (Supplementary Table 2) and 34 fecal (Supplementary Table 3) metabolites were significantly changed in mice fed BRBs compared to mice fed control diet. These metabolites were further analyzed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database to identify metabolic pathways modulated by BRBs, including amino acids, carbohydrates, lipids, nucleotides, cofactors, vitamins and xenobiotics. BRBs significantly increased and decreased multiple metabolites in various metabolic pathways in colonic mucosa (Figure 1A), liver (Figure 1B), and feces (Figure 1C).

BRBs significantly changed amino acid metabolism

Eight weeks of BRB administration to WT mice significantly increased several amino acids in colonic mucosa, liver, and fecal specimens (Supplementary Tables 1, 2 and 3). 3-[3-(sulfooxy) phenyl] propanoic acid, which is likely to be metabolized from phenolic amino acids, was increased 9.37-fold in colonic mucosa, 3.24-fold in liver, and 13.57-fold in feces, respectively. Similar increases of 3-[3-(sulfooxy) phenyl] propanoic acid were previously found in urinary specimens from colorectal cancer patients who consumed BRBs (15), as well as in colonic mucosa, liver and feces of BRB-treated $Apc^{Min/+}$ mice (14). These findings suggest that the increased levels of this metabolite might be directly attributable to BRB consumption.

Enhanced polyamine levels have been reported in the colonic mucosa of human colorectal cancer patients (19, 20) and in the $Apc^{Min/+}$ mouse model of intestinal cancer (14, 16, 21). Fecal levels of putrescine and spermidine, which are generated through the polyamine synthesis pathway, were decreased in WT mice fed BRBs (Supplementary Table 3). We observed similar results that BRBs fully reversed putrescine generation and inhibited the expression of ornithine decarboxylase (ODC), an enzyme that catalyzes decarboxylation of ornithine to form putrescine, in $Apc^{Min/+}$ mice (14). In addition, BRB-suppressed putrescine levels correlated with decreased colonic polyp size in $Apc^{Min/+}$ mice (14).

BRBs significantly increased benzoate metabolites

After eight weeks of BRB dietary administration to WT mice, multiple benzoate metabolites were significantly increased in colonic mucosa (Figure 2A and Supplementary Table 1), liver (Figure 2B and Supplementary Table 2) and feces (Figure 2C and Supplementary Table 3), such as 3,4-dihydroxybenzoate, catechol sulfate, hippurate and 3-hydroxybenzoate. We

previously observed similar increased levels of benzoate metabolites in urine and plasma specimens from colorectal cancer patients who consumed BRBs (15), and in colonic mucosa, liver and feces from BRB-treated *Apc^{Min/+}* mice (14). These increased benzoate metabolites could be derived from the polyphenols and anthocyanins in BRBs, which are absorbed by the large intestine and are metabolized by the gut microbiota (22, 23). 3-hydroxybenzoic acid, in particular, can be metabolized to 3,4-dihydroxybenzoate (protocatechuic acid, PCA), which is chemopreventive in the rat esophagus (24). Our previous studies showed that PCA is the major metabolite of anthocyanins (25). Though we have not measured PCA content in whole BRB powders, elevated PCA levels in both colonic mucosa (Supplementary Table 1) and feces (Supplementary Table 3) of BRB-fed mice suggest that they can be directly absorbed from BRBs or metabolized from anthocyanins.

BRBs significantly changed lipid metabolism

Lipid metabolism was significantly altered in different tissue matrices from WT mice fed BRBs. BRBs significantly decreased 34 lipid metabolites in colonic mucosa, including long-chain fatty acids, omega-6 PUFAs (ω -6 PUFAs), eicosanoids and lysolipids (Supplementary Table 1). BRBs were also shown to significantly decrease five lysolipids in liver (Supplementary Table 2). In contrast, five ω -3 PUFAs (linolenate, stearidonate, DHA, ω -3 DPA and EPA) in liver (Figure 3) and two ω -3 PUFAs (linoleate and linolenate) in fecal (Figure 4) specimens were markedly increased by BRBs. We previously observed significantly increased levels of linolenate, stearidonate, DHA, ω -3 DPA and EPA in colonic mucosa and liver specimens of BRB-treated *Apc^{Min/+}* mice (14), suggesting that increased ω -3 PUFA levels might be directly attributable to BRB consumption. As we previously observed a correlation between BRB-increased linolenate levels in colonic mucosa with decreased colonic polyp number in *Apc^{Min/+}* mice (14), these findings could provide further support for the chemopreventive properties of BRB diets. However, further investigation is needed to determine whether a reduction of fatty acid synthesis or a lower tissue uptake contributes to the consistent decrease of 34 lipid metabolites in colonic mucosa of BRB-fed WT mice.

BRBs contain higher levels of ω -3 fatty acids

Linoleate and linolenate are regarded as essential fatty acids, which must be consumed from diets (26). Linoleate is converted to arachidonate (AA), while linolenate is converted to EPA, ω -3 DPA, and DHA (26, 27). Linoleate can be found in many vegetable seeds and oils, such as soybean, coconut, corn and sunflower. Major dietary sources of linolenate are grape seeds, walnut, and dark green leafy plants. Given that we observed significantly increased levels of multiple ω -3 fatty acids in livers from BRB-fed mice, we measured the fatty acid contents in AIN-76A control diet pellets and BRB powder (Table 1). Surprisingly, the linolenate percentage of total fatty acid in BRBs is 30-fold higher than that in control diet. This is the first study to show that BRB powders contain high amount of linolenate. Similarly, it has been reported that black raspberry seed (BRS) oil contains approximately 5 times more linolenate compared to AIN-93G control diet (28). Although the levels of EPA, ω -3 DPA, and DHA were below the measurement limitation in both BRB and control diets in the current study, the increased levels of these ω -3 fatty acids in livers of BRB-fed mice

might be directly attributable to higher levels of linolenate (Figure 3A) or a potential higher conversion rate, which warrants further investigation.

Fish oil has been considered a good dietary source of ω -3 PUFAs. Multiple randomized, double-blind, placebo-controlled clinical trials with human cancer patients have been conducted to investigate the potential beneficial effects of fish oil as nutritional supplements of standard chemotherapy and/or radiotherapy (29). Patients with lung cancer (29–33), colorectal cancer (34, 35), breast cancer (36) and gastrointestinal cancer (37) were included. The dosages used in these clinical trials ranged from 0.3 g EPA + 0.4 g DHA to 2.4 g EPA + 1.2 g DHA, and the treatment duration varied from one week to twelve weeks. Different clinical outcome parameters were investigated, including body weight, body composition and quality of life (physical function, cognitive function, social function and fatigue). A significant improvement of body weight during active chemoradiotherapy was observed with daily supplementation of EPA + DHA in each study. Additionally, daily supplementation of EPA + DHA was found to significantly improve physical, cognitive and social functions in three studies (31, 32, 34). Therefore, the higher content of linolenate in BRB powder and the increased levels of EPA and DHA in liver after BRB administration suggest that BRBs may serve as a dietary source of EPA and DHA, and they may be beneficial to cancer patients undergoing chemoradiotherapy.

Conclusion

Many modifiable risk factors of cancer development, such as smoking and diet, can be targets for chemopreventive interventions. Indeed, many foodstuffs including BRBs, exhibit protective effects against various types of cancer. We investigated the metabolite changes in C57BL/6 mice produced by consumption of BRBs and found that 41 metabolites in colonic mucosa, 40 metabolites in liver and 34 fecal metabolites were significantly altered by BRBs. Particularly ω -3 PUFAs (EPA and DHA) were increased in liver. Considering this metabolomics profiling of BRB-fed WT mice combined with the metabolomics profiling of BRB-supplemented human colorectal cancer patients and BRB-treated Apc^{Min/+} mice, these studies suggest that BRBs may be chemopreventive and BRBs can serve as a dietary source of ω -3 PUFAs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

AA Arachidonate

BRBs	Black raspberries
DHA	Docosahexaenoate
ω-3 DPA	Docosapentaenoate
EPA	Eicosapentaenoate
GC-MS	Gas chromatography-mass spectrometry
KEGG	Kyoto Encyclopedia of Genes and Genomes
ODC	Ornithine decarboxylase
PCA	Protocatechuic acid
ω-3 PUFA	Omega-3 polyunsaturated fatty acid
UHPLC	Ultra-high-performance-liquid chromatography
WT	Wild-type

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Figure 1. Metabolic pathway classes for metabolites that were modulated by BRBs in colonic mucosa (A), liver (B) and feces (C)

Histograms represent the number of metabolites significantly increased (black) and decreased (white) in each metabolic pathway class.

Figure 2. BRBs significantly increased benzoate metabolites in colonic mucosa (A), liver (B) and feces (C)

Significantly increased levels of 3,4-dihydroxybenzoate and catechol sulfate in colonic mucosa (A), catechol sulfate and hippurate in liver (B), and 3-hydroxybenzoate and 3,4-dihydroxybenzoate in feces (C). * $p < 0.05$

Figure 3. BRBs significantly increased ω -3 PUFAs in liver
(A) linolenate, (B) stearidonate, (C) DHA, (D) ω -3 DPA, (E) EPA. * $p < 0.05$

Figure 4. BRBs significantly increased ω -3 PUFAs in feces
(A) linoleate, (B) linolenate. * $p < 0.05$

Table 1

Fatty acid contents in AIN-76A control diet and BRB powder.

	AIN-76A control diet		BRB powder	
	Fatty acid content g/100g diet	% of total fatty acids	Fatty acid content g/100g diet	% of total fatty acids
Total fatty acids	5.03		4.72	
Linoleate	2.77	55.07%	2.41	51.06%
Linolenate	0.05	0.99%	1.42	30.08%
Stearidonate	0.096	1.91%	0.045	0.95%
DHA	<0.007	<0.14%	<0.007	<0.14%
EPA	<0.007	<0.14%	<0.007	<0.14%
ω -3 DPA	<0.007	<0.14%	<0.007	<0.14%
AA	<0.007	<0.14%	<0.007	<0.14%

DHA, docosahexaenoate; EPA, eicosapentaenoate; ω -3 DPA, docosapentaenoate; AA, arachidonate.