JOURNAL OF MEDICINAL FOOD
J Med Food 14 (12) 2011, 1511–1518

DOI: 10.1089/jmf.2010.0292

Blueberry Intake Alters Skeletal Muscle and Adipose Tissue Peroxisome Proliferator-Activated Receptor Activity and Reduces Insulin Resistance in Obese Rats

E. Mitchell Seymour, Ignasia I. Tanone, Daniel E. Urcuyo-Llanes, Sarah K. Lewis, Ara Kirakosyan, Michael G. Kondoleon, Peter B. Kaufman, and Steven F. Bolling

Cardiovascular Center and the Michigan Integrative Medicine Program, University of Michigan Health System, Ann Arbor, Michigan, USA.

ABSTRACT Metabolic syndrome can precede the development of type 2 diabetes and cardiovascular disease and includes phenotypes such as obesity, systemic inflammation, insulin resistance, and hyperlipidemia. A recent epidemiological study indicated that blueberry intake reduced cardiovascular mortality in humans, but the possible genetic mechanisms of this effect are unknown. Blueberries are a rich source of anthocyanins, and anthocyanins can alter the activity of peroxisome proliferator-activated receptors (PPARs), which affect energy substrate metabolism. The effect of blueberry intake was assessed in obesity-prone rats. Zucker Fatty and Zucker Lean rats were fed a higher-fat diet (45% of kcal) or a lower-fat diet (10% of kcal) containing 2% (wt/wt) freeze-dried whole highbush blueberry powder or added sugars to match macronutrient and calorie content. In Zucker Fatty rats fed a high-fat diet, the addition of blueberry reduced triglycerides, fasting insulin, homeostasis model index of insulin resistance, and glucose area under the curve. Blueberry intake also reduced abdominal fat mass, increased adipose and skeletal muscle PPAR activity, and affected PPAR transcripts involved in fat oxidation and glucose uptake/oxidation. In Zucker Fatty rats fed a low-fat diet, the addition of blueberry also significantly reduced liver weight, body weight, and total fat mass. Finally, Zucker Lean rats fed blueberry had higher body weight and reduced triglycerides, but all other measures were unaffected. In conclusion, whole blueberry intake reduced phenotypes of metabolic syndrome in obesity-prone rats and affected PPAR gene transcripts in adipose and muscle tissue involved in fat and glucose metabolism.

KEY WORDS: • ***anthocyanins*** • ***cardiovascular disease*** • ***diabetes*** • ***insulin resistance*** • ***muscle*** • ***obesity*** • ***peroxisome proliferator-activated receptor***

INTRODUCTION

METABOLIC SYNDROME IS A CLUSTER of factors including hyperlipidemia, insulin resistance, and obesity that commonly precedes the development of type 2 diabetes and heart disease. A diet rich in plant foods is inversely correlated with cardiovascular morbidity and mortality, and non-nutritive phytochemicals in fruits, vegetables, and grains may participate in this benefit. Anthocyanins are a subclass of flavonoids that confer red, blue, purple, and black pigmentation in plants. Highbush blueberries (*Vaccinium corymbosum* L.) are a rich fruit source of anthocyanins. A recent epidemiology study showed that blueberry intake was correlated with reduced mortality from cardiovascular disease and coronary heart disease.[1] However, it is unclear how blueberry intake conferred these benefits.

Manuscript received 17 October 2010. Revision accepted 21 April 2011.

Address correspondence to: E. Mitchell Seymour, Ph.D., Cardiovascular Center and the Michigan Integrative Medicine Program, University of Michigan Health System, B560 MSRB2 0686, 1500 West Medical Center Drive, Ann Arbor, MI 48109, USA, E-mail: mitchell.seymour@gmail.com

In addition to observational studies, interventional clinical studies also suggest that blueberry intake affects cardiovascular risk factors. In adults with metabolic syndrome, blueberry intake for 8 weeks (50 g of freeze-dried berries/day, equal to approximately 350 g or 2.5 cups of fresh berries), reduced blood pressure and oxidized low-density lipoprotein (LDL).[2] In another study, subjects with metabolic syndrome consumed blueberry powder for 6 weeks (45 g of freeze-dried powder, approximately equal to 2 cups of fresh blueberries/day). Results indicated improved insulin sensitivity without significantly changed adiposity or inflammatory biomarkers.[3] The mechanisms of these compelling effects are unknown but may be explored in relevant animal models.

Several studies have suggested that anthocyanin-rich botanical extracts can modify lipid dynamics[4–7] and glucose metabolism.[8–10] Isolated anthocyanins and anthocyanin-rich extracts increase the activity of the transcription factor peroxisome proliferator-activated receptor (PPAR).[11,12] PPAR agonist drugs in humans and animals stimulate fat metabolism, inhibit fat storage, and improve insulin resistance.[13] However, PPAR agonist drugs such as fibrates

and thiazolidinediones may cause undesirable side effects that increase relative risk and reduce patient compliance. It is then compelling that a diet intervention may provide a nutraceutical effect on PPARs and reduce risk factors for diabetes and heart disease.

The Zucker Fatty rat has a mutation in the leptin receptor and develops spontaneous obesity, hyperlipidemia, insulin resistance, and systemic inflammation. These phenotypes are worsened by a high-fat diet and are reduced by PPAR agonist drugs.[14–16] Our central hypothesis was that blueberry-enriched diets reduce the development of metabolic syndrome in Zucker Fatty rats. Furthermore, blueberry intake would alter PPAR isoform activity and activity of genes related to glucose and lipid metabolism in skeletal muscle and abdominal fat, two tissues affected by metabolic syndrome.

MATERIALS AND METHODS

Study design and animal care

The factorial design includes the factors of rat strain (Zucker Lean and Zucker Fatty), diet fat content (15% or 45%), and diet supplementation (2% [wt/wt] blueberry powder or 2.8% glucose:fructose [1:1]). Male Zucker Fatty and Zucker Lean rats (6 weeks old) were acquired from Harlan (Indianapolis, IN, USA) and were housed two per cage. Rats were housed on 12-hour light:dark cycles. At 7 weeks of age, both strains of rats were randomized to four groups ($n = 12$ each): high-fat (45% kcal) diet with 2% blueberry powder; high-fat diet with 1.84% additional carbohydrate (dextrose:fructose 1:1); low-fat (10% kcal) diet with 2% blueberry powder; and low-fat diet with 1.84% additional carbohydrate (dextrose:fructose 1:1). Semipurified diets are as described in Supplementary Tables S1 and S2 (Supplementary Data are available online at www.liebertonline.com/jmf). The blueberry product was a freeze-dried powder from whole highbush blueberries (Tifblue and Rubel cultivars, 1:1) provided by the U.S. Highbush Blueberry Council (Folsom, CA, USA). Nutrient content of the blueberry powder is described in Table 1. Anthocyanin analysis was conducted using liquid chromatography–mass spectrometry (protocol in the Supplementary Data, results in Supplementary Fig. S1 and Supplementary Table S3). Diets were mixed weekly, vacuum-sealed, and stored at 4°C. Blueberry-fed rats were pair-fed to their respective control group and were provided 13–20 g of diet/day per rat, adjusted for increasing intake during maturation. The daily provision was approximately 10% below *ad libitum* intake to ensure complete consumption and equal food intake among all rats in the study. Water was provided *ad libitum*. Rats were sacrificed after 90 days of diet consumption. This protocol was approved by the University of Michigan's University Committee on the Use and Care of Animals.

Dual X-ray absorptiometry

Lean body mass and fat mass were measured by dual X-ray absorptiometry using a Norland Medical Systems (Fort Atkinson, WI, USA) peripheral dual X-ray absorptiometry scanner with pDEXA Sabre software version 3.9.4 set in ''research mode.'' Rats were anesthetized with 4% isoflurane and maintained with 1% isoflurane. After calibration, each rat was placed on the platform and scanned from nose to anus at 30 mm/second with a resolution of 1.0×1.0 mm. Results for percentage fat mass and lean body mass were determined relative to body weight on the day of the scan.

Table 1. Nutrient Composition, Oxygen Radical Absorbance Capacity, and Total Phenolic and Total Anthocyanin Contents of Blueberry Powder

Property	*Value*
Per 100 g	
Calories (kcal)	347.32
Protein (g)	3.47
Carbohydrates (g)	84.66
Fat (g)	0
Total sugars (g)	58.90
Dietary fiber (g)	18.67
Vitamin C (mg)	172.21
Calcium (mg)	30.39
Iron (mg)	1.01
Potassium (mg)	408.10
Sodium (mg)	16.06
ORAC (μmol of TE/g)	356
Total phenolics (mg of GAE/g)	32.48
Total anthocyanins (mg/g)[a]	14.84

[a]pH differential method.

GAE, gallic acid equivalents; ORAC, oxygen radical absorbance capacity; TE, Trolox equivalents.

Serum and plasma measures

Blood was obtained by lancet to the saphenous vein in conscious, restrained animals. Glucose was measured with a handheld glucose meter (TRUE™, Homediagnostics, Fort Lauderdale, FL, USA). Glucose area under the curve (AUC) was determined approximately 1 week before the study conclusion. Fasting blood glucose was determined after an overnight fast. Afterward, water gavage (1 mL) was administered, and fasting glucose was again determined after 30 minutes. The mean of the two values served as the baseline fasting glucose value. A glucose bolus (2 g/kg of body weight) was then given orally, and blood glucose was measured at 30-minute intervals for 3 hours. Glucose AUC was determined using GraphPad Prism version 5.0 (GraphPad Software, La Jolla, CA, USA).

For lipids and insulin, serial blood samples (0.5 mL) were obtained from the saphenous vein in restrained, conscious animals. Whole blood was collected in serum-separator tubes, allowed to clot at room temperature, and spun at 5,000 *g* for 7 minutes at room temperature. Fasting serum free fatty acids, total cholesterol, high-density lipoprotein (HDL), and total triglycerides were measured by the automated IDEXX Laboratories (Westbrook, ME, USA) VetTest® chemistry analyzer. Fasting insulin was measured by

radioimmunoassay (Bachem, Torrance, CA, USA), and insulin resistance was measured by the homeostasis model index of insulin resistance (HOMA-IR), the formula for which is (fasting insulin [mU/L] × fasting glucose [mmol/L]/22.5). After 90 days of feeding, rats were fasted for 18 hours and sacrificed by decapitation. The first fraction of trunk blood (~8 mL) was collected in EDTA tubes, spun for 15 minutes at 6,000 *g* at 4°C, and stored at −80°C until further analysis. Plasma interleukin-6, tumor necrosis factor-α, and C-reactive protein were measured using commercial immunoassays according to the manufacturers' instructions. The remaining fraction of trunk blood was collected (~0.5 mL) in a beaker, allowed to clot at room temperature, transferred to serum-separator tubes, and spun at 5,000 *g* for 7 minutes at room temperature. The serum was used for terminal measures of insulin, glucose, free fatty acids, and lipoproteins as conducted for earlier serial measures.

Tissue PPAR activity

Tissues were snap-frozen in liquid nitrogen and stored at −80°C until further analysis. Tissues were fractionated to obtain nuclear and cytosolic homogenates using the nuclear extraction kit (Cayman Chemical, Ann Arbor, MI, USA) as we previously described.[17,18] Once successful fractionation was confirmed by immunoblot, PPAR-α and PPAR-γ activities were determined in nuclear extracts using transcription factor DNA binding assays (Cayman Chemical) according to the manufacturer's instructions. A specific, proprietary oligonucleotide containing PPAR response elements was immobilized onto the bottom of the wells of a 96-well plate. If present in the nuclear extract (loaded at 10 μg per well), PPAR isoforms bind to the well-bound oligonucleotide PPAR response elements. Binding was then detected by addition of specific primary antibodies directed against the individual PPAR isoforms. A secondary antibody conjugated to horseradish peroxidase was added to enable colorimetric detection by reaction with the substrate tetramethylbenzidine/hydrogen peroxide and measurement of subsequent color development at 450 nm. Values were expressed as optical density relative to total protein as determined by the BCA assay (Pierce Chemical, Rockford, IL, USA).

Reverse transcription–polymerase chain reaction

Total RNA from frozen retroperitoneal fat was isolated with RNeasy™ Lipid Tissue Midi kit (Qiagen, Valencia, CA, USA), and skeletal muscle RNA was isolated using the RNeasy FibrousTissue Mini kit following the manufacturer's protocol. Samples ($n = 6$ per group) were analyzed by reverse transcription–polymerase chain reaction with an ABI 7900 HT real-time polymerase chain reaction instrument (Applied Biosystems, Carlsbad, CA, USA) using a custom rat RT2 Profiler polymerase chain reaction array and its proprietary reagents (SABiosciences, Frederick, MD, USA). Relative expression was determined by the $\Delta\Delta CT$ method as described by Livak and Schmittgen.[19] ΔCT for each gene (averaged across six animals per group) was calculated relative to the average of five housekeeping genes that were unaffected by treatment (P1 large ribosomal protein, hypoxanthine guanine phosphoribosyl transferase, ribosomal protein L13A, lactate dehydrogenase A, and β-actin). $\Delta\Delta CT$ values were back-transformed ($2^{-\Delta\Delta CT}$) to calculate the fold change of each transcript.

Statistical methods

Transcript differences were determined ± SD using the $\Delta\Delta CT$ method as described by Livak and Schmittgen,[19] using the polymerase chain reaction array data analysis web portal of SABiosciences. Results are expressed as mean fold change. All other experimental values were expressed ± SEM and compared using analysis of variance followed by Bonferroni's post test for between-group comparisons. Specifically, comparisons were made between the control diet and blueberry-fed animals within one diet type (low-fat or high-fat diet). Statistical analysis was conducted by GraphPad Prism version 5.0. For all measures, a value of $P < .05$ was considered statistically significant.

RESULTS

In Zucker Fatty rats prone to obesity and fed a high-fat diet, the incorporation of blueberry powder reduced abdominal fat but did not significantly reduce body weight or total fat mass (Table 2). In addition, the blueberry diet reduced plasma triglycerides and improved insulin sensitivity as reflected by reduced fasting insulin, reduced HOMA-IR, and reduced glucose AUC following an oral glucose bolus. Finally, blueberry diet did not affect plasma inflammatory markers despite the blueberry-associated reduction of abdominal fat, a known source of pro-inflammatory cytokines. It is possible that the reduction in abdominal fat was insufficient to reduce systemic inflammation. In animals prone to obesity and fed a low-fat diet, the incorporation of blueberry powder appears to have the same benefits as in the higher-fat diet, but with additional benefits of reduced total percentage fat mass, liver mass, and free fatty acids. As such, in animals prone to obesity, a low-fat diet with blueberry is the most beneficial approach to reduce indices of metabolic syndrome.

In Zucker Lean rats fed a high-fat diet, blueberry intake increased body weight and reduced serum triglyceride (Table 3). Zucker Lean rats fed a low-fat diet showed no impact from blueberry on any measured parameters. Given the limited effect on Zucker Lean rats on metabolic syndrome phenotypes, the remaining molecular assays were conducted exclusively in the Zucker Fatty rats fed a high-fat diet or a low-fat diet with or without blueberry.

Blueberry intake affected PPAR DNA binding activity in abdominal adipose tissue and skeletal muscle (Figs. 1 and 2, respectively). In abdominal fat, blueberry intake increased PPAR-α activity and PPAR-γ activity (high-fat blueberry-fed vs. high-fat control and low-fat blueberry-fed vs. low-fat control). In skeletal muscle, blueberry intake increased PPAR-α activity (high-fat blueberry-fed vs. high-fat control)

Table 2. Treatment Effects in Zucker Fatty Rats

	HF		*LF*	
	HF-CON	*HF-BB*	*LF-CON*	*LF-BB*
Body weight	489.6 ± 30.9	483.6 ± 20.9	413.5 ± 18.8	444.5 ± 33.2
Body weight/TL	139	138	125	111
Lean BM (g)/cm^2	3.53 ± 0.7	3.40 ± 0.36	3.42 ± 0.4	3.49 ± 0.24
Fat mass (g)/cm^2	6.5 ± 0.26	6.3 ± 0.34	6.4 ± 0.32	6.0 ± 0.26*
Liver (g)/TL	7.61 ± 1.1	7.86 ± 0.6	8.0 ± 0.93	6.7 ± 0.79*
RP fat (g)/TL	6.28 ± 0.33	5.8 ± 0.29*	4.79 ± 0.6	4.18 ± 0.31*
EPI fat (g)/TL	4.12 ± 0.3	3.6 ± 0.23*	3.97 ± 0.28	3.55 ± 0.26*
Fasting glucose	138 ± 8	134 ± 9	121 ± 8	125 ± 7
Glucose AUC	23,789 ± 1,136	22,842 ± 1,345*	21,646 ± 1,265	20,227 ± 1,312*
Insulin	6.6 ± 0.8	5.3 ± 1*	4.4 ± 0.5	3.8 ± 0.5*
HOMA-IR	64.3 ± 2.3	50.3 ± 2.4*	37.7 ± 1.8	33.6 ± 1.2*
Total triglyceride	293 ± 17	278 ± 14*	323 ± 24	310 ± 24*
Total cholesterol	220 ± 18	223 ± 18	214 ± 15	210 ± 15
Free fatty acids	0.9 ± 0.02	0.8 ± 0.1	0.6 ± 0.03	0.3 ± 0.02*
HDL	0.4 ± 0.04	0.5 ± 0.04	0.3 ± 0.04	0.4 ± 0.05
IL-6	301 ± 28	294 ± 36	179 ± 27	153 ± 18
TNF-α	47 ± 11	37 ± 7	39 ± 7	36 ± 6

Data are mean ± SEM values. Body weight is in g. Tibial length (TL) is in cm. Fasting glucose, total triglyceride, and total cholesterol are in mg/dL. Homeostasis model index of insulin resistance (HOMA-IR) was determined by the equation (fasting glucose [mmol/L] × fasting insulin [mU/L])/22.5. High-density lipoprotein (HDL) is in mg/mL. Free fatty acids are in mmol/L. Insulin, interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α) are in ng/mL.

Comparisons are between the blueberry-fed (BB) and control (CON) diet for each diet (high-fat diet [HF] and low-fat diet [LF]): *$P < .05$ versus the respective CON group.

AUC, area under the curve; BM, body mass; EPI, epididymal abdominal; RP, retroperitoneal abdominal.

and PPAR-γ activity (high-fat blueberry-fed vs. high-fat control and low-fat blueberry-fed vs. low-fat control).

Blueberry-associated changes in PPAR activity paralleled changes in PPAR mRNA and PPAR-regulated gene transcripts (Table 4). In abdominal fat of high-fat-fed rats, blueberry intake increased PPAR-α and PPAR-γ co-activator-1α mRNA and mRNA transcripts related to fat burning (acyl-coenzyme A [CoA] oxidase) and to glucose uptake (glucose transporter 4) but reduced mRNA related to fat storage (fatty acid synthase). In low-fat-fed rats, blueberry intake increased PPAR-α, PPAR-γ, and PPAR-γ co-activator-1α mRNA and mRNA transcripts related to fat burning (acyl-CoA oxidase and lipase) and to glucose uptake (insulin receptor substrate 1 and glucose transporter 4) but reduced

Table 3. Treatment Effects in Zucker Lean Rats

	HF		*LF*	
	HF-CON	*HF-BB*	*LF-CON*	*LF-BB*
Body weight	449.5 ± 21	462 ± 30*	390 ± 16	382 ± 33
Body weight/TL	104	105	100	97
Lean BM (g)/cm^2	4.5 ± 0.6	4.3 ± 0.7	6.5 ± 0.3	6.3 ± 0.3
Fat mass (g)/cm^2	4.3 ± 0.3	4.6 ± 0.4	3.6 ± 0.26	3.5 ± 0.34
Liver (g)/TL	3.13 ± 0.049	2.99 ± 0.41	3.03 ± 0.23	2.8 ± 0.3
RP fat (g)/TL	3.8 ± 1.19	3.9 ± 0.56	3.3 ± 0.61	3.1 ± 0.52
EPI fat (g)/TL	2.94 ± 0.64	3.1 ± 0.4	2.6 ± 0.32	2.4 ± 0.61
Fasting glucose	107 ± 8	103 ± 12	105 ± 9	101 ± 12
Glucose AUC	18,679 ± 1,234	17,934 ± 1,158	14,634 ± 1,202	14,425 ± 1,223
Fasting insulin	1.1 ± 0.3	0.9 ± 0.2	0.4 ± 0.1	0.4 ± 0.06
HOMA-IR	8.3 ± 0.3	6.6 ± 0.4	3.0 ± 0.3	2.9 ± 0.2
Total triglyceride	65 ± 4	52 ± 3*	47 ± 6	43 ± 4
Total cholesterol	80 ± 12	93 ± 13	64 ± 15	60 ± 13
Free fatty acids	0.5 ± 0.1	0.4 ± 0.1	0.4 ± 0.04	0.3 ± 0.05
HDL	0.3 ± 0.03	0.3 ± 0.06	0.4 ± 0.05	0.4 ± 0.03
IL-6	89 ± 14	80 ± 26	57 ± 15	63 ± 14
TNF-α	12 ± 4	15 ± 5	17 ± 6	14 ± 6

Data are mean ± SEM values.

Comparisons are between the BB and CON diet for each diet (HF and LF): *$P < .05$ versus the respective CON group.

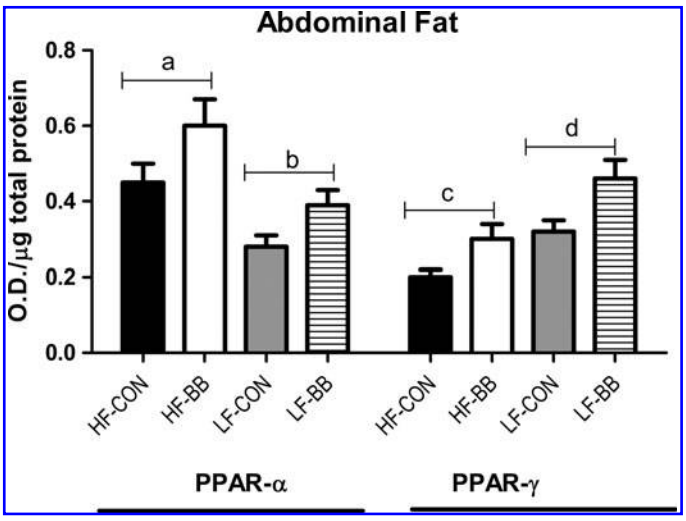

FIG. 1. Peroxisome proliferator-activated receptor (PPAR)-α and PPAR-γ activity in RP fat nuclear extracts. Data are mean ± SEM values ($n = 6$ per group). [abcd]Different letters indicate significant difference ($P < .05$). O.D., optical density.

mRNA related to fat storage (fatty acid synthase and fatty acid-CoA ligase). If translated into altered protein expression, these collective mRNA changes by blueberry would favor reduced fat storage and improved glucose utilization, effects supported by the blueberry-altered phenotypes listed in Table 4.

In skeletal muscle, blueberry intake affected mRNA related to PPAR isoforms and energy substrate metabolism (Table 5). In high-fat-fed rats, the intake of blueberry increased mRNA transcripts related to glucose transport and utilization (glucose transporter 4, phosphofructokinase, and pyruvate dehydrogenase kinase 4). Blueberry intake also increased PPAR-α and PPAR-γ co-activator-1α mRNA and insulin receptor substrate 1 mRNA. In low-fat-fed rats,

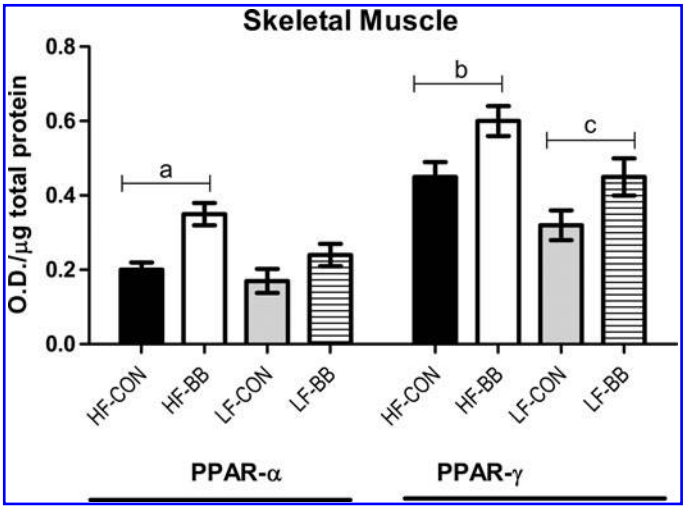

FIG. 2. PPAR-α and PPAR-γ activity in skeletal muscle nuclear extracts. Data are mean ± SEM values ($n = 6$ per group). [abc]Different letters indicate significant difference ($P < .05$).

Table 4. Zucker Fatty Rat Retroperitoneal Fat mRNA Changes

	Fold change by BB	
RP fat mRNA	*HF-BB*	*LF-BB*
PPAR-α	1.8*	2.0*
PPAR-γ	1.3	1.5*
PPAR-γ co-activator 1α	2.5*	1.9*
Acyl-CoA oxidase	1.9*	1.7*
Lipase, hormone sensitive	1.3	2.2*
Fatty acid synthase	0.74*	0.85
Fatty acid-CoA ligase	0.9	0.72*
Pyruvate dehydrogenase kinase 4	1.2	1.1
Glucose transporter 4	1.6*	1.8*
Insulin receptor substrate 1	1.7*	1.3*

Data are mean ± SEM values.
*$P < .05$ versus the respective HF or LF CON diet group.
CoA, coenzyme A.

blueberry intake increased PPAR-α, PPAR-γ, and PPAR-γ co-activator-1α mRNA and mRNA transcripts related to glucose uptake/metabolism (insulin receptor substrate 1 and glucose transporter 4). If translated into altered protein expression, these collective mRNA changes by blueberry would favor improve glucose utilization, effects supported by the blueberry-altered phenotypes listed in Table 4.

DISCUSSION

The intake of fruits and vegetables is inversely related to cardiac risk factors and cardiovascular disease, but certain fruits and vegetables may provide more benefit for specific risk factors. The Iowa Women's Health Study recently revealed that among postmenopausal women ($n = 34,489$) followed over 16 years, regular intake of certain diet-derived phytochemicals was inversely related to cardiovascular disease mortality.[1] Phytochemicals like anthocyanins, flavones, flavanones, and proanthocyanidins reduced cardiovascular disease mortality after adjustment for subject age and energy intake. However, of all phytochemical classes, only anthocyanin intake sustained significant benefit for

Table 5. Zucker Fatty Skeletal Muscle mRNA Changes

	Fold change by BB	
Skeletal muscle mRNA	*HF-BB*	*LF-BB*
PPAR-α	1.3	1.5*
PPAR-γ	1.6*	1.8*
PPAR-γ co-activator 1α	2.7*	2.5*
Fatty acid synthase	0.9	0.8
Uncoupling protein-3	2.3*	1.6*
Pyruvate dehydrogenase kinase 4	2.5*	1.7*
Phosphofructokinase	2.1*	1.3
Glucose transporter 4	1.9*	1.4*
Insulin receptor substrate 1	1.4*	1.6*

Data are mean ± SEM values.
*$P < .05$ versus the respective HF or LF CON diet group.

cardiovascular disease mortality after 11-point multivariate analysis with factors relevant to metabolic syndrome (age, energy intake, marital status, education, blood pressure, diabetes, body mass index, waist-to-hip ratio, physical activity, smoking, and estrogen use). Specifically, one or more servings of blueberry per week were associated with significant reductions in coronary heart disease and cardiovascular disease after adjustment for subject age and energy intake. Currently, a knowledge gap exists for mechanisms associated with this profound benefit of blueberry intake, including tissue-specific effects.

PPARs as a therapeutic target

The ligand-activated transcription factors of the PPAR family are involved in the regulation of energy homeostasis and represent important targets for treating hyperinsulinemia and insulin resistance. The PPAR isoforms (α, γ, and δ) share a common mode of action that involves heterodimerization with the nuclear receptor retinoid X receptor and subsequent binding to specific PPAR-responsive elements in the promoter region of target genes. PPAR-α is highly expressed in skeletal muscle and the liver, and it impacts genes involved in lipid oxidation and fatty acid transport such as acyl-CoA oxidase, acyl-CoA synthetase, and lipoprotein lipase.[20] PPAR-α agonist drugs like fibrates are prescribed to reduce hyperlidemia; they reduce levels of very LDL and LDL and increase that of HDL and the ratio of fatty acid oxidation:synthesis. PPAR-γ is expressed mainly in fat but also to a lesser extent in muscle[21] and is important for adipogenesis, lipid metabolism, and glucose control. PPAR-γ agonist drugs like thiazolidinedione are prescribed to improve glucose tolerance; they affect insulin-stimulated glucose disposal and alter peripheral insulin sensitivity. Thiazolidinediones also promote adipocyte lipogenesis and insulin-mediated suppression of lipolysis; they therefore decrease serum free fatty acids and reduce the insulin-desensitizing effects of free fatty acids in muscle.

The current results suggest dual PPAR agonism by blueberry intake. Activation of multiple PPAR isoforms may be optimal for effects on metabolic syndrome phenotypes. Selective PPAR-α agonists do not impact hyperglycemia, and selective PPAR-γ agonists can cause significant weight gain. Dual-acting PPAR agonists or pan agonists are aggressively pursued by the drug industry because they could simultaneously improve lipoproteins and insulin resistance without causing significant weight gain. Future studies could compare blueberry effects with those of selective and dual PPAR agonists on metabolic syndrome.

Anthocyanins and metabolic syndrome phenotypes

Although this study used a whole food model with a complex phytochemical profile, anthocyanins in blueberry may be critical to the observed effects because other animal studies with anthocyanin-rich extracts suggest similar effects. Rats fed a high-fat diet supplemented with anthocyanin-rich mulberry powder showed reduced serum and liver triglyceride, total cholesterol, and serum LDL cholesterol and increased HDL.[22] In high-fat-fed mice, intake of an anthocyanin-rich extract from purple corn significantly reduced fat mass, hyperlipidemia, hyperinsulinemia, and blood glucose; the extract also significantly reduced activity of enzymes in adipose tissue that affect fatty acid and triacylglycerol synthesis.[23] In atherosclerosis-prone mice, intake of an anthocyanin-rich extract from black rice significantly reduced atherosclerosis, total cholesterol, triglyceride, and tissue cholesterol and increased HDL lipoprotein.[6,24] In healthy mice given a high-fat diet, intake of an anthocyanin-rich extract from Cornelian cherry (*Cornus mas*) decreased weight gain and hepatic lipid content and improved hyperglycemia.[25] In mice with diet-induced obesity, intake of blueberry-derived anthocyanins in drinking water reduced body fat, abdominal fat, fasting glucose, and insulin resistance.[9] Finally, acute gavage of high-fat-fed mice with an anthocyanin-rich blueberry extract significantly lowered blood glucose.[8] However, the molecular mechanisms of these effects of anthocyanins were not explored.

Anthocyanins, whole foods, and PPAR activation

Our working hypothesis was that blueberry intake would affect tissue PPARs and that these effects may be due to blueberry anthocyanins. The current effects of blueberry on PPAR isoforms and related genes are supported by previous findings with concentrated anthocyanins or anthocyanin-rich extracts. Anthocyanins induced cholesterol efflux from macrophages in a PPAR-γ-dependent manner, and anthocyanins increased both PPAR-γ expression and PPAR-γ transcriptional activity in a dose-dependent fashion.[12] We previously showed that diets enriched with anthocyanin-rich tart cherry significantly reduced fasting glucose, insulin, total cholesterol, triglycerides, and hepatic steatosis in lean Dahl-SS hypertensive rats.[26] Furthermore, changes in liver total cholesterol and triglyceride were associated with changes in PPAR activity and the expression of PPAR-regulated genes that affect fatty acid synthesis and oxidation. In the Zucker Fatty rat model, tart cherry intake also affected glucose and lipoproteins but also reduced body weight, total fat mass, and abdominal fat mass.[17] Finally, in the lean Dahl-SS hypertensive rat, anthocyanin-rich whole grape powder increased cardiac PPAR activity and the expression of PPAR-related genes.[18] The current study explored skeletal fat and abdominal fat due to their known roles in insulin resistance and cardiac risk.

Compare and contrast studies with blueberry

Human studies with blueberry reveal both supportive and unique results to those observed here. A recent human study in subjects with metabolic syndrome showed that blueberry intake (blended beverage made with freeze-dried berries) for 8 weeks decreased blood pressure, plasma oxidized LDL, and the serum oxidative stress markers malondialdehyde and hydroxynonenal. However, unlike the results presented here, serum glucose concentration, HOMA-IR, and lipid profiles were not affected by blueberry

intake.[2] In another study, subjects with metabolic syndrome consumed blueberry powder for 6 weeks (45 g of freeze-dried powder, approximated to equal 2.5 cups of fresh blueberries/day). Results indicated that blueberry improved insulin sensitivity as measured by the hyperinsulinemic–euglycemic clamp method but did not change plasma inflammatory markers or dual X-ray absorptiometry-measured adiposity.[3] This short study in a clinically relevant population suggested that blueberry can reduce cardiac risk factors, but the effects of an extended intervention or the nutrigenomic mechanisms of effect are unknown.

Animal studies have also explored the benefits of blueberry intake and blueberry anthocyanins on phenotypes of metabolic syndrome. These studies vary by model, dietary fat content, amount of blueberry, source of blueberry, and concentration of anthocyanins. In a mouse model of diet-induced obesity, Prior *et al*.[27,28] showed that whole blueberry powder added to a high-fat diet (10% [wt/wt] blueberry and 45% kcal from fat) increased body weight gain, total fat, and abdominal fat but did not impact glucose dynamics. However, blueberry-fed mice consumed approximately 12% more energy/day than the control high-fat group, which may have contributed to these outcomes. It is interesting that another group fed an extract of blueberry anthocyanins did not show these adverse phenotypes. In another cohort fed 60% kcal from fat, blueberry anthocyanin intake also reduced serum cholesterol, triglycerides, and leptin.[27,28] In the same study model, Prior *et al*.[9] compared the effect of blueberry juice and blueberry anthocyanins in drinking water: blueberry juice intake reduced body fat and serum leptin, but the blueberry anthocyanin group had additional reduction of abdominal fat, glucose, and insulin resistance as measured by the homeostasis model index of β-cell assay. Of note is that the groups had similar energy intake, unlike those in the earlier study. In the same mouse model of diet-induced obesity, DeFuria *et al*.[29] showed that compared with a calorie- and carbohydrate-matched control group, whole blueberry powder added to a high-fat diet (4% [wt/wt] blueberry and 60% kcal from fat) reduced glucose AUC and modestly reduced fasting glucose ($P = 0.07$). Food intake per day, abdominal fat weight, and body weight were not significantly different after 8 weeks of diet. In high-fat-fed lean mice, acute gavage with a phenolic-rich extract and an anthocyanin-rich extract from wild blueberries (*Vaccinium angustifolium* Aiton) lowered blood glucose levels at 6 hours post-gavage by 33% and 51%, respectively.[8] The greater hypoglycemic activity of the anthocyanin-enriched fraction compared with the phenolic-rich extract suggests that the bioactivity was due to the anthocyanin components.

Collectively, these studies suggest that blueberry intake—specifically the intake of blueberry anthocyanins—can affect several risk factors for metabolic syndrome and that matching for sugar and calorie content in the control group(s) is optimal for examining the effects of blueberry phytochemicals. Although the literature reveals disparate effects of blueberry on body weight and adiposity, effects on glucose metabolism (improved glucose sensitivity and/or reduced fasting glucose) appear to be more consistent.[9,29] Variations exist in the degree and scope of benefit and concurrent nutrigenomic mechanisms were not explored as conducted here. The current study then presents important mechanistic insight that can be further explored.

Study limitations and future directions

Currently, the specific mechanisms of PPAR activation by anthocyanins are unknown. Studies suggest that anthocyanins interact with kinase signaling that then affects PPAR nuclear translocation and DNA binding. Future studies may attempt to assess kinase involvement in anthocyanin-mediated PPAR activity. This discussion focuses on anthocyanins, but results may be impacted by the complex polypharmacy of bioavailable blueberry phytochemicals. As such, we cannot exclude the contribution of these other phytochemicals to our observed phenotypes. Furthermore, the use of radiolabeled energy substrates could confirm blueberry-mediated changes in glucose and lipid metabolism and determine which organs are involved in this effect. Finally, comparisons of effect against Food and Drug Administration–approved PPAR agonists would be useful to determine the scope of nutraceutical effects of blueberry intake for greater public health relevance.

In summary, in an obesity-prone model, blueberry-enriched diets reduced abdominal fat, blood lipids, and fasting glucose in rats fed a high-fat or a low-fat diet, but rats only showed reduced body weight and total fat mass when fed with a low-fat diet. In addition, blueberry intake was associated with increased PPAR activity and PPAR-related mRNA in abdominal fat and skeletal muscle. Given these effects, it is possible that blueberry intake could reduce eventual pathologies related to type 2 diabetes, including cardiac sequelae. Further studies are needed in animal models and human subjects with metabolic syndrome to ascertain the degree and spectrum of blueberry-derived clinical benefits.

ACKNOWLEDGMENT

This study was funded by an unrestricted grant from U.S. Highbush Blueberry Council (Folsom, CA, USA), which did not participate in data analysis or manuscript preparation.

AUTHOR DISCLOSURE STATEMENT

E.M.S. had partial salary support by an unrestricted grant from the U.S. Highbush Blueberry Council. No competing financial interests exist for I.I.T., D.E.U.-L., S.K.L., A.K., M.G.K., P.B.K., and S.F.B.

REFERENCES

1. Mink PJ, Scrafford CG, Barraj LM, *et al*.: Flavonoid intake and cardiovascular disease mortality: a prospective study in postmenopausal women. *Am J Clin Nutr* 2007;85:895–909.
2. Basu A, Du M, Leyva MJ, *et al*.: Blueberries decrease cardiovascular risk factors in obese men and women with metabolic syndrome. *J Nutr* 2010;140:1582–1587.

3. Stull AJ, Cash KC, Johnson WD, Champagne CM, Cefalu WT: Bioactives in blueberries improve insulin sensitivity in obese, insulin-resistant men and women. *J Nutr* 2010;140:1764–1768.
4. Finné Nielsen IL, Elbøl Rasmussen S, Mortensen A, *et al.*: Anthocyanins increase low-density lipoprotein and plasma cholesterol and do not reduce atherosclerosis in Watanabe Heritable Hyperlipidemic rabbits. *Mol Nutr Food Res* 2005;49:301–308.
5. Kadar A, Robert L, Miskulin M, Tixier JM, Brechemier D, Robert AM: Influence of anthocyanoside treatment on the cholesterol-induced atherosclerosis in the rabbit. *Paroi Arterielle* 1979;5:187–205.
6. Xia X, Ling W, Ma J, *et al.*: An anthocyanin-rich extract from black rice enhances atherosclerotic plaque stabilization in apolipoprotein E-deficient mice. *J Nutr* 2006;136:2220–2225.
7. Valcheva-Kuzmanova S, Kuzmanov K, Mihova V, Krasnaliev I, Borisova P, Belcheva A: Antihyperlipidemic effect of *Aronia melanocarpa* fruit juice in rats fed a high-cholesterol diet. *Plant Foods Hum Nutr* 2007;62:19–24.
8. Grace MH, Ribnicky DM, Kuhn P, *et al.*: Hypoglycemic activity of a novel anthocyanin-rich formulation from lowbush blueberry, *Vaccinium angustifolium* Aiton. *Phytomedicine* 2009;16:406–415.
9. Prior RL, Wilkes SE, Rogers TR, Khanal RC, Wu X, Howard LR: Purified blueberry anthocyanins and blueberry juice alter development of obesity in mice fed an obesogenic high-fat diet. *J Agric Food Chem* 2010;58:3970–3976.
10. Martineau LC, Couture A, Spoor D, *et al.*: Anti-diabetic properties of the Canadian lowbush blueberry *Vaccinium angustifolium* Ait. *Phytomedicine* 2006;13:612–623.
11. Muñoz-Espada AC, Watkins BA: Cyanidin attenuates PGE2 production and cyclooxygenase-2 expression in LNCaP human prostate cancer cells. *J Nutr Biochem* 2006;17:589–596.
12. Xia M, Hou M, Zhu H, *et al.*: Anthocyanins induce cholesterol efflux from mouse peritoneal macrophages: the role of the peroxisome proliferator-activated receptor γ-liver X receptor α-ABCA1 pathway. *J Biol Chem* 2005;280:36792–36801.
13. Nehlin JO, Mogensen JP, Petterson I, *et al.*: Selective PPAR agonists for the treatment of type 2 diabetes. *Ann N Y Acad Sci* 2006;1067:448–453.
14. Lanne B, Dahllöf B, Lindahl C, *et al.*: PPARalpha and PPARgamma regulation of liver and adipose proteins in obese and dyslipidemic rodents. *J Proteome Res* 2006;5:1850–1859.
15. Oakes ND, Thalén P, Hultstrand T, *et al.*: Tesaglitazar, a dual PPARα/γ agonist, ameliorates glucose and lipid intolerance in obese Zucker rats. *Am J Physiol Regul Integr Comp Physiol* 2005;289:R938–R946.
16. Dana SL, Hoener PA, Bilakovics JM, *et al.*: Peroxisome proliferator-activated receptor subtype-specific regulation of hepatic and peripheral gene expression in the Zucker diabetic fatty rat. Metabolism 2001;50:963–971.
17. Seymour EM, Lewis SK, Urcuyo-Llanes DE, *et al.*: Regular tart cherry intake alters abdominal adiposity, adipose gene transcription, and inflammation in obesity-prone rats fed a high fat diet. *J Med Food* 2009;12:935–942.
18. Seymour EM, Bennink MR, Watts SW, Bolling SF: Whole grape intake impacts cardiac peroxisome proliferator-activated receptor and nuclear factor κB activity and cytokine expression in rats with diastolic dysfunction. *Hypertension* 2010;55:1179–1185.
19. Livak KJ, Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_T}$ method. *Methods* 2001;25:402–408.
20. Inoue I, Katayama S: The possible therapeutic actions of peroxisome proliferator-activated receptor alpha (PPAR alpha) agonists, PPAR gamma agonists, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, angiotensin converting enzyme (ACE) inhibitors and calcium (Ca)-antagonists on vascular endothelial cells. *Curr Drug Targets Cardiovasc Haematol Disord* 2004;4:35–52.
21. Zierath JR, Ryder JW, Doebber T, *et al.*: Role of skeletal muscle in thiazolidinedione insulin sensitizer (PPARgamma agonist) action. *Endocrinology* 1998;139:5034–5041.
22. Yang X, Yang L, Zheng H: Hypolipidemic and antioxidant effects of mulberry (*Morus alba* L.) fruit in hyperlipidaemia rats. *Food Chem Toxicol* 2010;48:2374–2379.
23. Tsuda T, Horio F, Uchida K, Aoki H, Osawa T: Dietary cyanidin 3-O-beta-D-glucoside-rich purple corn color prevents obesity and ameliorates hyperglycemia in mice. *J Nutr* 2003;133:2125–2130.
24. Xia M, Ling WH, Ma J, Kitts DD, Zawistowski J: Supplementation of diets with the black rice pigment fraction attenuates atherosclerotic plaque formation in apolipoprotein E deficient mice. *J Nutr* 2003;133:744–751.
25. Jayaprakasam B, Olson LK, Schutzki RE, Tai MH, Nair MG: 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.
26. Seymour EM, Singer AA, Kirakosyan A, Urcuyo-Llanes DE, Kaufman PB, Bolling SF: Altered hyperlipidemia, hepatic steatosis, and hepatic peroxisome proliferator-activated receptors in rats with intake of tart cherry. *J Med Food* 2008;11:252–259.
27. Prior RL, Wu X, Gu L, *et al.*: 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.
28. Prior RL, Wu X, Gu L, Hager TJ, Hager A, Howard LR: 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.
29. DeFuria J, Bennett G, Strissel KJ, *et al.*: 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.

This article has been cited by:

1. Nidhi Chaudhary , Kiran Kumar Nakka , Nilanjana Maulik , Samit Chattopadhyay . Epigenetic Manifestation of Metabolic Syndrome and Dietary Management. *Antioxidants & Redox Signaling*, ahead of print. [Abstract] [Full Text HTML] [Full Text PDF] [Full Text PDF with Links]