JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

J. Agric. Food Chem. 2010, 58, 3977–3983 3977
DOI:10.1021/jf9030772

Dietary Black Raspberry Anthocyanins Do Not Alter Development of Obesity in Mice Fed an Obesogenic High-Fat Diet†

Ronald L. Prior,*[,§,#] Sam Wilkes,[#] Theo Rogers,[#] Ramesh C. Khanal,[⊥,#] Xianli Wu,[#,⊗] Tiffany J. Hager,[⊥] Aaron Hager,[#] and Luke Howard[⊥]

[§]USDA-ARS and [#]Arkansas Children's Nutrition Center, 15 Children's Way, Little Rock, Arkansas 72202, [⊥]Department of Food Science, University of Arkansas, Fayetteville, Arkansas 72704, and [⊗]Department of Physiology and Biophysics, University of Arkansas for Medical Sciences, Little Rock, Arkansas 72205

Anthocyanins (ACNs) from various foods have been shown to minimize the development of obesity in some animal models. The objective of the current study was to compare the effects of feeding purified black raspberry (BRB) ACNs or the freeze-dried whole BRB on the development of obesity. Male C57BL/6J mice (25 days of age) were assigned at random to treatments (7/treatment; 3/cage). The treatments included (1) control low-fat diet (10% calories from fat) (LF); (2) LF plus BRB juice in place of drinking water; (3) LF diet plus purified BRB ACNs in drinking water (1.25 mg/mL); (4) control high-fat diet (60% calories from fat) (HF60); (5) HF60 diet + BRB juice in place of drinking water; (6) HF60 diet + ACNs in drinking water (1.25 mg/mL); and (7) HF60 + freeze-dried whole BRB powder (21.7 g/kg of diet). Body weight gains in mice fed HF60 diet plus purified BRB ACNs tended to be lower after 56, 63, and 70 days than in mice fed HF60 alone. Body weights were increased at time of sacrifice, but heart, liver, and kidney weights as a percentage of body weight were decreased in mice fed HF60 diet compared to LF fed mice. Weights (g or g/body weight) of epididymal and retroperitoneal fat were increased in the HF60 fed mice compared to LF fed mice. Fasting serum glucose, leptin, and insulin levels as well as homeostasis assessment of insulin resistance (HOMA-IR) were elevated in mice fed the HF60 diet relative to LF-fed controls. Serum cholesterol, triglycerides, and monocyte chemoattractant protein-1 (MCP-1) were not altered by diet. Serum levels of resistin were increased in mice fed the HF60 diet compared to mice fed the LF diet. None of the responses measured were altered by whole BRB powder included in the diet relative to the HF60 control diet. Cyanidin containing di- or triglycosides in BRB was ineffective in altering the development of obesity in contrast to cyanidin-monoglycosides, which have been shown to be effective. The sugar moiety on the anthocyanidins may be an important factor in determining the response in the development of obesity.

KEYWORDS: Obesity; leptin; black raspberry; anthocyanins

INTRODUCTION

Black raspberries (BRB, *Rubus occidentalis* L.) contain relatively high concentrations of anthocyanins (ACNs) compared to other fruits (*1*, *2*). The high levels of ACNs, or possibly other polyphenolics, in BRB may play an important role in the anticancer effects that have been observed (*3*−*5*). The major ACNs in black raspberries consist primarily (∼86%) of cyanidin-3-rutinoside and cyanidin-3-sambubioside-5-rhamnose and small amounts of cyanidin-3-glucoside (∼13%) (*1*, *6*). ACNs with complex sugar conjugates are not cleared from the blood as rapidly as cyanidin-3-glucoside. These same ACNs also were more stable in the gastrointestinal tract, which is also related to increased absorption and excretion in the urine (*1*). The phenolic acids in black raspberry have also been characterized (*7*), with 3,4-dihydroxybenzoic acid (protocatechuic acid) predominating. Black raspberries also contain ellagitannins, which are also contained in strawberries. Because of this somewhat unique mix of polyphenolics in black raspberry, we undertook the present study to evaluate their effects on the development of obesity in a mouse model fed a high-fat diet.

Previously, purified ACNs from blueberries or strawberries (*8*) and other sources (*9*−*12*) have been shown to have antiobesity effects in vivo. Most of these studies used a concentrated extract of ACNs containing cyanidin-based monoglycoside as the predominant ACN from food sources other than berries. In our studies of ACNs in berries, feeding whole powdered berries from

†Part of the Berry Health Symposium 2009.

*Address correspondence to this author at the USDA, Arkansas Children's Nutrition Center, 15 Children's Way, Little Rock, AR 72202 [e-mail priorronaldl@uams.edu; telephone (501) 364-2747; fax (501) 364-2818].

Published on Web 03/04/2010

blueberries or strawberries in a high-fat diet, compared to purified ACNs, was not effective in preventing obesity (*8*, *13*, *14*) and in some cases tended to promote obesity when fed as part of a high-fat but not a low-fat diet. Whole strawberries did not promote obesity when fed in a high-fat diet, and some measures indicated possible antiobesity effects (*8*). Whole freeze-dried powders of Concord grapes and BRB were ineffective in preventing obesity (*8*). Thus, there appear to be components in berries (i.e., ACNs) that can have beneficial effects in preventing obesity but other factors that may interact in a way to prevent any protective effect against obesity. Exactly what these factors are and how they interact is not known. There are no data are available on BRB and/or purified ACNs from BRB.

Mechanism(s) whereby purified ACNs exert antiobesity effects are not clear. Possible mechanisms suggested have included (1) amelioration of hyperglycemia and insulin insensitivity via the reduction of retinol binding protein 4 (RBP4) expression in white adipose tissue; (2) down-regulation of the inflammatory adipocytokines (monocyte chemoattractant protein-1 and tumor necrosis factor-α (TNF-α)) in white adipose tissue; (3) suppression of mRNA levels of enzymes involved in fatty acid and triacylglycerol synthesis; and (4) reduced sterol regulatory element binding protein-1 mRNA level in white adipose tissue (*9*).

Studies to date have not provided any explanation for why the purified ACNs are effective in preventing obesity but not the whole berry powder containing similar amounts and types of ACNs. Processing of blueberries into juice results in a significant loss of ACNs (*16*), but still provides a concentrated source of ACNs that have been separated from other components in the whole berry. Blueberry juice fed to mice in place of drinking water was effective in slowing the development of obesity in mice fed a high-fat diet (*17*). Unlike other studies using primarily monoglycoside ACNs, cyanidin 3-rutinoside and cyanidin 3-xylosylrutinoside account for 85−90% of the total ACNs in BRB (*1*, *2*, *6*). The effects of the cyanidin aglycone containing a di- or triglycoside have not been studied relative to the development of obesity.

The objectives of the current study were to compare effects of feeding purified ACNs, BRB juice, and whole freeze-dried BRB powder on the development of obesity in mice fed an obesogenic high-fat diet.

MATERIALS AND METHODS

Chemicals and Reagents. Standards of the 3-*O*-*β*-glucosides of pelargonidin, cyanidin, peonidin, delphinidin, petunidin, and malvidin (six mixed ACN standards, HPLC grade) were obtained from Polyphenols Laboratories (Sandnes, Norway). Formic acid was purchased from Sigma-Aldrich (St. Louis, MO). All other solvents were purchased from Fisher (Fair Lawn, NJ). All chemicals and solvents were of HPLC grade.

Preparation of Purified ACNs. The extraction and purification of ACNs from BRB was similar to that described previously for blueberries (*8*, *13*). Briefly, BRB powder was weighed (500 g) and extracted two times with methanol/water/formic acid (85:15:0.5, v/v). The filtrates were combined and subjected to vacuum evaporation (Büchi, Germany) to remove methanol. The concentrated extracts were loaded onto an Amberlite XAD-7 resin column (Sigma-Aldrich). The resin was washed with 0.5% formic acid in water, and subsequently the absorbed ACNs were recovered with 0.5% formic acid in methanol. The methanol eluent was subjected to vacuum evaporation again to remove most of the methanol. To remove other phenolic acids, the concentrated eluents were extracted one time with ethyl acetate (EtOAc). After EtOAc extraction, the aqueous layer was subjected to vacuum evaporation to remove residual organic solvents. The final concentrated extracts were analyzed with an Agilent 1100 series HPLC (Palo Alto, CA) equipped with an autosampler/injector and diode array detector to determine the concentration of ACNs as well as other phenolic acids. Total volume of the final BRB extract was measured, distributed appropriately, and lyophilized to yield dry purified ACN powders.

Table 1. Composition of Modified AIN-93G Diets Fed to Mice

ingredient	10% kcal fat (LF)	60% kcal fat (HF)	powder, 60% kcal fat
casein, 80 mesh	200	200	200
L-cystine	3	3	3
corn starch	315	0	0
maltodextrin 10	35	125	125
sucrose	350	68.8	58
cellulose, BW200	50	50	47
soybean oil	25	25	25
lard	50	245	245
Mineral Mix S10026	10	10	10
dicalcium phosphate	13	13	13
calcium carbonate	5.5	5.5	5.5
potassium citrate, 1 H_2O	16.5	16.5	16.5
Vitamin Mix V10001	10	10	10
choline bitartrate	2	2	2
black raspberry powder	0	0	13.3
total, g	1055	773.9	773.3
anthocyanins, mg			
cyanidin-3-glucoside	0	0	74.4
Cy-3-xyl-rut	0	0	166.4
Cy-3-rutinoside	0	0	494.4
Pel-3-rutinoside	0	0	10.7
total ACNs, mg	0	0	746.0
g			
protein	179	179	179
carbohydrate	700	193.8	193.6
fat	45	270	270
fiber	50	50	49.7
g %			
protein	17.0	17.0	17.0
carbohydrate	66.3	18.4	18.4
fat	4.3	25.6	25.6
fiber	4.7	4.7	4.7
kcal			
protein	716	716.0	716.0
carbohydrate	2800	775.2	774.6
fat	405	2430	2430
fiber	3921	3921	3921
kcal %			
protein	18.3	18.3	18.3
carbohydrate	71.4	19.8	19.8
fat	10.3	62	62
fiber	100	100	100

Preparation of BRB Fractions. Black raspberries were processed into juice as described previously (*15*). The BRB juice was freeze-dried and ground if necessary into a powder. BRB juice powder was reconstituted in water and provided to the mice in place of drinking water. Whole BRB powder was incorporated into the powdered diet. The ACN content in the BRB whole powder and purified juice powder and in purified ACN extract was determined as described previously (*2*).

Animals and Diets. All experimental animal protocols were approved by the Animal Care and Use Committee of the Arkansas Children's Hospital Research Institute. BRBs (cv. Munger) harvested at the fully ripe stage (shiny black) were obtained from a commercial grower in Oregon in July 2005. Freeze-dried BRB powder was provided by the Oregon Raspberry and Blackberry Commission. Purified diets were prepared by Research Diets (New Brunswick, NJ), and the composition is presented in **Table 1**. All diets were balanced for energy, protein, and fiber. The compositions of the BRB juice and powder are presented in **Table 2**.

Male C57BL/6J mice (25 days of age) (Jackson Laboratories, Bar Harbor, ME) were assigned at random to treatments such that there were nine animals per treatment (three per cage). All treatments were balanced

for initial body weight (18.8 ± 0.3 g) of the animals. The treatments included (1) control low-fat diet (10% calories from fat) (LF); (2) LF plus BRB juice in place of drinking water; (3) LF diet plus purified BRB ACNs in drinking water (1.25 mg/mL); (4) control high-fat diet (60% calories from fat) (HF60); (5) HF60 diet + BRB juice in place of drinking water; (6) HF60 diet + ACNs in drinking water (1.25 mg/mL); and (7) HF60 + freeze-dried whole BRB powder (21.7 g/kg of diet). All diets were fed in powdered form. Fresh deionized water containing purified ACNs was provided every other day, and the volume of liquid consumed was recorded. Preliminary studies indicated that the ACNs were stable in water over a 48 h period (*8*). Weekly weights and estimates of feed, water, or juice intake were recorded. Whole body composition (fat and lean tissues) was determined using nuclear magnetic resonance technology with an EchoMRI Analyzer system by Echo Medical Systems (Houston, TX) on days 50 and 67. On days 70−73 of the experiment, the animals were sacrificed after euthanization with isofluorane, and serum, heart, liver, kidney, and adipose tissue (epididymal and retroperitoneal) were weighed and their samples collected. All samples were stored at 70 °C prior to analysis.

Cytokine and Insulin Analysis. Serum cytokines (leptin, MCP-1, resistin) were analyzed using Luminex xMAP technology with Linco LINCOplex multiplex immunodetection kits and reagents (Millipore Corp., Billerica, MA). Cytokines were quantitated with Bio-Plex Manager software (Bio-Rad Laboratories, Inc., Hercules, CA) Serum insulin was determined by enzyme-linked immunosorbent assay (ELISA) using commercially available kits (Linco Research Inc., St. Charles, MO) in a Benchmark Plus microplate spectrophotometer (Bio-Rad Laboratories).

Serum Glucose and Lipid Analysis. Serum glucose (IR070), triglycerides (IR140), and cholesterol (IR060) were analyzed using commercially available kits (Synermed, Westfield, IN) in a 96-well plate format using a dual-pump Fluostar Galaxy (BMG Labtech, Durham, NC) microplate reader.

Insulin Resistance and β Cell Function. The degree of insulin resistance was estimated by a homeostasis assessment model (HOMA-IR), which was calculated according to the formula

$$\text{HOMA-IR} = [\text{serum glucose (mmol/L)} \times \text{serum insulin (mU/L)}/22.5$$

β cell function was assessed by the β cell homeostasis assessment (HOMA-BCF) score and calculated as follows:

$$\text{HOMA-BCF} = [20 \times \text{serum insulin (mU/L)}]/[\text{serum glucose (mmol/L)} - 3.5]$$

Insulin values were expressed in international units (1 IU = 0.04167 mg) (*18*, *19*).

Statistical Analysis. Data from treatments 1−6 were analyzed using a two-way (diet and fat level) analysis of variance (ANOVA) with a post hoc comparison using Sigmastat for Windows, ver. 3.5 (San Jose, CA). Treatments 7−10 plus treatment 4 were analyzed using one-way analysis of variance.

Table 2. ACN Composition of Different Components Derived from Black Raspberries during the Juicemaking Process[a]

ACN	BRB powder[c]	BRB juice
Cy-3-glucoside	5.59	5.40
Cy-3-xylosylrutinoside[b]	12.51	16.54
Cy-3-rutinoside	37.17	48.93
Pel-3-rutinoside	0.81	1.70
total	56.09	72.56

[a] Composition expressed as mg/g dry matter. [b] Also has been identified as cyandin-3-sambubioside-5-rhamnoside on the basis of HPLC/MS/MS data (*25*). [c] Freeze-dried powder of whole black raspberries.

RESULTS

Initial body weights of the mice averaged 18.8 g (**Table 3**). The main effects of feeding the high-fat diet were significant for subsequent measures of body weight, cumulative weight gain, and percent body lean and fat (**Table 3**). Cumulative weight gains are presented in **Figure 1**. Gains of mice fed the HF60 diet plus purified BRB ACNs tended to be lower at days 56, 63, and 70 days than those of mice fed the HF60 alone. However, they were not significantly different ($p > 0.05$) from mice fed the LF diet plus BRB ACNs. This decreased gain of the HF60 (+ACNs)-fed mice may merely be a reflection of the lower diet and caloric intake (11.0 kcal/day vs >12 kcal/day in the other HF60 treatments) (**Table 3**). HF60-fed mice had greater fat mass and lower lean body mass and gained more weight over the 69 days compared to LF-fed mice. Liquid consumption was decreased in the HF60 fed mice compared to the LF-fed mice, and those mice fed BRB juice consumed more liquid than the other treatments (**Table 4**). Total ACN intake in the water was higher for the LF-fed mice compared to the HF60-fed mice (**Table 4**).

Body weights were increased at time of sacrifice, but heart, liver, and kidney weights as a percentage of body weight were decreased in mice fed the HF60 diet compared to LF-fed mice (**Table 5**). Weights, as well as weights as a percentage of body weight, of epididymal and retroperitoneal fat were increased in the HF60-fed mice compared to LF-fed mice (**Figure 2**).

Fasting serum glucose, leptin, resistin, insulin and HOMA-IR were elevated in mice fed the HF60 diet relative to the LF fed controls (**Figure 3**; **Table 6**). Serum cholesterol, triglycerides and monocyte chemoattractant protein-**1** (MCP-1) were not altered by diet (**Table 6**).

Table 3. Body Weights, Cumulative Weight Gains, Body Composition (MRI), and Food Intake in Male C57BL/6J Mice Fed a Low-Fat (LF) or High-Fat (HF60) Diet with BRB Juice or Purified ACNs from BRB (1.25 mg/mL) in the Drinking Water[a]

item	LF	LF + juice	LF + ACN	HF60	HF60 + juice	HF60 + ACN	fat	ACN	fat × ACN
initial body wt (g)	19.1 ± 0.4	19.0 ± 0.3	18.5 ± 0.3	18.7 ± 0.3	18.8 ± 0.3	18.7 ± 0.3	NS	NS	NS
MRI data									
lean %, day 50[b]	60.3 ± 2.4	63.0 ± 2.1	61.0 ± 2.1	53.0 ± 2.1 *	53.7 ± 2.1 *	54.7 ± 2.1 *	<0.001	NS	NS
lean %, day 67[b]	57.0 ± 1.9	58.2 ± 1.7	56.4 ± 1.7	49.0 ± 1.7 *	48.3 ± 1.7 *	51.4 ± 1.7	<0.001	NS	NS
fat %, day 50[b]	28.0 ± 2.4	26.7 ± 2.1	28.1 ± 2.1	37.0 ± 2.1 *	36.3 ± 2.1 *	35.0 ± 2.1 *	<0.001	NS	NS
fat %, day 67[b]	29.2 ± 2.2	26.7 ± 1.9	28.6 ± 1.9	37.4 ± 1.9 *	37.9 ± 1.9 *	35.5 ± 1.9 *	<0.001	NS	NS
body wt, day 49	31.7 ± 1.4	31.1 ± 1.1	30.5 ± 1.1	34.5 ± 1.1	35.1 ± 1.1 *	30.1 ± 1.2	0.002	NS	NS
cumulative gain, day 49	12.6 ± 1.1	12.1 ± 0.9	12.1 ± 0.9	15.8 ± 0.9 *	16.3 ± 0.9 *	14.4 ± 1.0	<0.001	NS	NS
body wt (g), day 69	34.7 ± 1.4	35.0 ± 1.2	34.4 ± 1.2	40.1 ± 1.2 *	38.7 ± 1.2 *	36.1 ± 1.3	<0.001	NS	NS
cumulative gain (g), day 69	15.7 ± 1.2	15.9 ± 1.0	16.0 ± 1.0	20.0 ± 1.0 AB	21.4 ± 1.0 A	17.5 ± 1.1 B	<0.001	NS	NS
intake									
g/mouse/day	3.32 ± 0.09	3.41 ± 0.08	3.53 ± 0.08	2.38 ± 0.08 *	2.46 ± 0.08 *	2.18 ± 0.08 *	<0.001	NS	0.039
kcal/mouse/day	12.4 ± 0.4	12.7 ± 0.4 [c]	13.1 ± 0.4	12.0 ± 0.4	12.4 ± 0.4 [c]	11.0 ± 0.4 *	0.015	NS	0.052

[a] Values are presented as means ± SEM. LF, low-fat diet, 10% kcal; HF60, high-fat diet, 60% kcal; ACN, 1.25 mg/mL ACN content in liquid diet; HF diet group means within rows with * differ significantly from the comparable low-fat group; $p \leq 0.05$, ANOVA. Means within rows without a common letter differ among HF treatments; $p \leq 0.05$, ANOVA. [b] Body weights were taken after a 12 h fast. [c] kcal calculations do *not* include caloric contribution from BRB juice.

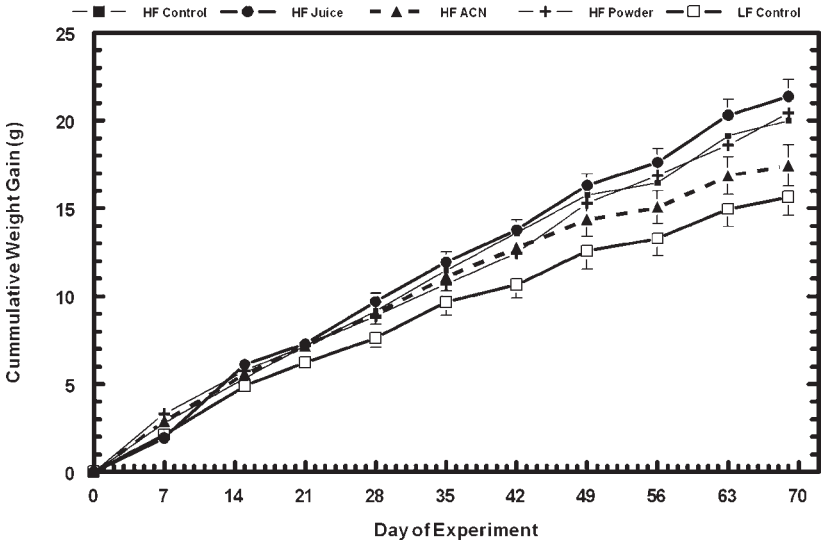

Figure 1. Cumulative body weight gains in male C57BL/J6 mice fed a low-fat (LF) or high-fat (HF) (60% kcal from fat) diet with or without BRB juice (J) or BRB purified ACNs (1.0 mg/mL) in place of drinking water.

Table 4. Liquid Consumption by Male C57BL/6J Mice Fed a Low-Fat (LF) or High-Fat (HF60) Diet Consuming BRB Juice or BRB Purified ACNs (1.25 mg/mL) in the Drinking Water or Water as the Liquid Source[a]

liquid consumed	LF (H_2O)	LF + juice	LF + ACN	HF60 (H_2O)	HF60 + juice	HF60 + ACN	fat	ACN	fat × ACN
mL/mouse/day	2.36 ± 0.09	3.50 ± 0.09	2.67 ± 0.09	1.85 ± 0.09	2.99 ± 0.09	2.13 ± 0.09	<0.001	<0.001	NS
liquid ACN intake (mg/day)	0	4.38	3.34 ± 0.11	0	3.74	2.66 ± 0.11			

[a] Values are presented as means ± SEM. LF, low-fat diet, 10% kcal; HF, high-fat diet, 60% kcal; ACN, 1.25 mg/mL ACN content in liquid diet.

Table 5. Body Weights and Tissue Weights in Male C57BL/6J Mice Fed a Low-Fat (LF) or High-Fat (HF60) Diet with BRB Juice or Purified ACNs from BRB (1.25 mg/mL) in the Drinking Water[a]

	LF	LF + juice	LF + ACN	HF60	HF60 + juice	HF60 + ACN	fat	ACN
body wt[b] (g)	32.2 ± 1.4	32.6 ± 1.1	31.8 ± 1.1	36.5 ± 1.1 *	38.0 ± 1.1 *	34.1 ± 1.2 *	<0.001	NS
heart (% BW)	0.495 ± 0.027	0.504 ± 0.022	0.466 ± 0.022	0.410 ± 0.022 *	0.383 ± 0.022 *	0.438 ± 0.024	<0.001	NS
liver (% BW)	3.99 ± 0.15	4.15 ± 0.12	3.93 ± 0.12	3.22 ± 0.12 *	3.27 ± 0.12 *	3.07 ± 0.13 *	<0.001	NS
kidneys (% BW)	1.08 ± 0.05	1.13 ± 0.04	1.052 ± 0.04	0.921 ± 0.04 *	0.910 ± 0.04 *	0.95 ± 0.04	<0.001	NS
epididymal fat (% BW)	4.54 ± 0.37	4.33 ± 0.30	4.56 ± 0.30	6.47 ± 0.30 *	6.83 ± 0.30 *	6.37 ± 0.32 *	<0.001	NS
retroperitoneal fat (% BW)	1.32 ± 0.12	1.21 ± 0.09	1.33 ± 0.09	1.90 ± 0.09 *	1.91 ± 0.09 *	1.94 ± 0.10 *	<0.001	NS
total fat (E+R) (% BW)	5.86 ± 0.44	5.54 ± 0.36	5.88 ± 0.36	8.37 ± 0.36 *	8.74 ± 0.36 *	8.31 ± 0.38 *	<0.001	NS
heart (g)	0.159 ± 0.009	0.163 ± 0.007	0.148 ± 0.007	0.149 ± 0.007	0.146 ± 0.007	0.148 ± 0.008	NS	NS
liver (g)	1.29 ± 0.09	1.36 ± 0.08	1.25 ± 0.08	1.19 ± 0.08	1.25 ± 0.08	1.05 ± 0.08	0.040	NS
kidneys (g)	0.348 ± 0.013	0.364 ± 0.010	0.334 ± 0.010	0.334 ± 0.010	0.344 ± 0.010	0.319 ± 0.011	NS	0.040
epididymal fat (g)	1.47 ± 0.17	1.44 ± 0.14	1.45 ± 0.14	2.35 ± 0.14 *	2.60 ± 0.135 *	2.19 ± 0.14 *	<0.001	NS
retroperitoneal fat (g)	0.425 ± 0.048	0.404 ± 0.040	0.423 ± 0.040	0.690 ± 0.040 *	0.725 ± 0.040 *	0.666 ± 0.0419 *	<0.001	NS
total fat (E+R) (g)	1.90 ± 0.20	1.85 ± 0.16	1.87 ± 0.16	3.04 ± 0.163 *	3.33 ± 0.163 *	2.86 ± 0.17 *	<0.001	NS

[a] Values are presented as means ± SEM of nine mice per treatment; data from sacrifice. Statistical interactions of fat × ACN were not significant ($p > 0.05$). LF, low-fat diet, 10% kcal; HF, high-fat diet, 60% kcal; ACN, 1.25 mg/mL ACN content in liquid diet; HF60 diet group means within rows with * differ significantly from the comparable low-fat group ($p \leq 0.05$, ANOVA). [b] Body weight values are after a 12 h fast.

Data on body weights and gains in mice fed the HF60 diet or the HF60 diet containing whole BRB powder is presented in **Table 7**. None of the traits measured were altered by the BRB powder in the diet relative to the HF60 control diet (**Table 7**).

DISCUSSION

The fact that purified BRB ACNs did not have the same effect as purified ACNs from blueberry or strawberry in slowing the development of obesity is somewhat unexpected. BRB contains ~67% of the total ACNs as cyanidin-3-rutinoside and~22% as cyanidin-3-xylosylrutinoside (**Table 2**), a diglycoside and triglycoside, respectively, whereas both blueberry and strawberry contain mainly monoglycosides. Previous studies with ACNs preventing obesity have been with monoglycosides, primarily cyanidin-3-glucoside (*8*−*10*, *17*). If it is the intact ACN molecule that is the active component, the size and perhaps conformation of a diglycoside or larger triglycoside may prevent the molecule from accessing a receptor or other binding sites to signal the changes that result in decreased adipose tissue deposition.

The competitive inhibition of cyclic nucleotide phosphodiesterase (PDE), an elevation in cAMP level, and subsequent activation of protein kinase A (cAMP-dependent protein kinase) by flavonoids have been proposed (*20*) as a mechanism to induce neutral lipid hydrolysis from lipid stores in adipose tissue and liver. Indeed, the three-dimensional structure of many flavonoids is sterically and electrostatically compatible with the catalytic site

of cAMP PDE3 and PDE4. Flavonoid-mediated PDE inhibition is dependent on the ability of the flavonoid to sterically fit in the cyclic nucleotide binding pocket (*21*). Findings from molecular docking investigations support the contention that many of the biological effects of plant flavonoids are attributable to competitive inhibition of specific cyclic nucleotide PDE isoforms (*20*). What has not been demonstrated is whether the glycoside forms of anthocyanidins would be able to dock in the catalytic site of PDE. Increased steric hindrance with diglycosides of cyanidin could well prevent inhibitory effects of BRB ACNs in this process.

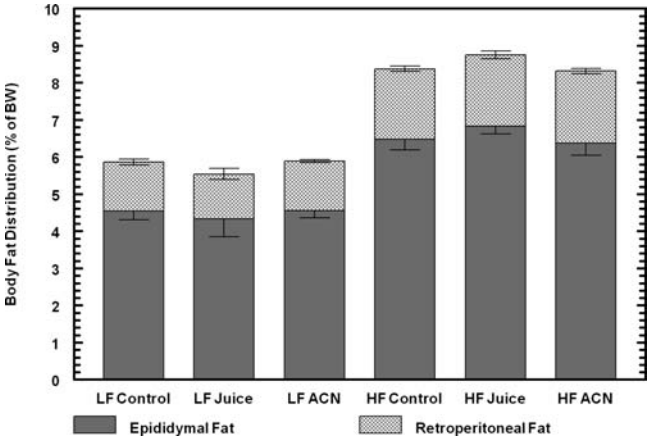

Figure 2. Epididymal and retroperitoneal fat weights (as percent of body weight) in fasted male C57BL/6J mice fed a low-fat (10% kcal from fat) or high-fat (45% kcal from fat) diet with blueberry juice or purified blueberry ACNs in place of drinking water.

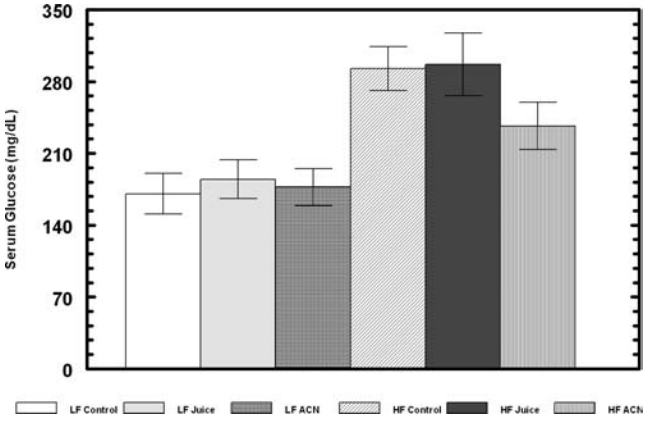

Figure 3. Serum glucose in fasted male C57BL/6J mice fed a low-fat (10% kcal from fat) or high-fat (60% kcal from fat) diet with BRB juice or purified BRB ACNs (1.25 mg/mL) in place of drinking water.

Fasted versus Fed State. Serum parameters affected by HF feeding differ between the fed state and fasting. In the fed state (*8*) in mice fed a high-fat diet, postprandial serum triglycerides and cholesterol are elevated relative to mice fed the LF control diet, which were normalized by purified ACNs, but not whole berry powders. No consistent modulation of postprandial serum glucose and insulin was observed by HF feeding (*8*). However, in the fasted state in this study, differences in serum triglycerides and cholesterol were not observed with HF feeding (**Table 6**) (*17*), but increases in glucose were observed (**Table 6**) (*17*). In the fasted state, serum insulin and resistin were elevated with HF60 feeding (**Table 6**), but not in the fed state (*8*). In the postprandial fed state in mice fed a HF45 diet, serum insulin and resistin were elevated relative to a LF diet.

Changes in HOMA-IR were apparent in mice fed the HF60 diet (**Table 6**) but in our previous study with feeding a HF45 diet, alterations in β cell function were present as indicated by the HOMA-BCF (*17*). These calculations are most appropriately applied to the fasted state.

Leptin and Other Hormones and Obesity. Changes in serum leptin have been consistent across experiments in that feeding a high-fat diet has elevated leptin levels, and in those treatments that have decreased or slowed the development of obesity, the leptin levels were decreased (*8*, *17*). Although rates of body weight gain tended to decrease after 56 days and beyond (**Figure 1**) in mice fed the purified ACNs and a high-fat diet, serum leptin was not altered in that treatment group. It is not clear whether the decreases in leptin observed with purified ACN treatments are merely a result of decreased fat mass, because leptin is secreted by adipose tissue and levels are proportional to the amount of total adipose tissue mass as well as perirenal fat mass (*22*) or whether the ACNs have an effect on adipose tissue cells to decrease the production of leptin in the cell. In one study (*17*), the ratio of leptin to adipose tissue mass, as reflected in the epididymal plus retroperitoneal fat, was decreased in the mice consuming purified blueberry ACNs, which was true whether they were fed a LF or HF diet. This observation suggests that the ACNs were having a direct effect on the adipose tissue and its production of leptin. Tsuda and co-workers (*9*) observed a much larger response in leptin to a HF diet (7.7 times) and also a much larger leptin to fat ratio (3.2 times). There was no indication in the ACN treatment that leptin production relative to adipose tissue mass was decreased below normal levels.

Resistin is a cysteine-rich protein secreted by adipose tissue of mice and is reputed to contribute to insulin resistance. Serum resistin was elevated in mice fed the HF60 diet compared to mice

Table 6. Fasting Serum Glucose, Lipids, and Cytokines in Male C57BL/6J Mice Fed a Low-Fat (LF) or High-Fat (HF60) Diet with BRB Juice or Purified ACNs from BRB (1.25 mg/mL) in the Drinking Water[a]

	LF	LF + juice	LF + ACN	HF60	HF60 + juice	HF60 + ACN	fat	ACN	fat × ACN
glucose (mg/dL)	170 ± 27	185 ± 22	177 ± 22	293 ± 22 *	297 ± 22 *	237 ± 23	<0.001	NS	NS
cholesterol (mg/dL)	215 ± 26	163 ± 21	161 ± 21	204 ± 21	179 ± 21	167 ± 23	NS	NS	NS
triglycerides (mg/dL)	208 ± 26	170 ± 21	157 ± 21	177 ± 21	167 ± 21	178 ± 22	NS	NS	NS
leptin (pg/mL)	4431 ± 1676	4244 ± 1530	4479 ± 1530	9134 ± 1417 *	9305 ± 1417 *	10560 ± 1417 *	<0.001	NS	NS
leptin/fat ratio	2397 ± 612	2163 ± 559	2388 ± 559	3130 ± 518	2845 ± 518	3582 ± 518	NS	NS	NS
MCP-1 (pg/mL)	150 ± 12	149 ± 11	174 ± 11	157 ± 10	162 ± 10	176 ± 10	NS	NS	NS
resistin (pg/mL)	891 ± 30	944 ± 28	898 ± 28	1071 ± 26 *	1095 ± 26 *	1016 ± 26 *	<0.001	NS	NS
insulin (pg/mL)	315 ± 121	401 ± 99	346 ± 99	419 ± 92	689 ± 92 *	463 ± 92	0.045	NS	NS
HOMA-IR	3.38 ± 2.60	4.32 ± 2.12	4.03 ± 2.12	7.82 ± 1.97	12.55 ± 1.97 *	6.78 ± 1.97	0.006	0.278	0.404
HOMA-BCF	27.1 ± 9.4	37.3 ± 7.7	27.0 ± 7.7	15.4 ± 7.1	28.8 ± 7.1	24.2 ± 7.1	NS	NS	NS

[a] Values are presented as means ± SEM. LF, low-fat diet, 10% kcal; HF60, high-fat diet, 60% kcal; ACN, 1.25 mg/mL ACN content in liquid diet; HF diet group means within rows with * differ significantly from the comparable low-fat group, $p \leq 0.05$, ANOVA.

Table 7. Body Weights, Weight Gains, Tissue Weights, and Serum Metabolites at Sacrifice in Mice Fed a High-Fat Diet or High-Fat Diet plus Freeze-Dried BRB Powder[a]

	HF60	HF60 + BRB powder	$p <$
initial body wt (g)	18.7 ± 0.4	18.9 ± 0.3	NS
body wt, day 49	34.5 ± 1.6	34.2 ± 1.3	NS
cumulative gain, day 49	15.8 ± 1.3	15.3 ± 1.2	NS
body wt (g), day 69	38.7 ± 1.6	39.3 ± 1.5	NS
cumulative gain (g), day 69	20.0 ± 1.3	20.4 ± 1.4	NS
g/mouse/day intake	2.38 ± 0.08	2.35 ± 0.04	NS
kcal/mouse/day intake	12.0 ± 0.4	11.9 ± 0.2	NS
data from sacrifice[b]			
body wt	36.5 ± 1.1	37.2 ± 1.4	NS
heart (% BW)	0.410 ± 0.012	0.415 ± 0.012	NS
liver (% BW)	3.22 ± 0.17	3.32 ± 0.19	NS
kidneys (% BW)	0.92 ± 0.03	0.89 ± 0.03	NS
epididymal fat (% BW)	6.47 ± 0.28	6.65 ± 0.27	NS
retroperitoneal fat (% BW)	1.90 ± 0.07	1.92 ± 0.07	NS
total fat (% BW)	8.37 ± 0.31	8.57 ± 0.28	NS
heart (g)	0.149 ± 0.006	0.153 ± 0.004	NS
liver (g)	1.19 ± 0.11	1.25 ± 0.12	NS
kidneys (g)	0.334 ± 0.009	0.329 ± 0.009	NS
epididymal fat (g)	2.35 ± 0.11	2.47 ± 0.13	NS
retroperitoneal fat (g)	0.690 ± 0.031	0.718 ± 0.047	NS
total fat (g)	3.04 ± 0.12	3.19 ± 0.15	NS
serum			
glucose (mg/dL)	293 ± 21	268 ± 21	NS
cholesterol (mg/dL)	204 ± 34	200 ± 14	NS
triglycerides (mg/dL)	177 ± 22	173 ± 18	NS

[a] Values are means ± SEM. [b] Body weights were taken after a 12 h fast.

fed the LF control diet but was not altered significantly ($p > 0.10$) by the BRB juice or purified ACN treatments in this study (**Table 6**). In a previous experiment, resistin was not increased in mice fed a HF diet (45% kcal from fat) fed mice but was increased in mice fed the HF45 diet plus freeze-dried whole blueberry or strawberry powder relative to the LF control containing the same berry (*8*).

The increase in serum insulin and glucose in mice fed the HF60 diet in this experiment and the increase in the HOMA-IR measure of insulin resistance indicate that conditions of insulin resistance are present in the HF60-fed mice.

The positive effect that purified ACNs have in slowing the development of obesity in animal models and the opposite effect observed with whole berries presents an interesting dilemma, the solution of which may provide important insights into preventing this disease process.

The lack of effect of purified anthocyanidins containing cyanidin di- or triglycosides from BRB but positive effects of cyanidin-monoglycosides in other studies poses a challenge in understanding how ACNs may alter the development of obesity. Apparently all anthocyanins cannot be considered equal from this perspective. Structural differences, in either the aglycone or sugar moiety, may have marked differences in their health effects. Metabolic products from the aglycones in terms of phenolic acids and other metabolites are most likely different. However, differences due to the sugar moiety most likely are due to the intact molecule.

ABBREVIATIONS USED

ACNs, anthocyanins; BRB, black raspberry; HF60, high-fat diet with 60% kcal from fat; LF, low-fat diet with 10% kcal from fat.

ACKNOWLEDGMENT

Appreciation is expressed to the Oregon Berry Commission for providing the freeze-dried whole BRB powder.

LITERATURE CITED

(1) Wu, X.; Pittman, H. E.; Prior, R. L. Fate of anthocyanins and antioxidant capacity in contents of the gastrointestinal tract of weanling pigs following black raspberry consumption. *J. Agric. Food Chem.* **2006**, *54*, 583–9.

(2) Wu, X.; Beecher, G. R.; Holden, J. M.; Haytowitz, D. B.; Gebhardt, S. E.; Prior, R. L. Concentrations of anthocyanins in common foods in the United States and estimation of normal consumption. *J. Agric. Food Chem.* **2006**, *54*, 4069–75.

(3) Harris, G. K.; Gupta, A.; Nines, R. G.; Kresty, L. A.; Habib, S. G.; Frankel, W. L.; LaPerle, K.; Gallaher, D. D.; Schwartz, S. J.; Stoner, G. D. Effects of lyophilized black raspberries on azoxymethane-induced colon cancer and 8-hydroxy-2′-deoxyguanosine levels in the Fischer 344 rat. *Nutr. Cancer* **2001**, *40*, 125–33.

(4) Casto, B. C.; Kresty, L. A.; Kraly, C. L.; Pearl, D. K.; Knobloch, T. J.; Schut, H. A.; Stoner, G. D.; Mallery, S. R.; Weghorst, C. M. Chemoprevention of oral cancer by black raspberries. *Anticancer Res.* **2002**, *22*, 4005–15.

(5) Huang, C.; Huang, Y.; Li, J.; Hu, W.; Aziz, R.; Tang, M. S.; Sun, N.; Cassady, J.; Stoner, G. D. 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.

(6) Tulio, A. Z., Jr.; Reese, R. N.; Wyzgoski, F. J.; Rinaldi, P. L.; Fu, R.; Scheerens, J. C.; Miller, A. R. Cyanidin 3-rutinoside and cyanidin 3-xylosylrutinoside as primary phenolic antioxidants in black raspberry. *J. Agric. Food Chem.* **2008**, *56*, 1880–8.

(7) Wu, X.; Pittman, H. E.III; Hager, T.; Hager, A.; Howard, L.; Prior, R. L. Phenolic acids in black raspberry and in the gastrointestinal tract of pigs following ingestion of black raspberry. *Mol. Nutr. Food Res.* **2009**, *53*, S76–84.

(8) Prior, R. L.; Wu, X.; Gu, L.; Hager, T.; Hager, A.; Wilkes, S.; Howard, L., Purified berry anthocyanins but not whole berries normalize lipid parameters in mice fed an obesogenic high fat diet. *Mol. Nutr. Food Res.* **2009**, *53*, 1406−1418.

(9) Tsuda, T.; Horio, F.; Uchida, K.; Aoki, H.; Osawa, T. Dietary cyanidin 3-*O*-β-D-glucoside-rich purple corn color prevents obesity and ameliorates hyperglycemia in mice. *J. Nutr.* **2003**, *133*, 2125–2130.

(10) Jayaprakasam, B.; Olson, L. K.; Schutzki, R. E.; Tai, M. H.; Nair, M. G. Amelioration of obesity and glucose intolerance in high-fat-fed C57BL/6 mice by anthocyanins and ursolic acid in cornelian cherry (*Cornus mas*). *J. Agric. Food Chem.* **2006**, *54*, 243–248.

(11) Tsuda, T. Regulation of adipocyte function by anthocyanins; possibility of preventing the metabolic syndrome. *J. Agric. Food Chem.* **2008**, *56*, 642–646.

(12) Sasaki, R.; Nishimura, N.; Hoshino, H.; Isa, Y.; Kadowaki, M.; Ichi, T.; Tanaka, A.; Nishiumi, S.; Fukuda, I.; Ashida, H.; Horio, F.; Tsuda, T. Cyanidin 3-glucoside ameliorates hyperglycemia and insulin sensitivity due to down regulation of retinol binding protein 4 expression in diabetic mice. *Biochem. Pharmacol.* **2007**, *74*, 1619–1627.

(13) Prior, R. L.; Wu, X.; Gu, L.; Hager, T. J.; Hager, A.; Howard, L. R. Whole berries versus berry anthocyanins: interactions with dietary fat levels in the C57BL/6J mouse model of obesity. *J. Agric. Food Chem.* **2008**, *56*, 647–653.

(14) DeFuria, J.; Bennett, G.; Strissel, K. J.; Perfield, J. W.2nd; Milbury, P. E.; Greenberg, A. S.; Obin, M. S. Dietary blueberry attenuates whole-body insulin resistance in high fat-fed mice by reducing adipocyte death and its inflammatory sequelae. *J. Nutr.* **2009**, *139*, 1510–1516.

(15) Hager, A.; Howard, L. R.; Prior, R. L.; Brownmiller, C. Processing and storage effects on monomeric anthocyanins, percent polymeric color, and antioxidant capacity of processed black raspberry products. *J. Food Sci.* **2008**, *73*, H134–H140.

(16) Brownmiller, C.; Howard, L. R.; Prior, R. L. Processing and storage effects on monomeric anthocyanins, percent polymeric color, and antioxidant capacity of processed blueberry products. *J. Food Sci.* **2008**, *73*, H72–H79.

(17) Prior, R. L.; Wilkes, S.; Rogers, T.; Khanal, R.; Wu, X.; Howard, L. R. Purified blueberry anthocyanins and blueberry juice alter

development of obesity in mice fed an obesogenic high fat diet. *J. Agric. Food Chem.* **2010**, doi: 10.1021/jf902852d.
(18) Al-Awwadi, N. A.; Araiz, C.; Bornet, A.; Delbosc, S.; Cristol, J. P.; Linck, N.; Azay, J.; Teissedre, P. L.; Cros, G. Extracts enriched in different polyphenolic families normalize increased cardiac NADPH oxidase expression while having differential effects on insulin resistance, hypertension, and cardiac hypertrophy in high-fructose-fed rats. *J. Agric. Food Chem.* **2005**, *53*, 151–157.
(19) Matthews, D. R.; Hosker, J. P.; Rudenski, A. S.; Naylor, B. A. Homeostasis model assessment: Insulin resistance and β-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia* **1985**, *28*, 412–419.
(20) Peluso, M. R. Flavonoids attenuate cardiovascular disease, inhibit phosphodiesterase, and modulate lipid homeostasis in adipose tissue and liver. *Exp. Biol. Med.* **2006**, *231*, 1287–1299.
(21) Ferrell, J. E., Jr.; Chang Sing, P. D.; Loew, G.; King, R.; Mansour, J. M.; Mansour, T. E. Structure/activity studies of flavonoids as inhibitors of cyclic AMP phosphodiesterase and relationship to quantum chemical indices. *Mol. Pharmacol.* **1979**, *16*, 556–568.
(22) Ebal, E.; Cavalie, H.; Michaux, O.; Lac, G. Visceral fat and total body fat mass correlate differently with hormones in rat. *Pathol. Biol.* **2008**, *56*, 283–285.
(23) Brownmiller, C.; Howard, L. R.; Prior, R. L. Processing and storage effects on procyanidin composition and concentration of processed blueberry products. *J. Agric. Food Chem.* **2009**, *57*, 1896–1902.
(24) Hager, T. J.; Howard, L. R.; Prior, R. L. Processing and storage effects on monomeric anthocyanins, percent polymeric color, and antioxidant capacity of processed blackberry products. *J. Agric. Food Chem.* **2008**, *56*, 689–695.
(25) Wu, X.; Prior, R. L. Systematic identification and characterization of anthocyanins by HPLC-ESI-MS/MS in common foods in the United States: fruits and berries. *J. Agric. Food Chem.* **2005**, *53*, 2589–2599.

Received for review September 8, 2009. Revised manuscript received February 17, 2010. Accepted February 19, 2010. Financial support for these studies was provided in part by the National Research Initiative of the USDA Cooperative State Research Service, Grant 2005-35503-15409, USDA, Agricultural Research Service, and Arkansas Biosciences Institute. Mention of a trade name, proprietary product, or specific equipment does not constitute a guarantee by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products that may be suitable.