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Dietary Anthocyanins Mitigate High-Fat Diet-Induced Hippocampal Inflammation in Mice

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A B S T R A C T

Background: Obesity and consumption of high-fat diets (HFD) are associated with intestinal permeabilization and increased paracellular transport of endotoxins, which can promote neuroinflammation. Inflammation can affect the hypothalamic pituitary adrenal (HPA) axis, which controls responses to stress and downregulates the brain-derived neurotrophic factor (BDNF), which can promote anxiety and depression, conditions frequently found in obesity. We previously showed that consumption of anthocyanins (AC) mitigate HFD-induced insulin resistance, intestinal permeability, and inflammation.

Objectives: This study investigated if a dietary supplementation with a cyanidin- and delphinidin-rich extract (CDRE) could counteract HFD/obesity-induced hippocampal inflammation in mice.

Methods: C57BL/6J male mice were fed for 14 wk on one of the following diets: *1*) a control diet containing 10% total calories from fat (C), *2*) a control diet supplemented with 40 mg AC/kg body weight (BW) (CAC), 3) a HFD containing 60% total calories from fat (lard) (HF), or 4) the HFD supplemented with 2, 20, or 40 mg AC/kg BW (HFA2, HFA20, and HFA40, respectively). In plasma and in the hippocampus, parameters of neuroinflammation and the underlying cause (endotoxemia) and consequences (alterations to the HPA and BDNF down-regulation) were measured.

Results: Consumption of the HFD caused endotoxemia. Accordingly, hippocampal *Tlr4* mRNA levels were 110% higher in the HF group, which were both prevented by CDRE supplementation. Consumption of the HFD also caused: *1*) microgliosis and increased expression of genes involved in neuroinflammation, that is, *Iba-1*, *Nox4*, *Tnfα*, and *Il-1β*, *2*) alterations of HPA axis regulation, that is, with low expression of mineralocorticoid (MR) and glucocorticoid (GR) receptors; and *3*) decreased *Bdnf* expression. Supplementation of HFD-fed mice with CDRE mitigated neuroinflammation, microgliosis, and MR and BDNF decreases.

Conclusions: CDRE supplementation mitigates the negative effects associated with HFD consumption and obesity in mouse hippocampus, in part by decreasing inflammation, improving glucocorticoid metabolism, and upregulating BDNF.

Keywords: high-fat diet, anthocyanidins, BDNF, neuroinflammation, endotoxemia, hippocampus, HPA axis, C57BL/6J mice

Introduction

Obesity is a global pandemic that is characterized by low-grade systemic inflammation and excess adiposity caused by chronic imbalances between energy intake and energy expenditure [1, 2]. Growing research shows that not only the imbalance of food intake, but the macronutrient ratio of meals plays a vital role in the pathophysiology of obesity [3–5]. Along with chronic over nutrition, diets have moved away from being high in fruits, vegetables, and whole grains toward processed foods with high content of saturated fats and refined sugars. Obesity and excessive consumption of energy dense foods cause systemic inflammation, which can adversely impact the brain. In this regard, different events can contribute to obesity/high-fat diet

Abbreviations: AC, anthocyanins; BDNF, brain-derived neurotrophic factor; BW, body weight; CDRE, cyanidin- and delphinidin-rich extract; GC, glucocorticoid; GFAP, glial fibrillary acidic protein; GR, glucocorticoid receptor; HFD, high-fat diet; HPA, hypothalamic pituitary adrenal; Iba-1, ionized calcium-binding adapter molecule-1; LPB, LPS-binding protein; LPS, lipopolysaccharides; MR, mineralocorticoid receptor; NOX4, NADPH oxidase 4; TLR4, toll-like receptor 4; TNFα, tumor necrosis factor alpha; TrkB, tropomyosin receptor kinase B; *11β-HSD1*, 11β-hydroxysteroid dehydrogenase type I.

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(HFD)-induced neuroinflammation, including intestinal permeabilization [6], brain-derived neurotrophic factor (BDNF) downregulation [7] and altered homeostasis of glucocorticoids (GC) [8]. Chronic consumption of HFDs alters intestinal physiology, which has been shown to adversely affect the peripheral and central nervous system [9,10]. Intestinal permeabilization can lead to an increased paracellular transport of luminal lipopolysaccharides (LPS) from food or bacteria to enter the bloodstream (metabolic endotoxemia) and trigger systemic inflammation [11,12]. LPS can cross the blood brain barrier, bind to the TLR4 receptor in microglial cells, activate signaling pathways leading to cytokine production and release, for example, IL-1β and tumor necrosis factor alpha (TNFα), and to an upregulation of oxidant-generating enzymes, for example, NADPH oxidase 4 (NOX4) [13,14]. Importantly, inflammation also downregulates the expression of BDNF, a peptide involved in neurogenesis, neuroprotection, and metabolic regulation [15–17]. In fact, diet-induced obesity decreases brain and circulating levels of BDNF [18–21]. In the context of obesity, decreased hippocampal BDNF levels are associated with anxiety, depression, and impaired memory [22].

The hypothalamic pituitary adrenal (HPA) axis is responsible for controlling responses to stressors. In inflammatory processes, TNFα, IL-1β, and IL-6 are the main stimulators of corticotropin-releasing hormone synthesis which promotes the production and release of adrenocorticotropic hormone, which stimulates the synthesis and release of GC. GC bind to the glucocorticoid receptor (GR) and mineralocorticoid receptor (MR), which play vital roles in neural physiology [23,24]. These receptors also act as immunosuppressants and effectively turn off the feedback loop of GC release [25]. In the hippocampus, GC preferentially bind to MR with respect to GR [26], promoting neuronal cell survival and synaptic plasticity [23]. In addition, short-term MR activation enhances long-term potentiation through mechanisms involving α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA) receptor trafficking [27]. In the context of obesity, we recently observed that HFD-induced obesity causes hippocampal inflammation and downregulation of GR and MR in association with increased anxiety in mice [28].

Anthocyanidins (AC), a flavonoid subfamily, are abundant in red and purple pigmented fruits and vegetables. These compounds are characterized by a 3-ring chemical structure with different patterns of hydroxyl, sugar and aliphatic or aromatic acid substitutions [29]. AC consumption is associated with several benefits at the gastrointestinal tract [9,30] which differ depending on each AC type. In this regard, cyanidin and delphinidin were the most protective against TNFα-induced permeabilization of differentiated Caco-2 cell monolayers [30]. In in vivo studies, we previously showed that supplementation with cyanidin- and delphinidin-rich extract (CDRE) mitigates HFD-induced intestinal permeabilization, tight junction dysfunction, endotoxemia, and related inflammation [9,31,32]. Suggesting a potential AC action at the brain level, recent evidence showed that the flavan-3-ol (−)-epicatechin, which has a similar chemical structure to that of cyanidin, decreased endotoxemia, mitigated neuroinflammation and increased hippocampal BDNF expression in HFD-fed/obese mice [6].

Although cyanidin and delphinidin supplementation has been shown to mitigate HFD/obesity-induced intestinal permeabilization, endotoxemia and systemic inflammation in mice and humans [9,31,32], there is no current evidence on their potential capacity to mitigate hippocampal inflammation. Thus, this work investigated the capacity of CDRE to counteract HFD/obesity-induced hippocampal inflammation in mice, characterizing potential protective mechanisms. We observed that CDRE supplementation mitigated HFD-triggered hippocampal inflammation in part by decreasing endotoxemia and TLR4 expression, and by preventing HFD-induced decreased expression of the MR and BDNF.

Methods

Materials

The primary antibody for glial fibrillary acidic protein (GFAP) (Z0334) was obtained from Dako. Ionized calcium-binding adapter molecule-1(Iba-1) (ab5076), and BDNF (ab213323), were obtained from Abcam. Secondary antibodies anti-goat (705-165-003) and anti-rabbit (711-165-152) were obtained from Jackson Immunoresearch. The ELISA Kit for LPS (abx150357) was obtained from Abbexa. The ELISA Kit for LPS-binding protein (LBP) (ab213876) was purchased from Abcam. All the reagents and materials for the quantitative PCR were purchased from BioRad. The CDRE was provided by NSE Products, Inc.

Animals and animal care

All procedures were in agreement with standards for care of laboratory animals as outlined in the NIH Guide for the Care and Use of Laboratory Animals. Before implementation, experimental protocols were approved by the University of California, Davis Animal Use and Care Administrative Advisory Committee. All procedures were supported by the Animal Resource Services of the University of California, Davis. Healthy 5-wk-old male C57BL/6J mice (20–25 g) were purchased from The Jackson Laboratories (Sacramento) and acclimated for 1 wk to the control diet. Then, they were divided into 6 groups (10 animals/group) that were fed for 14 wk: *1*) a control diet (C) containing ~10% total calories from fat, *2*) a control diet supplemented with 40 mg AC/kg body weight (BW) (CAC), *3*) an HFD (HF) containing ~60% total calories from fat (lard), or *4*) the HF diet supplemented with either 2 (HFA2), 20 (HFA20), or 40 (HFA40) mg AC/kg BW. The composition of the control and HF diets is described in Supplemental Table 1. The AC-containing diets were prepared weekly to take into consideration the changes in BW and to prevent AC degradation. All diets were stored at −20°C until use. Allometric scaling is an appropriate method to compare bioactive consumption in different species [33]. Based on the above, the human equivalent dose for 2, 20, and 40 mg AC/kg BW would be 12, 120, and 240 mg, respectively, for a 70 kg adult. These amounts can be reached through diet. Thus, based on the AC content, 12 mg AC can be found in 50 g of a fresh plum; 120 mg AC can be found in <½ cup (72 g) of raw blackberries; and 240 mg in ½ cup (75 g) of black currants [34]. CDRE was analyzed using a liquid chromatography method and its composition was previously described [35]. CDRE was composed of 3 different natural extracts from bilberry, black current, and black rice provided by NSE Products, Inc. On the base of the composition, it was estimated that the AC-containing diets provided AC glycosides in the following percentages: 66%

cyanidin, 28% delphinidin, and 5.6% peonidin of the total AC content. Food intake and BW were measured weekly throughout the study and results were previously reported [35]. After 14 wk on the diets, mice were killed by cervical dislocation, blood was drawn from the submandibular vein into EDTA tubes, and plasma was collected after centrifugation at 3000 × *g* for 15 min at room temperature. Brains were dissected and left and right hippocampi were isolated.

Determination of plasma LPS and LBP

Plasma LPS and LBP concentrations were determined using kits purchased from Abbexa and Abcam, respectively, following the manufacturer's protocols.

RNA isolation and quantitative PCR (qPCR)

For quantitative PCR studies, RNA was extracted from hippocampi using the TRIzol reagent protocol (Invitrogen). cDNA was generated using high-capacity cDNA Reverse Transcription Kit (Applied Biosystems). mRNA levels of *Bdnf*, tropomyosin receptor kinase B (*TrkB*), GR (*Nr3c1),* MR (MR*, Nr3c2)*, 11β-hydroxysteroid dehydrogenase type I (*11β-Hsd1*), *Iba-1*, *Tlr4*, *Il-1β*, *Il-18*, and *Nox4* were assessed by qPCR (iCycler, Bio-Rad) using the primers listed in Table 1. The Ct values were normalized by the housekeeping gene *β-actin*. Gene expression was determined using the $2^{-\Delta\Delta Ct}$ method [36].

Immunohistochemistry

Brains were fixed overnight in 4% (wt/vol) solution of paraformaldehyde in PBS. Subsequently, brains were washed in PBS, cryoprotected in 30% (wt/vol) sucrose in PBS, and frozen. Free-floating coronal sections (30 μm) were obtained using a Leica CM 1850 cryotome (NuBloch). For immunohistochemical analysis, cryotome sections were rinsed twice in PBS, pH 7.4, once in PBS containing 0.1% (vol/vol) Triton-X100 (PBST) and blocked for 2 h with a solution containing 1% (wt/vol) donkey serum in PBST. Sections were then incubated overnight at 4°C with primary antibodies for GFAP (1:500) to detect astrocytes, Iba-1 (1:500) to detect microglia cells and BDNF (1:5000). Subsequently, sections were incubated in the presence of the corresponding fluorescent conjugated anti-rabbit or anti-goat antibodies for 2 h at room temperature. Cell nuclei were stained with 1 μg/mL Höechst 33342 following the methods described by Oberhammer et al. [37]. Microscopic observations were carried out in an Olympus BX50 epifluorescence microscope (Olympus) equipped with a Cool-Snap digital camera (Olympus IMS). Pictures of 2 fields from the hippocampus areas CA1, CA2, CA3, and dentate gyrus were taken, and 3 sections per animals were analyzed. The sections were obtained from −1.46 mm to −2.46 mm from Bregma to include the hippocampus [38]. Images from hippocampus areas CA1, CA2, CA3, and dentate gyrus were analyzed with Image pro software and ImageJ (National Institutes of Health). The quantifications of all the sections were done by 2 independent researchers.

Statistical analysis

Hippocampal mRNA levels, immunohistochemistry results, and plasma parameters were analyzed by 1-way analysis of variance and Fisher's least significant difference post hoc analysis using Statview 5.0 (SAS Institute Inc.). Differences were considered statistically significant at $P < 0.05$. Data are shown as mean ± SE. Correlations were calculated using The Pearson Correlation 2-tailed method. Correlations were deemed statistically significant at $P < 0.05$.

Results

Effects of CDRE supplementation on plasma LPS and LBP concentrations and hippocampal *Tlr4* expression in HFD-fed mice

To determine if the HFD induced metabolic endotoxemia, we measured LPS and LBP concentrations in plasma. LBP binds circulating LPS and enhances its binding affinity to TLR4 [39]. In agreement with our previous findings, mice fed the HFD for 14 wk had significantly higher levels of plasma LPS and LBP (100% and 18%, respectively) compared with the control (C) group (Figure 1A and B). Supplementation with CDRE prevented both increases at all the tested concentrations (2–40 mg AC/kg BW). In HFD-fed mice, Tlr4 mRNA levels were 110% higher compared with controls, which was prevented by supplementation with 20 and 40 mg AC/kg BW (Figure 1C). Supporting an association between endotoxemia and neuroinflammation, plasma LPS concentrations were positively correlated with hippocampal Tlr4 mRNA levels ($r = 0.524$, $P = 0.001$) (Figure 1D).

Effects of CDRE supplementation on hippocampal *Iba-1* mRNA levels and microgliosis in HFD-fed mice

Iba-1 is a protein abundant in microglial cells, its expression increases in association with microgliosis [40]. Iba-1 mRNA

TABLE 1
List of primers used to perform the qPCR analysis in the mouse hippocampus

Gene	Forward primer (5′→3′)	Reverse primer (5′→3′)
β-actin	TCATGAAGTGTGACGTGGACATCCGC	CCTAGAAGCATTTGCGGTGCACGATG
Il-1β	AATCTCACAGCAGCACATCAA	CCATACTTTAGGAAGACA
Il-18	ACCAAGTTCTCTTCGTTGAC	ACCAAGTTCTCTTCGTTGAC
Tlr4	GGAAGTTCACATAGCTGAATGAC	CAAGGCATGTCCAGAAATGAGA
Tnfa	CCCCTCAGCAAACCACCAAGT	CTTGGGCAGATTGACCTCAGC
Nox4	TGAGGAGTCACTGAACTATGAAGTTAATC	TGACTGAGGTACAGCTGGATGTTCACA
GR *(Nr3c1)*	TGGAGAGGACAACCTGACTTCC	ACGGAGGAGAACTCACATCTGG
MR *(Nr3c2)*	TGTGTGGAGATGAGGC	GGACAGTTCTTTCTCCGAAT
11β-Hsd1	GGGATAATTAACGCCCAAGC	TCAGGCAGGGTTCTAAG
Bdnf	ATGGGACTCTGGAGAGCCTGAA	CGCCAGCCAATTCTCTTTTTGC
TrkB	GACTACTACAGGGTCGGTGG	CAGTATGGCCCCATTGTAGA
Iba-1	GTCCTTGAAGCGAATGCTGG	CATTCTCAAGATGGCAGATC

Abbreviation: qPCR, quantitative PCR.

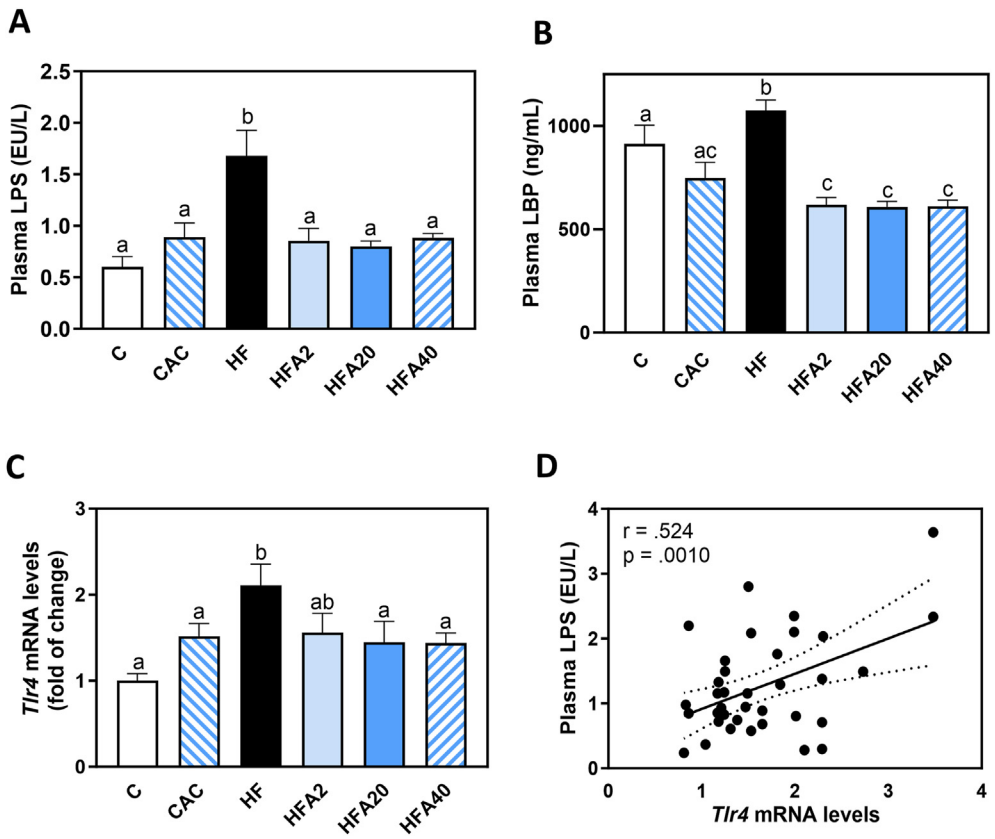

FIGURE 1. CDRE supplementation mitigated HFD-induced endotoxemia and hippocampal T*lr*4 expression. Mice were fed a control diet (C), a control diet supplemented with 40 mg AC/kg BW (CAC), a HFD (HF), or a HFD supplemented with 2 (HFA2), 20 (HFA20), and 40 mg (HFA40) AC/kg BW. At week 14 on the corresponding diets the following parameters were measured in plasma and in the hippocampus. (A) Plasma LPS and (B) plasma LPS-binding protein (LBP) were measured using a commercial ELISA kit; (C) hippocampal *Tlr4* mRNA levels were determined by qPCR. *β-actin* was used as housekeeping gene; (D)correlation between plasma LPS and *Tlr4* mRNA levels. The solid line represents the regression line and dashed lines delineate the 95% confidence interval. Results are shown as mean ± SE 5–10 animals/group. Values having different superscripts are significantly different ($P < 0.05$, 1-way ANOVA). ANOVA, analysis of variance; BW, body weight; AC, anthocyanins; C, control diet; CAC, C diet supplemented with 40 mg AC/kg BW; CDRE, cyanidin- and delphinidin-rich extract; HFD, high-fat diet; HFA 2, 20, and 40, HFD supplemented with 2,20, and 40 mg AC/kg BW.

levels were 34% higher in mice fed the HFD compared with the C group, and CDRE supplementation (2 and 40 mg AC/kg BW) mitigated this increase (Figure 2B). Hippocampal microgliosis was evaluated by immunohistochemical analysis of Iba-1+ cells per area of the hippocampus (Figure 2C). In the whole hippocampus, dentate gyrus, CA1, and CA2 regions, HFD did not significantly increase microglial population when compared with the C group (Figure 2C–F and Supplemental Figures 1–3). However, in the whole hippocampus, CDRE supplementation led to a decrease in the number of Iba-1+ cells by 49%, 48%, and 40% at doses of 2, 20, and 40 mg AC/kg BW, respectively when compared with HFD-fed mice (Figure 2D). Values in the dentate gyrus were similar among groups (Figure 2E). In the CA1 region, CDRE supplementation with 2 and 40 mg AC/kg BW had a lower amount of Iba-1+ cells, 26% and 32%, respectively, compared with the CAC group (Figure 2F). In the CA2 region, mice supplemented with 20 mg/kg BW of CDRE had 85% lower number of Iba-1+ cells compared with HF mice (Figure 2G). HFD induced significant microgliosis in the CA3 region of the hippocampus compared with the C group. This effect was prevented by CDRE supplementation (20 and 40 mg AC/kg BW) (Figure 2H). As microglia and astrocytes are key players in the initiation of innate immunity in neurodegeneration, we also evaluated the astrocytosis by immunohistochemical analysis of GFAP+ cells per area of the hippocampus, and no significant differences were observed between the different experimental groups (Supplemental Figures 4–7).

Effects of CDRE supplementation on parameters of inflammation and oxidative distress in HFD-fed mice

Activation of proinflammatory microglia is characterized by an increased expression of cytokines, for example, TNFα, IL-1β, and oxidant-generating enzymes, for example, NOX4. Consumption of the HFD for 14 wk led to an increased expression of *Tnfα* (Figure 3A), *Il-1β* (Figure 3B), and *Nox*4 (Figure 3D) (100%, 43%, and 46%, respectively) compared with the C group, while *Il-18* was not affected (Figure 3C). Supplementation with 20 and 40 mg AC/kg BW prevented HFD-mediated increase of *Tnfα* gene expression.

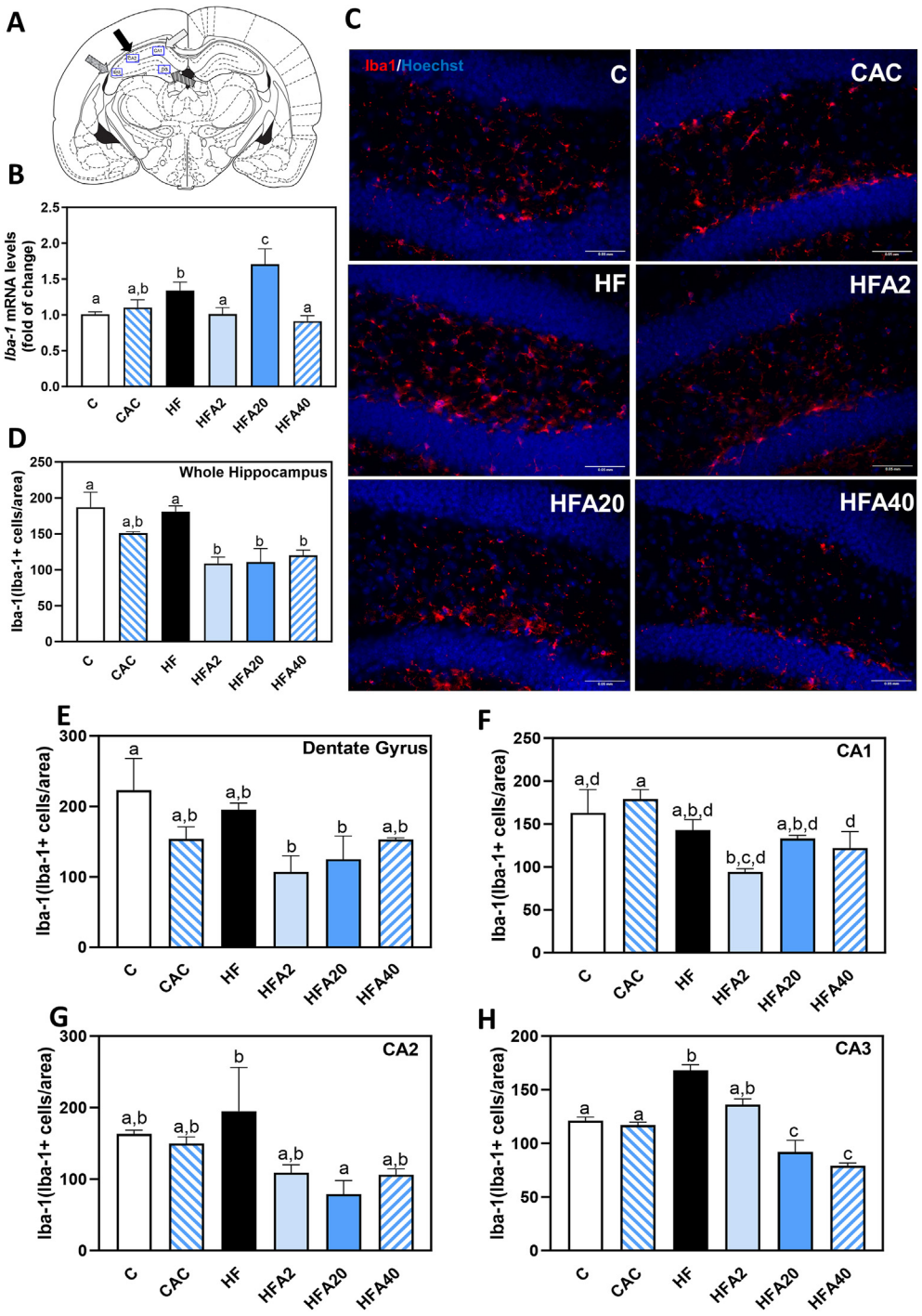

FIGURE 2. CDRE supplementation improved HFD-increased hippocampal *Iba-1* mRNA levels and microgliosis. Mice were fed a control diet (C), a control diet supplemented with 40 mg AC/kg BW (CAC), a HFD (HF), or a HFD supplemented with 2 (HFA2), 20 (HFA20), and 40 mg (HFA40) AC/kg BW. At week 14 on the corresponding diets, the following parameters were measured in the hippocampus: (A)location of dentate gyrus (striped arrow), CA1 (white arrow), CA2 black arrow), and CA3 (gray arrow) regions in the brain; (B) *Iba-1* mRNA levels were measured by qPCR. *β-actin* was used as housekeeping gene; (C) representative immunohistochemistry images showing Iba-1+ cells in hippocampal Dentate Gyrus in the different treatments' groups. Quantification of Iba-1+ cells/area in the (D) whole hippocampus; (E) dentate gyrus region; (F) hippocampal CA1 region; (G) hippocampal CA2 region; (H) hippocampal CA3 region. Results are shown as mean ± SE of 5–10 animals/group for the qPCR analysis and 2 animals/group for the immunohistochemistry analysis. Values having different superscripts are significantly different ($P < 0.05$, 1-way ANOVA). ANOVA, analysis of variance; BW, body weight; AC, anthocyanins; C, control diet; CAC, C diet supplemented with 40 mg AC/kg BW; CDRE, cyanidin- and delphinidin-rich extract; HFD, high-fat diet; HFA 2, 20, and 40, HFD supplemented with 2,20, and 40 mg AC/kg BW.

A trend was observed for the group supplemented with 2 mg AC/kg BW ($P = 0.06$) (Figure 3A). Regarding *Il-1β* mRNA levels, CDRE mitigated HFD-increased expression only at the highest concentration (40 mg AC/kg BW) (Figure 3B). CDRE supplementation did not have any significant effect on *Nox4* mRNA levels.

Effects of CDRE supplementation on parameters of GC homeostasis in HFD-fed mice

Next, we measured the effects CDRE supplementation on hippocampal GR (*Nr3c1*) and MR (*Nr3c2*) mRNA levels of mice fed an HFD. HFD-fed mice exhibited significantly lower levels of hippocampal GR and MR expression (79% and 78%, respectively) when compared the C group (Figure 4A and B). CDRE supplementation had no effect on HFD-mediated decreased GR mRNA levels (Figure 4A). Supplementation with 40 mg AC/kg BW prevented HFD-mediated decrease of MR mRNA levels (Figure 4B). Despite there not being significant differences between the HF group and the CDRE supplemented HFA2 and HFA20 groups, we observed a positive correlation between MR mRNA levels with increasing AC doses ($P = 0.012$, $r = 0.506$) (data not shown). No significant differences among treatment groups were observed for *11β-Hsd1* mRNA levels (Figure 4C). Suggesting a negative impact of inflammation on GR expression, a negative correlation was observed between GR and *Tlr4* mRNA levels ($r = 0.592$, $P = 0.0002$, Figure 4D).

Effects of CDRE supplementation on hippocampal *Bdnf* and *TrkB* mRNA levels, and BDNF expressing cells in HFD-fed mice

BDNF is known to have brain protective and anti-inflammatory effects [41]. Thus, we next evaluated the effects of CDRE supplementation on hippocampal *Bdnf* mRNA levels in mice fed an HFD. Mice from the HF group exhibited significantly lower levels of hippocampal *Bdnf* mRNA compared with the C, CAC, and HFA40 groups (Figure 5A). Within HFD-fed mice, a dose-dependent relationship was observed between CDRE supplemented and hippocampal *Bdnf* mRNA levels ($r = 0.74$, $P < 0.0001$) (data not shown). CDRE supplementation with 40 mg AC/kg BW in both, mice fed the control and HFD increased *Bdnf* mRNA levels above control values (2.6- and 1.9-fold increase, respectively) (Figure 5A). BDNF binds to the TrkB receptor activating a canonical intracellular kinase pathway involved in hippocampal neuron survival, plasticity, and growth. Consumption of the HFD did not affect *TrkB* mRNA levels compared with the C group (Figure 5B). Within HFD-fed mice, a dose-dependent relationship was observed between CDRE

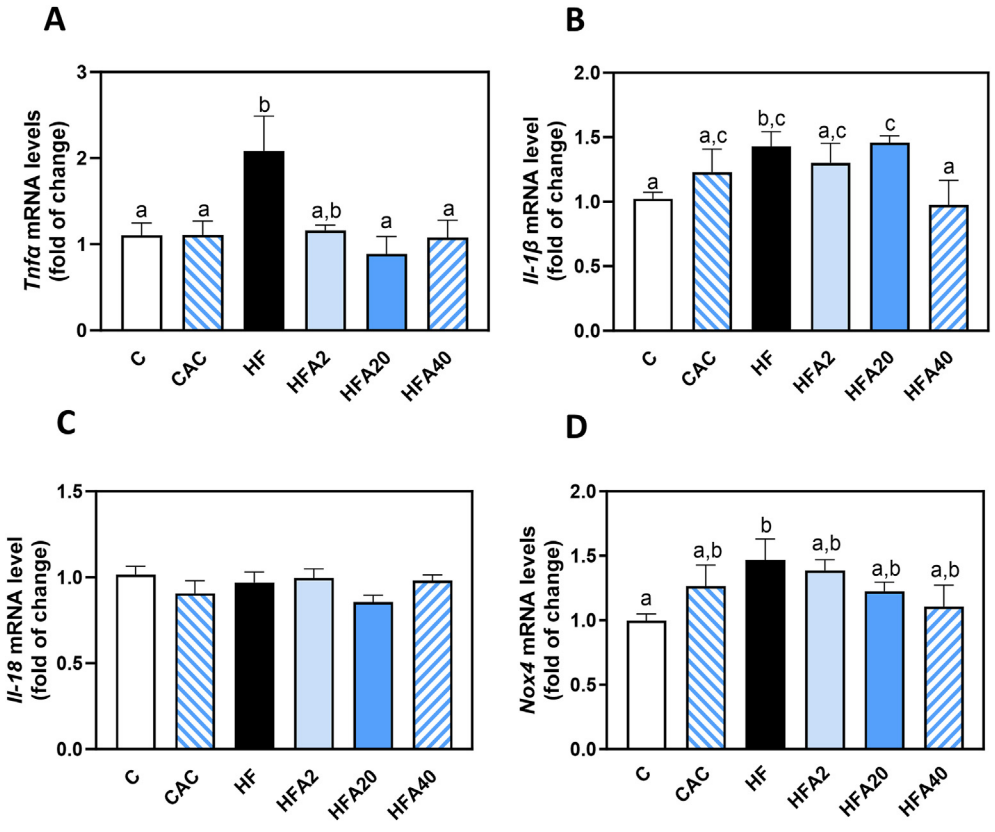

FIGURE 3. CDRE supplementation improved HFD-increased hippocampal inflammation and *Nox4* gene expression. Mice were fed a control diet (C), a control diet supplemented with 40 mg AC/kg BW (CAC), an HFD (HF), or an HFD supplemented with 2 (HFA2), 20 (HFA20), and 40 mg (HFA40) AC/kg BW. At week 14 on the corresponding diets, the following parameters were measured in the hippocampus. (A–D) mRNA levels of *Tnfα*, *Il-1β*, *Il-18*, and *Nox4* were assessed by qPCR. *β-actin* was used as housekeeping gene. Results are shown as mean ± SE of 5–10 animals/group. Values having different superscripts are significantly different ($P < 0.05$, 1-way ANOVA). ANOVA, analysis of variance; BW, body weight; AC, anthocyanins; C, control diet; CAC, C diet supplemented with 40 mg AC/kg BW; CDRE, cyanidin- and delphinidin-rich extract; HFD, high-fat diet; HFA 2, 20, and 40, HFD supplemented with 2,20, and 40 mg AC/kg BW.

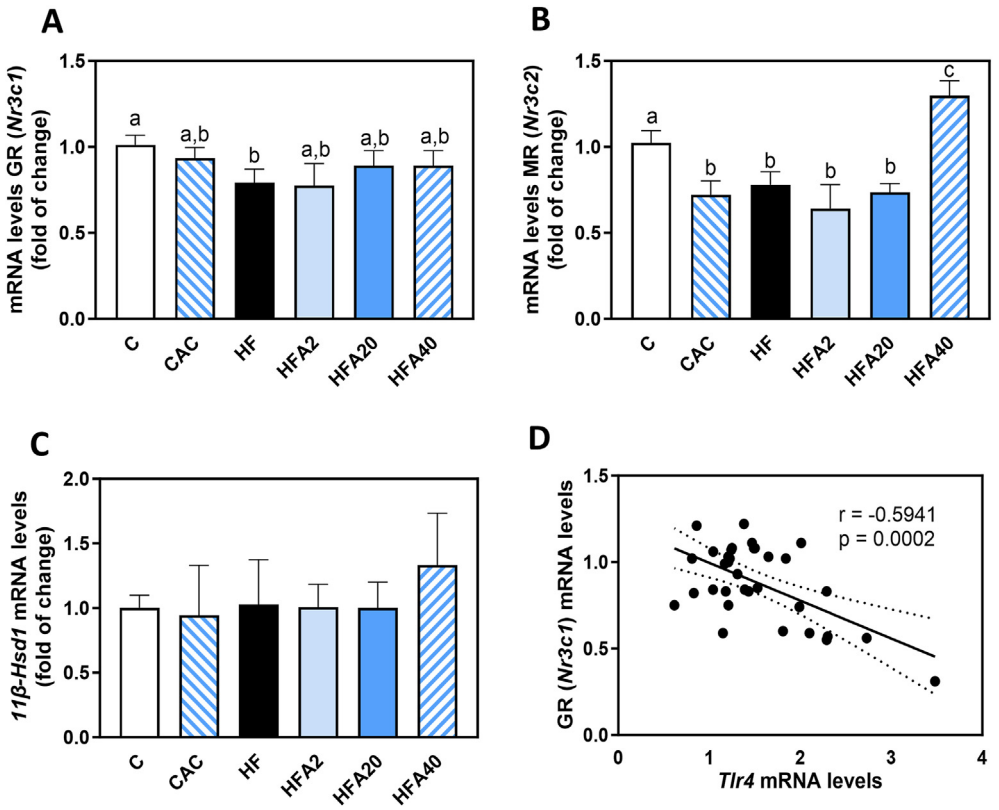

FIGURE 4. CDRE supplementation ameliorated HFD-altered GC homeostasis. Mice were fed a control diet (C), a control diet supplemented with 40 mg AC/kg BW (CAC), an HFD (HF), or a HFD supplemented with 2 (HFA2), 20 (HFA20), and 40 mg (HFA40) AC/kg BW. At week 14 on the corresponding diets, the following parameters were measured in the hippocampus. (A–C) mRNA levels of the glucocorticoid receptor (GR; *Nr3c1*), mineralcocorticoid receptor; (MR, *Nr3c2*), and *11β-Hsd1* were determined by qPCR. *β-actin* was used as housekeeping gene. (D) Correlation between plasma GR and *Tlr4* mRNA levels. The solid line represents the regression line and dashed lines delineate the 95% CI. Results are shown as mean ± SE of 5–10 animals/group. Values having different superscripts are significantly different ($P < 0.05$, 1-way ANOVA). ANOVA, analysis of variance; BW, body weight; AC, anthocyanins; C, control diet; CAC, C diet supplemented with 40 mg AC/kg BW; CDRE, cyanidin- and delphinidin-rich extract; HFD, high-fat diet; HFA 2, 20, and 40, HFD supplemented with 2,20, and 40 mg AC/kg BW.

supplementation dose and hippocampal *TrkB* mRNA levels ($r = 0.70$, $P < 0.0005$) (data not shown). Immunohistochemistry staining was used to evaluate BDNF+ cells per area to determine how HFD and CDRE supplementation affects neural expression of the protein (Figure 5D and E). In the dentate gyrus, no significant differences in the number of BDNF+ cells per area were observed between the HF and C group. CDRE supplementation (40 mg AC/kg BW) of control- and HFD-fed mice led to a higher number of BDNF+ cells per area compared with HF values (56% and 74%, respectively (Figure 5E). There were no significant differences among the groups for BDNF+ cells in the hippocampal CA3 region (Supplemental Figure 8).

Discussion

This work shows that CDRE supplementation mitigated the hippocampal inflammation associated with the consumption of high dietary fat and obesity in mice. Thus, HFD consumption induced metabolic endotoxemia and upregulated hippocampal inflammatory markers, which were both mitigated by CDRE supplementation. The beneficial effects of AC can be in part due to the prevention of endotoxemia and to the restoration of MR and BDNF expression. Results suggest a potential benefit of eating AC-rich diets against HFD/obesity-induced neuroinflammation.

This study expands upon previous work [9,35] that utilized the same mouse cohort to examine the effects of CDRE on metabolic, intestinal, and liver outcomes. Previous results showed that AC consumption mitigates HFD-induced altered lipid and glucose homeostasis, liver inflammation and insulin resistance. Consistent with those findings [42,43], we currently observed that consumption of the HFD for 14 wk caused hippocampal neuroinflammation. This was evidenced by the development of microgliosis and the associated upregulation of proinflammatory mediators, including Tlr4, Tnfα, and Il-1β, which were mitigated by supplementation with CDRE. These protective effects could be due to AC-mediated: *1*) preservation of the intestinal barrier function and prevention of endotoxemia, *2*) mitigation of altered GC metabolism, and *3*) upregulation of hippocampal BDNF.

The intestinal barrier function is regulated by the tight junctions, which integrity is compromised by HFD consumption [9,30,32]. Intestinal barrier permeabilization allows the passage of LPS

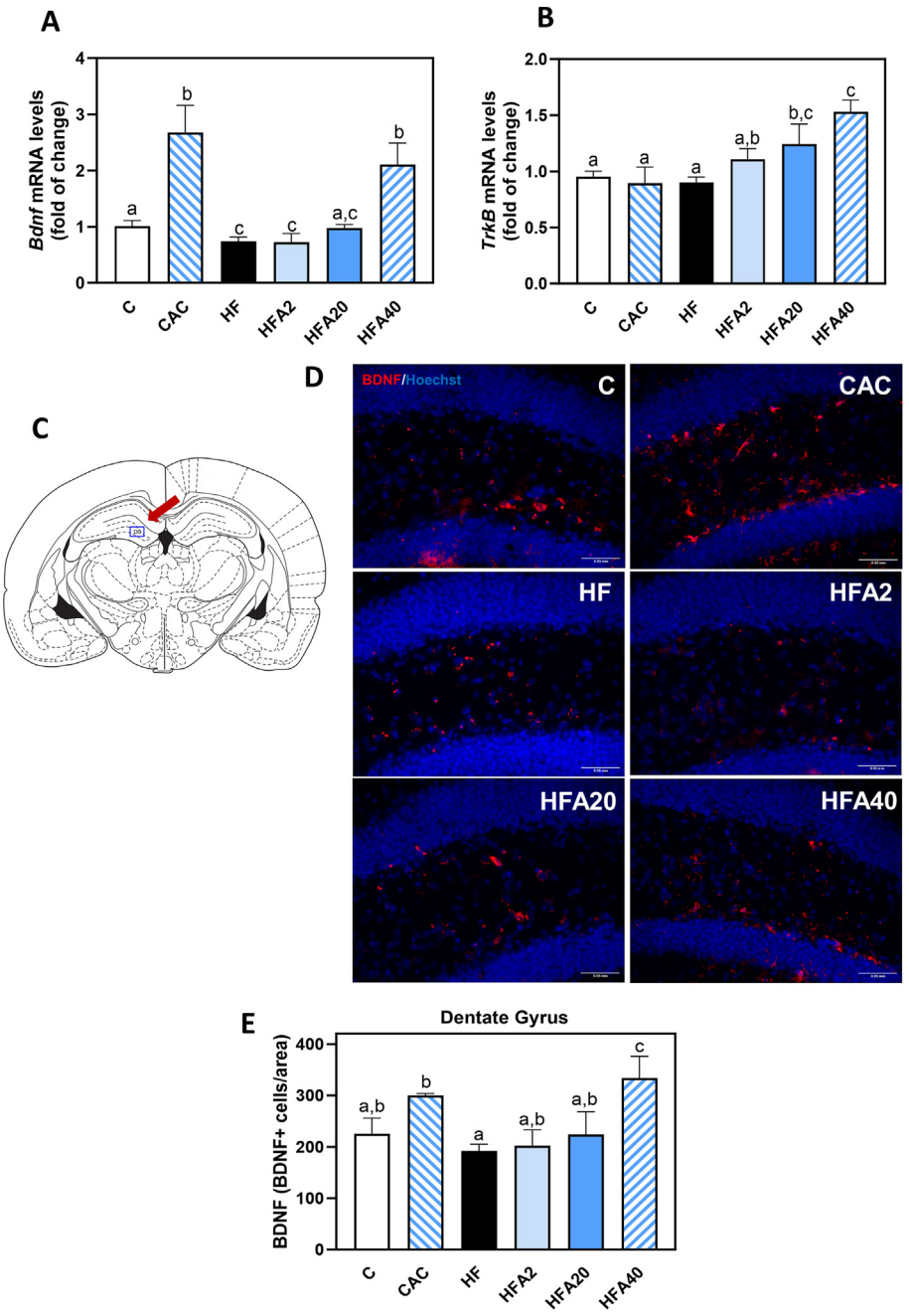

FIGURE 5. CDRE supplementation improved BDNF homeostasis. Mice were fed a control diet (C), a control diet supplemented with 40 mg AC/kg BW (CAC), an HFD (HF), or an HFD supplemented with 2 (HFA2), 20 (HFA20), and 40 mg (HFA40) AC/kg BW. At week 14 on the corresponding diets, the following parameters were measured in the hippocampus: mRNA levels of (A) *Bdnf* and (B) *TrkB* were determined by qPCR. *β-actin* was used as housekeeping gene. (C) Location of dentate gyrus region in the brain. (D) Representative immunohistochemistry images showing BDNF+ cells in the different treatments' groups. (E) Quantification of BDNF+ cells/area in the dentate gyrus region of the hippocampus. Results are shown as mean ± SE of 5–10 animals/group for the qPCR analysis, and 2 animals/group for the immunohistochemistry analysis. Values having different superscripts are significantly different ($P < 0.05$, 1-way ANOVA). ANOVA, analysis of variance; BW, body weight; AC, anthocyanins; C, control diet; CAC, C diet supplemented with 40 mg AC/kg BW; CDRE, cyanidin- and delphinidin-rich extract; HFD, high-fat diet; HFA 2, 20, and 40, HFD supplemented with 2,20, and 40 mg AC/kg BW.

from the intestinal lumen into the circulation (metabolic endotoxemia), which among other adverse effects, can trigger neuroinflammation. In agreement with our previous report [9,32], consumption of the HFD caused endotoxemia as indicated by increased plasma LPS and LBP concentrations. Supplementation with CDRE mitigated HFD-mediated increases in both LPS and LBP. This can be due to AC capacity to protect tight junctions against disruption and permeabilization through the regulation of the NF-κB and ERK1/2 pathways, as previously observed both in vitro and in vivo [9]. Findings of higher hippocampal expression of Tlr4 in HFD-fed mice and of a positive correlation between plasma LPS and hippocampal Tlr4 mRNA levels strongly suggest

that metabolic endotoxemia is in part involved in HFD-induced hippocampal inflammation and that mitigation of endotoxemia by CDRE supplementation in part contributes to the decreased neuroinflammation. Activation of Iba-1+ microglia, which express TLR4 receptors, leads to the production of cytokines as a neuroprotective mechanism [40]. However, chronic microglial activation and the resultant sustained pro inflammatory cytokine production may conversely damage neural tissues and inhibit BDNF production [40,44]. Consistent with a protective effect of AC through the modulation of endotoxemia, CDRE mitigated HFD-mediated microgliosis, as evidenced by the prevention of HFD-mediated increases in hippocampal Iba-1 mRNA levels and Iba-1+ cells in the C3 region. CDRE also decreased HFD-mediated microglia activation as evidenced by its capacity to prevent HFD-induced increases of Tnfα and Il-1β gene expression.

The current study provides evidence that CDRE supplementation decreases HFD/obesity-induced neuroinflammation in part by mitigating endotoxemia. However, effects of CDRE supplementation on the gut microbiota, and of absorbed AC and their metabolites on the brain could also contribute to the observed CDRE beneficial actions on neuroinflammation. We previously observed that, in the same animal cohort, CDRE supplementation improved HFD-induced dysbiosis. Thus, CDRE supplementation prevented HFD-mediated alterations of cecal microbiota by restoring a normal *Firmicutes/Bacteroidetes* ratio and relative abundance of *Akkermansia muciniphila* [9]. In addition, AC-mediated decrease in intestinal gram negative bacteria could potentially decrease the generation of endotoxins given that they are components of the outer membrane of this particular bacteria population. In terms of direct AC effect on the brain, evidence suggests that AC and their metabolites can cross the blood brain barrier. In this regard, pre-clinical studies showed that AC, particularly cyanidin, and AC metabolites can be found in the brain after oral AC consumption [45,46]. Thus, after 15 min of cyanidin-3-O-glucoside oral consumption, this cyanidin was found in the brain, achieving the highest concentration after 45 min [45]. In rodents and after consumption of strawberries and black currants, while at a lesser extent than other polyphenols, AC and their metabolites were detected in the brain [47]. Overall, besides protecting intestinal barrier function and preventing endotoxemia, initial evidence supports the concept that effects on intestinal microbiota and direct brain effects of AC and their metabolites could contribute to mitigate HFD/obesity-induced neuroinflammation.

The HPA axis is affected by stress and inflammation [48,49]. Consumption of a HFD impairs the expression of the GR and MR in mouse hippocampus [28]. Although both MR and GR bind to GC, in the hippocampus the MR has a binding affinity over 10 times higher for GC and is more abundant than the GR [26]. Because of this, it is thought that in the hippocampus the MR plays a different role than the GR in regulating physiologic hippocampal GC activity. Although we observed that the HFD caused a decrease in MR expression, supplementation with 40 mg AC/kg BW not only prevented this decrease but also increased MR mRNA levels above control levels. The observed increase is important because MR expression is linked to an increase in long-term potentiation via AMPA receptor trafficking, suggesting a mechanism for AC to exert potential cognitive benefits [27]. It has been shown that GR and TrkB interact to stimulate the BDNF/PLC-ϒ signaling pathway, which is important for glutamate release [4,50] and long distance signaling [51]. One reason for the suppression of the pathway is through a decrease in GR expression [50]. GR's capacity to interact with TrkB link GR and BDNF neurotrophic effects [52,53]. Although CDRE supplementation did not significantly prevent the decrease in hippocampal GR mRNA levels in mice fed the HFD, values were similar to those of C mice. Overall, the beneficial effect of CDRE supplementation on HFD-mediated alterations of the HPA axis could contribute to preserve hippocampal function by regulating GCs actions, enhancing long-term potentiation, and fostering BDNF-like tropic effects through GR and TrkB interactions.

Consumption of energy dense foods and obesity are associated to BDNF downregulation [6,15]. On the other hand, higher BDNF circulating levels have been found to have protective effects on cognition, behavioral challenges [16,54], and cardiovascular health [55]. Hippocampal BDNF mRNA levels are low in mice fed a high sucrose diet [56] and in HFD-fed obese mice [6]. In humans, there is an inverse relationship between serum BDNF levels and systemic inflammation in obese women [57] and children [58]. We observed that CDRE supplementation not only prevented HFD/obesity-mediated decrease in hippocampal Bdnf expression, but also increased Bdnf mRNA levels above control values and caused a dose-dependent increase in the expression of both Bdnf and its receptor (TrkB). Consistent with these findings, in a mouse model of chronic unpredictable mild stress, supplementation with an AC-rich extract from purple cauliflower increased hippocampal BDNF levels and dendrite thickness [59]. BDNF levels decrease in the hippocampus after prolonged inflammation [17]. Our data showing the capacity of AC to mitigate hippocampal inflammation suggests that this could be in part involved in the prevention of HFD/obesity-mediated decrease in BDNF expression by CDRE supplementation. However, additional mechanisms must be involved given the capacity of CDRE supplementation to increase Bdnf mRNA levels above control values in mice fed either the control or the HFD.

The link among neuroinflammation, alterations of the HPA axis [49], and BDNF decrease [17] provides a comprehensive view of the possible interplay between neuroinflammation and AC supplementation's potential to counteract HFD/obesity-induced negative effects in the hippocampus. Findings highlight the potential benefits of incorporating AC-rich foods into diets to counteract the adverse effects of HFD- and obesity-associated chronic inflammation and potential cognitive and behavioral alterations. Given the limitation of the current study in terms of lacking supporting behavioral data, further investigations are needed to explore the link between the actions of AC modulating neuroinflammation, HPA axis dysregulation and BDNF physiology, to HFD/obesity-associated alterations in behavior and cognition.

Author contributions

The authors' responsibilities were as follows – IM, EC, PM: conducted research; EC, PIO: designed research; IM, EC, PM, AMA: performed statistical analysis; IM, AMA, PIO: wrote the article; PIO: provided essential reagents and materials and had primary responsibility for the final content; and all the authors: read and approved the final manuscript.

Conflict of interest

EC has received a research grant from NSE Products Inc. PIO has received research grants from NSE Products Inc. and is a member of the NSE Products Inc. Advisory Board. PIO has also received research grants from other food companies and government agencies with an interest in health and nutrition. All other authors report no conflicts of interest.

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Data availability

Data described in the manuscript will be made available from the corresponding author upon reasonable request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tjnut.2024.07.028.

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