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Anti-inflammatory activity of aronia berry extracts in murine splenocytes

Derek A. Martin [a], *Rod Taheri* [a], *Mark H. Brand* [b], *Andrew Draghi II* [c], *Francisco A. Sylvester* [c,d], *Bradley W. Bolling* [a,*]

[a] *Department of Nutritional Sciences, University of Connecticut, Storrs, CT, USA*
[b] *Department of Plant Science and Landscape Architecture, University of Connecticut, Storrs, CT, USA*
[c] *Department of Pediatrics, University of Connecticut Health Center, Farmington, CT, USA*
[d] *Department of Immunology, University of Connecticut Health Center, Farmington, CT, USA*

ARTICLE INFO

Article history:
Received 13 January 2014
Received in revised form 28 February 2014
Accepted 5 March 2014
Available online 28 March 2014

Keywords:
Aronia
Chokeberry
Anti-inflammatory
Immune
Polyphenol
Anthocyanin

ABSTRACT

Aronia berries are a rich source of dietary polyphenols, with diverse polyphenol profiles among its genotypes. The objective of this work was to characterize the anti-inflammatory effects of underutilized aronia berries and their polyphenols using primary C57/BL6 mouse splenocytes. At 125 μg gallic acid equivalents/mL, the commercial 'Viking' aronia berry and underutilized aronia extracts inhibited LPS-stimulated IL-6 to a similar extent. 'Viking' extracts inhibited IL-6 predominately in $CD4^-$ lymphocytes. The primary polyphenol constituents of extracts were subsequently evaluated for inhibition of LPS-stimulated IL-6. Cyanidin-3-arabinoside, but not the primary aronia anthocyanin cyanidin-3-galactoside, inhibited IL-6 at 10 μg/mL. Quercetin, but not its 3-galactoside or glucoside, inhibited LPS-stimulated IL-6. Quercetin also inhibited LPS-stimulated IL-10, whereas 'Viking' extract increased splenocyte IL-10 in the absence of LPS. Thus, the capacity of aronia extracts to modulate LPS-stimulated splenocyte IL-6 and IL-10 in vitro was not attributed to its principal polyphenols.

1. Introduction

Inflammation is central to the etiology of many chronic diseases including cancer, cardiovascular disease, and diabetes (Coussens, Zitvogel, & Palucka, 2013; Patel, Buras, & Balasubramanyam, 2013; Pearson et al., 2003). Obesity and autoimmune diseases such as multiple sclerosis, arthritis, and inflammatory bowel diseases are characterized by unresolved inflammation of adipose tissue, nervous tissue, joints, and the intestines, respectively (Abraham & Cho, 2009; Gregor & Hotamisligil, 2011; Park et al., 2011; Reynolds et al., 2011). Therefore, there is increasing interest in identifying dietary components that could prevent or mitigate chronic inflammation.

Berries are promising candidates for dietary interventions targeting chronic inflammation. A diet enriched in blackberries (10% w/w) had antiobesity and anti-inflammatory effects in ovariectomized rats (Kaume, Gilbert, Brownmiller, Howard, & Devareddy, 2012). Spontaneously hypertensive rats fed cranberry and lingonberry had reduced mRNA expression of aortic angiotensin-converting enzyme 1, cyclooxygenase 2, and monocyte chemoattractant protein 1 (Kivimaki et al., 2012). A polyphenol-rich wild blueberry extract inhibited

* *Corresponding author.* Tel.: 860-486-2180; fax: 860-486-3674.
E-mail address: bradley.bolling@uconn.edu (B.W. Bolling).
http://dx.doi.org/10.1016/j.jff.2014.03.004
1756-4646/

lipopolysaccharide (LPS)-induced nuclear factor-κB (NF-κB) protein-DNA binding activity in BV2 murine microglial cells (Lau, Joseph, McDonald, & Kalt, 2009).

Aronia berries, juices, and extracts exhibit anticancer, cardioprotective, antihypertensive, antidiabetic, anti-inflammatory, and immunomodulatory activities in cellular and animal models (Hellstrom et al., 2010; Kim et al., 2013a; Kim, Park, Wegner, Bolling, & Lee, 2013b; Kokotkiewicz, Jaremicz, & Luczkiewicz, 2010; Sharif et al., 2013). Short-term human intervention studies have also demonstrated reduction in inflammatory biomarkers following aronia consumption. A randomized, placebo-controlled trial of 44 older adults that survived a myocardial infarction demonstrated that 6 week supplementation of 255 mg aronia extract reduced serum interleukin-6 (IL-6), C-reactive protein (CRP), soluble intercellular adhesion molecule, vascular cell adhesion molecule, and monocyte chemoattractant protein-1 compared to the placebo (Naruszewicz, Laniewska, Millo, & Dluzniewski, 2007). Consumption of citrus juice fortified with aronia extract reduced CRP, oxidized LDL, and 8-hydroxydeoxyguanosine and improved glutathione status in adults with metabolic syndrome at 4 and 6 months relative to a placebo drink (Bernabe et al., 2013; Mulero et al., 2012). Despite the promising anti-inflammatory effects of aronia consumption, little is known about the bioactivity of underutilized aronia genotypes.

Aronia berries belong to the Rosaceae family and have 4 species: *Aronia arbutifolia* which produce red berries; *Aronia melanocarpa* and *Aronia mitschurinii* which produce black berries; and *Aronia prunifolia* which produce purple berries (Brand, 2010). Aronia berries have varying content of phenolic acids, anthocyanins, flavonoids, and proanthocyanidins based on genotype and sample origin (Taheri, Connolly, Brand, & Bolling, 2013). Despite their enrichment and diversity of polyphenols among aronia berries, little is known about how their polyphenol profiles impact their ability to modulate inflammatory cytokines. Therefore, the aim of this investigation was to examine the effects of aronia berries and their representative phytochemicals on IL-6 and IL-10 production in murine splenocytes in vitro.

2. Materials and methods

2.1. Chemicals and reagents

HPLC grade acetone, acetic acid, and methanol; ammonium chloride, potassium carbonate, and sodium azide were purchased from Fisher Scientific (Pittsburg, PA, USA). Dulbecco's phosphate buffered saline (DPBS), minimum essential medium (MEM), and fetal calf serum (FCS) were purchased from Thermo Scientific Hyclone (Waltham, MA, USA). Antibiotic/antimycotic, L-glutamine, amino acids, and sodium pyruvate were purchased from Gibco (Life Technologies, Carlsbad, CA, USA). Lipopolysaccharide (LPS), 2-mercaptoethanol, >95% purity chlorogenic acid (Cga), >98% purity neochlorogenic acid (nCga), >95% purity quercetin, >90% purity quercetin-3-glucoside (Q3Glu), >97% purity quercetin-3-galactoside (Q3Gal), and analytical standard grade proanthocyanidin B2 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Cyanidin-3-arabinoside (Cy3A) chloride and cyanidin-3-galactoside (Cy3Gal) chloride of >98% purity were obtained from WuXi Apptec (Shanghai, China). Phorbol myristate acetate (PMA) was from Calbiochem (Gibbstown, NJ, USA). Ionomycin was from Invitrogen (Carlsbad, CA, USA). LIVE/DEAD® Fixable Far Red Dead Cell Stain Kit, for 633 or 635 nm Excitation antibody was purchased from Life Technologies (Carlsbad, CA, USA). Brefeldin A (Golgi Plug™) and anti-CD16/CD32 were both from BD Biosciences (San Jose, CA, USA). Antibodies for flow cytometry were obtained from eBioscience (San Diego, CA, USA). FITC conjugated anti-CD4 (clone: GK 1.6) and PE conjugated anti-IL-6 (clone: MP5-20F3). Isotype controls were FITC conjugated rat anti-IgG2b κ (clone: eB149/10HS) and PE-conjugated rat anti-IgG1 (clone: eBRG1).

2.2. Mice

Three-week-old male C57BL6/J mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). Mice were kept at 20–26 °C under a 12 h light/dark cycle and were fed standard chow diet upon arrival to the animal facility. The protocol was approved by the University of Connecticut Institutional Animal Care and Use Committee.

2.3. Aronia berry extracts

Extracts from aronia berries were prepared as previously described (Taheri et al., 2013). Briefly, fresh *A. mitschurinii* 'Viking', *A. arbutifolia* (UC021 and UC057 accessions), and *A. prunifolia* (UC011 and UC047 accessions) berries were harvested at apparent ripeness, frozen at −80 °C, and then lyophilized. Lyophilized berries were powdered and stored at −80 °C until extraction. Extracts were prepared by suspending berry powder in 1:20 (w/v) of 70% acetone, 29.5% ultrapure water, and 0.5% acetic acid. The suspensions were sonicated for 5 min and supernatants collected, and the procedure was repeated twice. Then, the extract was agitated in fresh extract solution for 12 h at room temperature. The supernatants were recombined and dried at 40 °C by rotary evaporation. Aliquots were resuspended in methanol, dried, and stored at −80 °C until use. The polyphenol content of aronia berry extracts was previously reported (Taheri et al., 2013). Total phenols of extracts were determined using the Folin–Ciocalteu method as previously described, and expressed as gallic acid equivalents (GAE) (Kim et al., 2013a; Singleton, Orthofer, & Lamuela-Ravent, 1999). Doses of extracts used in subsequent experiments were normalized to polyphenol content based on GAE.

2.4. In vitro splenocyte cultures

Mice were anesthetized under isofluorane and sacrificed by cervical dislocation. Splenocytes were obtained as previously described, with modifications (McAleer, Saris, & Vella, 2011). Spleens were removed and strained through a 70 μm cell strainer. Cells were pelleted in MEM by centrifuging at 400 × g for 5 min at 4 °C, and resuspended in 2 mL MEM. Red blood cells were lysed by adding 5 mL 0.15 M ammonium chloride with 10 mM potassium carbonate. After 5 min, 30 mL DPBS was added, cells were pelleted, and washed twice with MEM. Cells were counted in a Bio-Rad (Hercules, CA, USA) TC-10 cell counter or by a hemacytometer following Trypan blue staining. Cells were then plated at 10^6 cells/well with 250 μL of MEM containing 10% FCS and supplemented with amino acids,