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Dietary cocoa reduces metabolic endotoxemia and adipose tissue inflammation in high-fat fed mice☆

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Abstract

In diet-induced obesity, adipose tissue (AT) is in a chronic state of inflammation predisposing the development of metabolic syndrome. Cocoa (*Theobroma cacao*) is a polyphenol-rich food with putative anti-inflammatory activities. Here, we examined the impact and underlying mechanisms of action of cocoa on AT inflammation in high fat-fed mice. In the present study, male C57BL/6 J mice were fed a high fat diet (HF), a HF diet with 8% (w/w) unsweetened cocoa powder (HFC), or a low-fat diet (LF) for 18 weeks. Cocoa supplementation decreased AT mRNA levels of tumor necrosis factor-α, interleukin-6, inducible nitric oxide synthase, and EGF-like module-containing mucin-like hormone receptor-like 1 by 40–60% compared to HF group, and this was accompanied by decreased nuclear protein levels of nuclear factor-κB. Cocoa treatment reduced the levels of arachidonic acid in the AT by 33% compared to HF controls. Moreover, cocoa treatment also reduced protein levels of the eicosanoid-generating enzymes, adipose-specific phospholipase A_2 and cycloxygenase-2 by 53% and 55%, respectively, compared to HF-fed mice. Finally, cocoa treatment ameliorated metabolic endotoxemia (40% reduction in plasma endotoxin) and improved gut barrier function (as measured by increased plasma levels of glucagon-like peptide-2). In conclusion, the present study has shown for the first time that long-term cocoa supplementation can reduce AT inflammation in part by modulating eicosanoid metabolism and metabolic endotoxemia.

Keywords: Cocoa; *Theobroma cacao*; Polyphenol; Obesity; Inflammation; Adipose tissue

1. Introduction

Obesity and related health problems (e.g. type 2 diabetes, hypertension, and atherosclerosis) are the most prevalent nutrition-related issues in the United States [1], affecting over 50% of the adult population [2]. The cluster of obesity-related metabolic diseases is known as the metabolic syndrome. One emerging feature of the metabolic syndrome is its linkage with chronic inflammation in adipose tissue (AT) that becomes systemic [1,3]. This chronic systemic inflammation is driven by the infiltration of macrophages into AT, which, together with adipocytes, perpetuate a cycle of macrophage recruitment and secretion of free fatty acids and deleterious cytokines/chemokines that predispose that drive the development of metabolic syndrome [4].

During the progression of obesity, the adipocytes undergo hyperplasia and hypertrophy: these enlarged adipocyte begin recruiting macrophages [5,6]. Adipose tissue macrophages (ATMs) secrete pro-inflammatory cytokines such as tumor necrosis factor-α, interleukins (e.g., IL-6), and recruitment of additional macrophages by secreting chemokines including monocyte chemotactic protein-1 (MCP-1) [5,7]. These newly released inflammatory cytokines can interact with their receptors at the surface of nearby adipocytes to signal a further activation of nuclear factor-κB (NF-κB), the key transcription factor that drives the inflammatory responses of the innate immune system[8]. Pro-inflammatory genes such as inducible nitric oxide synthase (*Nos2*) and cyclooxygenase-2 (*Cox2*) are activated by NF-κB, and contribute to the progression of systemic inflammation [3].

Inflammatory lipid mediators play a role in the development of obesity-induced AT inflammation [4,9]. Eicosanoids, a large family of compounds generated from arachidonic acid (AA), represent one of the most potent classes of endogenous inflammatory mediators. In AT, upon activation of adipose-specific phospholipase A_2 (AdPLA), AA is released from membrane phospholipids and becomes available as a

Abbreviations: AA, arachidonic acid; AdPLA, adipose-specific phospholipase A_2; AT, adipose tissue; ATMs, adipose tissue macrophages; COX-2, cycloxygenase-2; DP, degree of polymerization; FAME, fatty acid methyl ester; GC, Gas Chromatography; GLP-2, glucagon-like peptide-2; HF, high-fat; HFC, high-fat diet with 8% (w/w) unsweetened cocoa powder; Nos2, inducible nitric oxide synthase; LF, low-fat diet; LOX, lipoxygenase; LPS, lipopolysaccharide; LT, leukotriene; MCP-1, monocyte chemotactic protein-1; NF-κB, nuclear factor-κB; PAC, proanthocyanidin; PG, prostaglandin; PUFA, polyunsaturated fatty acid; SVF, stromal vascular fraction;TLR4, toll-like receptor 4; TNF-α, tumor necrosis factor-α.

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substrate for the intracellular biosynthesis of eicosanoids through two major enzymatic routes: the cyclooxygenase (COX) and lipoxygenase (LOX) pathways[9]. Studies have shown that mice that are deficient in key eicosanoid-generating enzymes including COX-2, 5-LOX and 12/15-LOX exhibit decreases in adipocyte differentiation, macrophage infiltration and are protected from high-fat (HF) diet-induced elevation of inflammatory cytokines[10–12]. Thus, the metabolism of eicosanoids represents a novel target for the prevention or treatment of obesity-associated inflammation.

An increasing number of studies have suggested that metabolic endotoxemia, characterized by an excess of circulating bacterial wall lipopolysaccharide (LPS), is also associated with obesity and systemic inflammation [13,14]. Studies have shown that consumption of a HF diet can alter the composition of gut microbiota, increase the incorporation of LPS into chylomicrons as well as affect the intestinal permeability, which allow excess endotoxin to enter systemic circulation [15,16]. Metabolic endotoxemia is believed to trigger the AT inflammation via CD14 and toll-like receptor 4 signaling [14,17].

One potential strategy to reduce obesity-related inflammation is consumption of polyphenol-rich foods, which have been reported to have anti-inflammatory properties in a number of model systems [1]. Cocoa (*Theobroma cacao*) and cocoa-based products are among the richest food sources of polyphenols. Cocoa polyphenols are primarily composed of monomeric (epicatechin and catechin) and oligomeric (proanthocyanidins, PACs) flavan-3-ols or flavanols. PACs with a degree of polymerization up to 10 (i.e., decamer) have been identified in cocoa[18]. In addition to polyphenols, cocoa also contains a significant amount of dietary fibers (approximately 40%) as well as theobromine (2–3%) and a small amount of caffeine is also present (0.2%) [19]. Over the last decade, a growing number of studies have reported the health benefits of cocoa and cocoa flavan-3-ols, particularly reduced risk of cardiometabolic diseases by promoting nitric oxide bioavailability, as well as, through antioxidant, anti-inflammatory, and anti-platelet activities [20–25]. The impact of cocoa on eicosanoid metabolism and metabolic endotoxemia *in vivo* remains an understudied area.

A previous study from our laboratory has demonstrated that dietary cocoa supplementation (8% w/w) for 10 weeks can significantly ameliorate the body weight gain, nonalcoholic fatty liver disease and systemic inflammation in HF-fed obese mice, principally through modulation of cytokine secretion and inhibition of dietary fat absorption [26]. In addition, cocoa extracts can dose-dependently inhibit activity of digestive enzymes including secreted phospholipase A_2 (PLA_2, IC_{50} <20 μg/ml) in vitro [27]. In the present study, we investigated the preventative effects of a long-term dietary cocoa powder supplementation on AT inflammation though the regulation of pro-inflammatory gene expression, eicosanoid metabolism and metabolic endotoxemia in HF-fed C57BL/6J mice.

2. Materials and methods

2.1. Diets and chemicals

The composition of the low-fat (LF, 10% kcal from fat), high-fat (HF, 60% kcal from fat) and HF diet supplemented with 80 mg/g unsweetened cocoa powder (HFC) diet was described previously [26]. The unsweetened cocoa powder used in this study was provided by Blommer Chocolate (Chicago, IL, USA). The composition of the cocoa powder was previously reported [26]. All other chemicals were of the highest grade commercially-available.

2.2. Animals and treatment

The animal experiment was conducted in accordance with a protocol (IACUC# 37115) approved by the Institutional Animal Care and Use committee at the Pennsylvania State University (University Park, PA, USA). Male C57BL/6 J mice (4 weeks old) were purchased from Jackson Laboratories (Bar Harbor, ME) and maintained on 12 h light/dark with access to food and water ad libitum. After a 2-week acclimatization period, mice were randomized to LF diet (n=23), HF diet (n=21) or HFC diet (n=24) treatments. Body weight and food intake were recorded weekly. At the end of week 18, mice were food-deprived for 7 h (7 a.m.–2 p.m.), anesthetized, and sacrificed by exsanguination via cardiac puncture. Hearts, livers, spleens, kidneys and visceral fat depots (epididymal, retroperitoneal and mesenteric) were harvested, rinsed and weighed. Plasma samples were isolated by centrifugation at 3200×g for 15 min. All samples were snap-frozen and stored at −80 °C until further analysis.

2.3. Glycemic markers

Fasting blood glucose, plasma insulin levels were assessed as previously described [26]. Briefly, fasting blood glucose were measured using a hand-held Contour glucose monitor (Bayer Healthcare, Tarrytown, NY, USA) after 7 h of fasting. Fasting plasma insulin was determined at the end of the experiment using an ELISA kit (Crystal Chem, Downers Grove, IL, USA) according to the manufacturer's protocol. Homeostasis model assessment of insulin resistance (HOMA-IR) was estimated based on the final blood glucose and insulin values [28].

2.4. Fasting plasma triglycerides and free fatty acids

Fasting plasma concentration of triglycerides was measured by a commercial L-type triglyceride kit (Wako Diagnostics, Richmond, VA, USA). Non-esterified free fatty acids levels were quantitatively determined by an enzymatic colorimetric method (λ_{max}=546 nm, Zen-bio, Research Triangle, NC, USA).

2.5. Gene expression analysis in stromal vascular fraction (SVF) of AT

Isolation of SVF of epididymal AT and gene expression analysis by quantitative reverse transcriptase polymerase chain reaction were conducted as previously described [26]. In brief, epididymal AT were minced and filtered through a cell strainer and lysed, and the SVF was collected after centrifugation. Total RNA of SVF was extracted and genomic DNA contamination was removed using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. Gene expression of *Tnfa*, *Il6*, *Nos2*, and *Emr1* was analyzed by reverse-transcriptase real-time polymerase chain reaction using commercial TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA, USA), normalized to *Gapdh* as an endogenous control. Information of the Taqman probes used in this study was reported previously [26]. Gene expression was analyzed according to the delta Ct ($^{\Delta\Delta C}{}_T$) method normalized to *Gapdh*, and expression levels were calculated as $2^{-\Delta\Delta C}{}_T$.

2.6. Adipocyte cell size image analysis

Samples from epididymal AT were fixed in paraformaldehyde, embedded in paraffin, cut into 5 μm sections, and stained with hematoxylin and eosin. The sections were viewed at 100× magnification and images were obtained with a DV-130 digital camera (Hawking Technology, Irvine, CA, USA). A measure scale was added to each image using LissView program (Hawking Technology, Irvine, CA, USA). The images were pre-edited using Picasa 3 (Google, Mountain View, CA, USA). The images were conditioned by changing "Invert Color", "Fill Light", "Highlights" and "Shadows". Minor adjustments were also made by "Sharpen" to make image crisper and less fuzzy. After pre-editing the images, Adobe PhotoShop CS 8.0 (Adobe systems, San Joes, CA, USA) was used to optimize the images for adipocyte measurement. The following commands were used for the conversion: "Bilevel Thresholding", "Erode", "Dilate" and "Watershed Segmentation". The binary black and white images were compared with the original images to ensure an accurate conversion and minor adjustments were made using following commands: "Fill Holes" and "Paintbrush". The total number and cell diameter of adipocytes were calculated with the command "Measure All" after measure scale calibration. Results were directly loaded into Excel (Microsoft Inc., Redmond, WA, USA) for analysis.

2.7. Arachidonic acid quantification by gas chromatography

AA levels were determined in retroperitoneal AT. A one-step lipid extraction and methylation procedure was conducted according to Garces and Mancha [29]. Briefly, AT was heated at 80 °C for 2 h in a reagent containing methanol, heptane, toluene, 2,2-dimethoxypropane, and H_2SO_4. Fatty acid methyl esters were extracted in heptane and quantified using a GC (Agilent 6890A, Atlanta, GA, USA) equipped with a silica-fused capillary column (SP-2560, Sigma-Aldrich) and a flame ionization detector. Fatty acid peaks are identified using standard mixtures (GLC 68D, 461, and 780; NU-CHEK Prep) and tissue fatty acid concentration determined using internal standards (C13:0 and C19:0). Data were corrected for recovery and methyl ester mass.

2.8. Western blot

Frozen retroperitoneal AT was homogenized in tissue protein extraction buffer containing a cocktail of protease inhibitors and phosphatase inhibitors (Sigma-Aldrich, St. Louis, MO, USA). The nuclear fraction of AT was prepared using the Nuclear Extract Kit (Active Motif, Rixensart, Belgium). After centrifugation, the protein concentration in supernatant is determined by Bradford assay (Sigma-Aldrich, St. Louis, MO, USA). Whole cell lysates and nuclear lysates were then combined with an equal volume of laemmli