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The impact of chronic blackberry intake on the neuroinflammatory status of rats fed a standard or high-fat diet

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Abstract

Neuroinflammation has been suggested as a central mediator of central nervous system dysfunction, including in dementia and neurodegenerative disease. Flavonoids have emerged as promising candidates for the prevention of neurodegenerative diseases and are thought to be capable of antiinflammatory effects in the brain. In the present study, the impact of a chronic intake of an anthocyanin extract from blackberry (BE) on brain inflammatory status in the presence or absence of a high-fat diet was investigated. Following intake of the dietary regimes for 17 weeks neuroinflammatory status in Wistar rat cortex, hippocampus and plasma were assessed using cytokine antibody arrays. In the cortex, intake of the high-fat diet resulted in an increase of at least 4-fold, in expression of the cytokine-induced neutrophil chemoattractant CINC-3, the ciliary neurotrophic factor CNTF, the platelet-derived growth factor PDGF-AA, IL-10, the tissue inhibitor of metalloproteinase TIMP-1 and the receptor for advanced glycation end products RAGE. BE intake partially decreased the expression of these mediators in the high-fat challenged brain. In standard-fed animals, BE intake significantly increased cortical levels of fractalkine, PDGF-AA, activin, the vascular endothelial growth factor VEGF and agrin expression, suggesting effects as neuronal growth and synaptic connection modulators. In hippocampus, BE modulates fractalkine and the thymus chemokine TCK-1 expression independently of diet intake and, only in standard diet, increased PDGF-AA. Exploring effects of anthocyanins on fractalkine transcription using the neuronal cell line SH-SY5Y suggested that other cell types may be involved in this effect. This is the first evidence, in *in vivo* model, that blackberry extract intake may be capable of preventing the detrimental effects of neuroinflammation in a high-fat challenged brain. Also, fractalkine and TCK-1 expression may be specific targets of anthocyanins and their metabolites on neuroinflammation.

Keywords: Anthocyanins; Cytokines; Flavonoids; Fractalkine; High-fat diet; Neuroinflammation

1. Introduction

The incidence of neurodegenerative diseases, such as Alzheimer's disease (AD) and Parkinson's disease (PD) and dementia, have become more prevalent due, in part, to the exponential increase in life expectancy, making the discovery of novel treatments most pressing [1]. Both typical and atypical brain aging are associated with an increase in neuroinflammation and may contribute to the dysfunction of the central nervous system (CNS) and lead to the early onset of dementia and/or neurodegenerative diseases [2,3]. In support of this, the use of nonsteroidal antiinflammatory drugs has been shown to partly delay or prevent the onset of some neurodegenerative diseases [4,5]. On the other hand, modifiable lifestyle factors, such as diet, have also been postulated to have the potential to protect or stimulate neuroinflammation [6,7]. Notably, high-fat diets, combined with physical inactivity, may lead to obesity [6], a major risk factor for metabolic and cognitive dysfunction [8–10]. For example, intake of a typical 'western' diet (high fat; high sugar) has been shown to compromise normal cognitive functioning [10,11]. Furthermore, high-fat diets have been shown to induce markers of brain inflammation, with proinflammatory actions in the cerebral cortex (prostaglandin E2 levels and cyclooxigenases 1 and 2 expression) and hippocampus (tumor necrosis factor TNF-α, interleukins IL-6 and IL-1β and macrophage chemoattractant protein MCP-1) [12–14].

A growing body of evidence suggests that flavonoid intake may attenuate the progression of neurodegenerative disorders [15,16]. In particular, prospective studies showed a positive association between flavonoid intake and reduction in cognitive decline with age [17] and decrease risk of AD [18]. Furthermore, the higher intake of flavonoids (flavanones, anthocyanins, flavan-3-ols, flavonols, flavones) has been shown to be associated with a reduced risk of developing PD, in

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particular, with anthocyanins and the intake of anthocyanin-rich foods such as berries [19]. Although the molecular mechanisms underlying such protective effects remain unclear, the antiinflammatory properties of flavonoids are likely to play an important role in driving these positive outcomes [20–23]. Indeed, flavonoids and their *in vivo* metabolites have been shown to protect neurons against neuroinflammatory injury [20,24].

Despite the growing body of studies investigating the impact of flavonoids in brain function, only a few animal investigations have focussed on their specific actions in the context of neuroinflammation [25–28]. Anthocyanins from berries have aroused particular interest as potential neuroprotectants [29,30]. As such, the present study was designed to investigate the impact of long-term blackberry anthocyanin intake on neuroinflammation status of both high-fat-fed and low-fat-fed rats. A simultaneous multiple cytokine detection approach was used first in two important regions of CNS, cortex and hippocampus, and extended to plasma to explore relation with systemic cytokine profile. From the results of cytokine arrays, further hypotheses were explored, namely, the interaction of blackberry extract with microglia phenotype activation and the modulation of the cytokine fractalkine in an *in vitro* model of neurons.

2. Methods

2.1. Blackberry extract and anthocyanin metabolite synthesis

Preparation of anthocyanin blackberry extract was achieved using previously described methods [31,32]. Briefly, blackberries (*Rubus fruticosus*) were extracted with 50% aqueous ethanol (pH 1.5, acidified with HCl) for 24 h at 22°C. The solution obtained was filtered (50-μm nylon membrane) and concentrated using a rotary evaporator under at 30°C. The concentrated extract was added to a polyamide gel column (mesh 100–120) to remove sugars. The sugar-free anthocyanin extract was freeze-dried and stored at −20°C. The anthocyanin extract was analyzed by HPLC at 520 nm, as previously described [33]. Following preparative HPLC, the purified extract was characterized for anthocyanin content by analytical HPLC coupled to UV–vis, DAD-ESI/MS and NMR techniques. The mixture 3′ and 4′-Me-Cy3glc (Peo3glc and IsoPeo3glc) was obtained by enzymatic hemisynthesis. Briefly, Cy3glc was incubated with rat liver cytosol protein fraction and *S*-adenosyl-L-methionine during 2 h at 37°C. The identity of the purified compounds present in the mixture was established by UV–vis, HPLC-DAD-ESI/MS and NMR techniques as previously described [34].

2.2. Study design

Twenty-four male Wistar rats (200–250 g body weight) were acquired from Harlan Laboratories (Santiga, Spain). Animals were maintained at 23–25°C on a 12/12 h light–dark cycle and housed two per cage. Animal handling and housing protocols followed European Union guidelines (86/609/EEC) and Portuguese Act (129/92) for the use of experimental animals. The study was approved by Ethical Committee of Faculty of Medicine University of Porto and São João Hospital Center. After 2 weeks of acclimatization, animals were divided into four groups (n=6 rats per group): standard diet (C); standard diet+blackberry anthocyanin extract (BE); high-fat diet (HF); and high-fat diet+blackberry anthocyanin extract (HFBE). Animals were fed *ad libitum* with a standard maintenance diet (4% fat) from Harlan (2014S, Teklad Diets; Harlan Laboratories) or a high-fat chow diet (45% fat) from Research Diet D12451 (New Brunswick, NJ, USA) for 17 weeks. The blackberry anthocyanin dose (25 mg kg^{-1} body weight; adjusted weekly for weight changes) was incorporated into the animal feeds and was changed daily. Animal body weight and food ingestion were measured twice a week. Following the 17-week intervention, animals were anesthetized with ketamine+xylazine (50 mg kg^{-1}+1 mg kg^{-1}) and kept under isoflurane during blood collection from left ventricle. Blood was collected with heparinized needles and the protease inhibitor PMSF (100 μM) was added. After blood centrifugation (2000*g*; 15 min), plasma was collected and stored at −20°C. Rats were decapitated for brain removal. Hippocampus and cortex were immediately frozen in liquid nitrogen and kept at −80°C until use.

2.3. Cytokine antibody arrays

In order to have a global view of cytokines expression in brain, predefined cytokine arrays were used in cortex (RayBio Rat Cytokine Antibody Array 2; RayBiotech, Norcross, GA, USA) and plasma (Rat Cytokine Array Panel A; R&D Systems Europe, UK). Thirty microliters of plasma from each animal treatment was mixed, diluted and used according to instructions. For the brain cortex array, cortex tissue from animals in each experimental group was mixed, proteins were extracted according to kit and was quantified with Bradford reagent (Bio-Rad Laboratories, Hertfordshire, UK) and a 200-μg protein were used. Chemiluminescent detection was achieved using a luminescent image analyzer (ImageQuant LAS 4000mini from GE Healthcare, Life Sciences) and data were analyzed with ImageQuant TL array software, version 8.1.

Protein extraction from hippocampus was made with a Mammalian Tissue Lysis, CelLyticMT (C-3228; Sigma-Aldrich, Dorset, UK). A ratio of 1:20 was used (1 mg tissue/20 μl reagent), and PMSF (phenylmethylsulfonyl fluoride; Sigma-Aldrich, Dorset, UK), a protease inhibitor, was added to the reagent before use, at a final concentration of 100 μM. After a centrifugation of 15,000*g* for 10 min, supernatant was collected and proteins were measured with Bradford reagent (Bio-Rad Laboratories), according to previous literature [29]. For the determinations in the hippocampus, a glass-based array (Quantibody Rat Cytokine Array; RayBiotech) was performed according to manufacturer's instructions. Arrays were incubated with the samples (250 μg ml^{-1}) or standards and cytokine concentration in samples was determined using a standard curve. Fluorescent detection was analyzed with laser scanner (Genepix Professional 4200A; Molecular Devices) and data were extracted with GenePix Pro 6 Microarray Acquisition & Analysis Software (from Molecular Devices).

2.4. Measurements of microglia activation markers in cortex

In order to characterize microglia polarization, expression of IL-1β (marker of M1 status) and arginase activity (marker of M2 activation) were explored [35]. Cortex tissue was lysed and homogenized with Mammalian Tissue Lysis, CelLyticMT (C-3228; Sigma-Aldrich, Dorset, UK) in a ratio of 1 mg tissue:20 μl lysis buffer. The protease inhibitor PMSF (100 μM) was added, homogenates were centrifuged at 15,000*g* for 10 min and the supernatant was retained. Protein content of the supernatants was assessed by the Bradford assay (Bio-Rad Laboratories). Arginase activity was measured using a commercial arginase assay kit (KA1609; Abnova, Taipei City, Taiwan). Standards, controls and 5-fold diluted samples were incubated with arginase substrate for 2 h at 37°C then reaction was stopped with urea reagent and kept for 60 min before optical measurement at 450 nm.

Levels of IL-1β were measured by a commercial enzyme-linked immunosorbent assay (ELISA) kit (RayBio Rat IL-1; tebu-bio, Portugal). Cortex supernatant 4-fold diluted, standards and controls were plated in duplicate on a 96-well microplate coated with anti-rat IL-1β polyclonal antibody and incubated for overnight at 4°C. Biotinylated anti-IL-1β monoclonal antibody was then added for 1 h. After washing away unbound biotinylated antibody, HRP-conjugated streptavidin was added for 45 min. The wells were again washed, and a TMB substrate solution was added allowing color to develop in proportion to the amount of IL-1β