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Polyphenol-rich blackcurrant extract prevents inflammation in diet-induced obese mice

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Abstract

Obesity is closely associated with chronic, low-grade inflammation. We investigated if polyphenol-rich blackcurrant extract (BCE) can prevent inflammation *in vivo*. Male C57BL/6J mice were fed a modified AIN-93M control diet containing high fat/high cholesterol (16% fat, 0.25% cholesterol by weight) or the control diet supplemented with 0.1% BCE (wt/wt) for 12 weeks. In BCE-fed mice, the percentage of body weight and adipocyte size of the epididymal fat were significantly lower than those of control mice. There were fewer crown-like structures (CLS) with concomitant decreases in F4/80, cluster of differentiation 68 and inhibitor of nuclear factor κB kinase ε (IKKε) mRNA in the epididymal adipose of BCE-fed mice. F4/80 and IKKε mRNA levels were positively correlated with CLS number. In the skeletal muscle of mice fed with BCE, mRNA expression of genes involved in energy expenditure and mitochondrial biogenesis, including PPARα, PPARδ, UCP-2, UCP-3 and mitochondrial transcription factor A, were significantly increased. When splenocytes from BCE-fed mice were stimulated by lipopolysaccharides, tumor necrosis factor α and interleukin-1β mRNA were significantly lower than control splenocytes. Together, the results suggest that BCE supplementation decreases obesity-induced inflammation in adipose tissue and splenocytes, at least in part, by modulating energy metabolism in skeletal muscle.
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Keywords: Blackcurrant; Anthocyanins; Inflammation; Obesity; Macrophage infiltration; Crown-like structure

1. Introduction

Obesity rates continue to rise in the U.S. and as many as 42% of adults are projected to become obese by 2030 [1]. Chronic low-grade inflammation is causally related to the pathogenesis of obesity-associated metabolic diseases, such as insulin resistance, type 2 diabetes, cardiovascular disease (CVD) and nonalcoholic fatty liver disease (NAFLD) [2]. Metabolic stress due to fat overload and consequent monocyte recruitment to adipose tissue causes chronic local inflammation by inducing the production of proinflammatory mediators in lipid-laden adipose tissue [3]. Nonsteroidal anti-inflammatory drugs are commonly used to treat acute and chronic inflammatory conditions, but not without their adverse effects [4]. Furthermore, the etiology and physiological characteristics of obesity-induced chronic inflammation differ from those of other inflammatory disorders such as arthritis and ulcerative colitis [5,6]. Therefore, identification of anti-inflammatory food components with minimal side effects yet effective in inhibiting chronic inflammation is critically needed.

Epidemiological studies have suggested that diets high in fruits and vegetables are inversely associated with the development of obesity and CVD [7]. The beneficial effects are largely attributed to the high polyphenol contents in fruits and vegetables [8]. Among the polyphenols found in fruits, anthocyanins are the most abundant [9] and a wide variety of edible berries, including blueberry, cranberry, raspberry, blackberry, chokeberry and acai berry, are rich in anthocyanins [10]. These edible berries have been found to provide health benefits for the prevention of cancer, diabetes and other inflammatory diseases [11–13].

Blackcurrant (*Ribes nigrum*) is a rich source of anthocyanins [14], and its potential health benefits against hypertension, CVD, neurodegenerative disease and ocular disease have been suggested [15].

Abbreviations: ACOX-1, acyl-CoA oxidase 1; AdipoQ, adiponectin; BCE, black currant extract; CD68, cluster of differentiation 68; CLS, crown-like structures; CTP-1, carnitine palmotyoltransferase 1; CVD, cardiovascular disease; DIO, diet-induced obesity; FAS, fatty acid synthase; HF/HC, high fat/high cholesterol; IKK, inhibitor for nuclear factor kB kinase; IL, interleukin; IRF, interferon regulatory factor; LPS, lipopolysaccharide; NAFLD, nonalcoholic fatty liver disease; NF-κB, nuclear factor-κB; PGC-1α, PPARγ coactivator 1α; PPAR, peroxisome proliferator activated receptor; RPLP0, ribosomal protein large P0; SCD-1, stearoyl CoA desaturase 1; SREBP-1c, sterol regulatory element-binding protein 1c; TBK1, TANK-binding kinase 1; TFAM, mitochondrial transcription factor A; TNFα, tumor necrosis factor α; UCP, uncoupling protein.

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Blackcurrant farming was federally banned in the early 1900s in the U.S. because blackcurrant was identified as a possible vector for a fungus that negatively impacted white pines, but the ban has been lifted recently in several states [15]. Due to its high phenolic contents, blackcurrant has become popular for human consumption [14]. Blackcurrant also contains substantial amounts of vitamin C and γ-linolenic acid [15]. However, the effect of blackcurrant on obesity-associated inflammation has never been investigated. We sought to determine the anti-inflammatory effect of polyphenol-rich blackcurrant extract (BCE) in adipose tissue and primary splenocytes specifically and to evaluate its role in muscle, as it relates to diet-induced obesity (DIO) *in vivo*.

2. Methods and materials

2.1. Extraction of anthocyanins

The anthocyanins of polyphenol-rich BCE (Artemis International, Inc., Fort Wayne, IN, USA) were extracted by using the solvent mixture of formic acid (8.5%)/acetonitrile/methanol (90:8.5:1.5). BCE (300mg) in 20ml of the extracting solvent was vortexed for 3min and then sonicated for 5min. The extract was centrifuged at 3000rpm for 5min. The supernatant was carefully decanted and collected. The precipitate was re-extracted twice by repeating the same procedure mentioned above. The supernatants were combined together and used for UHPLC/HR-MS/MS analysis as described below.

2.2. Identification of anthocyanins using UHPLC/HR-MS/MS

Anthocyanins were analyzed by using a high-resolution LTQ-Orbitrap XL mass spectrometer connected to an Accela UHPLC system (Thermo Fisher Scientific, USA). The column ACQUITY BEH C_{18} (150×2.1mm, 1.7μm; Waters, Ireland) was used for the separation of anthocyanins. The flow rate and injection volume were at 0.5ml/min and 2μl, respectively. The solvent gradient conditions of binary mobile phases consisted of solvent A (ddH_2O with 4% formic acid) and solvent B (methanol with 4% formic acid) were as follows: 99% A/1% B at 0min, 95% A/5% B at 2min, 80% A/20% B at 6min, 80% A/20% B at 10min, 77% A/23% B at 12min, 70% A/30% B at 30min, 55% A/45% B at 18min, 47% A/53% B at 20min, 20% A/80% B at 25min, 0% A/100% B at 25.1min and 0% A/100% B at 27min. Anthocyanins were detected with PDA at 200–600nm. The MS conditions in positive ion mode of heated electrospray ionization probe as ionization source were optimized as follows: spray voltage at 5.0kV, capillary voltage at 30V and capillary temperature at 275ºC. Full scan mass spectra were acquired in an *m/z* range of 100–1000 at positive ion mode. The Orbitrap analyzer was used for full scan mass spectra data acquisition with a mass resolving power of 30,000 FWHM at *m/z* 400. The MS/MS spectra were acquired in data-dependent mode by collision-induced dissociation.

2.3. Animal care and diet

Male C57BL/6J mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA) and after 1 week of acclimation, they were randomly assigned into a control or BCE group (n=11 for control; n=13 for BCE) at age of 15 weeks. The control group of mice was fed a modified AIN-93M high fat/high cholesterol (HF/HC) diet (16% fat, 0.25% cholesterol by weight; 55.7%, 125.5% and 31.8% energy from carbohydrate, protein and fat, respectively; 4529kcal/Kg). BCE group was on the HF/HC diet supplemented with 0.1% of BCE by weight. The standardized BCE powder containing 25% anthocyanins and 40% polyphenols was kindly provided by Artemis International, Inc. Based on body surface normalization to a 70-kg individual [16], 0.1% BCE containing 25% anthocyanins is equivalent to daily consumption of ~500mg BCE and 120mg anthocyanins in humans. As the average daily intake of anthocyanins per person has been estimated to be ~200mg in the U.S. [17], we believe that the dietary level of BCE is attainable in humans. Mice were fed BCE while they were developing HF-induced obesity to evaluate the preventive effects of BCE on the development of obesity and its associated dysfunctions. Mice were housed in a controlled environment with 12h light/dark cycles and had free access to food and water throughout the study. Body weight and food consumption were recorded weekly, and blood draws were performed monthly from the lateral tail vein. After 12 weeks on the experimental diets, mice were fasted for 8h and anesthetized by injecting ketamine/xylazine (100/10 mpk). Epididymal and retroperitoneal fat pads were harvested, weighed and snap frozen in liquid nitrogen for gene analysis or fixed in 10% formalin for histological analysis. Soleus/gastrocnemius muscle samples were also collected, snap frozen in liquid nitrogen and stored at −80°C for gene analysis. All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Connecticut.

2.4. Adipocyte size and number of crown-like structures (CLS) in epididymal fat pad

Formalin-fixed epididymal fat pads were embedded in paraffin, cut into 5-μm sections and stained with hematoxylin and eosin. These procedures were conducted at the Connecticut Veterinary Medical Diagnostic Laboratory (Storrs, CT, USA). The stained tissue sections were viewed at magnification ×10, and images were taken with AxioCam MRc (Zeiss, Thornwood, NY, USA). Diameter of at least 100 adipocytes from each mouse was measured using ImageJ software (National Institutes of Health). The number of CLS in one field of a slide at magnification ×10 was manually counted. One slide from each mouse was analyzed for adipocyte diameter and CLS number.

2.5. Splenocyte isolation

Splenocytes were isolated from the spleens of mice on experimental diets as we previously described [18]. Splenocytes were resuspended in RPMI-1640 complete media and plated at a density of 1×10^6 cells/0.5ml. The cells were stimulated by 500ng/ml of lipopolysaccharide (LPS) for 20h and the expression of inflammatory genes, including tumor necrosis factor α (TNFα), interleukin (IL)-1β and IL-6, were measured.

2.6. Gene expression analysis by quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from epididymal fats, muscle tissue of the gastrocnemius and splenocytes using TRIzol reagent (Invitrogen, Grand Island, NY, USA) and qRT-PCR analysis was conducted as previously described using the SYBR Green procedure and CFX96 real-time PCR detection system (BioRad, Hercules, CA, USA) [19,20]. Primer sequences were designed according to GenBank database using the Beacon Designer software (Premier Biosoft, Palo Alto, CA, USA) and primer sequences are listed in Table 1. Two internal controls, i.e., ribosomal protein large P0 (RPLP0) and β-actin, were used for the calculation of $2^{-\Delta\Delta Ct}$ to ascertain the validity of the chosen internal control for the analysis. Data analyzed using each internal control showed similar trends of change of changes in gene expression and the data reported in this study used RPLP0 as an internal control.

2.7. Statistical analysis

Student's t tests were performed to compare mean difference between groups and Pearson correlation was conducted to measure linear dependence between two variables using GraphPad InStat 6 (GraphPad Software, La Jolla, CA, USA). An α-level of P<.05 was considered statistically significant and all data are expressed as means±S.E.M.

3. Results

3.1. Anthocyanin compositions of BCE

Based on UHPLC/HR-MS/MS analysis, BCE is composed of four major anthocyanins including delphinidin-3-*O*-glucoside, delphinidin-3-*O*-rutinoside, cyanidin-3-*O*-glucoside and cyanidin-3-*O*-rutinoside in order of greatest amount, which contribute to ~98% of anthocyanins in BCE (Fig. 1 and Table 2).

3.2. Effect of BCE supplementation on weights of fat pads

After 12 weeks on an HF/HC control or a BCE-supplemented diet, both groups of mice gained ~10g of body weight with no significant differences between groups (initial body weight for control 29.9±0.6g vs. for BCE 30.3±0.9g; final body weight for control 40.3±1.0g vs. for BCE 39.1±1.0g). The percentage body weight of epididymal fat weight of BCE-fed mice was significantly lower than control mice (Fig. 2A). There was a trend toward a decrease in both epididymal and retroperitoneal adipose fat pad weights in mice fed BCE compared with control mice (Fig. 2).

3.3. Decreased cell size and CLS number in epididymal adipose tissue by BCE supplementation

Adipocyte diameter of epididymal fats was significantly smaller in mice fed BCE for 12 weeks compared with control mice (Fig. 3A), and therefore, the number of adipocytes per a microscopic field was significantly increased with BCE supplementation (data not shown).

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