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Gene expression microarray analysis of the effects of grape anthocyanins in mice: a test of a hypothesis-generating paradigm

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Abstract

The mechanism(s) through which fruits, vegetables, and whole grains favorably affect health is not well established. Using an anthocyanin-rich grape as a model, we examined the ability of an agnostic analytical approach using gene expression microarrays to generate novel testable hypotheses regarding the mechanisms of action of potentially healthful foods and food components. C57BL/6 mice were divided into 2 groups and fed a proatherogenic diet with or without a semipurified anthocyanin extract (70% anthocyanins) incorporated at a level of 0.1 mg/mL into the drinking water. After 6 weeks, compared with control mice, mice supplemented with anthocyanins tended to gain more weight and have increased adipose tissue mass, although these effects did not achieve statistical significance. Anthocyanin-supplemented mice had significantly reduced relative liver weights and heart weights. Serum lipids and inflammatory cytokines were not different between the groups. Gene expression microarray analysis of the liver and skeletal muscle identified a number of molecular pathways significantly affected by anthocyanin treatment. Two distinct clusters emerged. The first cluster included down-regulated pathways in both muscle and liver involving cellular defense, whereas the second included hepatic genes involved in energy metabolism. From these data, 3 hypotheses were developed for future investigation.

1. Introduction

Increased consumption of fruits, vegetables, and whole grains and lower intakes of foods high in saturated fat are recommended by public agencies [1,2] largely because of supporting epidemiologic data. For saturated fat, the mechanism underlying the link between high intakes and disease (specifically, cardiovascular disease) is well established. Abundant evidence exists demonstrating an adverse effect of saturated fat on low-density lipoprotein cholesterol levels [3], a well-established risk factor for cardiovascular disease. However, the mechanism(s) through which fruits, vegetables, and whole grains favorably affect health is substantially less well established. We have previously suggested [4] that an agnostic approach (eg, without prior belief as to mechanism of action) using gene expression microarrays, proteomics, and metabolomic analytical methods could be used to generate novel testable hypotheses regarding the mechanisms of action of potentially healthful foods and food components.

In the present study, we apply gene expression microarray analysis to the investigation of the potential health benefits of an anthocyanin-rich grape extract (ACN-GE) as a partial test of this hypothesis-generating paradigm. Anthocyanins are polyphenolic compounds that provide color in berries such as grapes, blueberries, strawberries, and blackberries. Consumption in the United States is estimated at 12.5 mg/d [5]. Unlike other polyphenols, glycosides of anthocyanins are absorbed intact [6,7], suggestive of a potential unique role among polyphenols in human health. Previous work has focused on antioxidant and anti-inflammatory properties in relation to cardiovascular disease and maintenance of brain function with aging [8-13]. There is also evidence that anthocyanins may have

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anticarcinogenic, antiobesity, and antidiabetic effects as well [14-16]. The availability of prior data regarding the potential health effects of anthocyanins provides an opportunity to validate our approach through corroboration of hypotheses generated from our analyses with existing published hypotheses.

2. Methods

2.1. Anthocyanin-rich extract preparation

An ACN-GE was prepared from the highly pigmented wine grape A-1575. The extracts were prepared by solid-phase extraction using Amberlite XAD-7 (Sigma-Aldrich, St Louis, MO) resin. The final extract contained 67% anthocyanins (Table 1) as assessed by high-performance liquid chromatography analysis and was exceptionally rich in malvidin glucosides, with moderately high levels of petunidin and delphinidin glucosides. High-performance liquid chromatography analysis additionally confirmed the extract to be free of free sugars and organic acids.

2.2. Animals and diets

Twenty 5-week-old male C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME) were maintained at constant temperature (22°C-24°C) under an automated lighting with a 12-hour/12-hour light/dark cycle throughout the experiment. The mice were divided into 2 groups of 10 and fed for 6 weeks a proatherogenic diet (D01022601; Research Diets, New Brunswick, NJ) to increase oxidative stress. The diet provided 39.9% of energy as fat and 1.5 g/kg cholesterol (Table 2). Diets were provided ad libitum for the duration of the study.

A 10-mg/mL stock solution of the ACN-GE was prepared in ethanol. The ACN-GE stock solution was added to the drinking water of one group of mice (ACN group) to provide a final concentration of 0.1 mg/mL ACN-GE and 1% ethanol mice. The control group received drinking water with added ethanol alone. The ACN-GE–supplemented drinking water was provided in brown water bottles and changed every other day. Preliminary studies demonstrated that, under these conditions, the anthocyanin preparations remained stable. Water intake was monitored and did not differ between groups. The experimental design was approved by the Pennington Biomedical Research Center Institutional Animal Care and Use Committee.

Table 1
Anthocyanin content of A-1575 grape skin extract

Anthocyanin	Content (mg/g)
Cyanidin-(x)gluc	27
Delphinidin-(x)gluc	118
Malvidin-(x)gluc	317
Pelargonidin-(x) gluc	–
Peonidin-(x)gluc	69
Petunidin-(x)gluc	140
Total	671

Data are in milligrams per gram dry weight. "-(x)glucosides" includes 3-glucoside, 3-acetylglucoside, and 3-(*p*-coumaroyl)glucoside.

Table 2
Composition of base diet

Ingredient	g/kg diet
Casein	225
L-Cystine	3.4
Cornstarch	239
Maltodextrin 10	80
Sucrose	127
Cellulose	56
Soybean oil	28
Cocoa butter	175
Minerals [a]	51
Vitamins [b]	14
Cholesterol	1.5

[a] Minerals include mineral mix (S10021), dicalcium phosphate, calcium carbonate, and potassium citrate.
[b] Vitamins include vitamin mix (V10001) and choline bitartrate.

2.3. Collection of serum and tissues

Two days before the end of the experiment, body composition was determined by nuclear magnetic resonance (model mq10 NMR analyzer; Brucker, Milton, Ontario, Canada). At the end of the feeding period, the mice were fasted for 4 hours before blood was obtained by cardiac puncture under anesthesia. The liver, heart, kidney, spleen, adipose tissue depots (subcutaneous, retroperitoneal, epididymal, brown), and sample of thigh skeletal muscle were harvested, weighed, and flash frozen in liquid nitrogen. Serum was collected by centrifugation. All tissue and serum samples were stored at −70°C until used for assays.

2.4. Measurement of serum glucose, triglyceride, cholesterol, hormones, and cytokines

Serum glucose, triglycerides, and cholesterol were measured using commercially available kits. Serum cytokines were measured by multiplexed immunobeads (Luminex, Austin, TX) with reagents purchased from LINCO (LINCOplex; Millipore, St Charles, MO).

2.5. RNA preparation

Ribonucleic acid was isolated from liver and skeletal muscle using TRI Reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's protocol. Potential impurities were removed (RNeasy Mini Kit; Qiagen, Valencia, CA), and the quality of RNA was assessed using a 1.5% agarose gel stained with ethidium bromide.

2.6. Gene expression analysis

Microarrays were prepared by printing oligonucleotides (mouse library; QIAGEN Operon, Alameda, CA) suspended

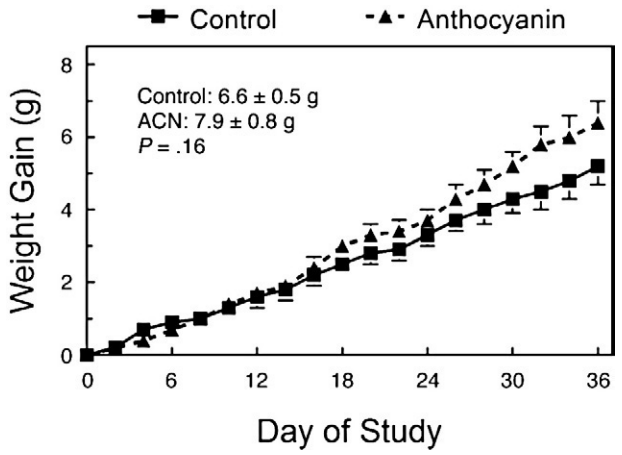

Fig. 1. Weight gain of control and ACN-GE–treated mice over the course of the study.

in 45% (vol/vol) dimethyl sulfoxide onto polylysine-coated, glass microscope slides using the GeneMachines OmniGrid microarrayer (San Carlos, CA). The mouse oligonucleotide library consists of 70mers that represent more than 13 000 well-characterized genes.

Gene-expression microarray analysis was performed using the MICROMAX TSA Labeling and Detection Kit protocol (PerkinElmer Life Sciences, Boston, MA). Samples from each treatment were pooled to yield a total of 4 to 6 μg of RNA per biotin (B) or fluorescein (F) label. A total of 6 slides were used per experiment with 3 for forward labeling (group 1 is B; group 2 is F) and 3 for reverse labeling (group 1 is F; group 2 is B). Slides were scanned using the ScanArray 5000 (Packard BioChip Technologies, Billerica, MA), and the data were normalized [17].

2.7. Statistical analysis

All phenotypic data are presented as mean ± SEM. Differences between means were assessed by Student *t* test. *P* values < .05 were considered significant.

Results from microarray array gene expression analysis were analyzed by GenMapp and MAPPFinder (Gladstone Institutes, University of California, San Francisco, CA) to identify molecular pathways or gene groupings significantly affected by the ACN-GE treatment. Pathways significantly affected at an unadjusted *P* value ≤ .001 were examined. Duplicate or synonymous pathways were removed, as were pathways in which the primary difference in gene expression resided in the "interaction partners" rather than the main metabolic pathway.

Table 3
Effect of ACN-GE on body composition and relative organ weights

Tissue	Control group	ACN group
	% of body weight	
Total fat	17.1 ± 0.9	19.7 ± 1.9
Liver	3.81 ± 0.07	3.57 ± 0.07 *
Heart	0.49 ± 0.02	0.44 ± 0.01 *
Kidney	1.18 ± 0.03	1.10 ± 0.04
Spleen	0.29 ± 0.01	0.27 ± 0.01
Brown fat	0.23 ± 0.01	0.22 ± 0.02
Retroperitoneal fat	0.54 ± 0.04	0.68 ± 0.10
Epididymal fat	2.47 ± 0.14	2.90 ± 0.34
Subcutaneous fat	1.31 ± 0.07	1.48 ± 0.12

* $P < .05$.

Table 4
Effect of ACN-GE on serum hormones

Hormone	Control group	ACN group
Insulin (pg/mL)	138 ± 17	178 ± 26
Adiponectin (pg/mL)	1473 ± 104	1446 ± 121
Leptin (pg/mL)	94 ± 15	135 ± 46

Control group, n = 10; ACN group, n = 5.

3. Results

3.1. Weight gain, body composition, and relative organ weights

As compared with control mice, mice supplemented with ACN-GE tended to gain more weight (1.3 g, Fig. 1), composed of 33% fat (0.43 g) and 67% fat-free mass (0.87 g). The ACN-GE mice tended to have increased adiposity, although these effects did not achieve statistical significance (Table 3). The ACN-GE–supplemented mice had significantly reduced relative liver and heart weights and near-significantly reduced kidney weights (P = .09). Retroperitoneal, epididymal, and subcutaneous fat depots tended to be higher in the ACN group, but were not significantly different from the control group.

Table 5
Molecular pathways significantly ($P \leq .001$) affected by ACN-GE in the liver

Pathway name	Number measured on pathway	Percent changed	Z score
Pathways significantly up-regulated			
All pathways (reference)	10 025	6.6	0
Wnt signaling	269	14.9	4.8
Pathways significantly down-regulated			
All pathways (reference)	10 025	6.0	0
Ribosomal proteins	69	34.8	9.4
Electron transport chain	61	24.6	5.6
Metabolism	182	16.5	5.4
Acute inflammatory response	67	20.9	5.1
Cholesterol biosynthesis	12	41.7	4.9
Fatty acid β-oxidation	28	28.6	4.7
TCA cycle	18	33.3	4.5
Selenium metabolism-selenoproteins	35	22.9	3.8
Energy derivation by oxidation of organic compounds	62	17.7	3.5

3.2. Serum glucose, cholesterol, triglycerides, hormones, and cytokines

Serum cholesterol, triglycerides, and glucose levels were not different between groups (data not shown). Insulin and leptin levels tended to be higher in the ACN group, but the differences were not significantly different (Table 4). Adiponectin levels were similar between the 2 groups. Levels of granulocyte macrophage colony-stimulating factor; interferon-γ; tumor necrosis factor–α; and interleukins 1b, 2, 4, 5, 6, 10, and 12 were all low and not different between the 2 groups (data not shown).

3.3. Gene expression microarray results

In the liver, only the Wnt signaling pathway met our criteria (unadjusted $P \leq .001$) for consideration as a pathway up-regulated by ACN-GE supplementation (Table 5). In contrast, 9 pathways met our criteria for down-regulation. Of these 9 pathways, 5 are directly related to energy metabolism (metabolism, energy derivation by oxidation of organic compounds, electron transport chain, fatty acid β-oxidation, and tricarboxylic acid [TCA] cycle), with 3 pathways (electron transport chain, fatty acid β-oxidation, and TCA cycle) specific to the mitochondria. The inclusion of the "acute inflammatory response" genes is in large part due to the down-regulation of a significant number of genes in the "complement activation" subgroup (24% of genes down-regulated, $P = .002$). The "selenium metabolism-selenoproteins" includes 3 significantly down-regulated antioxidant enzymes (glutathione peroxidase 4, ↓47%; selenoprotein K, ↓30%; selenoprotein X1, ↓40%) and glutathione peroxidase 1 (↓41%, $P = .07$).

In muscle, only the "translation reactome" pathway met our criteria for up-regulation (Table 6). Six pathways were significantly down-regulated. Of these, 4 pathways dealt directly or indirectly with cellular defenses (response to wounding and its subcategories inflammatory response and complement activation; immunoglobulin-mediated immune response).

Table 6
Molecular pathways significantly ($P \leq .001$) affected by ACN-GE in skeletal muscle

Pathway name	Number measured on pathway	Percent changed	Z score
Pathways significantly up-regulated			
All pathways (reference)	11 424	3.3	0
Translation reactome	280	8.2	4.501
Pathways significantly down-regulated			
All pathways (reference)	11 424	4.3	0
Extracellular matrix	205	12.7	5.7
Complement activation	15	33.3	5.4
Response to wounding	158	12.0	4.6
Carbohydrate binding	140	12.1	4.4
Inflammatory response	151	11.9	4.8
Immunoglobulin-mediated immune response	17	23.5	4.6

4. Discussion

The objective of this study was to determine if testable hypotheses could be developed regarding the potential health benefits of foods or food components by using an agnostic analytical and phenotyping approach. Our primary tool in this approach was gene expression profiling by microarray followed by pathway analysis. This allowed us to survey the effects of our dietary supplementation on more than 4500 physiological processes/gene groupings as defined by gene ontology terms. Our selection of an anthocyanin-rich grape skin extract was based in large part on the availability of a grape variety (A-1575) that is exceptionally rich in anthocyanins and on published literature suggesting positive health benefits of anthocyanins [18]. The study was conducted in the C57BL/6 mouse, a model used extensively in metabolic studies, allowing comparison with other studies. Our diet was high in fat and cholesterol, which has been used to stress a number of metabolic systems including those involved with lipid and carbohydrate metabolism, oxidative stress, and inflammatory response. The level of the anthocyanin-rich extract was relatively modest and was calculated to provide, on a metabolic body weight basis, an equivalent of approximately 150 mg anthocyanins per day for a 70-kg human. This amount approximates the intake of between 400 and 750 mL of red wine [19].

A number of physiological pathways were significantly affected by the addition of ACN-GE to the drinking water. Two distinct clusters emerged. The first cluster included down-regulated pathways in both muscle and liver involving cellular defense. The "inflammatory response" gene grouping was down-regulated in both liver and muscle. Secondary investigation of additional significantly ($P < .05$) down-regulated pathways in the liver identified genes involved in "response to oxidative stress" (16% of genes down-regulated, $P = .008$) and "response to unfolded proteins" (17% of genes down-regulated, $P = .008$) as additional targets of ACN-GE. From these data, we can logically hypothesize that *an anthocyanin-rich extract from grape decreases tissue inflammation by reducing oxidative stress*.

This first hypothesis, although generated de novo from our data, is not novel. It is well established that anthocyanins are potent antioxidants [10-14]. Furthermore, both in vitro and in vivo models have demonstrated that anthocyanins from a variety of sources can inhibit the inflammatory process [20-24]. That our agnostic approach independently yielded a hypothesis already under consideration by other investigators partially validates our hypothesis-generating paradigm.

The second cluster centered on energy metabolism in the liver with major metabolic pathways down-regulated including the TCA cycle, fatty acid β-oxidation, and cholesterol biosynthesis. These pathways are under control of a number of nuclear receptors, suggesting the hypothesis that *an anthocyanin-rich extract from grape through*

modulation of the activities of specific nuclear hormone receptors and transcription factors such as liver x-receptor, peroxisome proliferators–activated receptors (PPARα, PPARδ, PPARγ), sterol regulatory element binding protein (SREBP)–1c, and/or PPARγ coactivator–1α and –1β alter substrate metabolism in the liver. The ligands for several of these nuclear receptors are oxidized derivatives of sterols or fatty acids. By virtue of its antioxidant capacity, the ACN-GE may modulate the endogenous levels of specific ligand activators and affect the activities of their nuclear receptors. However, we cannot exclude the possibility of direct interactions of anthocyanins with the nuclear receptors.

Effects of polyphenolic compounds on metabolic pathways involved in energy metabolism are not without precedent. Sesamin, a polyphenolic lignan compound found in sesame oil, was shown to decrease both the enzyme activity and expression levels of enzymes involved in fatty acid synthesis, while increasing the activity of enzymes involved in fatty acid oxidation [25]. A reduction in the levels of SREBP-1c and conversion of SREBP-1c to its mature form led the authors to conclude that sesamin affected lipid metabolism through modulation of SREBP-1c activity. In a study of purple corn anthocyanin effects, Tsuda et al [26] demonstrated that isolated rat adipose tissue incubated with cyanidin for 24 hours had elevated expression levels of PPARγ. It is noteworthy that PPARγ in adipocytes is thought to induce lipogenesis through modulation of SREBP-1c activity [27]. Thus, both studies suggest involvement of nuclear receptors, consistent with our developed hypothesis.

However, we also note that many of the affected pathways in the liver are localized to the mitochondria. Thus, we have also developed a competing hypothesis that states that *an anthocyanin-rich extract from grape inhibits mitochondrial biogenesis through modulation of the activity of either PPARγ coactivator–1α or –1β or its downstream targets nuclear respiratory factor–1 and –2*. The PPARγ coactivator–1α and –1β are induced under conditions of oxidative stress [28]. Thus, the antioxidant properties of anthocyanins may decrease hepatic oxidative stress and PPARγ coactivator–1α and –1β expression, leading to reduced mitochondrial biogenesis.

Other pathways were clearly influenced by ACN-GE, and additional hypotheses may be developed. However, for the purpose of this proof-of-concept study, we have focused on the 2 largest clusters of affected pathways. We have demonstrated that an agnostic approach could be used to develop testable hypotheses for future investigation into the potential health benefits of foods and food components. This approach could be strengthened by the concomitant application of proteomic and metabolomic methods. In the present study, we also conducted a proteomic analysis of liver proteins. Preliminary analysis of these data confirms the effects of the ACN-GE on the levels of proteins involved in oxidative response and energy metabolism (Lefevre, manuscript in preparation).

Finally, it should be emphasized that the designed outcome of these studies is new hypotheses to be tested in future studies. The microarray gene expression data have not been confirmed by real-time polymerase chain reaction; and thus, these data should not be taken as definitive evidence of an effect of ACN-GE on the pathways discussed. Additional studies specifically designed to test these developed hypotheses are required.

Acknowledgment/Conflict of Interest

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