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Modulating the expression of genes associated with hepatic lipid metabolism, lipoperoxidation and inflammation by cocoa, cocoa extract and cocoa flavanols related to hepatic steatosis induced by a hypercaloric diet

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ABSTRACT

Cocoa and its bioactive compounds are widely known for its beneficial effects as attenuate insulin resistance, hepatic lipid deposition, inflammation and oxidative stress induced by hypercaloric diets. The present *in vivo* study investigated the effects of cocoa powder, cocoa extract and its main flavanols (epicatechin, catechin and procyanidin B2) on the expression of genes involved in the regulation of hepatic lipid metabolism, lipoperoxidation and inflammation associated to hepatic steatosis induced by a hypercaloric diet. This study demonstrates that oral treatment with cocoa powder, cocoa extract and cocoa flavanols significantly attenuate hepatic steatosis induced by hypercaloric feeding, in part, through downregulation of genes involved in hepatic fatty acid uptake (PPARγ, CD36) and lipogenesis (ACC) and upregulation of key regulators of mitochondrial function (PPARα, SIRT1) and FA oxidation (PGC-1α) as well as in attenuating hepatic oxidative stress and inflammation by upregulating hepatic antioxidant enzymes and decreasing gene expression of proinflammatory cytokines (TNF-α, IL-6).

1. Introduction

Cocoa and its bioactive compounds (mainly epicatechin, catechin and procyanidin B2) have increasingly attracted attention because of their beneficial effects in attenuating insulin resistance, hepatic lipid deposition, inflammation and oxidative stress induced by hypercaloric diets (Ali, Ismail, & Kersten, 2014). Some potential mechanisms that have been proposed for these effects include downregulation and inhibition of transcription factors (NF-kB, SREBP1-c), enzymes involved in oxidative stress and lipogenesis (NADPH oxidase, Fas) and proinflammatory mediators (TNF-α, IL-6, IL-1β, MCP-1), as well as upregulation and activation of endogenous antioxidant enzymes (GPx, SOD, CAT) and transcription factors (PPAR-α) (Cordero-Herrera, Martín, Goya, & Ramos, 2015; Cordero-Herrera, Martín, Fernández-Millán, et al., 2015).

Non-alcoholic fatty liver disease (NAFLD) is widely considered as the hepatic manifestation of the metabolic syndrome due to its association with central obesity, diabetes mellitus type 2, insulin resistance, hypertension and dyslipidemia (Marchesini et al., 2003). NAFLD is characterized by triglyceride (TG) accumulation in hepatocytes that results from an imbalance between the rate of fatty acids (FA) input (uptake and de novo lipogenesis) and the rate of FA output (oxidation and secretion as VLDL-TG) and hepatic inflammation (Chen, Varghese, & Ruan, 2014).

Experimental dietary models of NAFLD have demonstrated that hepatic peroxisome proliferator-activated receptor γ (PPARγ) overexpression promotes FA influx and upregulation of lipogenic genes (ACC and Fas) and its target gene CD36 (Ables, 2012; Inoue et al., 2005; Reddy & Rao, 2006). Hepatic transmembrane protein CD36 (FAT/CD36) plays an important role in long chain fatty acids uptake, intracellular trafficking and esterification into intrahepatic triacylglycerols (IHTG) (Koo, 2013).

In the setting of high caloric intake, fatty acids can be synthesized de novo from acetyl-CoA (derived from glycolysis), which is used as a substrate by acetyl-CoA carboxylase (ACC) to produce malonyl-CoA.

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The latter is considered as the major inhibitor of carnitine palmitoyltransferase 1 (CPT-1) and thus prevents the entry of FA into the mitochondrial matrix and inhibits β-oxidation (Koo, 2013).

Evidence suggests that mitochondrial dysfunction is a key player in the pathophysiology of NAFLD since its oxidative capacity becomes impaired and promotes IHTG accumulation. Peroxisome proliferator-activated receptor α (PPARα) is essential in the upregulation and activation of key enzymes involved in mitochondrial (CPT-1), peroxisomal (ACOX) and microsomal fatty acid β-oxidation (CYP4A). Therefore, downregulation of hepatic PPARα expression contributes to increased susceptibility to the development of NAFLD (Koo, 2013; Rolo, Teodoro, & Palmeira, 2012). Additionally, decreased hepatic peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) and Sirtuin 1 (SIRT1) levels have also been described in a variety of models of NAFLD and have been associated with alterations in mitochondrial biogenesis (both proliferation and differentiation) and impaired mitochondrial respiratory chain and oxidative phosphorylation.

IHTG accumulation and impaired mitochondrial function increases the production of reactive oxygen species (ROS: superoxide anions, hydrogen peroxide and hydroxyl radicals) and activate alternative oxidation pathways in the peroxisomes and microsomes (Ω-oxidation), resulting in additional ROS that can attack membrane polyunsaturated fatty acids (PUFAs) and initiate lipid peroxidation within the cell. This results in the formation of trans-4-hydroxy-2-nonenal and malondialdehyde (MDA), which in turn have the potential to amplify the effects of oxidative stress. Additionally, oxidative stress decreases superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase activities and triggers proinflammatory cytokines (TNF-α and IL-6) production, leading to inflammation and further development of non-alcoholic steatohepatitis (NASH) (Browning & Horton, 2004; Rolo et al., 2012).

Most studies have focused mainly on the antioxidant and anti-inflammatory mechanisms through which cocoa powder and/or cocoa extract ameliorate hepatic steatosis; however, a deeper understanding of the molecular mechanisms regulating hepatic lipid metabolism is needed. Therefore, the aim of the present study was to investigate the effects of cocoa powder, cocoa extract and its main flavanols (epicatechin, catechin and procyanidin B2) on the expression of genes involved in the regulation of hepatic lipid metabolism, lipoperoxidation and inflammation associated to hepatic steatosis induced by a hypercaloric diet (high fat-high sucrose).

2. Materials and methods

2.1. Chemicals

(+)-Catechin hydrate (C1251; ≥98%) and (−)-epicatechin (E1753; ≥90%) were purchased from Sigma–Aldrich (St. Louis, MO) and procyanidin B2 was purchased from Chempacific (Baltimore, Maryland).

2.2. Cocoa powder and cocoa extract

Unsweetened cocoa powder (CocoaVia®) and cocoa extract (CocoaVia®) were provided from Mars, Inc. (Hackettstown, NJ). By aluminum chloride and Folin–Ciocalteu methods were quantified the total flavonoids and polyphenols in cocoa powder and cocoa extract respectively, using (+)-catechin as a standard. (+)-Catechin, (−)-epicatechin and procyanidin B2 levels were assessed using HPLC-DAD, by a previously described method (Ortega et al., 2010). The dosage in this study of unsweetened cocoa powder (1 g/kg bw) and cocoa extract (100 mg/kg bw) contained 72.4 and 61.7 mg of total polyphenols, 55.5 and 44.1 of total flavonoids, 9.25 and 8.6 mg of (−)-epicatechin, 2.33 and 1.74 mg of (+)-catechin and 5.1 and 3.9 mg of procyanidin B2, respectively.

2.3. Animal study

Male Wistar rats (6-week-old; body weight: 180 ± 5 g) were purchased from the Animal House of Autonomous Metropolitan University, Xochimilco Campus (Mexico City) and individually housed in stainless steel cages at a controlled temperature (22 ± 2 °C), 40–60% humidity and under 12-h dark: 12-h light cycle. Rats were allowed free access to standard diet and purified water for a 5-day acclimatization period. After this period, rats were weighed and randomly assigned to the different experimental groups (n = 7 per group): standard diet (SD) (Rodent Diet 2018; Teklad Global Harlan Laboratories, Inc. Madison, WI) containing (wt/wt) 18.6% protein, 6.2% fat, and 44.2% carbohydrate, with an energy content of 3.1 kcal/g, 18% energy from fat; high-fat diet (HF) (TD. 88,137; Teklad Global Harlan Laboratories, Inc. Madison, WI) containing (wt/wt) 17.3% protein, 21.2% fat, and 48.5% of carbohydrate, with an energy content of 4.5 kcal/g, 42% of energy from fat; HF + cocoa powder (Co, 1 g/kg bw); HF + cocoa extract (Co-Ex, 100 mg/kg bw); HF + (−)-epicatechin (Epi, 10 mg/kg bw); HF + (+)-catechin (Cat, 10 mg/kg bw); HF + procyanidin B2 (PB2, 10 mg/kg bw).

Purified water (control groups) and treatment compounds were administered by oral gavage daily during the experimental period. The cocoa extract and epicatechin doses used in this study were selected on the basis of their estimated content per gram of cocoa powder (116.8 mg and 9.25 mg, respectively/g cocoa powder). This epicatechin oral dose has already shown to be efficiently absorbed. Catechin and procyanidin B2 were administered at the same dose as epicatechin in order to compare their effects. Rats were allowed *ad libitum* access to purified water and their respective diets for 8 weeks. Body weight and food intake (individually) were monitored daily. At the end of the experimental period, rats were anesthetized with pentobarbital sodium (35 mg/kg IP) after withholding food for 12 h. Blood samples were collected from abdominal aorta into 3.5 mL-sterile gold BD Vacutainer test tubes for serum isolation by centrifugation at 2500 rpm for 15 min at 4 °C. Serum was aliquoted and stored at −80 °C until biochemical analysis. Liver and retroperitoneal, epididymal and mesenteric adipose tissues were removed, rinsed with phosphate buffered saline (PBS), weighed and immediately frozen in liquid nitrogen and stored at −80 °C until further use. Experimental protocol followed the Ethics Code for Animal Studies of the Escuela Nacional de Ciencias Biológicas (ENCB) and the Guide for the Care and Use of Laboratory Animals of the Mexican Council for Animal Care (NOM-062-ZOO-1999).

2.4. Biochemical analyses

Levels of serum TG were determined using a semi-autoanalyser (Ekem control Lab, Mindray, China). Serum total cholesterol (TC) and non-esterified fatty acids (NEFAs) were measured using commercially available enzymatic colorimetric assay kits (Randox Laboratories, Crumlin, United Kingdom and Wako Chemicals, Neuss, Germany, respectively).

2.5. Measurement of liver triglycerides and total cholesterol

Triacylglycerol and total cholesterol were measured in the liver as described by Zhou et al. Briefly, hepatic tissue (100 mg) was homogenized in 1 mL ice-cold PBS using a MagNA Lyser Instrument (Roche Diagnostics, USA). Then 2 mL chloroform/methanol (2: 1, *v*/v) were added to the homogenate and vortexed for 60 s. After standing for 12 h, each sample was centrifuged at 4000 rpm and 4 °C for 15 min. The organic phase was dried under nitrogen gas and the residue was dissolved in 3% Triton X-100. The hepatic triglyceride and cholesterol contents were analyzed using the same semi-autoanalyser as used for the serum analysis (Zhou et al., 2015).