Cyanidin and delphinidin modulate inflammation and altered redox signaling improving insulin resistance in high fat-fed mice.
Research Paper

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\textbf{A B S T R A C T}

Consumption of diets high in fat and/or fructose content promotes tissue inflammation, oxidative stress, and insulin resistance, activating signals (e.g. NF-κB/JNK) that downregulate the insulin cascade. Current evidence supports the concept that select flavonoids can mitigate obesity and type 2 diabetes (T2D). This work investigated if supplementation with the anthocyanidins (AC) cyanidin and delphinidin could attenuate the adverse consequences of consuming a high fat diet (HFD) in mice. Consumption of an AC-rich blend mitigated HFD-induced obesity, dyslipidemia and insulin resistance (impaired responses to insulin and glucose). HFD-fed mice were characterized by increased liver lipid deposition and inflammation, which were also attenuated upon AC supplementation. HFD caused liver oxidative stress showing an increased expression of NADPH oxidases, generators of superoxide and H\textsubscript{2}O\textsubscript{2}, and high levels of oxidized lipid-protein adducts. This was associated with the activation of the redox sensitive signals IKK/NF-κB and JNK1/2, and increased expression of the NF-κB-regulated PTP1B phosphatase, all known inhibitors of the insulin pathway. In agreement with an improved insulin sensitivity, AC supplementation inhibited oxidative stress, NF-κB and JNK activation, and PTP1B over-expression. Thus, cyanidin and delphinidin consumption either through diet or by supplementation could be a positive strategy to control the adverse effects of Western style diets, including overweight, obesity, and T2D. Modulation of inflammation, oxidative stress, and NF-κB/JNK activation emerge as relevant targets of AC beneficial actions.

1. Introduction

Overweight and obesity put individuals at risk of major health problems including type 2 diabetes (T2D), nonalcoholic fatty liver disease (NAFLD) and cardiovascular disease. Consumption of Western style diets can be a major contributing factor to the increased rates of overweight and obesity in human populations, while consumption of select fruits and vegetables could attenuate these conditions. Evidence for the latter is conflicting when considering overall intakes, types of fruits and vegetables consumed, and other variables associated with population studies [1–3]. On the other hand, a large body of evidence in experimental animals suggests a benefit of select phytochemicals present in fruits and vegetables in the development of obesity and associated pathologies triggered by consumption of high fructose and/or high fat diets.

Among phytochemicals, anthocyanidins (AC) are flavonoids being actively investigated for their potential to mitigate unhealthy conditions, particularly metabolic disorders. In this regard, mounting evidence supports a potential beneficial action of AC consumption on T2D [4] and cardiovascular health [5]. Furthermore, AC-rich food

\textit{Abbreviations:} AC, anthocyanidins; GIP, gastric inhibitory polypeptide; GLP-1, glucagon-like peptide-1; GTT, glucose tolerance test; HFD, high fat diet; 4-HNE, 4-hydroxynonenal; IKK, IκB kinase; IRS1, insulin receptor substrate-1; ITT, insulin tolerance test; JNK, c-jun N-terminal kinase; MCP-1, monocyte chemotactic protein-1; NAFLD, nonalcoholic fatty liver disease; NOS2, inducible nitric oxide synthase; NOX, NADPH oxidase; PTP1B, protein tyrosine phosphatase 1B; TNF\textsubscript{α}, tumor necrosis factor alpha; T2D, type 2 diabetes.

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consumption is inversely correlated with overall mortality [6]. AC are flavonoids that exist in nature as anthocyanins, the glycosylated forms of AC. They provide color to grapes, berries, blueberries, black currants, bilberries, purple corn, and black rice, among other fruits and vegetables. With the basic three-ring structure of flavonoids, AC are characterized by double bonds in the three rings and a positive charge in the B ring on the oxygen atom [7]. Different hydroxyl substitutions in number and position define different AC, e.g. delphinidins, malvidins, andpeonins. These differences in substitutions can have a major impact on AC biological actions in animals. In this regard, we recently observed that 3-O-glucosides of cyanidin and delphinidin were more efficient than malvidin, petunidin and peonidin 3-O-glucosides at inhibiting tumor necrosis factor alpha (TNFα)-induced activation of transcription factor NF-κB in Caco-2 cells [8].

Dietary energy overload can cause tissue inflammation, oxidative stress, and insulin resistance. Excess fat consumption leads to the activation of inflammatory and redox-regulated events including: i) the IκB kinase (IKK), and downstream the transcription factor NF-κB; and ii) the mitogen activated kinase c-jun N-terminal kinase (JNK). Activation of both JNK [9,10] and IKK [11] and the increased expression of the NF-κB-regulated protein tyrosine phosphatase 1B phosphatase (PTP1B) [12] downregulate the insulin signaling pathway leading to insulin resistance. Inflammation, oxidative stress, and chronic NF-κB activation also contribute to other major adverse consequences of obesity, e.g. NAFLD and cardiovascular disease [13–15].

Identifying fruits and vegetables and their active components that can provide protection against the adverse effects of consuming Western style diets has the potential to have a major impact on human health. Moreover, understanding the mechanisms by which these components act modifying cell functions is crucial to define public recommendations in terms of diets and potential supplementation. This work investigated the capacity of a diet enriched in the AC cyanidin and delphinidin to mitigate in mice the development of obesity, dyslipidemia, steatosis, and insulin resistance promoted by the chronic consumption of a HFD. The beneficial effects of AC were mainly associated with the attenuation of liver inflammation, oxidative stress, and downregulation of the redox sensitive JNK and IKK/NF-κB. These findings stress the concept that cyanidins and delphinidins can provide benefits against excess fat consumption and its adverse health consequences.

2. Materials and methods

2.1. Materials

Cholesterol and triglyceride concentrations were determined using kits purchased from Wiener Lab Group (Rosario, Argentina). Glucose levels were measured using a kit purchased from Sigma-Aldrich Co (St. Louis, MO). Concentrations of insulin, glucagon-like peptide-1 (GLP-1), gastric inhibitory polypeptide (GIP), leptin and adiponectin were determined using kits purchased from Crystal Chem Inc. (Downers Grove, IL). Antibodies for monoocyte chemoattractant protein-1 (MCP-1) (sc-6254), nitric oxide synthase 2 (NOS2) (sc-649), phospho (Thr183/184) - JNK (sc-67005), phospho (Ser176/180) - IKKα/β (sc-25830), and F4/80 (ab213900) were from Cell Signaling Technology (Danvers, MA). Antibodies for F4/80 (sc-25830), nitric oxide synthase 2 (NOS2) (sc-649), phospho (Thr183/184)- JNK - (sc-6254) and NOX3 (sc-67005) were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies for 4-hydroxyxenononal (4-HNE) (ab46545), NOX4 (ab133303) and gp91phox (ab129068) were from Abcam, Inc. (Cambridge, MA). The antibody for PTP1B (ABS40) was from EMD Millipore (Hayward, CA). PVDF membranes were obtained from BIO-RAD (Hercules, CA). The Enhanced chemiluminescence (ECL) Western blotting system was from Thermo Fisher Scientific Inc. (Piscataway, NJ). All other chemicals were purchased from Sigma-Aldrich Co (St. Louis, MO). The AC-rich blend was provided by NSE Products, Inc. (Provo, UT) and its composition is shown in Table 1.

2.2. Determination of AC blend composition

The AC-rich blend was analyzed using a liquid chromatography method [16,17] as previously described [8]. Briefly, the blend was dissolved in water, filtered through PTFE (0.22 µm) membranes and separations performed using an Agilent series 1200 instrument (Agilent Technologies, Santa Clara, CA) with a Kinetex F5 pentfluorophenyl HPLC column (2.6 µm, 100 × 4.6 mm) and SecurityGuard® cartridge (PPF, 4.0 × 2.0 mm) (Phenomenex, Torrance, CA). A flow rate of 0.70 ml/min and a column temperature of 37°C was set. The injector temperature was 4°C with an injection volume of 7µl. Detection was done by UV–Vis DAD (wavelength monitored at 280 and 520 nm) and ESI-MS-MS for mass spectral results. Quantification was based on DAD peak area absorbance at 520 nm. A binary gradient was employed consisting of 1.0% formic acid (v/v) in water (mobile phase A) and 1.0% formic acid (v/v) in acetonitrile (mobile phase B). Gradient was as follows: 1% B at 0 min, 7.5% B at 7 min, 7.6% B at 14 min, 10% B at 17 min, 12% B at 18.5 min, 30% B at 24 min, 90% B at 25 min, 1% B at 26–30 min. Mass spectral data were acquired using an Agilent 6430 triple-quadrupole mass spectrometer with electrospray injection (Agilent Technologies, Santa Clara, CA, USA) set to scan mode with the following optimal MS/MS source parameters: nebulizer at 40 psi, capillary voltage + 4000 V, gas temperature 325 °C, and flow of 5 l/min. Sheath gas was 250°C and sheath flow of 11 l/min. AC were identified after ionization in their molecular cation form under MS positive ion mode. Parent ions (m/z) were previously described [8].

2.3. 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; experimental protocols were approved before

| Compounds               | mg/g dry weight extract |
|-------------------------|-------------------------|
| **Anthocyanidins**      |                         |
| Cyanidin                | 95                      |
| Delphinidin             | 40                      |
| Peonidin                | 8                       |
| Malvidin                | ND                      |
| Petunidin               | ND                      |
| Pelargonidin            | ND                      |
| **Benzoic acids**       |                         |
| 3-methylgallic         | ND                      |
| 4-hydroxybenzoic acid   | ND                      |
| Ferulic acid            | 0.1                     |
| Fumaric acid            | ND                      |
| Gallic acid             | 0.8                     |
| Protocatechic acid      | 14.9                    |
| Quinic acid             | ≤ 0.02                  |
| Shikimic acid           | ND                      |
| Syringic acid           | ≤ 0.02                  |
| Vanillic acid           | 0.4                     |
| **Hydroxyccinnamates**  |                         |
| Caffeic acid            | 1.0                     |
| Chlorogenic acid        | 1.0                     |
| p-Guaiaric              | 3.4                     |
| Sinapinic acid          | 0.7                     |
| **Flavanols**           |                         |
| Kaempferol-3-galactose  | ND                      |
| Kaempferol-3-glucose    | 3.4                     |
| Kaempferol              | ≤ 0.05                  |
| Naringenin              | ≤ 0.05                  |
| Quercetin               | 0.1                     |
| Quercetin-3-galactose   | 1.2                     |
| Quercetin-3-Rutin       | 0.1                     |
| Catechin                | ND                      |
| Epicatechin             | ≤ 0.001                 |
| Gallocatechins          | ND                      |

ND: Not detected.
Implementation by the University of California, Davis Animal Use and Care Administrative Advisory Committee. Procedures were administered under the auspices of the Animal Resource Services of the University of California, Davis.

Healthy male C57BL/6J mice (20 g) (10 mice/group) were fed a control diet (empty triangles), the control diet supplemented with 40 mg AC/kg body weight (full triangles), a HFD (black circles), or the HFD supplemented with 2, 20, or 40 mg AC/kg body weight (light pink diamonds), 20 (dark pink diamonds), or 40 mg AC/kg body weight (CA40, HF). Cholesterol, triglycerides, glucose, and insulin were measured in plasma. Values are shown as means ± SE (n = 10). Values having different superscripts are significantly different (p < 0.05, one way ANOVA).

### Table 2

| Parameter                  | Control | CA40 | HF     | HFA2  | HFA20 | HFA40 |
|----------------------------|---------|------|--------|-------|-------|-------|
| Daily food intake (g/d)    | 3.7 ± 0.2a | 4.1 ± 0.1c | 3.0 ± 0.1b | 3.0 ± 0.1b | 2.8 ± 0.1b | 2.9 ± 0.03b |
| Body weight (g)            | 33.7 ± 0.8b | 35.8 ± 1.0a | 44.1 ± 1.8a | 41.6 ± 1.2c | 41.0 ± 1.2c | 38.1 ± 2.2a |
| Brown fat (mg)             | 149 ± 12a  | 213 ± 24b  | 259 ± 20b  | 204 ± 32c | 201 ± 20b  | 176 ± 20a  |
| Epididymal fat (g)         | 1.23 ± 0.10c | 1.45 ± 0.15a | 2.36 ± 0.10b | 2.28 ± 0.21b | 2.49 ± 0.09b | 2.22 ± 0.29a |
| Visceral fat (g)           | 0.53 ± 0.10a  | 0.55 ± 0.07ac | 1.28 ± 0.12b | 1.02 ± 0.12c | 1.16 ± 0.18b | 0.89 ± 0.12a |
| Subcutaneous fat (g)       | 0.98 ± 0.06a  | 1.59 ± 0.19a  | 3.54 ± 0.23b | 2.78 ± 0.36b | 3.43 ± 0.27b | 2.71 ± 0.49b |
| Retroperitoneal fat (g)    | 0.40 ± 0.27a  | 0.54 ± 0.07ab | 1.26 ± 0.09b | 0.95 ± 0.07ab | 1.25 ± 0.15b | 0.84 ± 0.13b |
| Total cholesterol (mg/dl)  | 126 ± 3a   | 134 ± 5b   | 160 ± 3b   | 147 ± 3c   | 156 ± 6b   | 138 ± 5b   |
| Triglycerides (mg/dl)      | 84 ± 2a    | 84 ± 2a    | 92 ± 1c    | 86 ± 2b    | 86 ± 3a    | 86 ± 2a    |
| Fasted glucose (mg/dl)     | 164 ± 8a  | 200 ± 12a  | 230 ± 11b  | 206 ± 17b  | 225 ± 11b  | 161 ± 13a  |
| Fasted insulin (ng/ml)     | 0.53 ± 0.04a | 0.47 ± 0.04c | 1.22 ± 0.10b | 0.88 ± 0.20a | 1.08 ± 0.24b | 0.61 ± 0.10c |

For insulin tolerance tests (ITT), mice were fasted for 4 h and injected i.p. with 1 U human insulin/kg body weight. Blood glucose values were measured before and at 15, 30, 45, 60, and 120 min post-injection. For glucose tolerance tests (GTT), overnight fasted mice were injected with i.p.-glucose (2 g/kg body weight), and blood glucose was measured before and at 15, 30, 60, and 120 min post-injection. For both tests, glucose levels were measured using a glucometer (Easy Plus II, Home Aid Diagnostics Inc, Deerfield Beach, FL). At the end of the study, plasma total cholesterol, triglycerides, glucose, insulin, adiponectin, leptin, GLP-1 and GIP concentrations were determined following manufacturer’s guidelines.

### 2.5. Determination of fecal and liver triglyceride content

Fecal triglyceride content was measured using a modified method to that proposed by Folch et al. [18]. Fecal samples were collected over 24 h from single cages (3–4 mice) and dried at 37 °C for 24 h. Dried feces (0.5 g) were ground to a fine powder using a mortar and pestle. The lipid extraction was performed by homogenizing the fecal powder with 500 ml of chloroform-methanol (2:1, v/v) solution. Samples were mixed for 5 min and centrifuged at 1000 × g for 15 min at 4 °C. Different adipose tissue pads, and liver were collected and weighed. Tissues were flash frozen in liquid nitrogen and then stored at −80 °C for further analysis.

### 2.4. Metabolic measurements

For insulin tolerance tests (ITT), mice were fasted for 4 h and injected i.p. with 1 U human insulin/kg body weight. Blood glucose values were measured before and at 15, 30, 45, 60, and 90 and 120 min post-injection. For glucose tolerance tests (GTT), overnight fasted mice were injected with i.p.-glucose (2 g/kg body weight), and blood glucose was measured before and at 15, 30, 60, and 120 min post-injection. For both tests, glucose levels were measured using a glucometer (Easy Plus II, Home Aid Diagnostics Inc, Deerfield Beach, FL). At the end of the study, plasma total cholesterol, triglycerides, glucose, insulin, adiponectin, leptin, GLP-1 and GIP concentrations were determined following manufacturer’s guidelines.
2.6. Histological analyses

The liver was removed and samples fixed overnight in 4% (w/v) neutralized paraformaldehyde solution. Samples were subsequently washed twice in phosphate buffer saline solution, dehydrated, and then embedded in paraffin for histological analysis. Sections (5 µm thickness) were obtained from paraffin blocks and placed on glass slides. Hematoxylin and eosin staining was performed following standard procedures. Sections were examined using an Olympus BX51 microscope (Olympus America Inc., Center Valley, PA). Hepatic histological examination was performed using the NAFLD activity score (NAS) described by Kleiner et al. [20]. Three randomly selected fields per animal were assessed and analyzed using Pro Plus 5.1 software (Media Cybernetics, Rockville, MD).

2.7. Western blot analysis

Livers were homogenized as previously described [21]. Aliquots of total homogenates containing 25–40 µg protein were denatured with Laemmli buffer, separated by reducing 7.5–12.5% polyacrylamide gel electrophoresis, and electroblotted to PVDF membranes. Membranes were blocked for 2 h in 5% (w/v) bovine serum albumin and subsequently incubated in the presence of the corresponding primary antibodies (1:1000 dilution) overnight at 4°C. After incubation for 90 min at room temperature in the presence of secondary antibodies (HRP conjugated) (1:10,000 dilution), the conjugates were visualized using enhanced chemiluminescence.

2.8. Electrophoretic mobility shift assay (EMSA)

NF-κB-DNA binding was assessed in the nuclear fractions obtained from liver as previously described [22,23]. The EMSA was performed by end labeling the oligonucleotide containing the consensus sequences for NF-κB with [γ-32P] ATP. The oligonucleotide was end-labeled using T4 polynucleotide kinase and purified using Chroma Spin-10 columns. Samples were incubated with the labeled oligonucleotide (20,000–30,000 cpm) for 20 min at room temperature in 1X binding buffer [5X binding buffer: 50 mM Tris-HCl buffer, pH 7.5, containing 20% (v/v) glycerol, 5 mM MgCl2, 2.5 mM EDTA, 2.5 mM DTT, 250 mM NaCl, and 0.25 mg/ml poly(dI-dC)]. The products were separated by electrophoresis in a 6% (w/v) non-denaturing polyacrylamide gel using 0.5 X TBE (45 mM Tris/borate, 1 mM EDTA) as the running buffer. The gels were dried and the radioactivity quantified in a Phosphoimager 840 (Amersham Pharmacia Biotech. Inc., Piscataway, NJ).

2.9. Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) using Statview 5.0 (SAS Institute Inc., Cary, NC). Fisher least significance difference test was used to examine differences between group means. A repeated measure ANOVA with Tukey-Kramer multiple comparison test was used to analyze changes in body weight and food intake. A P value < 0.05 was considered statistically significant. Data are shown as means ± SEM.

3. Results

3.1. Diets

To prepare the diets containing AC, in particular cyanidin and delphinidin, control and high fat diets were added with a blend of berry and black rice extracts. The polyphenol composition of the AC-rich blend was determined as described in Methods and is shown in Table 1. It can be 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.

3.2. Animal outcomes

Daily food intake in the groups fed the HFD was significantly lower than in those fed the control and CA diets (Table 2, Fig. 1A). However, the calorie intake was similar within groups (14.4 and 14.8 kcal/d for control- and HFD-fed mice, respectively). Weekly food intake did not significantly vary within groups (Fig. 1A).

Starting at week 4 and through the following weeks, the body weight gain for C, CA, and HFD40 groups was significantly lower than for the HF group (Fig. 1B). At week 14, there was a dose-dependent decrease in body weight depending on the amount of AC in the diet (Table 2). At the end of the study, consumption of the HFD caused a 31% higher body weight compared to controls, while the body weight of HFD40 mice was 14% lower than in HF mice.

HFD-induced obesity was associated with an increased weight of the different fat pads (brown, epididymal, visceral, subcutaneous and retroperitoneal) (Table 2). HFD40 mice showed a brown fat content similar to C mice, and 72% and 49% lower visceral and retroperitoneal fat accumulation, respectively, compared to HF mice. Brown fat weight in CA mice was 43% higher than in the C group.

Consumption of the HFD also caused dyslipidemia. Plasma cholesterol and triglyceride levels were 26% and 9% higher in HF than in C mice, respectively (Table 2). Supplementation of HFD-fed mice with AC prevented the increase of plasma triglyceride concentrations at all the AC concentrations tested, while plasma cholesterol increase was only prevented at the highest AC supplementation level, i.e. HFA40 group.

While there were no significant differences in the amount of triglycerides excreted with the feces among the control, HF and CA groups (10.2 ± 0.8, 12.0 ± 0.3 and 12.0 ± 0.1 mg/g feces, respectively), fecal triglyceride amount in the HFA40 group (15.6 ± 3.6 mg/g feces) was significantly higher than in the control group (p < 0.04). Fecal cholesterol content was significantly higher (p < 0.05) in the HF and HFA40 groups (8.0 ± 0.3 and 6.0 ± 0.8 mg/g feces, respectively) than in control and CA groups (2.7 ± 0.1 and 3.2 ± 0.3, respectively).

3.3. Supplementation with AC improved glucose homeostasis in mice fed a high fat diet

Fasting plasma glucose and insulin were 40% and 130% higher, respectively, in the HF compared to the C group. In the HFA40 group, both parameters showed values similar to those observed in the C and CA groups, while in the HFA2 group only plasma insulin was similar to control values (Fig. 2A, B). The area under the curve for the GTT and ITT was 41% and 24% higher, respectively, in HF than in C and CA mice. Supplementation with AC at the highest dose tested caused a 59% reduction of the increase in GTT area under the curve, and total prevention of the increase in ITT area under the curve. Given that supplementation with
40 mg AC/kg body weight was the AC level that provided the strongest level of protection against HFD-induced insulin resistance, subsequent experiments were focused only on the HFA40 group.

3.4. Supplementation with AC improved plasma hormone profiles

We next investigated the levels of select hormones, which are relevant to the regulation of glucose homeostasis, and that are produced by the adipose tissue, i.e. adiponectin and leptin, and by gastrointestinal enteroendocrine cells, i.e. GIP and GLP-1. While plasma adiponectin levels were similar among groups, plasma leptin was 4 times higher in the HF compared to the C group (Fig. 3A, B). The increase in leptin observed in HF mice was partially (63%) prevented in HFA40 mice. HFD consumption caused a significant increase (83%) in plasma GIP that was not observed in the HFA40 group (Fig. 3C). Plasma GLP-1 concentration was significantly higher in HF, HFA40 and CA groups (57%, 52%, and 98%, respectively) compared to the C group (Fig. 3D).

3.5. Supplementation with AC attenuated steatosis and inflammation in the liver of mice fed a high fat diet

Consumption of the HFD caused steatosis and liver inflammation. Liver triglyceride levels were 76% higher in the HFD-fed compared to the C group. This increase in liver triglycerides was not observed in the HFA40 group. (Fig. 4A). Lipid deposition was also assessed by histological analysis after hematoxylin-eosin staining (Fig. 4B). Consumption of the HFD caused an increased lipid deposition in the liver that was not observed in HFA40 mouse liver. The NAFLD activity score (NAS) was significantly higher (6.4 folds) in the liver from HF compared to C, CA, and HFA40 mice (Fig. 4C).

Several proteins involved in the inflammatory response were measured in liver by Western blot (Fig. 4D). The chemokine MCP-1, the cytokine TNFα, the macrophage marker F4/80, and the enzyme NOS2 were all upregulated in the liver of HF mice. Supplementation with AC either partially (MCP-1) or fully (F4/80, TNFα, and NOS2) prevented these increases. No significant differences were observed between C and CA in markers of steatosis and liver inflammation.

3.6. Supplementation with AC improved parameters of oxidative stress and redox signaling involved in insulin resistance in the liver of mice fed a high fat diet

HFD-associated alterations in glucose homeostasis can be due to an upregulation of NADPH oxidase (NOX) isoforms leading to hepatic oxidative stress, and the activation of redox-sensitive signals that promote insulin resistance. Consumption of the HFD caused the upregulation of liver NOX2 (gp91phox), NOX3 and NOX4 (Fig. 5). Thus, gp91phox, NOX3 and NOX4 levels were 43%, 51% and 80% higher, respectively, in HF compared to C mice. AC supplementation prevented
HFD-mediated upregulation of NOX3 and NOX4, but not that of gp91phox. The increased NOXs expression was paralleled by a 40% increase in the levels of 4-hydroxynonenal-protein adducts in HFD-fed mice, which was prevented by AC supplementation (Fig. 5).

The phosphorylation of the redox-sensitive signals JNK (Thr183/Tyr185) and IKK (Ser176/180) was 84% and 83% higher, respectively, in the liver of HF compared to C mice (Fig. 6A). The increased phosphorylation of both JNK and IKK was not observed in HFA40 mice. In agreement with the activation of IKK, an upstream event in the pathway, NF-κB-DNA binding, was 52% higher in liver nuclear fractions from HF than in C and HFA40 mice (Fig. 6B). Activation of the NF-κB signaling pathway was associated with an increased expression of PTP1B, which in the liver of HF mice was 31% higher than in C and HFA40 mice (Fig. 6A).

4. Discussion

Chronic consumption of a HFD by mice led to the development of obesity, adiposity, dyslipidemia, steatosis, liver inflammation and insulin resistance. Simultaneous consumption of a diet rich in AC, i.e. cyanidins and delphinidins, attenuated all these adverse effects. In addition, the AC-associated improvement of inflammation, oxidative stress, and insulin sensitivity was in part associated with their capacity to modulate NF-κB and JNK.

Diets rich in fat and carbohydrates are in part responsible for the increasing global burden of overweight and obesity. The dietary consumption of a HFD by mice mimics the consequences of Western style diets in humans. The amount of ACs provided are comparable in quality and amount to that achievable through food consumption and/or rational amounts of dietary supplements in humans. We observed that mice eating the HFD and AC gained less weight than those fed the HFD alone, despite consuming similar amounts of calories. In line with these results, AC at the highest amount provided, i.e. 40 mg/kg body weight, led to lower weight of brown, visceral, and retroperitoneal fat pads, but not epididymal or subcutaneous fat, compared to the non-supplemented mice.
HFD-fed mice. AC-mediated decrease in visceral fat is particularly relevant given the role of this fat pad in the development of systemic adverse effects through the release of adipokines, growth factors and inflammatory molecules. Thus, visceral fat accumulation is associated with the development of metabolic syndrome [24] and associated diseases, e.g., NAFLD [25] and cardiovascular disease [26]. AC supplementation also attenuated the hyperlipidemia and steatosis associated with HFD consumption. These results disagree with previous reports that pure AC but not AC in berry extracts have the capacity to improve weight loss and excess tissue lipid deposition may include the modulation of GLP-1, a hormone known to reduce adiposity [29], and/or a decreased fat absorption associated with the inhibition of pancreatic lipase, which is essential for dietary triglyceride absorption in the intestine [30]. With regard to the latter, both cyanidin and cyanidin-3,5-diglucoside were shown to inhibit the enzyme in vitro [31]. Pancreatic lipase inhibition would also be consistent with the current finding of high fecal triglyceride levels in the AC-supplemented and HFD-fed mice.

The increasing incidence of T2D worldwide has paralleled that of overweight and obesity. As previously reported [32,33]. HFD consumption by C57BL/6J mice led to insulin resistance as evidenced by high fasted plasma glucose and insulin levels, and impaired ITT and GTT tests. At the highest concentration tested, the AC blend improved all these parameters. Accordingly, an AC-rich blueberry extract was found to improve parameters of insulin sensitivity in HFD-fed mice, although under the tested conditions the ITT and GTT were not affected by fat consumption [34]. The above beneficial effects could be in part related to the capacity of AC to modulate hormones that regulate different aspects of glucose homeostasis. Adipokines (adiponectin, leptin) and incretins (GLP-1, GIP) contribute to the regulation of satiety and/or glucose homeostasis. While plasma adiponectin levels were not affected, plasma leptin was increased because of HFD consumption, as it is observed in diet-induced obesity [35]. The attenuation of hyperleptinemia by AC supplementation in HFD-fed mice may in part reflect a decreased fasted fat pad mass and an improved capacity to modulate food intake and energy balance. In terms of the incretins, GIP and GLP-1 increase insulin secretion after food consumption in

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