Title
Effects of (-)-Epicatechin on High-fat-induced Intestinal Permeability and Endotoxemia

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Effects of (-)-Epicatechin on High-fat-induced Intestinal Permeability and Endotoxemia

By

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DISSERTATION

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DAVIS

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2021

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During another recent peaceful and serene winter afternoon shrouded in warm sunlight for the thousandth time, I asked myself a question I had often pondered: what had I done to deserve my fruitful, unforgettable, and extraordinary life journey here at UC Davis? Before answering that, I must acknowledge how nothing would have been possible without me being brought into this wonderful world by my dearest mom and dad, Liping Zhang and Xifa Wang. Thank you for giving me life and allowing me to experience this adventurous and beautiful life, for bringing me up and raising me to become a functional and responsible adult, and for supporting me in pursuing my dreams at any cost.

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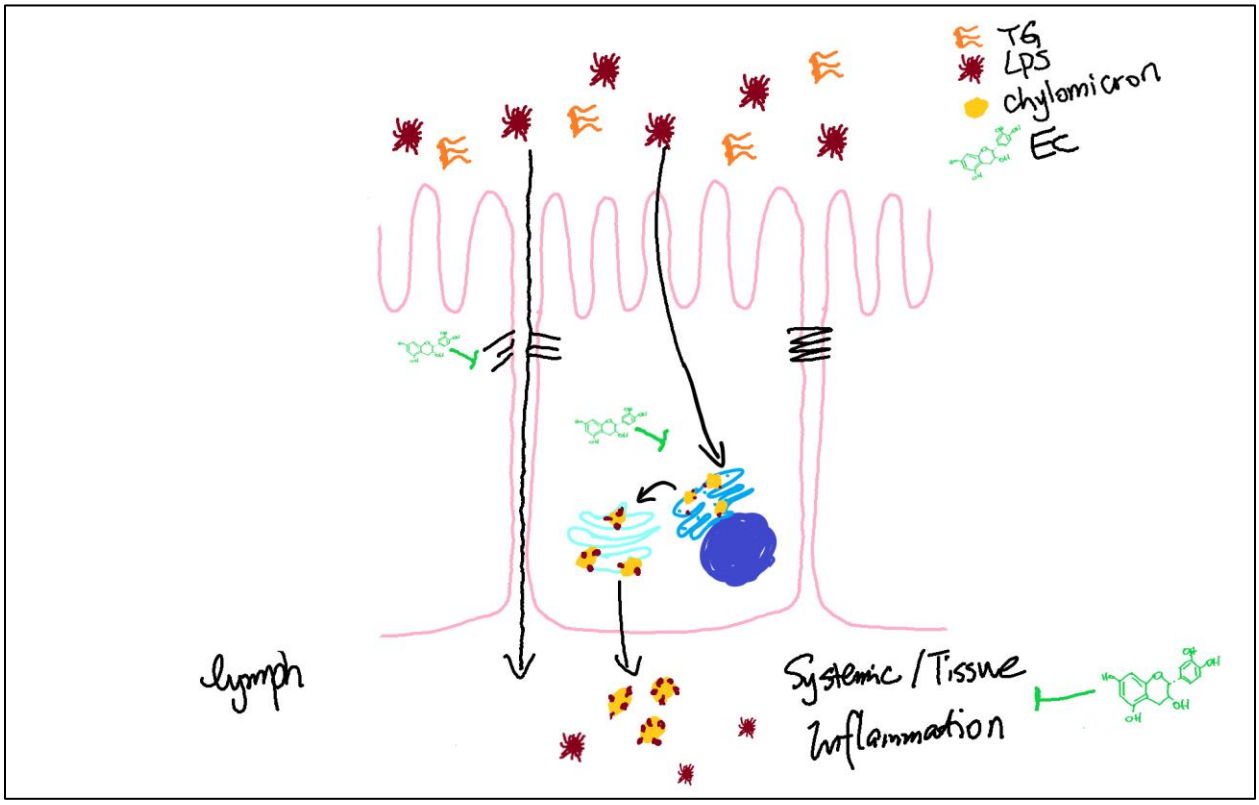
Abstract

Obesity constitutes a major global public health threat. It can lead to a cluster of metabolic disorders and a higher risk of developing various types of pathological conditions and diseases. Among multiple environmental factors, high fat diets (HFD) play an important role in the development of obesity. Flavonoids are one of the most abundant categories of bioactive compounds present in the human diet. My dissertation work identified mechanisms by which the flavan-3-ol (-)-epicatechin (EC), could mitigate high fat diet-induced obesity-associated intestinal permeabilization and metabolic endotoxemia. Chapter one looked at the protective effects of EC from HFD-induced intestinal barrier permeabilization and endotoxemia and its implications for steatosis and insulin resistance in vivo. We subsequently found that the luminal content of total bile acid and select secondary bile acids, particularly deoxycholic acid, were increased by HFD consumption. The second chapter focused on investigating if EC could prevent DCA-induced intestinal permeabilization in vitro. Such effect could in turn, help prevent the paracellular transport of lipopolysaccharides (LPS) from the lumen into the bloodstream. The last chapter provided additional mechanistic insight into how flavan-3-ols and procyanidins (PCAs) can mitigate HFD-induced endotoxemia through their capacity to prevent chylomicron-dependent transcellular transport of LPS in Caco-2 monolayers and high fat-induced postprandial endotoxemia in mice. In summary, this thesis works provide evidence that EC can decrease HFD-associated endotoxemia, characterizing the underlying mechanisms. This action would be critical to the capacity of EC to mitigate HFD- and obesity-associated comorbidities.

Graphical Abstract



Introduction

Diet is a lifestyle factor that plays a central role in sustaining human health. High fat and high sugar intake largely contribute to overweight and obesity, a current major global public health threat that is associated with a cluster of metabolic disorders and chronic diseases [1]. This public health crisis can also be in part attributed to current dietary patterns of low fruit and vegetable intake [2]. Plant-based foods are rich in naturally occurring bioactive compounds, such as polyphenols [3]. Current dietary guidelines recommend promoting fruit and vegetable intake, given mounting evidence shows inverse associations between their intake and weight-related adverse outcomes in adults [4, 5]. However, our understanding of the health effects of phytochemicals and the underlying mechanisms is still very limited to establish a clear recommendation to the public. Research efforts designed to characterize the beneficial effects of polyphenols and to identify their mechanisms of action are needed. In particular, characterizing the capacity of polyphenols to mitigate obesity-associated comorbidities will provide critical insights to improve food-derived polyphenol intake among individuals with obesogenic dietary patterns. Thus, this research project will contribute to the development of human dietary strategies to alleviate the adverse consequences of obesity worldwide.

1. (-)-Epicatechin: chemistry, metabolism, and function

Flavonoids are a class of polyphenols found in high concentrations in a variety of plants. Among them, the flavan-3-ol (-)-epicatechin (EC) is one of the most abundant flavonoids present in the human diet [6]. As summarized in our recent review article [7] (included

under the section of "Additional publications"), a significant body of evidence supports the capacity of EC and/or its metabolites to mitigate obesity-associated pathologies.

1.1. EC: sources, chemistry, and metabolism

EC is found in large amounts in select plant-based food, including but not limited to apples, berries, cherries, black grapes, broad beans, cocoa, and green tea. EC has the basic flavonoid chemical structure C6-C3-C6 (ring A-C-B), where ring A and B are aromatic rings linked by ring C, a heterocycle formed by an extra three-carbon chain and one oxygen atom. The EC molecule has two hydroxyl residues on ring A (C5 and C7), one on ring C (C3), and two on ring B (C3' and C4') (Fig. 1).

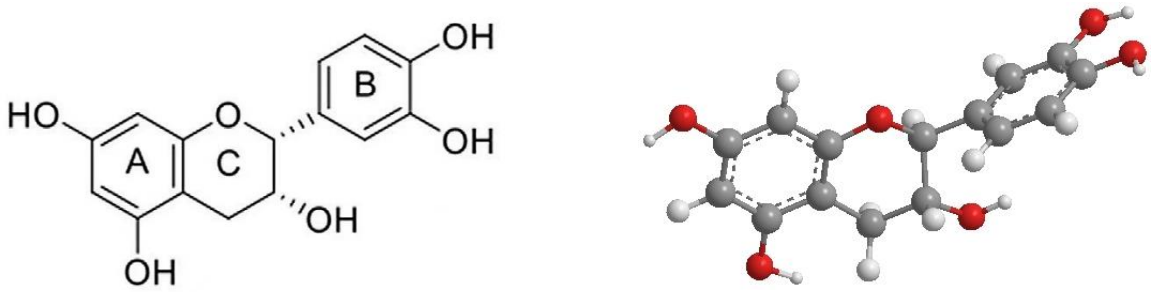


Figure 1. Planar chemical structure and tridimensional structure of (-)-epicatechin.

The absorption, distribution, metabolism, and excretion (ADME) of EC largely determine its biological functions and the sites of its actions in the body. Overall, in humans, ~95% of ingested EC (as parent compound or microbiota metabolites) is metabolized and absorbed, reaching the circulation mainly as a diversity of metabolites [8]. EC is first metabolized into structural-related EC metabolites (SREMs) and absorbed by the

small intestine, contributing to the first peak of plasma concentration at ~1 h following EC consumption. SREMs account for ~20% of EC intake, predominantly in the forms of EC-3'-O-glucuronide, 3'-O-methyl-EC-5-sulfate, and EC-3'-sulfate. Phase II metabolites, mainly 5C-ring fission metabolites (5C-RFMs), appear later in the circulation and reach the second peak concentration in plasma ~6 h after EC ingestion. This is a result of EC extensive metabolization by the colonic microbiota and accounts for ~42% of ingested EC [8]. The 3- and 1-carbon-side chain ring fission metabolites (3/1C-RFM) are found to be the major metabolites that appear in urine after 12-24 h of EC consumption, yet their levels are not detectable in the plasma [9]. The actions of EC in the body can be mediated by the parent compound and/or its metabolites both locally when they reach the gastrointestinal (GI) tract and systemically once they circulate around the body [7].

1.2. Health benefits of EC in obesity-associated comorbidities

Obesity is associated with the development of metabolic disorders and the progression of various types of diseases [1, 10]. The observed beneficial effects of EC in the mitigation of obesity-related pathologies can be explained by its capacity to i) prevent obesity progression by diminishing the digestion and absorption of calorie-providing nutrients; ii) decrease endotoxemia by limiting the transport of luminal LPS; iii) regulate gut microbiome composition and potentially improve obesity-associated microbial dysbiosis; iv) inhibit inflammation, oxidative stress, and endoplasmic reticulum (ER) stress; and v) modulate cell signaling. Detailed information on the actions of EC on the co-morbidities of obesity was extensively discussed in our recent review article [7].

1.3. EC and its isomers and oligomers

EC (with *cis* configuration) and its isomer (+)-catechin (CT) with *trans* configuration are two commonly seen catechin monomers [11, 12]. Procyanidins (PCAs) are oligomeric compounds that are formed from flavan-3-ol subunits [13] and which are present in high concentrations in flavonoids-rich food [14]. Depending on the chemical configuration and linkage between the monomers, PCAs can be categorized into A-type or B-type, with the latter form found to be the most abundant in the human diet [13]. PCAs can also be categorized by the degree of polymerization (DP). Dimeric PCAs can be absorbed as parent compounds at the GI tract. PCAs with higher DP are not absorbed but can still exert beneficial effects by interacting directly with the cell membranes of the intestinal enterocytes [15, 16]. PCAs can also be metabolized by the intestinal microbiota, and the generated metabolites, mostly 5-(3,4-dihydroxyphenyl)-c-valerolactone, be absorbed [17]. Previous research from our laboratory showed evidence on the beneficial effects of catechins as well as the B-type dimeric and hexameric PCAs in several biological systems [11, 18-21], including their protective effects on Caco-2 monolayer integrity [16, 21]. Therefore, characterization and identification of the capacity of EC, catechin, and PCAs to mitigate HFD-associated pathologies are of great significance.

2. High fat diet-induced obesity and associated comorbidities

Multiple lifestyle risk factors, including unhealthy eating behaviors, lack of physical activity, and high levels of stress are responsible for the development of obesity. Among unhealthy eating habits, the prevalence of the Western-pattern diet, characterized by high

fat, sugar, and sodium content and low fruits, vegetables, and fiber contents, plays an inevitable role in increasing obesity rates [22]. The World Health Organization (WHO) recommends limiting daily total fat intake to under 30% of total energy intake to avoid unhealthy weight gain. However, according to the Centers for Diseases Control and Prevention (CDC), the average daily total fat intake is ~35% of total calories in the US. Numerous studies have revealed that high dietary fat intake is directly related to the rising incidence of overweight and obesity [23-25]. The consumption of a high fat diet (HFD) can adversely affect health through direct and acute effects at the GI tract, as well as induce postprandial dysmetabolism and chronic systemic inflammation.

2.1. Systemic adverse effects of HFD

A typical HFD is characterized by being high in both total and saturated fat contents. The long-term consumption of HFD can lead to overweight and obesity, which is linked to the development and progression of a variety of pathological conditions including, but not limited to, hyperlipidemia, insulin resistance, type 2 diabetes (T2D), hypertension, cardiovascular diseases, stroke, non-alcoholic fatty liver disease (NAFLD), cognition impairment, alterations of gastrointestinal, musculoskeletal, renal, respiratory and immune systems, and certain types of cancer [26-33]. Elevated free fatty acid (FFA) levels are found in the circulation of obese individuals due to increased adipose tissue mass, which is associated with decreased insulin sensitivity [34]. FFA can also cause low-grade systemic inflammation, a condition that is commonly present in obesity and is recognized as one of the most relevant mechanisms underlying obesity-associated comorbidities [35, 36]. In addition, adipose tissue functions as a key endocrine organ include the release of a

variety of mediators with both pro-inflammatory and anti-inflammatory properties [37]. The accumulation of excess lipids in the enlarged adipose tissue promotes macrophage migration and infiltration. Together, it increases circulating levels of pro-inflammatory mediators such as tumor necrosis factor α (TNF-α) and interleukin 6 (IL-6) secreted by both adipocytes and macrophages [35]. Meanwhile, increased inflammatory mediators and saturated fatty acids can interact with innate pattern recognition receptors (PRRs) such as toll-like receptors (TLRs) and nod-like receptors (NLRs) in various metabolic tissues [38]. The activated receptors cause the downstream activation of intracellular signaling pathways, including the c-jun N-terminal kinase (JNK), inhibitor of K kinase (IKK), and protein kinase R (PKR) pathways, triggering additional inflammatory responses. Furthermore, obesity-related downregulation of adiponectin, an anti-inflammatory adipokine produced by adipose tissue, worsens the systemic chronic inflammation [35]. Besides, the obesity-associated inflammatory state is also partially attributed to HFD-induced endotoxemia [39] (further discussed in Section 3.1.).

2.2. Effects of HFD at the GI tract

The GI tract is the largest site where our body interacts directly with dietary components. A high dietary fat content can alter the GI tract's metabolic functions, ultimately leading to the development of metabolic disorders, obesity, and other diseases.

The intestinal barrier integrity is of great importance to prevent the unwanted penetration of potentially hostile bacteria and toxins. Excess dietary fat can induce intestinal permeabilization (further discussed in Section 3.1.), which is closely linked to

obesity-associated pathologies [32, 40], via several mechanisms that include: i) the stimulation of local immune responses [41], ii) alterations of bile acids metabolism [42], and iii) the promotion of dysbiosis [43]. HFD can directly modulate intestinal epithelial integrity by stimulating proinflammatory signaling pathways that affect the intestinal barrier function [41]. Indirectly, HFD can elevate the concentration of select hydrophobic secondary bile acids, which can *per se* trigger intestinal permeabilization by promoting oxidative stress and apoptosis of intestinal epithelial cells [42, 44, 45]. Notably, Western dietary patterns are commonly accompanied with low intakes or deficiencies of certain micronutrients, which are involved in sustaining intestinal barrier integrity [32, 46].

The morphology of the small intestine, as well as the profile of secreted pancreatic enzymes, are modified in response to a HFD in order to increase its capacity for fat digestion and absorption [47]. Additionally, gastric emptying and motoring are accelerated by high dietary fat [48]. Moreover, HFD also modulates the secretion and sensitivity of various appetite-regulating and energy-metabolizing gastrointestinal hormones such as cholecystokinin (CCK), glucagon-like peptide-1 (GLP-1), GLP-2, peptide YY (PYY), and ghrelin [47]. Together, these actions resulting from HFD intake will lead to imbalanced energy homeostasis, which further contributes to metabolic dysregulation.

Great attention has been given to HFD-induced GI dysbiosis, given that emerging evidence pointed out the inevitable role of microbiota in the development of obesity-related diseases. The capacity of HFD on modifying the microbiota profile can increase the population of LPS-producing Gram-negative bacteria, change energy harvest and expenditure, alter bile acids metabolism, activate local immune and inflammatory

responses, and consequently increase intestinal permeability and metabolic endotoxemia [7, 32, 49]. However, the above hypotheses remain largely inconclusive. Thus, more research efforts are needed to address the knowledge gap in the relationship between HFD, microbiota composition, obesity, and its comorbidities.

3. The role of the GI tract in health and disease

The GI tract serves as a critical front line of defense for our body with direct exposure to environmental molecules. The intestinal epithelium is covered by a layer of mucus, which serves as immune-sensing and regulatory molecules. The intestinal epithelium is formed by a monolayer of epithelial cells, including enterocytes, Paneth cells, Goblet cells, and neuroendocrine cells [50], which are lined and tightened up with junctional complexes, including tight junctions (TJs) and adherents junctions (AJs). TJs play a major role in regulating the paracellular transport of small molecules (water and ions), and the AJs physically adhere the IECs together to keep the intestinal barrier intact. The lamina propria, which lies beneath the intestinal epithelium, contains a variety of immune cells that aids in the immunological function of the GI tract [51].

The GI tract is involved in multiple essential physiological functions, including being a physical, chemical, and biological defense barrier against pathogens, nutrients digestion, absorption and metabolism, energy intake modulation, appetite regulation, and maintenance of microbiota homeostasis. Several pathological factors, including direct exposure to environmental triggers (e.g. change of dietary patterns, food hypersensitivity and allergy, and use of antibiotics) and genetic susceptibility can lead to structural and

functional disorders and diseases at the GI tract, such as celiac disease, irritable bowel syndrome (IBS) [52], and inflammatory bowel diseases (IBD), i.e. ulcerative colitis and Chron's disease [53]. Increased intestinal permeability, as well as the change of gut microbial composition, are reported to be associated with the development of these diseases as it triggers immunological responses that promote intestinal inflammation by exposing the lamina propria to luminal pathogens [28, 31, 33, 54, 55]. In addition, they can also adversely affect other organs and tissue (previously discussed in Section 2.1.). Current dietary patterns of high fat/high sugar and low fiber content present a particular challenge to GI tract physiology, which helps in part to explain the associated metabolic diseases.

3.1. Intestinal permeability and metabolic endotoxemia

The GI tract serves as a dynamic and semipermeable structure that selectively allows the passage and absorption of nutrients, while preventing the passage of pathogens and other hostile molecules from entering the circulation and activating immune responses. A "leaky gut" is proposed to be one of the pathophysiological events leading to obesity-associated metabolic dysregulations. It is well acknowledged that HFD induces intestinal permeabilization by altering TJ structure and function [41, 42, 45]. The long-term disruption of the TJ protein expression, structure and function can result in the undesirable transport of pathogenic substances across the intestinal epithelium causing local and, once in the circulation, systemic inflammation.

LPS, also known as endotoxin, is recognized as a major pathogenic substances [56]. Although healthy individuals have low but detectable circulating LPS levels, LPS plasma

concentration is found to be elevated in diabetic and obese subjects [40, 57, 58]. Moreover, Cani et al. found that HFD consumption caused plasma LPS concentration in mice to increase two to three times, a threshold that they defined as metabolic endotoxemia [59]. Endotoxemia can cause inflammation by triggering immune responses and actively participate in the development of obesity-associated pathologies. Historically, increased circulating LPS levels were considered as a consequence of HFD consumption and obesity. However, Cani et al. demonstrated that LPS could *per se* initiate obesity and insulin resistance [59]. This finding advanced our understanding of the bidirectional causal relationship between obesity and metabolic endotoxemia.

Given: i) the relevance of the GI tract in LPS production (microbiota) and in inhibiting LPS transport across the mucosa, and ii) the high concentration that EC can reach in the GI tract, the prevention of endotoxemia by EC emerge as a potential mechanism of action to explain in part the beneficial actions of EC on HFD-mediated obesity and associated diseases.

3.2. The microbiota and metabolic endotoxemia

The GI tract is colonized by trillions of bacteria which play roles in symbiosis, metabolism, inflammation, and maintenance of gut barrier integrity and homeostasis [60]. The microbiota community is established as early as at birth, while its composition is not always static, but dynamic, and can be modified under different conditions [61]. The effects of diets on microbiota composition can never be ignored, given that the survival of microbiota is dependent on undigested nutrients. Dysbiosis is considered a reflection of an

individual's long-term imbalanced diet and is implicated in multiple disease's progression [41].

It has been consistently observed that obese individuals harbor distinct microbiota profiles compared to lean individuals [61, 62]. This obesity-associated change in microbial diversity has been directly linked to high dietary fat intake, and is characterized by a shift in microbiota species and abundance [63]. The gut microbiota can affect the host's energy harvest and storage from the diet [64]. Moreover, a metagenome-wide association study has shown an association between T2D and increased bacterial expression of genes involved in oxidative stress, that create a proinflammatory environment in the gut [65]. Besides, a higher ratio of Gram-negative bacteria (LPS producing bacteria) to Gram-positive bacteria has also been dentified as a gut microbial signature for HFD consumption [43]. In addition, HFD-altered microbiota can increase the deconjugation process of bile acids, further leading to an increase in hydrophobic bile acid content, which can damage the intestinal epithelium [42, 66]. All these events can be contributing factors to the development of intestinal permeability and metabolic endotoxemia induced by HFD/obesity-induced microbiota alteration.

3.3. Triglycerides transport and metabolic endotoxemia

LPS is a large glycolipid found mostly in Gram-negative bacteria and composed of three domains: lipid A, the core oligosaccharide, and the O antigen [67] (Fig.2).

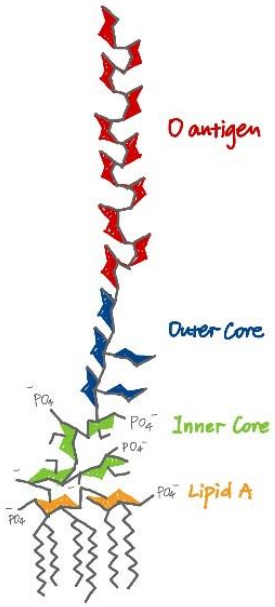


Figure 2. LPS structure

A study including 8 healthy individuals that were subjected to a Western-style diet for 1 month showed ~71% increase in plasma endotoxin activity [68], which suggested an association between high dietary fat and endotoxemia. Luminal LPS can be transported from the lumen to the circulation paracellularly when TJs are altered but also transcellularly incorporated into nascent chylomicrons due to the affinity of the lipid A domain for lipids [69, 70]. Chylomicron production is significantly upregulated following fat consumption due to its physiological function in the absorption of long-chain fatty acids, which in turn can increase the transport of LPS. Chylomicron-dependent transport of LPS is considered as a protective mechanism that enhances the hepatic clearance of endotoxin. On the other hand, the excess formation of chylomicrons in response to high dietary fat can increase tissue exposure to LPS and promote inflammation-related metabolic disorders [69].

4. Dissertation Objectives

Previous research from our laboratory showed that dietary EC supplementation could improve both high-fructose [72] and high-fat-induced [73] insulin sensitivity in rodents. However, the potential mechanisms underlying these beneficial effects of EC need further elucidation. My dissertation work investigated the capacity of EC to prevent high fat-induced metabolic disorders by alleviating endotoxemia. The objectives of this dissertation work were:

1- To investigate the capacity of EC to prevent high fat diet-induced intestinal barrier permeabilization and metabolic endotoxemia in mice, characterizing the effects of EC on the cell signaling that regulates TJs structure and function.

2- To investigate if EC can mitigate bile acid-induced intestinal permeability in Caco-2 cell monolayers, characterizing the effects of EC on the cell signaling that mediates deoxycholic acid-induced monolayer permeabilization.

3- To investigate if EC, its isomer catechin, and PCA dimer, and hexamer can mitigate fat-induced increased transcellular transport of endotoxins via chylomicrons both in Caco-2 monolayers and mice.

We concluded that EC could mitigate the intestinal translocation of LPS both paracellularly and transcellularly by preserving TJs integrity and decreasing the chylomicron-dependent transport of LPS.

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Chapter 1

(-)-Epicatechin protects the intestinal barrier from high fat diet-induced permeabilization: Implications for steatosis and insulin resistance

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(-)-Epicatechin protects the intestinal barrier from high fat diet-induced permeabilization: implications for steatosis and insulin resistance

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Abbreviations: ALT, alanine aminotransferase; AMPK, AMP activated protein kinase; EC, (-)-epicatechin; ERK1/2, extracellular signal-regulated kinase; GTT, glucose tolerance test; GLP-2, glucagon-like peptide-2; HFD, high fat diet; HNE, 4-hydroxynonenal; ITT, insulin tolerance test; LPS, lipopolysaccharides; MCP-1, monocyte chemoattractant protein-1; NAFLD, nonalcoholic fatty liver disease; NOS2, nitric oxide synthase 2; T2D, type 2 diabetes; TEER, transepithelial electrical resistance; TJ, tight junction; TNFα, tumor necrosis factor alpha; MLCK, myosin light chain kinase

Key words: intestinal permeability, (-)-epicatechin, steatosis, insulin resistance, endotoxemia

Abstract

Increased permeability of the intestinal barrier is proposed as an underlying factor for obesity-associated pathologies. Consumption of high fat diets (HFD) is associated with increased intestinal permeabilization and increased paracellular transport of endotoxins which can promote steatosis and insulin resistance. This study investigated whether dietary (-)-epicatechin (EC) supplementation can protect the intestinal barrier against HFD-induced permeabilization and endotoxemia, and mitigate liver damage and insulin resistance. Mechanisms leading to loss of integrity and function of the tight junction (TJ) were characterized. Consumption of a HFD for 15 weeks caused obesity, steatosis, and insulin resistance in male C57BL/6J mice. This was associated with increased intestinal permeability, decreased expression of ileal TJ proteins, and endotoxemia. Supplementation with EC (2–20 mg/kg body weight) mitigated all these adverse effects. EC acted modulating cell signals and the gut hormone GLP-2, which are central to the regulation of intestinal permeability. Thus, EC prevented HFD-induced ileum NOX1/NOX4 upregulation, protein oxidation, and the activation of the redox-sensitive NF-κB and ERK1/2 pathways. Supporting NADPH oxidase as a target of EC actions, in Caco-2 cells EC and apocynin inhibited tumor necrosis alpha (TNFα)-induced NOX1/NOX4 overexpression, protein oxidation and monolayer permeabilization. Together, our findings demonstrate protective effects of EC against HFD-induced increased intestinal permeability and endotoxemia. This can in part underlie EC capacity to prevent steatosis and insulin resistance occurring as a consequence of HFD consumption.

1. Introduction

Steatosis and insulin resistance are among the major and more severe consequences of obesity. Nonalcoholic fatty liver disease (NAFLD) is more frequent in individuals with diabetes, higher body mass index, and those frequently consuming fast foods [1]. One of the hypotheses on the mechanisms linking obesity and Western-style diets with NAFLD and insulin resistance is the associated increase in intestinal permeability[2, 3]. In this regard, it is proposed that an increased transfer of bacterial lipopolysaccharides (LPS) from the gut lumen into the circulation can cause tissue inflammation and damage, which in the liver would lead to steatosis and insulin resistance [3-5].

The intestinal barrier is composed of a single layer of intestinal epithelial cells sealed by the tight junctions (TJs). TJs modulate intestinal permeability by regulating the paracellular transport of water and ions. They also constitute the first line of defense against the entry of noxious bacteria/bacterial toxins (e.g. lipopolysaccharide (LPS)) and toxins/antigens present in food. Once transported via the paracellular route, they can initiate local inflammation, and once in the circulation they can also promote systemic tissue inflammation and damage [6-8]. Increased intestinal permeability is observed in different pathologies including celiac disease and inflammatory bowel diseases, and is present in obesity and type 2 diabetes (T2D) [3, 5, 9, 10]. Consumption of high fat diets (HFD) also causes intestinal permeabilization, impairs mucosal defenses [11], and alters the composition of the intestinal microbiota [11]. In murine models, these intestinal alterations have been attributed to the dietary fat per se [12], being present before the development of obesity and insulin resistance [11].

(-)-Epicatechin (EC) is a flavan-3-ol abundant in human diet [13]. Cumulative evidence has shown an improvement of insulin sensitivity in both humans and rodents upon consumption of EC or EC-containing foods [14-19]. We previously observed that EC protects rodents from dietary high fructose- and high fat-induced insulin resistance [18, 19]. EC prevented the increased liver triglyceride deposition associated with high fructose consumption in rats [18]. Although EC mitigates events involved in hepatic and adipose tissue insulin resistance (i.e. NADPH oxidase activation, oxidative stress, altered redox signaling, inflammation and endoplasmic reticulum stress) [18-21], the primary mechanisms involved in EC protective effects remain unknown.

In vitro, EC mitigated the permeabilization of Caco-2 intestinal cell monolayers induced by tumor necrosis factor alpha (TNFα) [22]. The underlying protective mechanisms of EC involved the prevention of TNFα-mediated NADPH oxidase activation, increased superoxide anion production, and altered TJ protein expression and organization [22]. Given the above, we hypothesize that EC can mitigate HFD-induced steatosis and insulin resistance by protecting the intestine from permeabilization. Thus, this study investigated the capacity of EC to prevent liver fat deposition and inflammation in HFD-fed mice, and its relationship to EC capacity to modulate intestinal permeability and the associated endotoxemia. We characterized the effects of EC on major events regulating intestinal permeability, including TJ protein expression, NADPH oxidases and modulatory redox sensitive signaling cascades (NF-κB and extracellular signal-regulated kinase (ERK1/2)), glucagon-like peptide-2 (GLP-2), and dysbiosis. The actions of EC on NADPH oxidases NOX1 and NOX4, protein oxidation, and intestinal permeability were further characterized

in Caco-2 cell monolayers. EC improved insulin sensitivity and mitigated the development of steatosis in HFD-fed mice. This was associated with the prevention of HFD-induced intestinal TJ disruption, permeabilization and endotoxemia. EC protected TJs in part by preventing intestinal NOX1/NOX4 upregulation, protein oxidation, and the activation of the redox-sensitive NF-κB and ERK1/2 signaling cascades, and increasing plasma GLP-2. The beneficial effects of EC at the gastrointestinal tract could in part underlie its capacity to improve insulin sensitivity, steatosis, and other obesity-associated pathologies.

2. Materials and methods

2.1. Materials

Cholesterol and triglycerides 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, alanine transaminase (ALT), GLP-2, and bile acids were determined using kits purchased from Crystal Chem Inc (Downers Grove, IL). Endotoxin levels were determined using a kit from Lonza (Basel, Switzerland). Antibodies for β-actin (#12620), monocyte chemoattractant protein-1 (MCP-1) (#2029), TNFα (#119487), phospho (Ser536) p65 (#3033), p65 (#3987), phospho (Thr172) AMPKα (#2535), AMPKα (#5832), phospho (Thr202/Tyr204) ERK (#4370), and ERK (#9102) were obtained from Cell Signaling Technology (Danvers, MA,). Antibodies for F4/80 (sc-25830), heterogeneous nuclear ribonucleoprotein (hnRNP) (sc-32301), HSC-70 (SC-1059), nitric oxide synthase 2 (NOS2) (sc-649), and NOX4 (sc-21860) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies for ZO-1 (33-9100), occludin (71-1500), and claudin-1 (71-7800) were from Invitrogen (Carlsbad, CA). Antibodies for 4-

hydroxinonenal (HNE) (ab46545) and NOX1 (ab55831) were from Abcam Inc. (Cambridge, MA). PVDF membranes were obtained from BIO-RAD (Hercules, CA, USA). The Enhanced chemiluminescence (ECL) Western blotting system was from Thermo Fisher Scientific Inc. (Piscataway, NJ). Apocynin, VAS-2780, EC, fluorescein isothiocyanate (FITC)-dextran (4 kDa) and all other chemicals were purchased from Sigma-Aldrich Co (St. Louis, MO).

2.2. Animals and animal care

All procedures were in agreement with standards for the care of laboratory animals as outlined in the NIH Guide for the Care and Use of Laboratory Animals. All procedures were administered under the auspices of the Animal Resource Services of the University of California, Davis. Experimental protocols were approved before implementation by the University of California, Davis Animal Use and Care Administrative Advisory Committee.

Healthy male C57BL/6J mice (20–25 g) (9–10 mice/group) were fed for 15 weeks either: **A**- a diet containing approximately 10% total calories from fat (Control) (TD.06416, Harlan Laboratories, Madison, WI), **B**- a diet containing approximately 60% total calories from fat (lard) (HF) (TD.06414, Harlan Laboratories, Madison, WI), **C**- the control diet supplemented with 20 mg EC/kg body weight (CE), and **D**- the HFD supplemented with 2 (HFE2), 10 (HFE10) or 20 (HFE20) mg EC/kg body weight. EC-containing diets were prepared every two weeks to account for changes in body weight and food intake, and to prevent potential EC degradation. All diets were stored at –20 °C until use. The highest amount of EC supplemented has been found to improve insulin resistance in rats fed high fructose levels [18] and in mice fed a HFD [19]. In comparison to EC intake in human

populations [23], the highest EC amounts supplemented are relatively high. However, they can be reached by supplementation or consumption of select EC-rich fruits/vegetables and derivatives [13].

Body weight and food intake were measured weekly throughout the study as previously described [19]. After 15 weeks on the dietary treatments, mice were euthanized by cervical dislocation. Blood was collected from the abdominal aorta into heparinized tubes, and plasma collected after centrifugation at 3000g for 10 min at room temperature. Tissues were dissected and flash frozen in liquid nitrogen and then stored at –80 °C for further analysis.

2.3. Metabolic measurements

For insulin tolerance tests (ITT), mice were fasted for 4 h and injected intraperitoneally with 1 U/kg body weight human insulin (Novolin R U-100, Novo Nordisk Inc, Princeton NJ). Blood glucose values were measured before and at 15, 30, 60 and 120 min post-injection. For glucose tolerance tests (GTT), overnight fasted mice were injected with D-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). Total cholesterol, triglycerides, glucose, insulin, GLP-2 and endotoxin concentrations, and alanine transaminase activity were determined following manufacturer's guidelines.

2.4. Intestinal permeability

Intestinal permeability was measured after 13 weeks on the diets as described previously [24] with minor modifications. Mice were fasted for 4 h then gavaged with fluorescein

isothiocyanate FITC-dextran 4 kDa (200 mg/kg body weight). After 90 min, 100 µL of blood were collected from the tip of the tail vein. The blood was kept in the dark and centrifuged at 3000g for 10 min at room temperature, and the serum collected. Serum aliquots (20 µL) and a standard curve of FITC-dextran were plated in 96-well plates and diluted to 200 µL with 0.9% (w/v) NaCl. Fluorescence was measured using a microplate spectrofluorometer (Wallac 1420 VICTOR2™, PerkinElmer Life Science, Waltman, USA) at λexc: 485 nm and λem: 520 nm.

2.5. Caco-2 cell culture and assessment of monolayer permeability

Caco-2 cells (at passages 3 through 15) were cultured as previously described [25]. Briefly, cells were used 21 d after reaching confluence to allow for differentiation into intestinal epithelial cells. All the experiments were performed in serum- and phenol red-free MEM.

Monolayer permeability was assessed measuring the transepithelial electrical resistance (TEER) and the paracellular transport of FITCdextran (4 kDa) as described [25]. Briefly, cells were grown on transwell inserts (12 mm, 0.4 µm pore polyester membranes) in 12-well plates (0.3×10^6 cells/transwell), and monolayers were used when TEER values were between 350–450 Ω cm^2. TEER was measured using a Millicell-ERS Resistance System (Millipore, Bedford, MA) and calculated as: $TEER = (Rm - Ri) \times A$ (Rm, transmembrane resistance; Ri, intrinsic resistance of a cell-free media; A, membrane surface area in cm^2). For the experiments, Caco-2 cell monolayers were preincubated for 24 h with interferon-γ (10 ng/ml) to upregulate the TNFα receptor. Monolayers were then incubated in the presence of 1 µM EC or apocynin added to the upper compartment and incubated for 30

min. Subsequently, cells were incubated in the absence or the presence of TNFα (5 ng/ml) added to the lower compartment, and cells were further incubated for 6 h. For TEER assessment, incubation media were removed from the upper and lower compartments, cells rinsed with HBSS 1X, and the same solution was added to both compartments.

The paracellular transport of FITC-dextran was measured after the 6 h incubation by adding 100 μM FITC-dextran (final concentration) to the upper compartment. After 3.5 h incubation, 100 μL of the medium in the lower compartment were collected, diluted with 100 μL HBSS 1X, and fluorescence was measured at λexc: 485 nm and λem: 520 nm.

2.6. Western blot analysis

Tissue and cell total homogenates and nuclear fractions were prepared as previously described [18, 21, 26]. Aliquots of total homogenates or nuclear fractions containing 25–40 μg protein were denatured with Laemmli buffer, separated by reducing 7.5–12.5% polyacrylamide gel electrophoresis, and electroblotted onto PVDF membranes. Membranes were blocked for 1 h in 5% (w/v) bovine serum albumin and subsequently incubated in the presence of the corresponding primary antibodies (1:500–1:1000 dilution) overnight at 4 °C. After incubation for 90 min at room temperature in the presence of the corresponding secondary antibodies (HRP conjugated) (1:10,000 dilution) the conjugates were visualized by ECL system using a Phosphoimager 840 (Amersham Pharmacia Biotech. Inc., Piscataway, NJ).

2.7. Histological analysis

The liver was removed and samples fixed overnight in 4% (w/v) neutralized paraformaldehyde buffer solution. Samples were subsequently washed twice in phosphate buffer saline solution, dehydrated, and then embedded in paraffin for histological analysis. Sections with a thickness of 5 μm 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, USA). Hepatic histological examination was performed using the histological scoring system for NAFLD as described by Kleiner et al. [27]. Three randomly selected fields per animal were assessed blindly by an independent pathologist who did not know the identities of the study groups. All liver specimens were analyzed using Pro Plus 5.1 software (Media Cybernetics, Rockville, MD).

2.8. Immunohistochemistry

Ileum samples were dissected out, fixed in 4% (w/v) solution of paraformaldehyde in PBS overnight, rinsed with PBS and stored in 70% (v/v) ethanol. Samples were embedded in paraffin and 5 μm sections were obtained. Once deparaffinized, sections were processed for antigen retrieval by incubation in 10 mM sodium citrate buffer (pH 6.0) containing 0.05% (v/v) Tween 20 at 95 °C for 10 min, washed twice with 0.1% (v/v) Triton X-100 in 0.1 M PBS, blocked for 50 min in 10% (v/v) donkey serum in 0.1% (v/v) Triton X-100 in 0.1 M PBS, and incubated overnight at 4 °C with primary antibodies for occludin (1:200), ZO-1 (1:100) or claudin-1 (1:200). Sections were washed in PBS and incubated for 2 h at room temperature with Cy2- or Cy3-conjugated donkey anti-mouse or anti-rabbit IgG (1:500) (Jackson ImmunoResearch Co. Laboratories West Grove, PA). After immunostaining, cell nuclei

were stained with Hoechst 33342 and sections were imaged using an Olympus FV 1000 laser scanning confocal microscope (Olympus, Japan). Olympus Fluoview version 4.0 software was used to merge images. Four slices per animal and four animals from each group were analyzed. The integrated optical density was measured using Image Pro Plus 5.1 software (Media Cybernetics, Rockville, MD) and expressed per area. Three randomly selected fields were measured per animal for each antibody and experimental condition.

2.9. Determination of NADPH oxidase activity

NADPH oxidase activity was measured using a lucigenin-enhanced chemiluminescence assay in membrane fractions from differentiated Caco-2 cells incubated in the absence or the presence of TNFα (5 ng/ml). For the isolation of membrane fractions, cells were homogenized in Krebs Buffer (20 mM HEPES, 119 mM NaCl, 4.7 mM KCl, 1 mM $MgSO_4$, 0.4 mM NaH_2PO_4, 0.15 mM Na_2HPO_4 and 1.25 mM $CaCl_2$) containing 1 mM PMSF, and Roche proteases inhibitor cocktail (Roche, Switzerland) and centrifuged at 800g for 10 min at 4 °C. The supernatant was subsequently centrifuged at 100,000g for 60 min at 4 °C, the pellet was collected (membrane fraction) and resuspended in Krebs buffer. Aliquots of membrane fractions (30 μg of protein) were added with or without 1 μM epicatechin or apocynin, and subsequently with 5 μM lucigenin and 50 μM NADPH. The reaction was followed under temperature-controlled conditions (37 °C). Light emission was measured every 30 s for 20 min using a Biotek Synergy H1 plate reader (BioTek Instruments, Inc.,Winooski, VT, USA) in the chemiluminescence mode. Results were expressed as the difference between the areas under the curve in the absence and in the presence of the NADPH oxidase inhibitor VAS-2780 (1 μM).

2.10. Microbiota analysis

Cecum content samples were collected in sterile tubes for subsequent investigation of the microbiome through high throughput sequencing. Samples were stored at –80 °C until processing for DNA extraction. Genomic DNA was extracted from cecal samples using the Zymo Research Fecal DNA Miniprep Kit per the manufacturer's instructions (Zymo Research, Irvine, CA, USA). The V4 region of the 16S rRNA gene was amplified by targeted barcoded primers F515 (5′-NNNNNNNNGTGTGCCAGCMGCCGCGGTAA-3′) and R806 (5′-GGACTACHVGGGTWTCTAAT-3′) as previously described [28]. Amplicons were then pooled and purified with the QIAquick PCR Purification Kit (Qiagen, Germantown, MD, USA) and taken to the UC Davis Genome Center DNA Technologies Sequencing Core for library preparation and paired-end sequenced on an Illumina Miseq. PEAR [29] was used to merge the paired end reads and they were subsequently demultiplexed using the FASTX Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/). Cutadapt was then used to trim off barcodes and primers from reads [30]. Read quality filtering, OTU picking using the implemented swarm method, filtering the OTU table, rarefaction, and beta diversity data analysis was carried out within the QIIME softwarepackage (University of Colorado, Boulder, CO, USA. version 1.9.1) [31]. Swarm was used within the QIIME package as the operational taxonomic unit clustering method [32]. Beta diversity metrics were calculated based on Unifrac distances.

2.11. 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 P value<0.05 was considered statistically significant. Data are shown as mean±SE.

3. Results

3.1. EC supplementation improves insulin sensitivity and dyslipidemia in HFD-fed mice

As previously observed [19], consumption of a HFD for 15 weeks caused obesity, dyslipidemia and insulin resistance in C57BL/6J mice (Table 1). Body weight of HFD-fed mice was 40% higher than controls (C and CE), and comparable to the three HFD-fed and EC-supplemented groups. Plasma cholesterol and triglyceride levels were 43% and 63% higher, respectively, in the HF group compared to controls, and both were decreased only in the HFE10 and HFE20 groups.

Dietary EC supplementation (2–20 mg/kg body weight) attenuated the hyperglycemia and hyperinsulinemia caused by HFD consumption (Table 1). Chronic consumption of HFD was associated with decreased insulin sensitivity and decreased glucose tolerance as evidenced by insulin (ITT) and glucose (GTT) tolerance tests, respectively (Fig. 1A and B). The area under the curve for the ITT in HF mice was 31% and 71% higher, and for the GTT, 54% and 73% higher, respectively, than in C and CE groups. The ITT area under the curve was similar in the HFE2, and significantly lower (26% and 44%, respectively) in the HFE10 and HFE20 compared to the C group. At all concentrations tested, EC supplementation

mitigated HFD-mediated increase in the GTT area under the curve. The above results confirm the capacity of dietary EC to improve insulin sensitivity in HFD-fed C57BL/6J mice even at the lowest concentration tested (2 mg/kg body weight). However, although most of the protective effects were observed in all the range of concentrations assessed (2–20 mg/kg body weight), the highest EC concentration was that consistently effective in all the different parameters tested. Thus, in most of the following experiments we have evaluated select parameters only in the groups supplemented with 20 mg/kg body weight (CE, HFE20).

3.2. EC supplementation attenuates HFD-induced steatosis and inflammation

The effects of EC on liver steatosis and inflammation triggered by the consumption of a HFD were assessed by measuring liver triglyceride content using a biochemical assay, inflammation markers by Western blot, and histology. Liver triglyceride content was 38% higher in HF mice. Values for EC-supplemented mice were similar to controls (Fig. 2A). Accordingly, hematoxylin-eosin stained liver sections showed higher NAFLD activity score in liver from HF mice compared to C, CE and HFE20 mice (Fig. 2C). Although not statistically different, the CE group showed a trend ($p<0.07$) for higher NAFLD activity score than the C group. Steatosis-associated cell damage was also evaluated by measuring plasma alanine aminotransferase (ALT) activity in plasma. In HF mice we observed 48% and 68% higher activity of plasma ALT compared to the C and CE groups (Fig. 2B). Supplementation with 20 mg EC/kg body weight prevented this increase. Values for HFE2 and HFE20 mice were not significantly different to those of controls and HF.

We next evaluated the capacity of EC to mitigate HFD-induced liver inflammation by measuring hepatic protein levels of the chemokine MCP-1, the cytokine TNFα, NOS2, and the macrophage marker F4/80 by Western blot. In HF mice we observed 81%, 210%, 40% and 58% higher levels of MCP-1, TNFα, NOS2 and F4/80, respectively, compared to the C group. Supplementation with EC (HFE20) mitigated the effects of the high fat feeding on the expression of all the tested proteins (Fig. 2D).

3.3. EC supplementation prevents intestinal permeabilization and endotoxemia in HFD-fed mice

Increased intestinal permeability and the associated increase in bacterial endotoxins in the bloodstream are significant contributors to HFD- and obesity-associated steatosis. Thus, we investigated the effects of EC supplementation on HF-induced permeabilization and increased plasma endotoxin levels. We measured intestinal permeability using FITC-dextran after 13 weeks on the corresponding diets. The intestinal paracellular permeability to FITC-dextran was 100% higher in the HF group compared to controls and all the EC-supplemented groups (Fig. 3A). Plasma endotoxin levels were 63% higher in HF mice than in controls. EC supplementation either partially (HFE10) or significantly (HFE20) mitigated endotoxemia (Fig. 3B). Plasma endotoxin levels positively correlated with plasma ALT activity (r: 0.84, $p = 0.04$), and with the area under the curve corresponding to the GTT (r: 0.89, $p = 0.02$).

3.4. EC prevents TNFα-induced permeabilization of Caco-2 cell monolayers

TNFα is a major inducer of intestinal barrier permeabilization. TNFα plasma concentration was 2.3-fold higher in HF mice compared to controls, and EC supplementation caused only a partial prevention (47%) of this increase (Fig. 3C). Thus, using Caco-2 monolayers as an in vitro model of intestinal barrier, we further assessed the capacity of EC to protect the monolayer from TNFα-induced permeabilization. For this purpose, we measured the FITC-dextran paracellular transport and the monolayer transepithelial electrical resistance (TEER). TNFα (5 ng/ml), added to the lower chamber, caused a 1.1-fold increase in FITC-dextran transport (Fig. 3D) and a 31% decrease in TEER (Fig. 3E) in Caco-2 cell monolayers. These changes were prevented by simultaneous incubation of cells with EC (1 μM) or with apocynin (1 μM) added to the upper chamber.

3.5. EC supplementation attenuates HFD-induced alterations in TJ protein expression

An increased intestinal permeability can be due to impaired TJ structure and/or function. To investigate the potential protective effects of EC on HFD-mediated alterations of TJs, we examined the expression of the TJ proteins ZO-1, occludin, and claudin-1 in mouse ileum by Western blot and by immunofluorescence. ZO-1, occludin and claudin-1 protein levels assessed by Western blot were 64%, 55% and 33% lower, respectively, in the ileum from HF mice compared to controls (Fig. 4A). Supplementation of the HF mice with 20 mg EC/kg body weight prevented the decrease in ZO-1, occludin and claudin-1. Similar trends were observed when TJ proteins were evaluated by immunofluorescence confocal microscopy (Fig. 4B and C).

3.6. Mechanisms underlying the protective effects of EC on HFD-induced increased intestinal permeability

Intestinal permeability can be regulated by different cell signaling pathways (ERK1/2, AMPK and NF-κB), some of which are susceptible to redox regulation. GLP-2 is a major regulator of epithelial monolayer permeability. To assess signaling activation we measured the phosphorylation of ERK1/2, NF-κB (p65) and AMPK, and the nuclear presence of p65 by Western blot (Fig. 5A and B). Higher levels of ERK1/2, p65, and AMPK phosphorylation were observed in HF, but not in HFE20 mice when compared to controls (Fig. 5A). In agreement with the pattern of p65 phosphorylation in total homogenates, nuclear p65 levels were significantly higher (98%) in the HF group compared to controls and HFE20 mice (Fig. 5B). Plasma GLP-2 levels were 1- and 1.5-fold higher in CE and HFE20 mice, respectively, compared to the C group (Fig. 5C). In HF mice, plasma GLP-2 concentration was not statistically different compared to the control groups. Significantly, plasma GLP-2 levels in both EC-supplemented groups (CE, HFE20) were significantly higher than in control mice.

Oxidative stress could contribute to intestinal permeabilization upon HFD consumption. Thus, we measured protein levels of ileum NADPH oxidases NOX1 and NOX4, and 4-hydroxynonenal (HNE)-protein adducts as parameter of oxidative stress (Fig. 6A). NOX4 and NOX1 levels were 50% higher in the ileum from HF mice than in controls, and this was prevented by EC supplementation (Fig. 6B). 4-HNE-protein adducts in the molecular weight corresponding to actin were 24% higher in HF mice than in all other groups. To assess the potential involvement of NADPH oxidases on intestinal

permeabilization, we measured the effects of TNFα, EC, and apocynin on the expression of NOX1 and NOX4, and on NADPH oxidase activity in Caco-2 cells. TNFα caused a 79% and 52% increase in NOX1 and NOX4 expression, respectively (Fig. 6B). Both EC and apocynin (1 μM) prevented both increases. Supporting a condition of increased oxidant production and oxidative stress, TNFα caused higher (38%) levels of 4-HNE-protein adducts in Caco-2 cells, which were prevented by EC and apocynin. At 1 μM concentration, both EC and apocynin added to Caco-2 membrane fractions inhibited NADPH oxidase activity (Fig. 6C). The above experiments support the capacity of EC to inhibit NADPH oxidase both at the level of expression and activity.

3.7. EC supplementation does not affect HFD-induced dysbiosis

As expected, the chronic consumption of a HFD caused dysbiosis in mice. Principal component analysis of cecum microbiota showed clearly separated clusters for the control and HF groups. EC supplementation did not normalize the observed microbiota cluster segregation (Fig. 7).

4. Discussion

This study presents evidence that EC supplementation mitigates intestinal permeabilization and endotoxemia induced by HFD consumption in mice. EC protected the integrity of the TJ and modulated signaling pathways that contribute to TJ normal function, including NOX1/NOX4 upregulation, oxidative stress, and NF-κB and ERK1/2 activation. The involvement of NADPH oxidase in EC-mediated protection of barrier integrity in HFD-fed mice was supported by evidence in Caco-2 cells. The beneficial effects

of EC supplementation on intestinal permeability could protect the liver from endotoxin-induced damage. This may explain EC capacity to improve the metabolic profile in HFD-induced obesity and T2D [19, 20].

The link between steatosis and a "leaky gut" is supported by a large body of evidence associating NAFLD to different conditions with increased intestinal permeability, including cardiometabolic disorders, obesity [2, 3], and inflammatory bowel diseases [33]. We currently observed that the chronic consumption of a HFD led to steatosis, liver inflammation (high tissue chemokines and cytokines, NOS2, and macrophage F4/80 protein levels) and damage (high ALT plasma activity) in mice. This can in part explain previous findings showing impaired hepatic response to insulin in HFD mice [19]. EC improved systemic glucose homeostasis, and mitigated HFD-induced liver triglyceride accumulation, inflammation and ALT release. Accordingly, in a rat model of high fructose-induced T2D, EC supplementation also prevented hepatic triglyceride accumulation and insulin resistance [18]. Overfeeding is recognized as a contributor of NAFLD [34]. Although EC can prevent steatosis in two models of overfeeding (high fat and high fructose diets) in mice, the underlying mechanisms are unknown. Evidence that EC can preserve Caco-2 cell monolayer permeability in an in vitro model of inflammation [22], points to the intestinal barrier as a potential target of EC beneficial actions on steatosis, and hepatic/systemic insulin resistance.

A permeable intestinal epithelium allows the paracellular transport of bacterial and dietary antigens leading to local and systemic inflammation [5, 7]. For example, endotoxemia occurs upon overfeeding in men [35], as a consequence of consuming a HFD

in T2D patients [36], and correlates with energy intake in healthy individuals [37]. One consequence of increased endotoxemia is liver inflammation, steatosis and insulin resistance. In support of this mechanism, all these symptoms manifest in mice i.p. injected with LPS for four weeks [5]. We observed that EC prevented HFD-induced barrier permeabilization and increased circulating endotoxin. Accordingly, an EC-containing cocoa extract decreased endotoxemia in high fat fed mice [38]. Suggesting a potential link between a leaky gut and liver dysfunction upon HFD consumption, we observed that plasma endotoxin concentrations positively correlated with plasma ALT activity (parameter of liver damage), and GTT area under the curve (parameter of glucose tolerance).

The protective capacity of EC on intestinal permeability is in part due to the preservation of TJ structure. HFD consumption caused a decreased expression of the TJ proteins occludin, claudin-1 and ZO-1 in the ileum, which was prevented by EC supplementation. Among several factors, TNFα is a central trigger of TJ structural/functional disruption. EC partially mitigated HFD-induced increase in circulating TNFα which can partially explain the protective action of EC on barrier function. This is in agreement with previous observations showing that EC protects Caco-2 monolayers from TNFα-induced decrease in ZO-1 expression, alters ZO-1 subcellular distribution, and increases paracellular transport [22]. NADPH oxidase is activated when TNFα binds to its receptor, and increased $O_2^{\cdot-}$ production contributes to TNFα-induced TJ disruption [22]. EC mitigated HFD-induced increased expression of NOX1 and NOX4 and protein oxidation in the ileum. Significantly, we also observed that the permeabilization of

Caco-2 monolayers by TNFα was associated with increased NOX1 and NOX4 expression and protein oxidation. As previously observed in other tissues [18, 20, 39, 40], EC mitigated NADPH oxidase overexpression (NOX1 and NOX4) in HFD-fed mice and Caco-2 cells, and inhibited the enzyme activity in cells. In vitro experiments showing the parallel action of a NADPH oxidase inhibitor (apocynin) and EC preventing TNFα-induced permeabilization and NADPH oxidase overexpression and activity, strongly support ileum epithelial NADPH oxidase as a significant target for the protective actions of EC on HFD-induced intestinal barrier permeabilization in mice. Both NOX1 and NOX4 have κB sites in their promoters, and NF-κB is a central regulator of both NOX1 and NOX4 expression [41]. Thus, a decrease in cellular $O_2^{\cdot-}$/H_2O_2 production as a consequence of NADPH oxidase inhibition, and subsequent inhibition of NF-κB activation, can explain the observed prevention by EC and apocynin of HFD- and TNFα-induced overexpression of both NOX1 and NOX4. On the other hand, it has been argued that apocynin is not a NADPH oxidase inhibitor unless the dimer is formed. However, apocynin inhibits NADPH oxidase in vitro both as monomer and as dimer, with an IC50 4-fold lower for the dimer than for the monomer [40]. The low (1 μM) concentration of apocynin that inhibited TNFα-induced Caco-2 monolayer permeabilization suggests that the involved mechanism is primarily the inhibition of NADPH oxidase rather than a direct antioxidant action. Overall, although the role of NADPH oxidase on TJ structure/function regulation is not completely understood, an increase in $O_2^{\cdot-}$/H_2O_2 production could increase intestinal permeability through the oxidation of TJ structural and modulatory proteins and, as described below, the activation of select redox-sensitive signals.

Different signaling cascades are involved in the regulation of TJ assembly. AMPK plays a key role in promoting the assembly of TJs [42, 43]. On the other hand, AMPK is also important for nutrient absorption [44]. In our model, AMPK activation by HFD consumption seems to be related to the local management of nutrient absorption and metabolism rather than to the regulation of intestinal permeabilization. Very importantly, TNFα activates pathways that increase TJ permeability, including NF-κB and ERK1/2. NF-κB regulates the transcription of myosin light chain kinase (MLCK) [45, 46], which increases TJ permeability by phosphorylating the myosin light chain protein. ERK1/2 activation increases TJ permeability by regulating the expression of TJ proteins [47] and promoting MLCK transcription via the ETS domain containing protein ELK-1 [48]. Thus, the above results are consistent with an upstream regulatory effect of EC (i.e. TNFα-triggered NADPH oxidase activation) in the inhibition of NF-κB and ERK1/2 activation and ultimately TJ disruption.

Different factors/events are proposed to contribute to the intestinal permeabilization triggered by HFD consumption including fat per se, an increase in luminal bile acids, and alterations in the microbiota [49]. In fact, consumption of HFDs with different fat sources leads to increased intestinal permeability in mice [50]. Dysbiosis, including increased Bacteroides and decreased Firmicutes, decreased bacterial diversity and altered representation of bacterial genes, is observed in obese individuals [51, 52]. Consumption of high energy foods, obesity and dysbiosis are linked to steatosis and NAFLD [24, 53-55]. Although the microbiota can be central to energy metabolism, intestinal permeability, endotoxemia and steatosis; mitigation of dysbiosis by EC through changes in microbiota

composition does not seem to be the mechanism involved in the observed EC beneficial effects. This is based on the lack of effects of EC on the overall microbiota changes observed as a consequence of HFD consumption.

GLP-2 is a 33 amino acid peptide that is generated from the cleavage of proglucagon. GLP-2 is involved in different aspects of intestinal physiology, including trophic actions and decreased barrier paracellular transport [56]. GLP-2 also protects the liver from steatosis. In this regard, mice fed a HFD and injected with an antagonist to the GLP-2 receptor showed worsening of triglyceride hepatic deposition and steatosis degeneration [57]. EC supplementation increased plasma GLP-2 levels both in control and HFD-fed mice. Thus, EC-mediated plasma GLP-2 increase could contribute to the mitigation of steatosis by both preserving the structure/function of TJs in HF mice, and also decreasing hepatic triglyceride deposition.

In summary, consumption of a HFD leads to intestinal permeabilization, steatosis, hepatic inflammation and insulin resistance in mice. EC supplementation mitigates all these adverse effects. HFD-induced endotoxemia, secondary to increased intestinal permeabilization can be in part involved in the development of steatosis. Indeed, HFD consumption caused alterations in TJ composition, and affected events involved in intestinal permeabilization, including dysbiosis and increased plasma TNFα. In the ileum, HFD consumption triggered a cascade of redox-related events: upregulation of NOX1/NOX4, protein oxidation, activation of NF-κB and ERK1/2, and TJ disruption. EC protection of barrier function is in part due to its capacity to preserve normal TJ composition in part by preventing NOX1/NOX4 upregulation, inhibiting NF-κB and ERK1/2,

and increasing GLP-2 expression. Current results support the concept that dietary EC might be important in mitigating the adverse effects of Western style diets.

Acknowledgements

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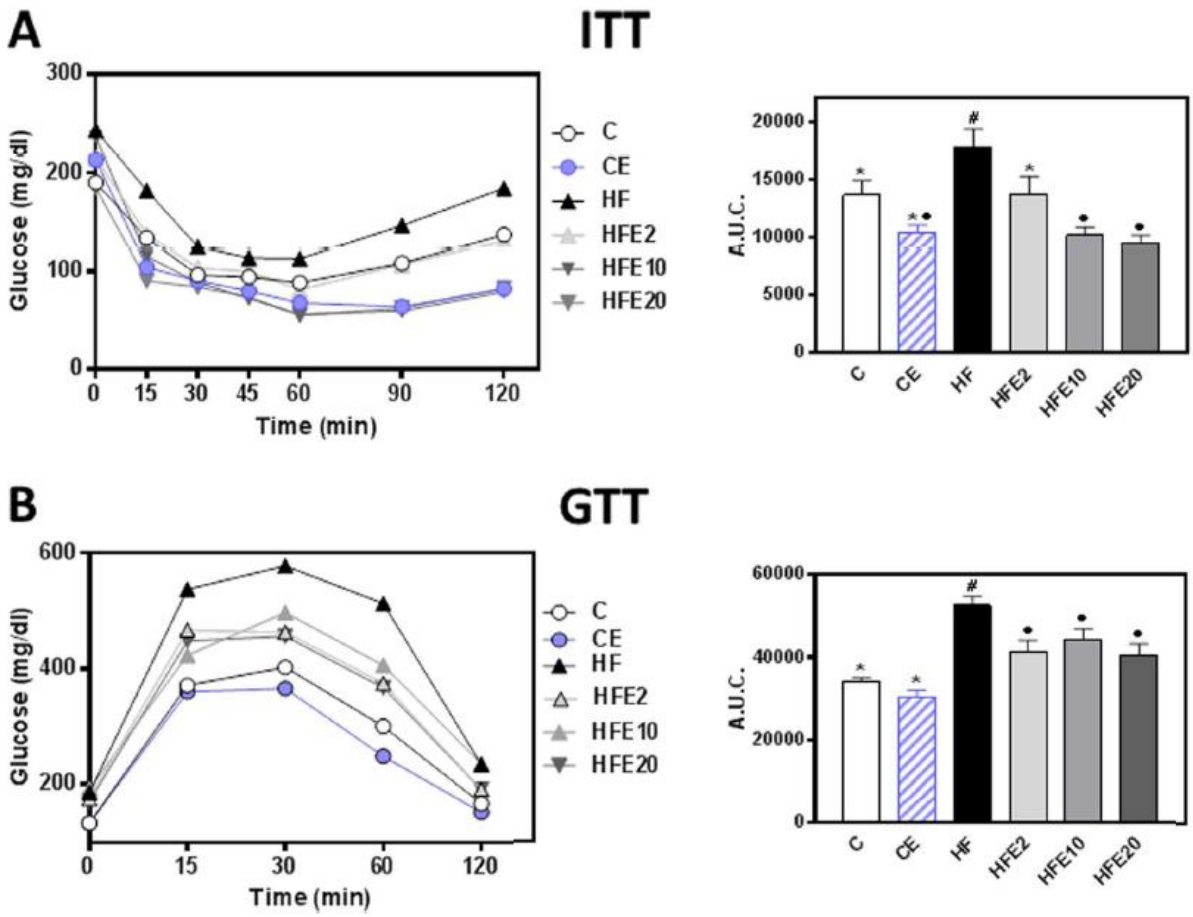
Tables and figures

Table 1. Metabolic parameters.

Parameter	C	CE	HF	HFE2	HFE10	HFE20
Body weight (g)	31.6 ± 0.9^{a}	33.0 ± 0.7^{a}	44.0 ± 1.6^{b}	40.9 ± 2.2^{b}	42.1 ± 2.6^{b}	39.8 ± 1.9^{b}
Food intake (g/d)	3.5 ± 0.19^{a}	4.1 ± 0.05^{b}	2.8 ± 0.17^{c}	3.0 ± 0.07^{c}	2.8 ± 0.09^{c}	2.8 ± 0.12^{c}
Fasted glucose (mg/dl)	96 ± 4^{a}	85 ± 12^{a}	161 ± 8^{b}	134 ± 9^{c}	$137 \pm 15^{b,c}$	130 ± 7^{c}
Fasted insulin (ng/ml)	0.15 ± 0.04^{a}	0.22 ± 0.10^{a}	0.87 ± 0.23^{b}	0.38 ± 0.11^{a}	0.47 ± 0.09^{a}	0.37 ± 0.07^{a}
Total cholesterol (mg/dl)	119 ± 6^{a}	$128 \pm 5^{a,d}$	170 ± 6^{b}	$156 \pm 10^{b,c}$	$146 \pm 7^{c,d}$	$136 \pm 10^{a,c}$
TG (mg/dl)	45.4 ± 2.1^{a}	$55.4 \pm 3.8^{c,d}$	73.8 ± 3.6^{b}	62.5 ± 3.2^{c}	$49.9 \pm 3.2^{a,d}$	62.7 ± 1.3^{c}

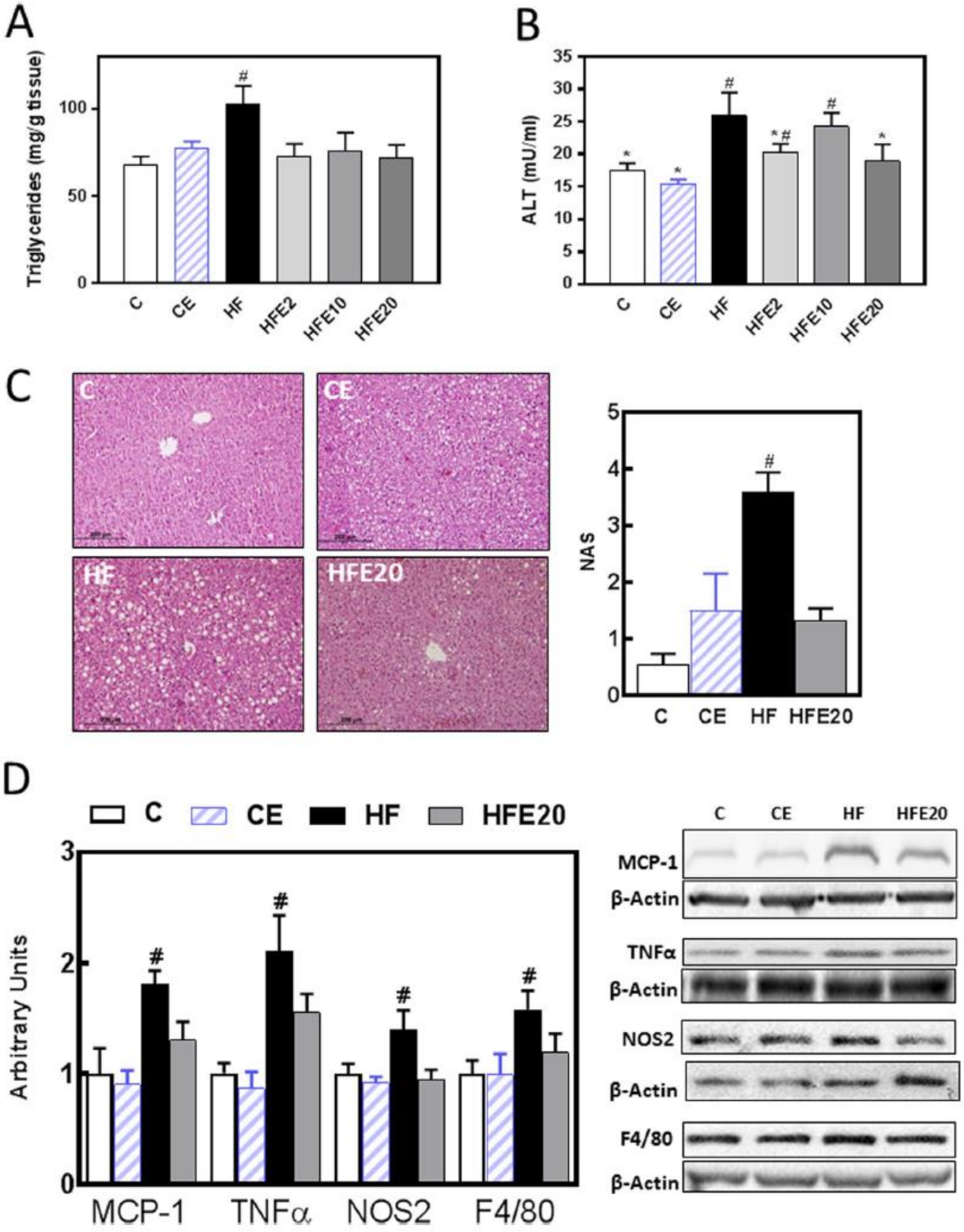
Results are shown as means±SE and are the average of 5-8 animals/group. Values having different superscripts are significantly different; ($p<0.05$, one way ANOVA).

Figure 1. Effects of EC supplementation on metabolic parameters in HFD-fed mice.



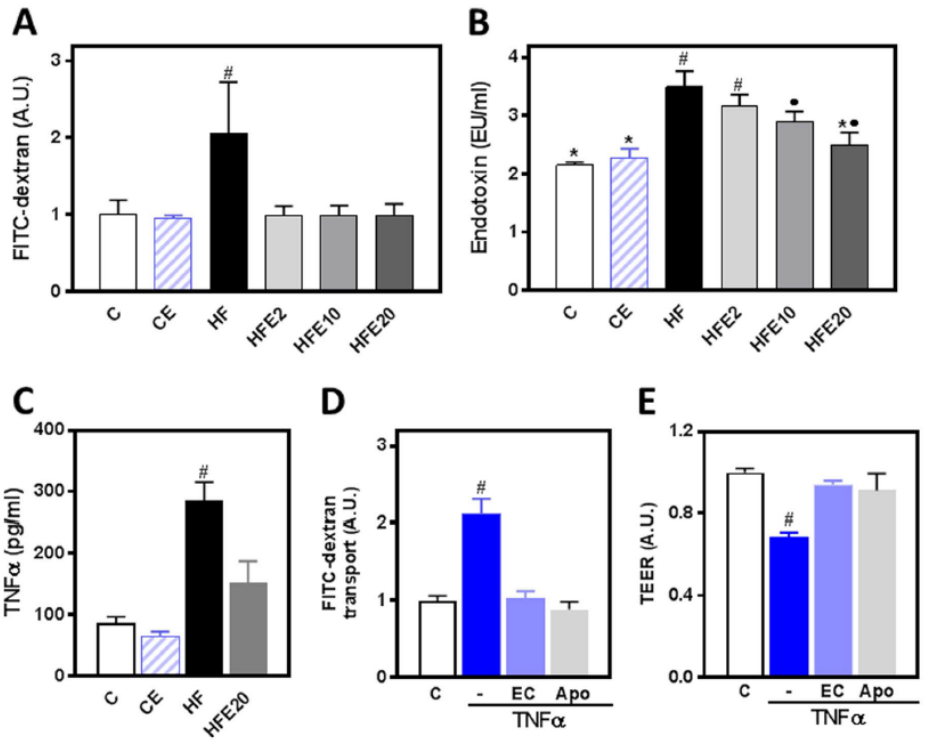
A- ITT and **B-** GTT (the corresponding area under the curve (A.U.C) is shown in the right panels), were performed on weeks 9 and 11 on the diets, respectively. Mice were fed a control diet (empty circles and empty bars), the control diet supplemented with 20 mg EC/kg body weight (blue circles and dashed bars), a HFD (HF) (black triangles and bars), or the HFD supplemented with 2 (HFE2), 10 (HFE10), or 20 (HFE20) mg EC/kg body weight (grey triangles and bars). Results are shown as means±SE and are the average of 5–8 animals/group. Values having different symbols are significantly different ($p<0.05$, one way ANOVA).

Figure 2. Effects of EC supplementation on steatosis and hepatic inflammation.



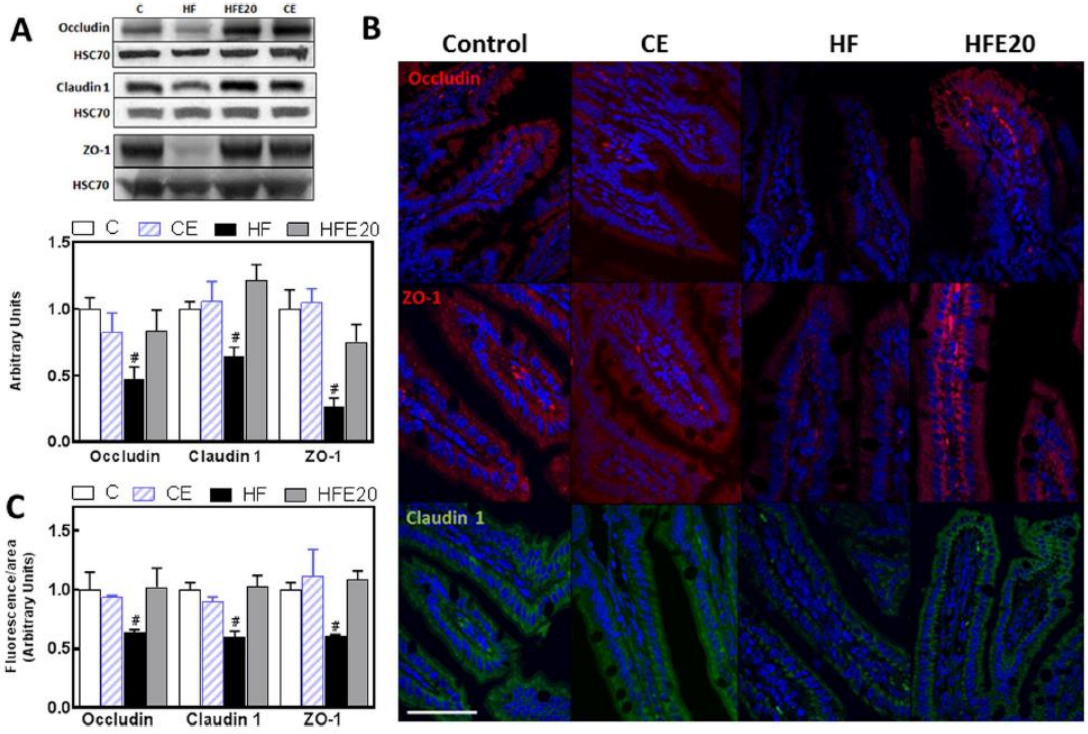
Mice were fed a control diet (empty bars), the control diet supplemented with 20 mg EC/kg body weight (dashed bars), a HFD (HF) (black bars), or the HFD supplemented with 2 (HFE2), 10 (HFE10), or 20 (HFE20) mg EC/kg body weight (grey bars). On week 15 on the corresponding diets the following parameters were measured: **A-** liver triglyceride content, **B-** plasma alanine amino transferase (ALT) activity, **C-** fat liver deposition and NAFLD activity score (NAS) evaluated by hematoxylin/eosin tissue staining, **D-** proteins involved in inflammation: MCP-1, TNFα, NOS2 and F4/80 protein levels were measured by Western blot. Bands were quantified and values referred to β-actin levels (loading control). Results for HF, HFE20 and CE were referred to control group values (C). Results are shown as mean±SE of 5–8 animals/group. **A,B-** Values having different symbols are significantly different; **C,D-** #Significantly different from all other groups; ($p<0.05$, one way ANOVA test).

Figure 3. Effects of EC on epithelial barrier permeabilization induced by HFD consumption in mice and by TNFα in Caco-2 cells.



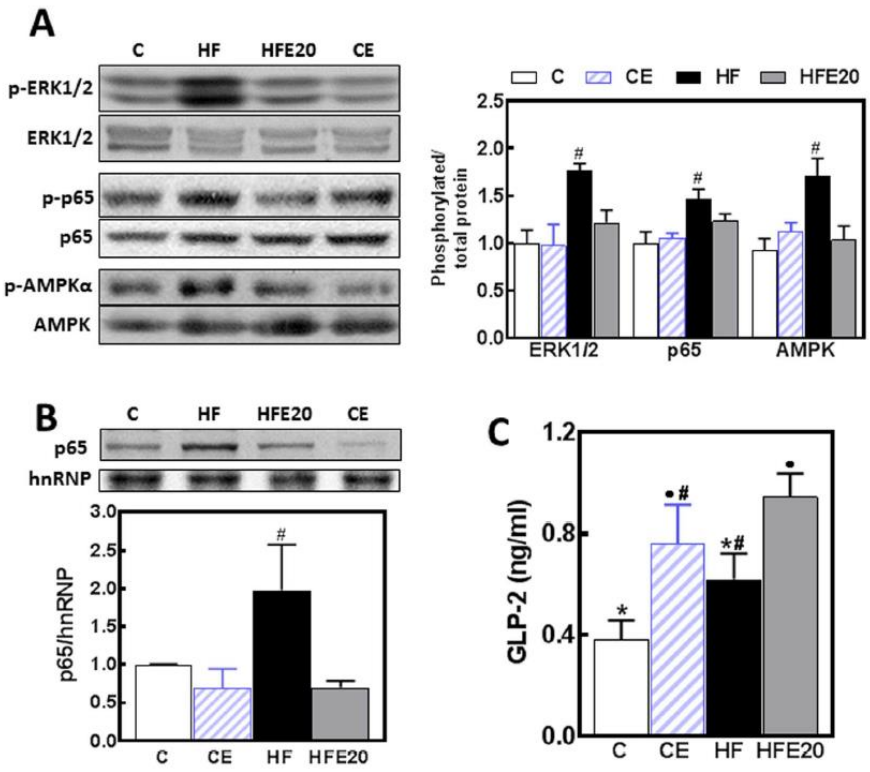
A,C- Mice were fed a control diet (empty bars), the control diet supplemented with 20 mg EC/kg body weight (dashed bars), a HFD (HF) (black bars), or the HFD supplemented with 2 (HFE2), 10 (HFE10), or 20 (HFE20) mg EC/kg body weight (grey bars). Intestinal permeability was evaluated by measuring at week 13 FITC-dextran permeability **(A)**, and at week 15 plasma endotoxin **(B)** and TNFα **(C)** concentrations. Results are shown as mean±SE of 5–8 animals/group. **D**- FITC-dextran transport and **E**- TEER in Caco-2 cells. Polarized cells were incubated for 6 h at 37 °C in the absence of additions (control, C); or after addition of 5 ng/ml TNFα to the lower chamber in the absence (TNF) or the presence of 1 μM EC or 1 μM apocynin (Apo) added to the upper chamber. Results are shown as mean±SE of 3 independent experiments. **A,C-E**- #Significantly different from all other groups; B- Values having different symbols are significantly different; ($p<0.05$,one way ANOVA test).

Figure 4. Effects of EC supplementation on HFD-induced alterations in ileum tight junction protein expression.



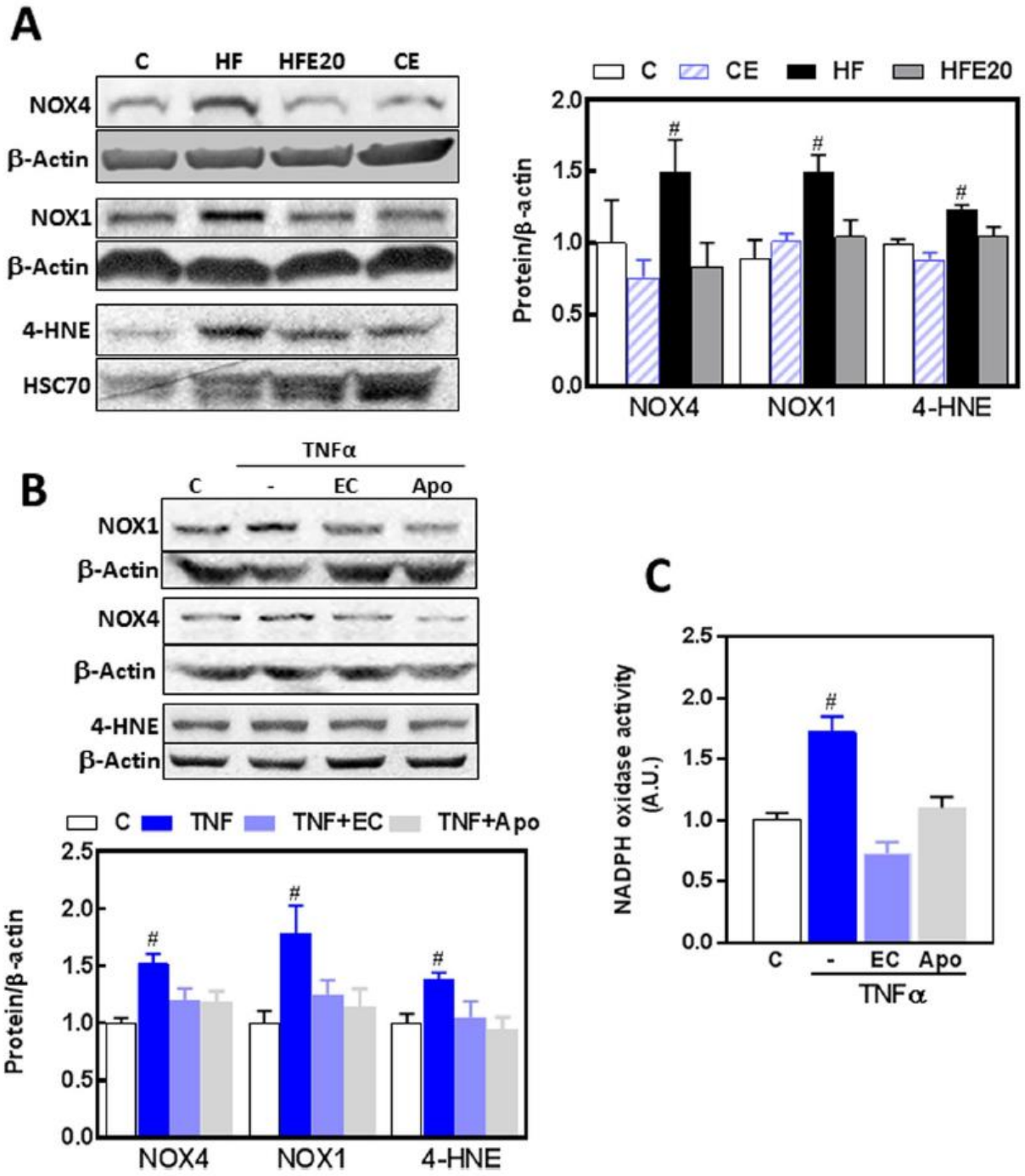
Mice were fed a control diet (empty bars), the control diet supplemented with 20 mg EC/kg body weight (dashed bars), a HFD (HF) (black bars), or the HFD supplemented with 20 mg (HFE20) EC/kg body weight (grey bars). **A-** TJ proteins occludin, claudin-1 and ZO-1, expression was measured by Western blot. Bands were quantified and values referred to HSC70 levels (loading control). Results for HF, HFE20 and CE were referred to control group values (C). Results are shown as mean±SE of 8 animals/treatment. **B-** Representative images for immunohistochemistry and confocal microscopy for occludin and ZO-1 (red fluorescence), and claudin-1 (green fluorescence). Hoechst was used for nuclear counterstaining (blue). Bar: 50 μm. **C-** Fluorescence intensity was measured as described in methods and results are shown as mean±SE of 4 animals/treatment. **A,C-** #Significantly different from all other groups; ($p<0.05$, one way ANOVA test).

Figure 5. Effects of EC supplementation on HFD-induced alterations on signaling events that regulate intestinal permeability.



Mice were fed a control diet (empty bars), the control diet supplemented with 20 mg EC/kg body weight (dashed bars), a HFD (HF) (black bars), or the HFD supplemented with 20 mg EC/kg body weight (HFE20) (grey bars). **A-** Phosphorylation levels of ERK1/2 (Thr202/Tyr204), p65 (Ser536) and AMPK (Thr172) in total ileum homogenates, and **B-** p65 and hnRNP levels in ileum nuclear fractions were measured by Western blot. Bands were quantified and values referred to either **(A)** the non-phosphorylated protein form or **(B)** hnRNP levels. Results for HF, HFE20 and CE were referred to control group values **(C)**. **C-** Plasma GLP-2 concentration was measured using a commercial kit. **A-C-** Results are shown as mean±SE of 6–8 animals/treatment. **A,B-** #Significantly different from all other groups; C- Values having different symbols are significantly different; ($p<0.05$, one way ANOVA test).

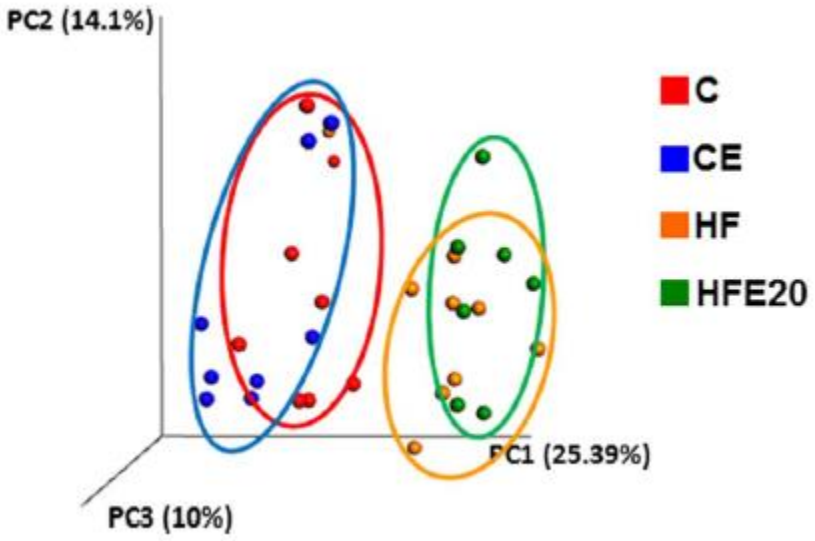
Figure 6. Effects of EC on the upregulation of intestinal NADPH oxidases and protein oxidation induced by HFD consumption in mice and by TNFα in Caco-2 cells.



Total protein levels of NOX4, NOX1, and HNE-protein adducts (MW: 40 kDa) were measured by Western blot in: **A**- the ileum of mice fed a control diet (empty bars), the

control diet supplemented with 20 mg EC/kg body weight (dashed bars), a HFD (HF) (black bars), or the HFD supplemented with 20 mg EC/kg body weight (HFE20) (grey bars); **B-** Caco-2 cells incubated for 6 h at 37 °C in the absence of additions (control, C) (empty bars); or after addition of 5 ng/ml TNFα in the absence (TNF) (dark blue bars) or the presence of 1 μM EC (TNF + EC) (light blue bars) or 1 μM apocynin (Apo) (grey bars). Bands were quantified and values referred to β-actin or HSC70 levels (loading controls). Results were referred to control group values (**C**). **C-** In vitro effects of 1 μM EC and apocynin (Apo) on NADPH oxidase activity were measured in membrane fractions isolated from Caco-2 cells incubated without or with 5 ng/ml TNFα for 6 h. Results are shown as mean±SE of **A-** 6–8 animals/treatment, and **B,C-** 4 independent experiments. #Significantly different from all other groups ($p<0.05$, one way ANOVA test).

Figure 7. Effects of EC on HFD-induced changes in cecal microbiota.



Changes in cecal microbiota assessed by clustering of samples based on diet. Principal coordinate analysis (PCA) was performed based on the weighted UniFrac distance matrix generated from sequencing fecal 16S rRNA gene in cecum samples from mice fed the corresponding diets for 15 weeks. The X-axis represents the tertiary coordinate, the Y-axis represents the secondary coordinate. Axis numbering represents the relative distance between samples based on the weighted UniFrac distance matrix.

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Chapter 2

(-)-Epicatechin and NADPH oxidase inhibitors prevent bile acid-induced Caco-2 monolayer permeabilization through ERK1/2 modulation

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(-)-Epicatechin and NADPH oxidase inhibitors prevent bile acid-induced Caco-2 monolayer permeabilization through ERK1/2 modulation

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Abbreviations: DCA, deoxycholic acid; DCFDA, 5-(and-6)-carboxy-2′7′-dichlorodihydrofluorescein diacetate; DHE, dihydroethidium; EC, (-)-epicatechin; EGF, epidermal growth factor; EGFR, EGF receptor; ERK, extracellular signal-regulated kinase; GI, gastrointestinal; MLC, myosin light chain; MLCK, MLC kinase; MEM, minimum essential medium; MMP, matrix metalloproteinase; NOX, NADPH oxidase; ROS, reactive oxygen species; TJ, tight junction

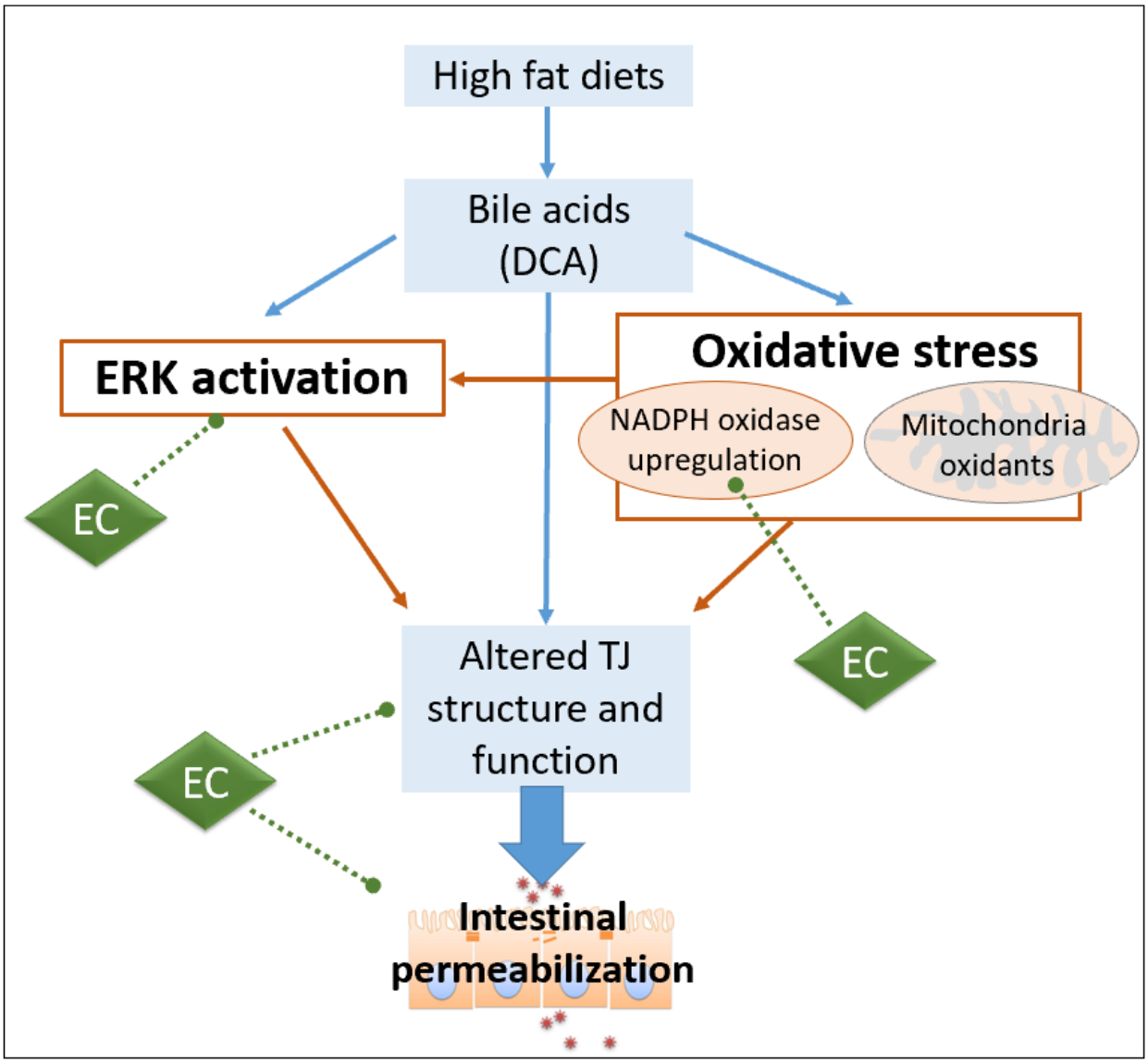
Key words: bile acids; deoxycholic acid; epicatechin; intestinal permeability; high fat

Abstract

Secondary bile acids promote gastrointestinal (GI) tract permeabilization both in vivo and in vitro. Consumption of high fat diets increases bile acid levels in the GI tract which can contribute to intestinal permeabilization and consequent local and systemic inflammation. This work investigated the mechanisms involved in bile acid (deoxycholic acid (DCA))-induced intestinal epithelial cell monolayer permeabilization and the preventive capacity of (-)-epicatechin (EC). While EC prevented high fat diet-induced intestinal permeabilization in mice, it did not mitigate the associated increase in fecal/cecal total and individual bile acids. In vitro, using differentiated Caco-2 cells as a model of epithelial barrier, EC and other NADPH oxidase inhibitors (VAS-2870 and apocynin) mitigated DCA-induced Caco-2 monolayer permeabilization. While EC inhibited DCA-mediated increase in cell oxidants, it did not prevent DCA-induced mitochondrial oxidant production. Prevention of DCA-induced ERK1/2 activation with EC, VAS-2870, apocynin and the MEK inhibitor U0126, also prevented monolayer permeabilization, stressing the key involvement of ERK1/2 in this process and its redox regulation. Downstream, DCA promoted myosin light chain (MLC) phosphorylation which was related to MLC phosphatase (MLCP) inhibition by ERK1/2. DCA also decreased the levels of the tight junction proteins ZO-1 and occludin, which can be related to MMP-2 activation and consequent ZO-1 and occludin degradation. Both events were prevented by EC, NADPH oxidase and ERK1/2 inhibitors. Thus, DCA-induced Caco-2 monolayer permeabilization occurs mainly secondary to a redox-regulated ERK1/2 activation and downstream disruption of TJ structure and dynamic. EC's capacity to mitigate in vivo the gastrointestinal permeabilization caused by

consumption of high-fat diets can be in part related to its capacity to inhibit bile-induced NADPH oxidase and ERK1/2 activation.

Graphical Abstract



1. Introduction

Consumption of Western type diets high in saturated fats has deleterious consequences on the gastrointestinal (GI) tract. They include, among others, a higher risk for colorectal cancer [1-3], altered intestinal immunity [4], inflammation [5], and intestinal permeabilization [5-7]. In fact, an increased intestinal permeability and the associated local and systemic inflammation can underlie several of high fat diet- and obesity-associated morbidities [8, 9].

The permeability of the GI tract is determined by the tight junction (TJ), which connect a single layer of epithelial cells [10]. Intercellular TJs are a complex of proteins including both integral (e.g. occludin, claudins) and intracellular (e.g. ZO-1, ZO-2) proteins that are linked to an actomyosin ring. TJs selectively allow the passage of water and ions and restrict the paracellular transport of larger molecules. The unrestricted intestinal passage of large molecules (e.g. pathogens, luminal food and microbial toxins) can have direct systemic effects, activating the GI immune system leading to local inflammation and the systemic release of proinflammatory cytokines. Accordingly, a loss of intestinal barrier integrity/function has been observed in several human pathologies [5].

Select bile acids increase GI permeability both in vivo and in vitro models. Thus, the intestinal permeabilization observed in rats fed a high-fat diet was found to be related to both the dietary fat itself and the associated increased intestinal levels of bile acids [11]. Deoxycholic acid (DCA), a hydrophobic secondary bile acid, promotes the permeabilization of Caco-2 cell monolayers [12]. DCA affects the physical properties of cell

membrane domains [13], which is associated to the activation of cell signals that regulate TJ permeability. In intestinal epithelial cells DCA causes the activation of the epidermal growth factor (EGF) receptor (EGFR) [13, 14], and downstream the ERK1/2 signaling pathway [15]. Together with transcription factor NF-κB, ERK1/2 upregulates myosin light chain (MLC) kinase (MLCK), a central physiological modulator of TJ permeability [10, 16, 17]. Bile acids also downregulate TJ proteins expression, which could contribute to DCA-mediated barrier permeabilization [12]. Overall, the increased bile acid production and luminal content required for fat absorption could explain the increased permeabilization of the GI tract observed with consumption of high-fat diets.

Diet has a major role in the development and progression of GI-associated pathologies. Among dietary factors, flavonoid-rich foods can exert GI protective and trophic effects through different mechanisms [18]. In particular, the flavan-3-ol (-)-epicatechin (EC) prevents inflammation- and high fat diet-induced intestinal permeabilization both in vitro and in vivo [19, 20]. In vitro, EC prevents tumor necrosis alpha (TNFα)-induced permeabilization of Caco-2 cell monolayers through the inhibition of NADPH oxidase, leading to a decrease in superoxide production, and inactivation of the transcription factor NF-κB [19]. In mice fed a high-fat diet, supplementation with EC prevented the increased GI permeability and the associated endotoxemia [20].

An increase in luminal bile acids can in part underlie the increased intestinal permeabilization associated with the consumption of diets rich in fat content, contributing to the associated co-morbidities. On the other hand, a diet rich in select bioactives (e.g. EC) could mitigate intestinal permeabilization by inhibiting bile-acid mediated barrier damage.

This work investigated the mechanisms involved in bile acid (deoxycholic acid (DCA))-induced intestinal epithelial cell monolayer permeabilization and the preventive capacity of EC. We initially studied if the capacity of EC to prevent high fat diet-induced intestinal permeabilization in mice could occur through the modulation of bile acid metabolism. Subsequently, and using Caco-2 cell monolayers as a model of intestinal barrier, we investigated the potential capacity of EC to prevent bile direct effects on the epithelium. The ERK1/2 signaling pathway emerges as a central mechanism in DCA-induced monolayer permeabilization. The protective action of EC is in part mediated by its capacity to inhibit NADPH oxidase, oxidant production, and downstream ERK1/2 activation.

2. Materials and Methods

2.1. Materials

Caco-2 cells were from the American Type Culture Collection (ATCC, Rockville, MA). Cell culture media and reagents, 5-(and-6)-carboxy-2′7′-dichlorodihydrofluorescein diacetate (DCFDA), MitoSOX Red mitochondrial superoxide indicator (M36008), and primary antibodies for ZO-1 (#339100), occludin (#331500), claudin-1 (#717800), and claudin-2 (#325600) were from Invitrogen/Life Technologies (Grand Island, NY). U0126 (#9903) and primary antibodies for phospho-p44/42 ERK1/2 (Thr202/Tyr204) (#4370), p44/42 ERK1/2 (#4695), phospho-p65 (Ser536) (#3033), p65 (#8242), phospho-MLC 2 (Ser19) (#3671), MLC 2 (#8505), phospho-MYPT1 (Thr696) (#5163); MYPT1 (#8574), phospho-EGFR (Tyr1068) (#3777), EGFR (#4267), and β-actin (#12620) were from Cell Signaling Technology (Danvers, MA). DCA, EC, apocynin, VAS-2870, FITC-dextran, and dihydroethidium (DHE) were from Sigma Chem. Co. (St. Louis, MO). The Amplex® Red

Hydrogen Peroxide/Peroxidase Assay Kit was from Thermo Fisher Scientific (Waltham, MA).

2.2. Animals and animal care

All procedures conducted during this study were in agreement with standards for the care of laboratory animals as outlined in the NIH Guide for the Care and Use of Laboratory Animals. All procedures were administered under the auspices of the Animal Resource Services of the University of California, Davis. Experimental protocols were approved before implementation by the University of California, Davis Animal Use and Care Administrative Advisory Committee.

Healthy male C57BL/6J mice (20–25 g) (7 mice/group) were divided into 2–3 mice per cage, housed at 21–22 °C and 53–55% humidity on a 12-h light-dark cycle. They were fed for 15 weeks either: A-a diet containing approximately 10% total calories from fat (Control) (TD.06416, Harlan Laboratories, Madison, WI), B- a diet containing approximately 60% total calories from fat (lard) (HF) (TD.06414, Harlan Laboratories, Madison, WI), and C-the high fat diet supplemented with 20 mg EC/kg body weight (HFE) as previously described [20]. The EC-containing diet was prepared every two weeks to account for changes in body weight and food intake, and to prevent potential EC degradation. All diets were stored at –20 °C until use. Details on metabolic parameters and in vivo evaluation of intestinal permeability for this animal study have been previously published [20]. After 15 weeks on the dietary treatments, mice were euthanized by cervical dislocation. The cecum content and feces were collected and stored at –80 °C until analysis for bile acid content.

2.3. Determination of total and individual bile acids

For total bile acid content determination, feces were weighed and dried in an oven at 37 °C for 24 h. Samples were subsequently powdered using a mortar, and bile acids were hydrolyzed under alkaline conditions at 220 °C. Subsequently, samples were neutralized with 12 N HCl, followed by three extractions in diethyl ether, as previously described [21, 22]. Total bile acids were measured using a 3-α hydroxysteroid dehydrogenase assay kit (Crystal Chem, Inc., IL, USA).

For individual bile acid analysis, mice cecum samples (20 mg) were homogenized in 1 ml of 70% (v/v) methanol containing 25 µl of 40 µg/ml d4 (deuterated)-DCA for 30 s at 6,000 rpm on a Precellys homogenizer (Bertin Technologies, UK). The slurry was then centrifuged at 1,000×g at 4 °C and the supernatant transferred to a new tube and added with 25 µl of 40 µg/ml d4-chenodeoxycholic acid. Samples were concentrated by centrifugal evaporation at 50 °C for 70 min to almost dryness using a SpeedVac™ concentrator, and then brought to 1 ml volume with 5% (v/v) methanol and added with 25 µl of 40 µg/ml d4-cholic acid. The reconstituted samples were passed through a hydrophilic-lipophilic balance clean-up cartridge (Waters Oasis Prime HLB, 1 ml, 30 mg), washed with 1 ml of 5% (v/v) methanol and eluted in 500 µl methanol added with 25 µl of 40 µg/ml d4-glycholic acid and d4-lithocholic acid. Of the internal standards added, d4-glycholic acid was the primary reference internal standard, with the others monitored as checks in the extraction procedure. The final sample was submitted for analysis by LC-MS/MS using an Agilent 1260 binary HPLC coupled to an AB Sciex 4000 QTrap triple quadrupole mass spectrometer. HPLC was carried out using a binary gradient of solvent A (water + 5 mM ammonium

acetate + 0.012% (v/v) formic acid) and solvent B (methanol + 5 mM ammonium acetate + 0.012% (v/v) formic acid) at a constant flow rate of 600 µl/min. Separation was achieved using a Supelco Ascentis Express C18 150 × 4.6, 2.7 µm column maintained at 40 °C. The mass spectrometer was operated in electrospray negative mode with capillary voltage of 4500V at 550 °C. Instrument specific gas flow rates were 25 ml/min curtain gas, GS1: 40 ml/min and GS2: 50 ml/min. Mass fragmentation was monitored in MRM mode. Quantification was applied using Analyst 1.6.2 software to integrate detected peak areas relative to the deuterated internal standards.

2.4. Cell culture and incubations

Caco-2 cells were cultured at 37 °C and 5% (v/v) CO_2 atmosphere in minimum essential medium (MEM) supplemented with 10% (v/v) fetal bovine serum and antibiotics (50 U/ml penicillin, and 50 µg/ml streptomycin). For the experiments, cells seeded in semipermeable membranes or in regular dishes were differentiated for 18–21 or 9–12 days, respectively, after confluence. The cell culture medium was replaced every 3 days. Cells were then incubated in the absence or the presence of 100 µM DCA, with or without EC (1–10 µM), 1 µM apocynin, 1 µM VAS-2870, and 10 µM U0126, for the time period indicated for each experiment. After the corresponding incubations, the medium was collected for matrix metalloproteinase (MMP) determination, and cells collected and processed accordingly for the different determinations.

2.5. Cell viability

Cell viability was evaluated using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay which is based on the conversion of MTT into formazan crystals by living cells. Cells were treated with DCA and/or EC as described above, and at the end of the incubation cells were added with a 0.5 mg/ml MTT solution in PBS. After 2 h of incubation at 37 °C, the reaction was stopped by addition of DMSO, and plates were incubated overnight. Absorbance (λ 570–690 nm) was measured using a BioTek Synergy H1 plate reader (BioTek Instruments, Winooski, VT) and expressed as percentage of untreated (control) cell values.

2.6. Transport of fluorescein isothiocyanate-dextran (FITC-dextran)

Cells were differentiated into polarized monolayers by culture on transwell inserts (12 mm, 0.4 μm pore polyester membranes) placed in 24-well plates at a seeding density of 0.3×10^6 cells/transwell. The volume of medium added to the upper and lower compartments was 400 μl and 600 μl, respectively. The paracellular transport through Caco-2 cell monolayers was determined by measuring the apical-to-basolateral clearance of FITC-dextran (4 kDa). DCA (100 μM) and EC (1–10 μM), the NADPH oxidase inhibitors apocynin and VAS-2870 (1 μM) or U0126 (10 μM) were added to the apical compartment. FITC-dextran was subsequently added to this compartment (100 μg/ml final concentration), and 100 μl of the medium in the lower compartment were collected every hour for 4 h. Aliquots were diluted with 100 μl of MEM without phenol red, and the fluorescence was measured at λexc: 485 nm and λem: 530 nm in a plate reader (Biotek Synergy H1 plate reader, BioTek Instruments, Winooski, VT). The FITC-dextran clearance (CLFITC) was calculated using the equation fFITC/(FFITC/A), where fFITC is flux of FITC-dextran (in

fluorescence units/h); FFITC, the fluorescence of FITC-dextran in the upper compartment at zero time (in fluorescence units per nl); and A, the surface area of the membrane (1 cm2). Arbitrary units (AU) were calculated based on the CLFITC value for the non-added (control) cells which showed a mean CLFITC = 75 nl/h/cm^2.

2.7. Cell oxidant levels

Cell oxidant levels were estimated using the probes DCFDA, DHE and Amplex® Red. DCFDA and DHE enter cells, and when oxidized are converted into fluorescent compounds. Caco-2 cells were grown and differentiated in 96 well plates. Cells were incubated for 0.5–4 h in the absence or presence of 100 μM DCA with or without the addition of 5 μM EC. At the corresponding times, cells were added with 20 μM DCFDA or 10 μM DHE, and after 30 min incubation the medium was removed, cells rinsed with PBS, and fluorescence measured, for oxidized DCFDA at λexc: 485 nm; λem: 535 nm, and for oxidized DHE at λexc: 485 nm; λem: 535 nm. To normalize for the number of cells, DCFDA and Amplex® Red fluorescence was referred to the DNA content measured with propidium iodide as previously described [19], and DHE fluorescence was referred to protein content. H_2O_2 released to the medium was measured at the corresponding time points with the Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit following the manufacturer's protocol.

2.8. Mitochondria oxidant production

Mitochondria oxidants production was determined using the MitoSOX Red reagent, which is oxidized in the mitochondria mainly by superoxide, and exhibits red fluorescence. Caco-2 cells were grown and differentiated in 24 well plates. Cells were incubated for 0.5–

4 h in the absence or presence of 100 μM DCA with or without the addition of 5 μM EC. Cells were dissociated by treatment with trypsin, washed once with PBS and the pellet resuspended in 0.4 ml PBS containing 5 μM MitoSOX Red (from a 5 mM reagent stock solution prepared in DMSO). Cells were subsequently incubated for 30 min at room temperature, protected from light. Appropriate controls were prepared: a positive control incubated with Antimycin A (20 μM), known to produce a burst of superoxide (and therefore an increase in MitoSOX fluorescence), and a negative control of cells incubated in the absence of MitoSOX. Cell fluorescence was measured in a FACSCanto II Flow Cytometer (BD Biosciences, San Jose, CA). Data were acquired by CellQuest Software (BD Biosciences, San Jose, CA) and analyzed using FlowJo (BD Biosciences, San Jose, CA).

2.9. RNA isolation and real-time PCR (RT-PCR)

For quantitative RT-PCR studies, RNA was extracted from cells using TRIzol reagent (Invitrogen, Carlsbad, CA). cDNA was generated using high-capacity cDNA Reverse Transcriptase (Applied Biosystems, Grand Island, NY). Expression of NOX1, NOX4, MLCK and β-actin was assessed by quantitative real-time PCR (iCycler, Bio-Rad, Hercules, CA) with the following primers:

Primer NOX1 Forward: 5′-GTACAAATTCCAGTGTGCAGACCAC-3′

Primer NOX1 Reverse: 5′-GTACAAATTCCAGTGTGCAGACCAC-3′

Primer NOX4 Forward: 5′-CTCAGCGGAATCAATCAGCTGTG-3′

Primer NOX4 Reverse: 5′-AGAGGAACACGACAATCAGCCTTAG-3′

Primer MLCK Forward: 5′-GAGGTGCTTCAGAATGAGGACG -3′

Primer MLCK Reverse: 5′- GCATCAGTGACACCTGGCAACT -3′

Primer β-Actin Forward: 5′-TCATGAAGTGTGACGTGGACATCCGC-3′

Primer β-Actin Reverse: 5′-CCTAGAAGCATTTGCGGTGCACGATG-3′

2.10. Western blot analysis

Total fractions were prepared as previously described [15]. Protein concentration was measured [23] and aliquots containing 25–100 μg protein were separated by reducing 10% (w/v) polyacrylamide gel electrophoresis and electroblotted to PVDF membranes. Colored (Biorad Laboratories, Hercules, CA) and biotinylated (Cell Signaling Technologies, Danvers MA) molecular weight standards were run simultaneously. Membranes were blotted for 1 h in 5% (w/v) non-fat milk, incubated overnight in the presence of the corresponding antibodies (1:1,000 dilution) in 5% (w/v) bovine serum albumin in TBS buffer (50 mM Tris, 150 mM NaCl, pH 7.6), containing 0.1% (v/v) Tween-20. After incubation for 90 min at room temperature in the presence of the secondary antibody (HRP-conjugated) (1:10,000 dilution) the conjugates were visualized by chemiluminescence detection in a Phosphoimager 840 (Amersham Pharmacia Biotech. Inc., Piscataway, NJ).

2.11. Detection of matrix metalloproteinases by gelatin zymography

Analysis of MMPs gelatinolytic activity was performed using 7.5% (w/v) polyacrylamide gels impregnated with 0.1% (w/v) gelatin. After 2 h incubation in the corresponding conditions, same volumes of medium were collected from all wells, samples were

centrifuged at 800×g for 8 min to eliminate cell debris, and concentrated 7X using a Vacufuge concentrator (Eppendorf, Germany). Non-reducing sample buffer was added to 75 µl of the concentrated medium, and proteins were separated by SDS-PAGE. Gels were washed 3 times in washing buffer (2.5% (v/v) Triton X-100, 50 mM Tris-HCl pH 7.5, 5 mM $CaCl_2$, 1 µM $ZnCl_2$) for 20 min at room temperature to remove the SDS. Gels were subsequently equilibrated with incubation buffer (1% (v/v) Triton X-100, 50 mM Tris-HCl pH 7.5, 5 mM $CaCl_2$, 1 µM $ZnCl_2$) for 10 min at 37 °C, which was subsequently replaced with fresh buffer, and gels incubated for 24 h at 37 °C. Gels were stained with Coomassie Brilliant Blue R-250 (Biorad Laboratories, Hercules, CA) overnight, and destained in a solution containing 10% (v/v) acetic acid and 40% (v/v) methanol, until the gelatinase activity was clearly seen. Bands were visualized in MyECL Imager (Thermo Scientific, New York, NY, USA).

2.12. Statistical analysis

All values are shown as means ± standard error of the means (SEM). Data were analyzed by one-way analysis of variance (ANOVA) using GraphPad Prism 7.0 (GraphPad Software, San Diego, CA, USA). Fisher least significance difference test was used to examine differences between group means. A p value < 0.05 was considered statistically significant.

3. Results

3.1. Bile acid profiles in mice fed a high-fat diet: effects of EC

We previously observed that EC prevents high-fat diet-triggered intestinal permeabilization [20]. In the same group of C57BL/6J mice, consumption of the high-fat diet for 14 weeks led to a significant ($p < 0.05$) increase in total fecal bile acids compared to levels found in mice fed the control diet (Fig. 1A). Dietary supplementation with EC (20 mg/kg body weight) did not affect high-fat diet-mediated increase in total fecal bile acid levels (Fig. 1A). Cecal unconjugated and conjugated bile acids were subsequently characterized by HPLC-MS in MRM mode (Fig. 1B–E). Compared to controls, consumption of the high-fat diet caused significantly higher levels of cecal β-muricholic acid, cholic acid, deoxycholic acid, taurochenodeoxycholic acid, glycholic acid, taurodeoxycholic acid and glycohyodeoxycholic acid. EC supplementation did not prevent those increases (Fig. 1B–E).

3.2. EC prevents DCA-induced Caco-2 cell monolayer permeabilization

We next investigated if EC could inhibit DCA-induced monolayer permeabilization of human Caco-2 cells differentiated into intestinal epithelial cells. Under the experimental conditions used (100 μM DCA and/or 1–10 μM EC) and upon 4 h incubation, neither DCA nor EC affected cell viability (Fig. 2A). Changes in monolayer permeability were evaluated by measuring the paracellular transport of the fluorescent probe FITC-dextran (Fig. 2B–D). DCA (100 μM) caused a time (0–4 h)-dependent increase in FITC-dextran transport from the upper to the lower chamber (Fig. 2B). EC (1–10 μM) inhibited DCA-mediated increase in FITC-dextran clearance at 2 h (Figs. 2C) and 3 h (data not shown). After 4 h incubation, 1 μM EC had no effects while 5 and 10 μM EC inhibited DCA-induced increase in FITC-dextran paracellular transport (Fig. 2D).

3.3. DCA increases NADPH oxidase expression and ROS production: effects of EC

We previously observed that DCA-induced Caco-2 monolayer permeabilization was associated with increases in reactive oxygen species (ROS) production [12, 15]. EC has been shown to both inhibit NADPH oxidase activation and prevent NOX1/NOX4 overexpression [20]. Thus, we next investigated if the capacity of EC to prevent DCA-induced permeabilization could be associated with the modulation of ROS production, characterizing the two main ROS cellular sources, NADPH oxidase and mitochondria.

NOX1 and NOX4 are the main NADPH oxidases present in Caco-2 cells (data not shown). DCA did not affect NOX1 expression within the studied period (0.5–4 h), while a significant increase in NOX4 mRNA levels was observed after 3 and 4 h incubation with DCA (Fig. 3A). EC (5 μM) prevented the increase in NOX4 expression after 3 h incubation with DCA (Fig. 3B). Given that EC has been shown to also inhibit NADPH activity we next assessed the capacity of EC to prevent DCA-mediated increase in cellular ROS. This was assessed using three different probes; Amplex® Red, DCFDA and DHE. While Amplex® Red fluorescence increase only reached significance after 4 h incubation with DCA, DCA-induced DCFDA fluorescence increase was significant at 2 and 4 h and DHE fluorescence increase was significant at 0.5, 1 and 4h (Fig. 3C). EC prevented DCA-mediated increases in oxidant production at all time points for the three probes used (data not shown). Fig. 3D shows EC inhibition of ROS increase at the corresponding time points of highest DCA effect, i.e. 4 h for Amplex® Red and DCFDA fluorescence (12 and 75% higher in DCA-treated than

in control cells, respectively) and 30 min for DHE fluorescence (45% higher in DCA-treated than in control cells) (Fig. 3D).

DCA increased mitochondrial ROS production as evaluated by reaction with MitoSOX and subsequent FACS (Fig. 3E–G). DCA caused a significant increase in mitochondrial ROS production starting at 30 min incubation with DCA, which remained high for the following 3.5 h (Fig. 3F). After 2 h incubation with DCA, MitoSOX fluorescence increased to 2.1-fold over control values, and EC (5 μM) showed no preventive effects (Fig. 3G).

3.4. The ERK1/2 pathway is involved in DCA-induced Caco-2 cell monolayer permeabilization: inhibition by EC and other NADPH oxidase inhibitors

The redox-sensitive signals NF-κB and ERK1/2 are the main pathways controlling intestinal epithelium permeability. We next investigated the effects of DCA on the activation of NF-κB and ERK1/2 by measuring the phosphorylation of p65 (Ser536) and ERK1/2 (Thr202/Tyr204) by Western blot. p65 phosphorylation levels were not affected by treatment with 100 μM DCA along a 4 h incubation period (Fig. 4A). On the other hand, ERK1/2 phosphorylation significantly increased (34–84%) within 1–4 h incubation with DCA, when compared to basal levels. Upstream ERK1/2, DCA increased (100% over control values) the activating phosphorylation (Tyr1068) of the EGFR, which was prevented by EC (Fig. 4B). After 2 h incubation, EC (5 μM), apocynin (1 μM) and VAS-2870 (1 μM), fully prevented DCA-mediated increase of ERK1/2 phosphorylation (Fig. 4B and C).

To understand the role of ERK1/2 on DCA-induced Caco-2 monolayer permeabilization we inhibited the pathway with the MEK inhibitor U0126. At 10 μM concentration, U0126

not only fully inhibited DCA-mediated increase in ERK1/2 phosphorylation, but values were 64% lower than basal levels (Fig. 4C). Stressing a central role of ERK1/2 in the permeabilization induced by DCA, U0126 also prevented the increase in FITC-dextran clearance (Fig. 4D). The NADPH oxidase inhibitors Apocynin and VAS-2870 were also effective inhibiting DCA-mediated increase in ERK1/2 phosphorylation and permeabilization of the monolayer (Fig. 4C and D).

3.5. Effects of DCA on MLC phosphorylation: role of EC

Phosphorylation of MLC is a central mechanism involved in TJ opening and barrier permeabilization. After 2 h incubation, DCA caused a 30% increase in the levels of MLC phosphorylation at Ser19. EC (5 μM), NADPH oxidase inhibitors (1 μM), and U0126 (10 μM) fully inhibited this increase (Fig. 5A). MLC is phosphorylated in Thr19/Ser19 by MLCK and dephosphorylated by MLC phosphatase. To evaluate the potential upregulation of MLCK by DCA, the mRNA levels of the kinase were measured by RT-PCR. DCA cause a time-dependent decrease in MLCK mRNA levels (Fig. 5B). After 2 h incubation, MLCK mRNA levels were similar among the DCA-treated cells in both the absence and the presence of the inhibitors (Fig. 5C). On the other hand, DCA caused a significant increase (27%) in the phosphorylation levels of the MLC phosphatase regulatory subunit MYPT1 at Thr696 (Fig. 5D). This increase was prevented by EC (5 μM), NADPH oxidase inhibitors (1 μM), and U0126 (10 μM).

3.6. DCA promotes the downregulation of tight junction proteins

The TJ is composed by several proteins including ZO-1, occludin and claudins. To assess if DCA could act permeabilizing the Caco-2 monolayer by affecting the expression of TJ proteins, the protein content of ZO-1, occludin and claudin 1 and 2 were measured by Western blot. DCA promoted a time-dependent decrease in ZO-1 and occludin protein levels, while not affecting those of claudins 1 and 2 (Fig. 6A). After 2 h incubation with DCA (100 μM), ZO-1 and occludin levels in total cell homogenates were 24 and 25% lower than in control cells, and this was prevented by co-incubation with EC (5 μM), apocynin (1 μM) and VAS-2870 (1 μM) (Fig. 6B and C). U0126 (10 μM) fully prevented the DCA-induced decreased expression of ZO-1, with a trend ($p = 0.06$) inhibiting DCA-mediated occludin decrease.

Metalloproteinases can degrade TJ proteins. Thus, MMP-2 activity was next measured in the cell culture medium by zymography. DCA caused a 2.1-fold increase in MMP-2 activity over control values (Fig. 6D). EC (5 μM), NADPH oxidase inhibitors (1 μM), and U0126 (10 μM) fully inhibited DCA-mediated MMP-2 activation.

4. Discussion

The capacity of EC to mitigate high fat diet-induced intestinal permeabilization in mice is not due to changes in bile acid metabolism, but it can be in part attributed to the prevention of bile (DCA)-induced barrier damage. DCA-induced Caco-2 cell monolayer permeabilization occurred secondary to NADPH oxidase and ERK1/2 activation. Permeabilization was paralleled by increased phosphorylation of MLC due to the inhibition of the MLC phosphatase. DCA also decreased the expression of the TJ proteins ZO-1 and

occludin, which can be related to their degradation by MMP-2. Stressing a central role for NADPH oxidase in DCA-induced permeabilization, EC and other NADPH oxidase inhibitors prevented DCA-mediated ERK1/2 activation and the downstream events. Thus, the observed capacity of EC to inhibit intestinal permeabilization in high fat-fed mice can be in part due to the inhibition of bile acid-induced alterations in TJ structure/dynamics.

Excess levels of select bile acids could explain the increased intestinal permeability associated to the consumption of high-fat diets [11, 20, 24]. The microbiota can contribute to this adverse effect given that it undergoes major changes upon chronic high fat consumption, and its central role in bile acid metabolism [25]. Thus, the microbiota deconjugate and dehydroxylate bile acids produced in the liver and secreted into the intestinal lumen, to generate unconjugated and secondary bile acids. While high-fat diet consumption caused major changes in mouse fecal bile acid profiles, EC supplementation did not affect neither total bile acid levels nor the individual types of bile acids. In agreement with a lack of effect of EC on unconjugated and secondary bile acids content, EC supplementation did not modify the altered microbiota profiles associated to the consumption of a high-fat diet [20]. On the other hand, the protective actions of EC on high-fat diet-induced GI loss of barrier function in mice [20], could be due to EC capacity to mitigate bile-induced intestinal monolayer permeabilization.

GI tract permeabilization can cause an increased paracellular transport of bacterial endotoxins, which trigger local and systemic inflammation, contributing to the development of pathologies, including insulin resistance, type 2 diabetes, and cardiovascular diseases [6, 26, 27]. The consumption of a high-fat diet per se, rather than

the associated obesity, have been proposed to trigger intestinal permeabilization in rats [11]. In fact, the bile juice isolated from rats fed a high-fat diet, as well as individual bile acids, promote permeabilization of Caco-2 cell monolayers [11, 19]. We observed that bile acids found at largest amounts in the cecum from high fat-fed mice are muricholic acids, cholic, and DCA. Among them, and also present in humans, DCA causes permeabilization of intestinal monolayers [12] and is a major player in pathologies associated to consumption of high-fat diets and obesity [28].

In agreement with previous evidence [12], DCA caused the permeabilization of Caco-2 cells differentiated into a monolayer of intestinal epithelial cells, and EC prevented it. It is important to consider that the use of Caco-2 cell monolayers as a model of intestinal epithelium has limitations [29]. Among others, the Caco-2 cell population is heterogeneous, the monolayer does not involve interactions of epithelial cells with other cell types, and the model lacks a mucus layer. However, the validity of the model for our study is supported by current and previous observations on the capacity of EC to protect Caco-2 monolayers from permeabilization [19, 20], which are in accordance with the GI barrier protective effects exerted by dietary EC in high fat-fed mice [20].

EC and the NADPH oxidase inhibitors apocynin and VAS-2870 inhibited DCA-mediated increase in paracellular transport. We previously observed that the NADPH oxidase inhibitors apocynin and diphenyleneiodonium inhibited DCA-stimulated oxidant production in Caco-2 cells [12]. Accordingly, we now observed that DCA increased NOX4 expression and cell oxidant levels, which were both prevented by EC. Consistently, EC prevented high-fat diet-induced intestinal permeabilization and NADPH oxidase

upregulation in mice [20]. EC, which has a structural similarity to apocynin, also directly inhibits NADPH oxidase activity in vascular endothelial cells [30], and in Caco-2 cells [19, 20]. On the other hand, while DCA increased mitochondrial ROS production, EC had no preventive effects. Thus, the above findings stress a relevant role for NADPH oxidase in DCA-mediated epithelial monolayer permeabilization, and this enzyme as a target of EC in the protection of GI barrier function.

Both NF-κB and ERK1/2 are major players in the regulation of TJ structure and dynamics. Under the current experimental conditions, 100 μM DCA did not affect NF-κB but promoted the activation of ERK1/2 in Caco-2 cells. ERK1/2 activation by DCA was previously described in hepatocytes [31, 32] and intestinal cells [15, 33]. This activation can be triggered by the interactions of DCA with the cell membrane, promotion of membrane physical alterations, and the associated activation of membrane proteins, i.e. the EGFR and cell membrane NADPH oxidases [12, 13, 15, 19, 32, 34]. In fact, we observed that DCA activated the EGFR and downstream the ERK1/2 pathway. Our findings that EC, VAS-2870, apocynin and the MEK inhibitor U0126 inhibit both ERK1/2 activation and Caco-2 monolayer permeabilization establishes a causal link between NADPH oxidase and ERK1/2 activation in DCA-induced increase in monolayer paracellular transport.

Phosphorylation of MLC is a required step in the physiological opening of the TJ and is regulated by its phosphorylation by MLCK and dephosphorylation by MLC phosphatase. DCA increased MLC phosphorylation that was inhibited by EC and by NADPH oxidase and ERK1/2 inhibitors. ERK1/2 modulates MLCK expression by phosphorylating the ETS domain-containing protein ELK-1. Phosphorylated ELK-1 subsequently binds to the

promoter of the MLCK gene increasing its transcription [16]. However, we did not observe an upregulation of MLCK by DCA indicating that this is not the mechanism causing increased MLC phosphorylation. On the other hand, the activity of MLC phosphatase is regulated by phosphorylation of the MYPT1 regulatory subunit at threonine 696 and 853. This is done by the kinases ERK1/2 and ROCK, leading to the inhibition of MLCP activity [35-37]. DCA increased MYTP phosphorylation which was inhibited by EC and the NADPH oxidase and ERK1/2 inhibitors. This suggests that the inhibition of MLCP is one mechanism involved in DCA/ERK1/2-mediated increased levels of phosphorylated MLC in Caco-2 cells, and consequent monolayer permeabilization.

Other adverse effect of bile acids is the downregulation of TJ proteins. In rodents chronically fed a high-fat diet there is a decreased expression of TJ proteins including ZO-1, occludin, claudin-1, claudin-3, and junctional adhesion molecule-1 [11, 20]. Accordingly, bile juice isolated from rats fed a high-fat diet promoted Caco-2 cell monolayer permeabilization and the downregulation of claudin-1, claudin-3, and junctional adhesion molecule-1 [11]. DCA-treated Apcmin/+ mice also show a decreased expression of ZO-1 [38]. We observed that DCA decreased the protein content of ZO-1 and occludin, which was prevented by EC, and both NADPH oxidase and ERK1/2 inhibitors. On the other hand, the EC isomer (+)-catechin did not prevent inflammation-induced decrease in ZO-1 and occludin expression and consequent monolayer permeabilization [39]. This stresses the critical importance of the stereospecificity of flavonoids on their biological actions. Metalloproteinases have been found to be involved in the degradation of TJ proteins [40]. The activation of MMP-9 and MMP-2 was found to be responsible for the low levels of ZO-

1, occludin and JAM-A in cholesterol oxidation products-treated Caco-2 cells [41]. Consistently, EC and NADPH oxidase and MEK/ERK inhibitors prevented DCA-mediated MMP-2 activation. Accordingly, ERK1/2 regulates the expression of MMP-2. MEK1 constitutive activation causes increased MMP-2 and MMP-9 activity and decreases ZO-1 and occludin levels in intestinal epithelial cells [42]. Thus, the protection of TJ structure via downregulation of MMP-2 is another mechanism involved in EC-mediated protection against the adverse effects of DCA.

We conclude that bile acids play a central role in high-fat diet-induced intestinal permeabilization. The capacity of EC supplementation to prevent high-fat diet-induced permeabilization and endotoxemia in mice can be in part explained by its preventive effects on bile acid-induced loss of TJ structure and function. Evidence suggest that EC acts in part preventing NADPH oxidase upregulation, increased oxidant production, and the activation of the redox-sensitive ERK1/2 signaling pathway. Through these mechanisms, diets rich in EC can contribute to protect the gastrointestinal tract from the adverse effects of high fat consumption.

Acknowledgements

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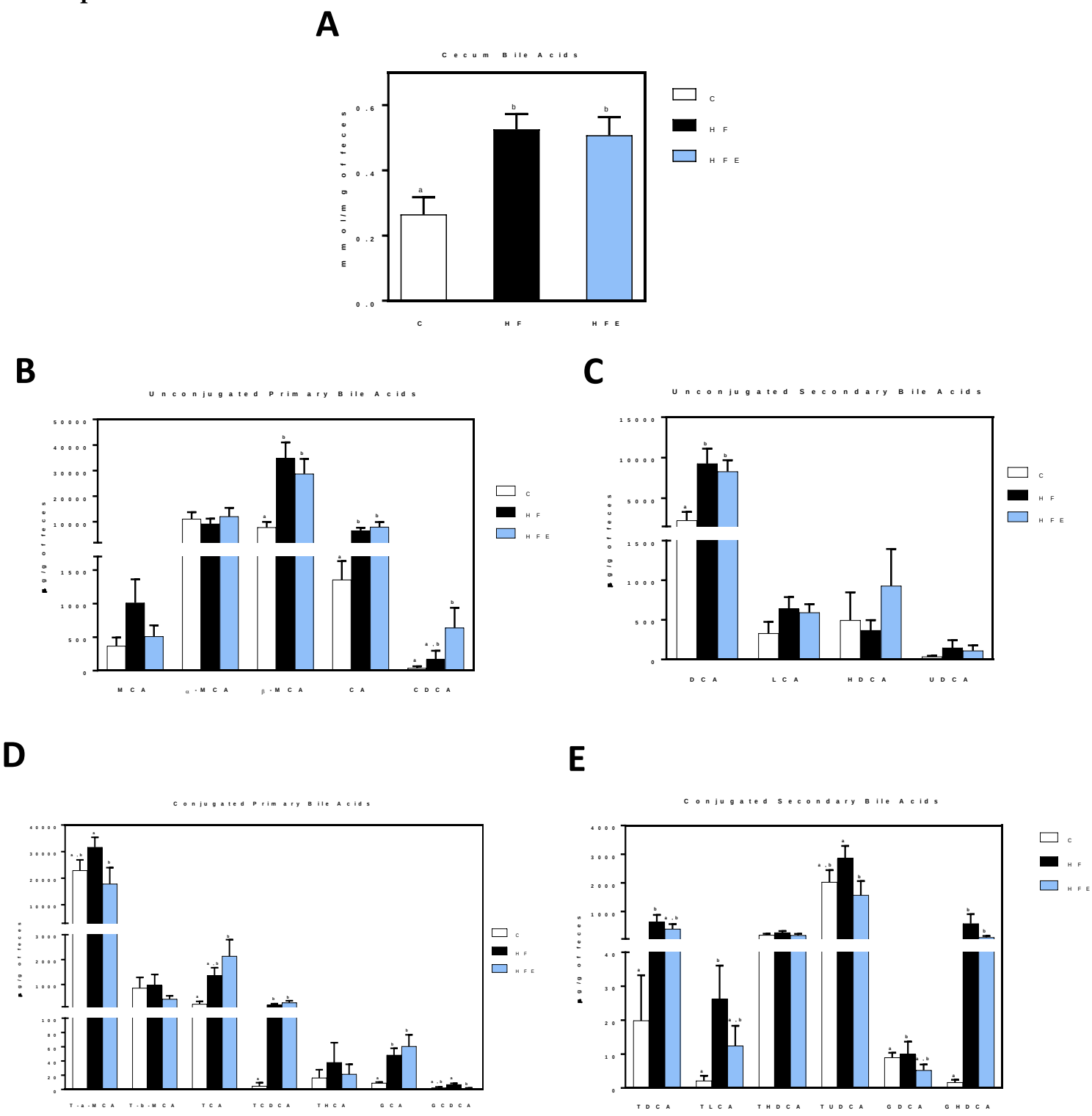
Tables and Figures

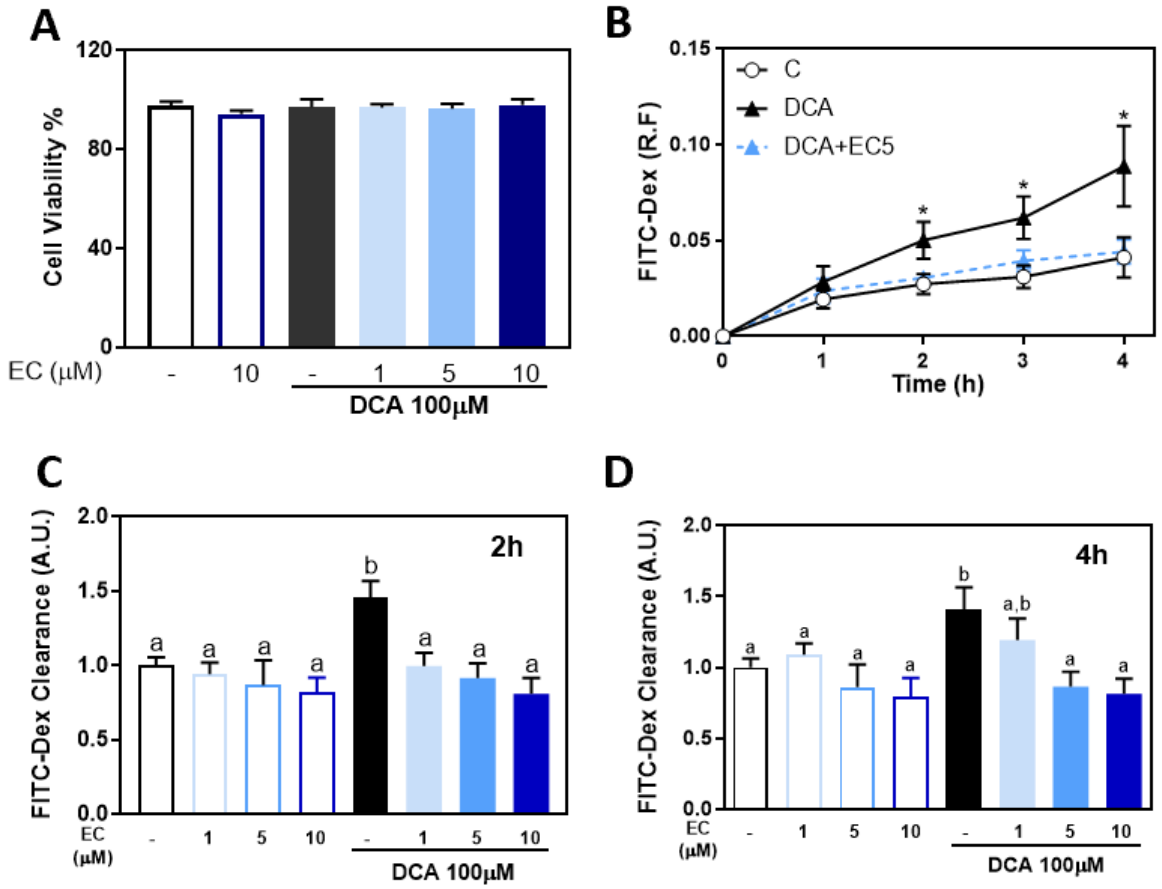
Figure 1. Effects of a high-fat diet and of EC supplementation on fecal/cecal bile acid profiles in mice.



Mice were fed a control diet (empty bars), a high-fat diet (HF) (black bars), or the high-fat diet supplemented with 20 mg EC/kg body weight (HFE) (blue bars). **A**- Total fecal bile acids and **B-E**- individual cecum bile acids were measured as described in methods. Results are shown as means ± SEM and are the average of 5–7 animals/group. Values having different superscripts are significantly different ($p < 0.05$, One-way ANOVA)

MCA: muricholic acid, CA: cholic acid, CDCA: chenodeoxycholic acid, DCA, deoxycholic acid; LCA: lithocholic acid, HDCA: hyodeoxycholic acid, UDCA: ursodeoxycholic acid, T-α-MCA: tauro-α-muricholic acid, T-β-MCA: tauro-β-muricholic acid, TCA: taurocholic acid, TCDCA: taurochenodeoxycholic acid, THCA: taurohyocholic acid, GCA: glycholic acid, GCDCA: glycochenodeoxycholic acid, TDCA: taurodeoxycholic acid, TLC: taurolithocholic acid, THDCA: taurohyodeoxycholic acid, TUDCA: tauroursodeoxycholic acid, GDCA: glycohyodeoxycholic acid and GHDCA: glycohyodeoxycholic acid. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

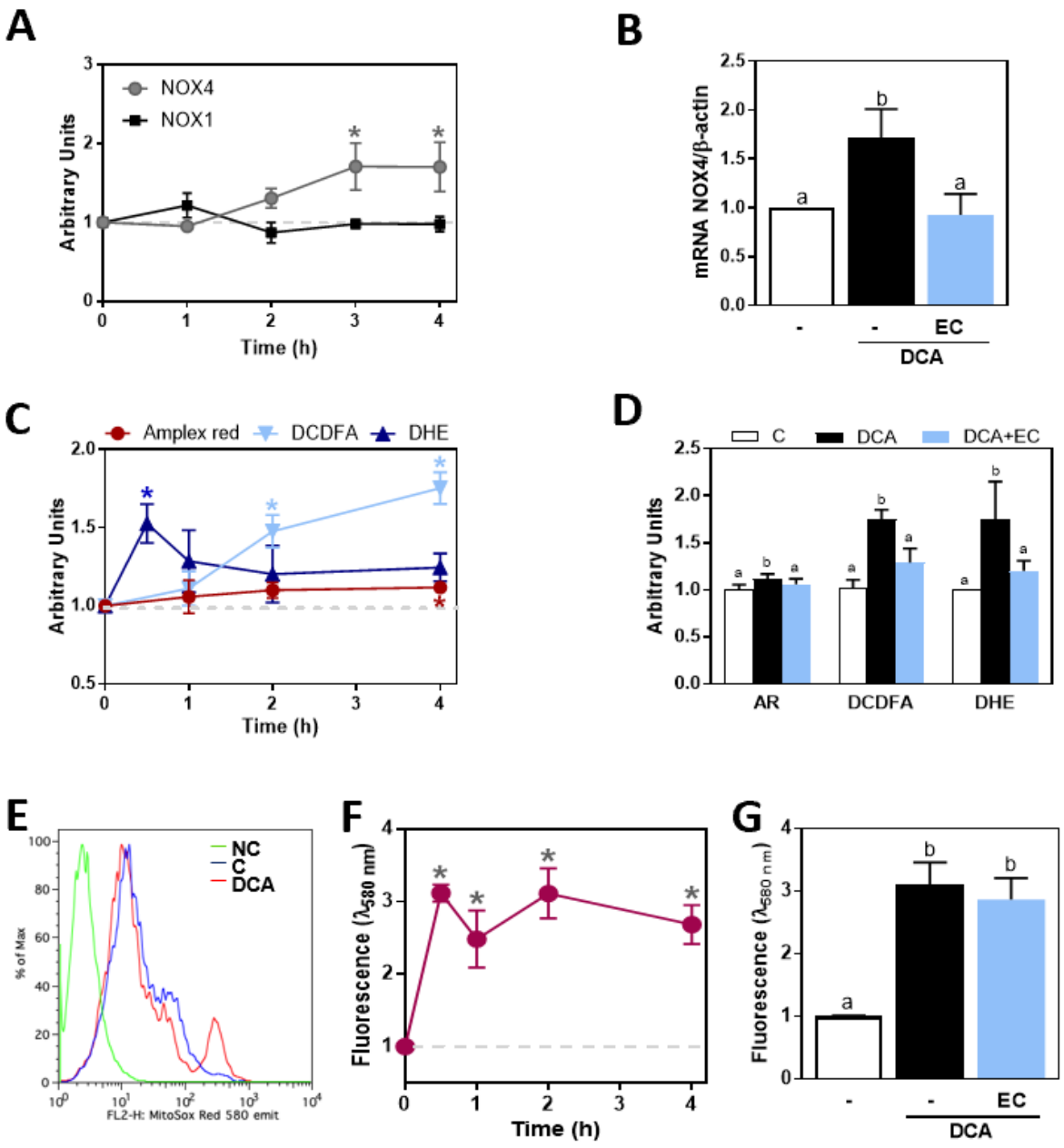
Figure 2. DCA causes an increase in Caco-2 cell monolayer paracellular permeability that is prevented by EC.



Caco-2 cell monolayers were treated with 100 μM DCA in the absence or the presence of 0–10 μM EC added to the upper chamber and cells incubated for 0–4 h. Caco-2 cell monolayer permeability was evaluated by measuring FITC-dextran paracellular transport. **A**- Cell viability of Caco-2 cells treated with DCA and in the absence or the presence of 1–10 μM EC for 6 h. **B**- Kinetics of FITC-dextran paracellular transport in Caco-2 monolayers in the absence of additions (empty circles), and in the presence of 100 μM DCA without (black triangles) or with (blue triangles) simultaneous addition of 5 μM EC. *Significantly different

from controls at the corresponding time point. **C,D**- Dose-dependent inhibition by EC of DCA-induced FITC-dextran paracellular transport after 2 h (**C**) or 4 h (**D**) incubation. Results are shown as mean ± SEM of 5 independent experiments. **A,C,D**- Data were normalized to control values. Values having different superscripts are significantly different ($p < 0.05$, One-way ANOVA). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

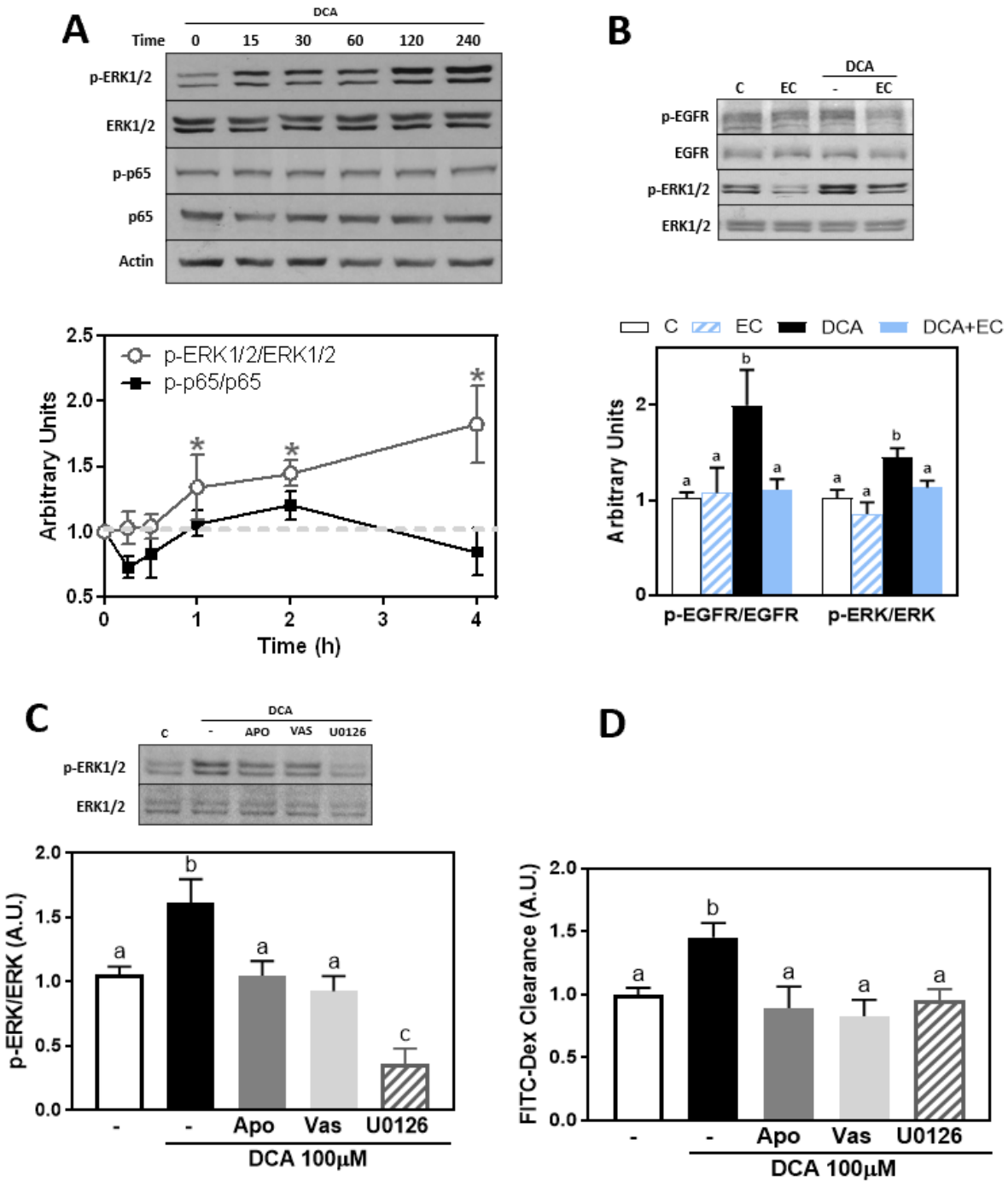
Figure 3. DCA causes an increase in Caco-2 cell oxidant: effects of EC.



Caco-2 cell monolayers were treated with 100 μM DCA in the absence or the presence of 5 μM EC for 0–4 h. **A,B**- mRNA levels of NOX1 and NOX4 were measured by RT-PCR and referred to β-actin mRNA content. mRNA levels were measured **A**- between 0-4 h for cells incubated without or with DCA, or **B**- for 2 h without or with addition of DCA and in the

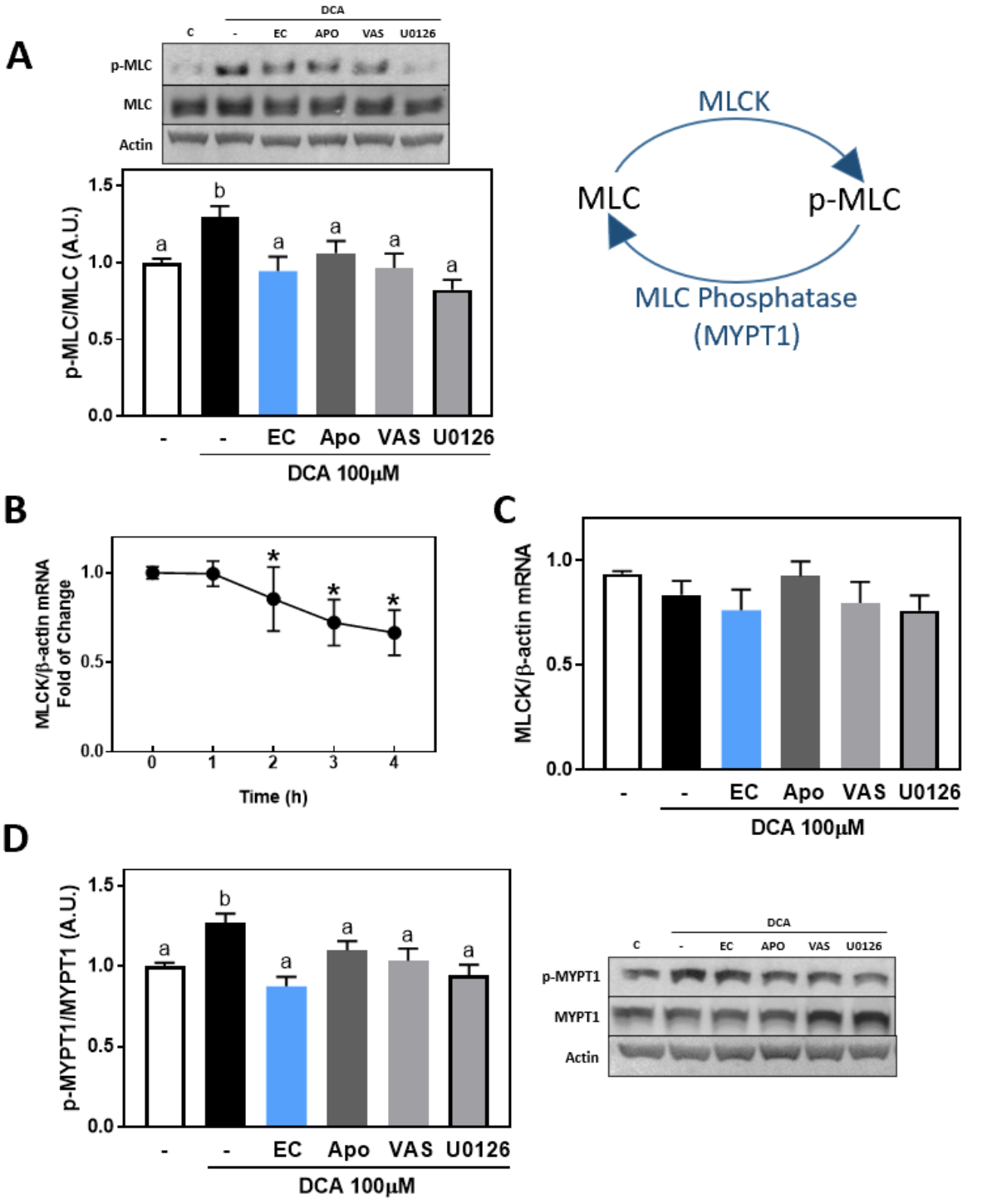
absence or the presence of 5 μM EC. **C**- Kinetics (0–4 h) of oxidant production evaluated using the probes Amplex® Red (red circles), DCFDA (grey triangles), and DHE (blue triangles) as described in methods. **D**- Effects of DCA and 5 μM EC on oxidant production as measured with Amplex® Red (AR) after 1 h incubation, or with DCFDA and DHE after 4 h incubation. **E-G**- Mitochondrial oxidant production was evaluated with MitoSOX. **E**- typical FACS scan profiles for cells incubated in the absence (**C**) or the presence of 100 μM DCA (DCA). NC: negative control of cells not added with MitoSOX. **F**- Kinetics of MitoSOX fluorescence of cells incubated in the presence of DCA. **G**- MitoSOX fluorescence in cells incubated without or with DCA and in the absence or presence of 5 μM EC for 2 h. Results are shown as mean ± SEM of 4–5 independent experiments. Data were normalized to control values. Kinetic graphs: control values are shown as a dashed grey line. *Significantly different from controls at the corresponding time point. Bars: values having different superscripts are significantly different ($p < 0.05$, One-way ANOVA). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Figure 4. ERK1/2 activation is involved in DCA-induced Caco-2 cell monolayer permeabilization: inhibitory actions of EC and other NADPH oxidase inhibitors.



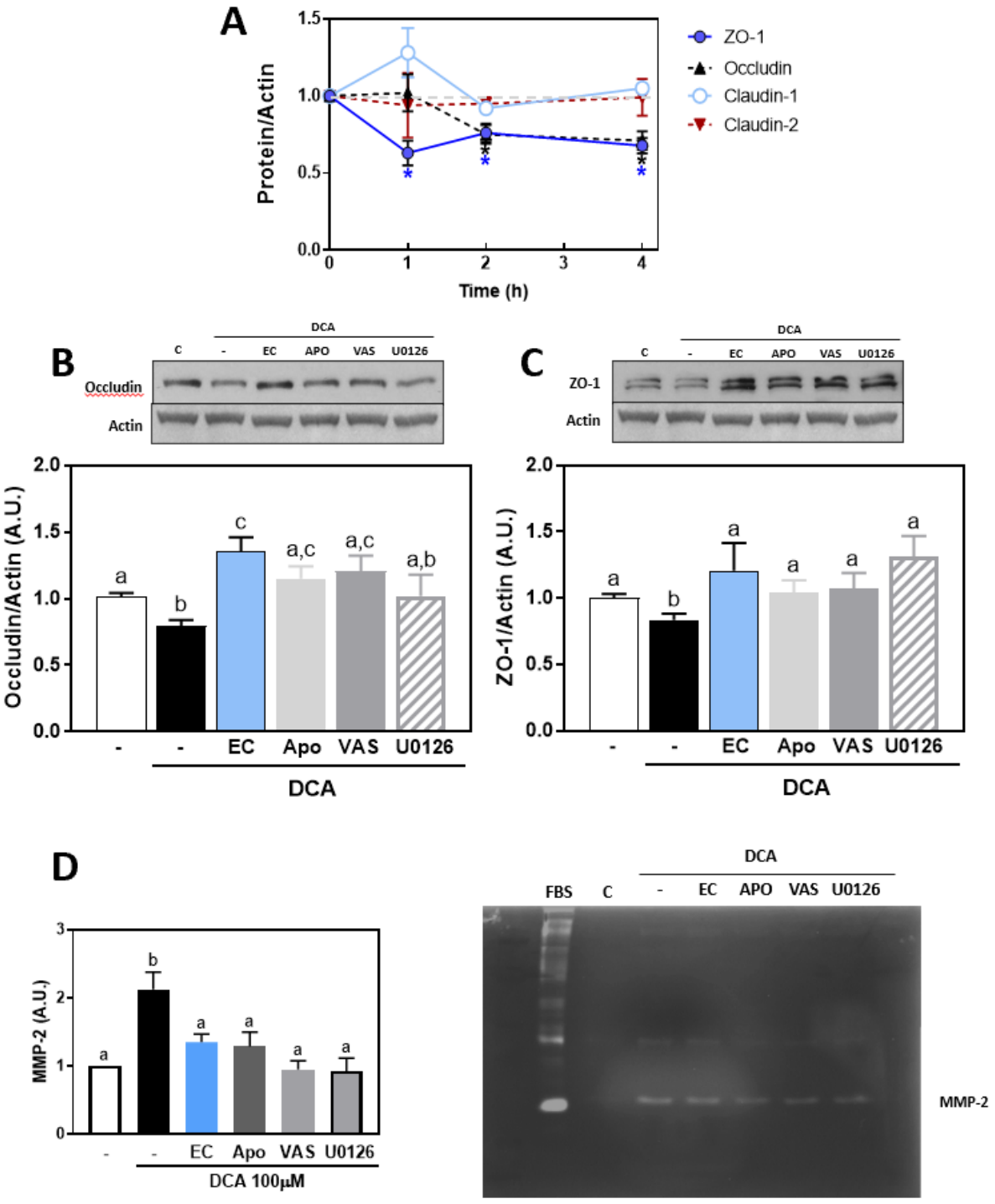
Caco-2 cell monolayers were incubated for 0–4 h with or without 100 µM DCA in the absence or the presence of 5 µM EC, 1 µM apocynin, 1 µM VAS-2870 or 10 µM U0126. **A**- The kinetics of ERK1/2 and NF-κB activation in the presence of 100 µM DCA was evaluated by Western blot by measuring the phosphorylation of ERK1/2 (T202/Y204) and p65 (Ser536), respectively. B- Cells were incubated with or without 100 µM DCA and in the absence or the presence of 1 µM EC for either 0.5 h to assess EGFR phosphorylation (Tyr1068) or 2 h for ERK1/2 phosphorylation. **C,D**- Cells were incubated with or without 100 µM DCA and in the absence or the presence of apocynin, VAS-2870 or U0126 for 2 h. **C,D**- Effects of the NADPH oxidase and ERK1/2 inhibitors on (**C**) DCA-induced ERK1/2 phosphorylation measured by Western blot, and (**D**) Caco-2 cell monolayer permeabilization evaluated as the FITC-dextran paracellular transport. Western blot bands were quantified and values for phosphorylated proteins were referred to the respective total protein content. Results are shown as mean ± SEM of 4–6 independent experiments. **A**- *Significantly different from controls at the corresponding time point. **B-D**- Values having different superscripts are significantly different ($p < 0.05$, One-way ANOVA).

Figure 5. DCA promotes an increase in MLC phosphorylation independently from MLCK but dependent on MLC phosphatase inhibition: effects of EC, and NADPH oxidase and ERK1/2 inhibitors.



Caco-2 cell monolayers were incubated for 2 h with or without 100 μM DCA and in the absence or the presence of 5 μM EC, 1 μM apocynin, 1 μM VAS-2870 or 10 μM U0126. (**A**) MLC (Ser19) and (**D**) MYPT1 (Thr696) phosphorylation levels were measured by Western blot. After quantification values for phosphorylated proteins were referred to the respective total protein content. β-actin was measured as loading control. **B,C**- MLCK mRNA levels were measured by RT-PCR and values referred to the β-actin mRNA content. **B**- Kinetics of DCA-mediated effects on MLCK mRNA levels, **C**- Effects of the inhibitors on the MLCK mRNA content after 2 h incubation with DCA. Results are shown as mean ± SEM of 4–6 independent experiments. Values having different superscripts are significantly different ($p < 0.05$, One-way ANOVA).

Figure 6. DCA decreases the expression of tight junction proteins: effects of EC, NADPH oxidase and ERK1/2 inhibitors.



Caco-2 cell monolayers were incubated for 0–4 h with or without 100 μM DCA and in the absence or the presence of 5 μM EC, 1 μM apocynin, 1 μM VAS-2870 or 10 μM U0126. The expression of TJ proteins (ZO-1, occludin, claudin-1 and claudin-2) was measured by Western blot. Bands were quantified and values referred to β-actin levels. **A**-Kinetics of DCA-mediated effects on ZO-1 (full circles), occludin (black triangles), claudin-1 (empty circles) and claudin-2 (red triangles) protein profiles. **B-D**- Effects of EC, apocynin, VAS-2870 and U0126 on occludin (**B**) and ZO-1 (**C**) protein levels and (**D**) MMP-2 activity measured by zymography after 2 h incubation with 100 μM DCA. Results are shown as mean ± SE of 4–6 independent experiments. Values having different superscripts are significantly different ($p < 0.05$, One-way ANOVA test). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

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Chapter 3

Effects of catechins and procyanidins on fat-induced transcellular transport of endotoxins via chylomicrons

In Preparation

Effects of catechins and procyanidins on fat-induced transcellular transport of endotoxins via chylomicrons

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Abbreviations: ApoB48, Apolipoprotein B48; B2, procyanidin B2 dimer; BA, butyric acid; BW, body weight; CD36, cluster determinant 36; CT, (+)-catechin; EC, (-)-epicatechin; ER, endoplasmic reticulum; Hex, hexamer; HFD, high fat diets; I.P., intraperitoneal; JNK, c-jun N-terminal kinase; LCFA, long-chain fatty acid; LPL, lipoprotein lipase; LPS, lipopolysaccharides; BA, butyric acid; MTP, triglyceride transfer protein; OA, oleic acid; OO, olive oil; PCAs, procyanidins; TEER, transepithelial electrical resistance; TG, triglyceride

Key words: High fat, endotoxemia, (-)-epicatechin, (+)-catechin, procyanidins, chylomicrons

Abstract

Metabolic endotoxemia is linked to the development and progression of obesity and its comorbidities. Consumption of high-fat diets (HFD) is associated with metabolic endotoxemia in part by facilitating the chylomicron-dependent transcellular transport of luminal lipopolysaccharides (LPS). This work investigated whether catechins and procyanidins (PCAs) can mitigate fat-induced transcellular transport of endotoxins via chylomicrons. Mechanisms underlying the protective effects of (-)-epicatechin (EC) on chylomicron-dependent LPS translocation were also studied. *In vitro,* using differentiated Caco-2 cells as a model of the intestinal epithelial barrier, catechins (EC and (+)-catechin (CT)) and PCAs (B2 dimer (B2) and hexamer (Hex)) decreased oleic acid (OA)-stimulated basolateral secretion of chylomicrons in Caco-2 monolayer. EC, CT, B2, and Hex also prevented OA-mediated and chylomicron-dependent transcellular transport of cell-associated LPS. Mechanistically, EC acted modulating JNK activation, which is a central regulator for fatty acid uptake and chylomicron basolateral release. EC prevented the transient overexpression of microsomal triglyceride transfer protein (MTP) but did not affect the OA-mediated recruitment of the fatty acid transport protein CD36 to the plasma membrane. *In vivo,* EC mitigated the postprandial increase in plasma triglycerides (TG) and LPS caused by an oral fat load. Together, high dietary fat can contribute to metabolic endotoxemia by promoting the transcellular translocation of endotoxins from the lumen to the circulation via a chylomicron-dependent way. The capacity of catechins and PCAs to mitigate HFD-associated pathologies can be in part related to its capacity to mitigate fat-induced transcellular transport of endotoxins via chylomicrons.

1. Introduction

Obesity currently constitutes a major global public health threat [1]. Consumption of a Western-pattern diet, characterized by high fat and sugar and low fiber content, is one of the major environmental factors associated with the development of obesity [2]. Obesity is associated with the onset and progression of, among others, hyperlipidemia, insulin resistance, type 2 diabetes, non-alcoholic fatty liver disease, heart diseases, cognitive impairment, and certain types of cancer [3-7]. These detrimental effects are in part attributed to high fat diet (HFD)- and obesity-associated metabolic endotoxemia [8, 9]. Endotoxemia is defined as a subclinical 0.5- to 2-fold elevation of lipopolysaccharides (LPS) levels in circulation, which contributes to the obesity-associated condition of systemic low-grade chronic inflammation [10, 11]. In this regard, LPS triggers inflammatory and immune responses throughout the body by recruiting primary inflammatory cells such as macrophages, lymphocytes, and plasma cells to the tissue site, which produce inflammatory cytokines, growth factors, and enzymes that can lead to tissue damage and/or dysfunction [12]. In human, high serum LPS level was found to be directly associated with the total energy intake and obesity prevalence [13]. Similar results were observed in mice subcutaneously administrated with LPS for 4 weeks, showing that LPS can initiate liver insulin resistance and obesity-related disease features [9].

The development of intestinal permeabilization induced by altered bile acid profiles and increased gut lumen concentration serves as one mechanism underlying the HFD-associated metabolic endotoxemia through LPS paracellular transport [14, 15]. High dietary fat also increases the production of chylomicrons, a lipoprotein that is assembled in

enterocytes and secreted to lymphatic system to facilitate postprandial lipid transport. Lipid A is a highly conserved and key structural component of LPS, helping anchor LPS to the outer membrane of Gram-negative bacteria [16, 17]. Thus, chylomicrons have a high affinity for LPS due to the hydrophobicity of its lipid A domain, which favors LPS transcellular transport through its incorporation into nascent chylomicrons [18]. As a consequence, an increased chylomicron production induced by high consumption of fat can promote the transcellular transport of LPS and lead to postprandial endotoxemia [19-22].

The biosynthesis and secretion of chylomicrons involve multiple steps starting with the luminal fatty acid absorption. While monoacylglycerol and short-chain fatty acid can go across the intestinal epithelium by passive diffusion, the uptake of luminal long-chain fatty acid (LCFA) requires apical plasma membrane transporters [23]. Several membrane proteins have been implicated as candidates to facilitate luminal fatty acid uptake, including the fatty acid translocase/cluster determinant 36 (FAT/CD36) [24, 25], the LCFA transport protein 4 (FATP4) [26], and the fatty acid-binding protein plasma membrane (FABPpm) [27]. Apolipoprotein B48 (ApoB48) and microsomal triglyceride transfer protein (MTP) and play key roles in lipidation and phospholipidation with triglycerides (TG) and cholesterol, assembling a prechylomicron in the endoplasmic reticulum (ER) [25, 28]. Through a budding and fusion process, prechylomicrons are transported to the Golgi, where mature chylomicrons are formed by the addition of apolipoprotein AI (ApoAI) and subsequently released at the basolateral surface of the enterocyte to the lymphatic circulation [29]. The c-jun N-terminal kinase (JNK) is proposed to modulate basal

chylomicron release by regulating fatty acid uptake [30]. Thus, the inhibition of JNK1 phosphorylation decreases the synthesis of fatty acid uptake complex proteins, ApoB48, and proteins involved in chylomicron excretion, consequently diminishing the basolateral secretion of chylomicrons [31].

EC, its isomer (+)-catechin (CT), and their oligomers (B-type linkage), also known as procyanidins (PCAs), are highly abundant flavonoids in many foods [32, 33]. The flavan-3-ol (-)-epicatechin (EC) can exert beneficial effects against obesity comorbidities [34]. In a mouse model of high fat diet-induced obesity, EC mitigated dyslipidemia and metabolic endotoxemia in part by modulating redox-sensitive signals including JNK and preserving the intestinal tight junction integrity [34-37]. The capacity of PCAs to mitigate hyperlipemia was proposed to be mediated by a decrease in in chylomicron secretion but its occurrence and underlying mechanisms have not been proven yet [38]. This work investigated the potential capacity of EC, CT, and low molecular weight (B2 dimer (B2)), and high molecular weight (hexamer (Hex)) PCAs to prevent high fat-induced endotoxemia. Using the Caco-2 cell monolayer model, we found that EC, CT, B2, and Hex mitigated the intestinal translocation of LPS by decreasing the chylomicron-dependent transport of LPS. The protective action of EC is in part by its capacity to modulate JNK activation. Additionally, EC prevented postprandial endotoxemia in mice challenged with high dietary fat.

2. Materials and Methods

2.1. Materials

Caco-2 cells were from the American Type Culture Collection (ATCC, Rockville, MA). Cell culture media and reagents were from Invitrogen/Life Technologies (Grand Island, NY). Cell culture transwell permeable supports were from MilliporeSigma (Burlington, MA). Primary antibodies for ApoB48 (sc-13538), CD36 (sc-7309), MTP (sc-515742), and flotillin-1 (H-104) (sc-25506) were from Santa Cruz Biotechnology (Santa Cruz, CA). Primary antibodies for phospho-SAPK/JNK (Thr183/Tyr185) (#9251), JNK2 (56G8) (#9258), β-actin (#12620), and lipopolysaccharide (LPS, #14011) were from Cell Signaling Technology (Danvers, MA). (-)-epicatechin (E1753), (+)-catechin (C1788), procyanidin B2 (42157), oleic acid (#O1383), butyric acid (B103500), sodium taurocholic hydrate (86339), and tyloxapol (T0307) were from Sigma Chem. Co. (St. Louis, MO). Hex was prepared and supplied by Mars Incorporated (Hackettstown, NJ). Based on fluorescence detection, it was determined that Hex was composed by 76% hexamers, 4.5% monomers, 2.2% dimers, 1.0% trimers, <1.0% tetramers, 11.5% pentamers, and 4.1% procyanidins higher than hexamers.

2.2. Cell culture and incubations

Caco-2 cells (at passages 7 through 25) were cultured at 37°C and 5% (v/v) CO_2 atmosphere in minimum essential medium (MEM) supplemented with 10% (v/v) fetal bovine serum and antibiotics (50 U/ml penicillin, and 50 μg/ml streptomycin). For the experiments, cells seeded in transwells or in dishes were differentiated for 9-12 days after confluence. The cell culture medium was replaced every 2-3 days. Cells cultured on transwells were incubated overnight (16-18 h) with or without 0.1 mg/mL LPS added to the upper chamber. Monolayers were washed twice to remove cell-unassociated LPS. Oleic acid (OA) or butyric acid (BA) were dissolved in MEM-based enriched medium (Opti-MEM)

containing 0.5 mM sodium taurocholate and incubated at 37 °C for 1 h with periodic vortexing. Cells were then added at the apical side with or without either 1.6 mM OA to induce chylomicron formation or BA as a negative control, and with or without the addition of EC, CT, B2 or Hex (0.1-1 μM) for 3-24 h. After the corresponding incubations, the basolateral medium was collected with endotoxin-free pipette tips and tubes and stored at -80 °C for LPS and ApoB48 determination. Cells cultured in dishes were incubated with or without either 1.6mM OA, with or without the addition of EC (0.1-5 μM) for 10min - 12 h. Cells were collected and processed for the different determinations.

2.3. Animals and animal care

All procedures conducted during this study were in agreement with standards for the care of laboratory animals as outlined in the NIH Guide for the Care and Use of Laboratory Animals. All procedures were administered under the auspices of the Animal Resource Services of the University of California, Davis. Experimental protocols were approved before implementation by the University of California, Davis Animal Use and Care Administrative Advisory Committee. Healthy male C57BL/6J mice (22-29 g) were housed at 21-22 °C and 53.0 ± 1.5% humidity on a 12-hour light-dark cycle with free access to water and standard chow diet LabDiet 5001 (LabDiet, Saint Louis, MO).

2.4. Oral fat tolerance test

Tyloxapol, an inhibitor for lipoprotein lipase (LPL), was dissolved by extensively vortexing in saline solution and sit at room temperature, covered from light for at least 3 h before use. EC solutions were prepared in a saline solution containing 5% Tween-80. Mice

were fasted for 4 h and followed by an initial blood sample collection. Mice then received intraperitoneal (IP) injections of tyloxapol (500mg/kg BW) and divided into six groups (4-6 mice/group): **1-** control (C), mice gavaged with 5% (v/v) Tween-80 saline solution 15min and saline solution 30 min after tyloxapol injection, respectively; **2-** olive oil (OO), mice gavaged with 5% (v/v) Tween-80 saline solution 15 min and olive oil (10 µL/g body weight (BW)) 30min after tyloxapol injection, respectively; and **3-6-** mice gavaged with 5 (EC5), 10 (EC10), 20 (EC20), and 40 (EC40) mg/kg BW EC 15 min and olive oil 30 min after tyloxapol injection, respectively. Blood samples were collected at 1 h and 2 h form the tail vein, and 3 h from the submandibular vein into EDTA tubes. Plasma was isolated by centrifugation at 3,000×g for 15 min at room temperature. All animals remained in a fasted state for the duration of the experiment.

2.5. Determination of parameters in cell culture medium

LPS in the basolateral medium from cell transwell culture was measured using the LAL Kinetic-QCL assay kit from Lonza (Basel, Switzerland) following the manufacturer's protocol. To determine ApoB48 concentration, cell culture medium samples were diluted with 4X Laemmli buffer (Biorad Laboratories, Hercules, CA) containing 10% (v/v) 2-mercaptoethanol (Thermo Fisher Scientific, Waltham, MA) followed by Wester-blot analysis.

2.6. Determination of plasma LPS and TG concentration

Plasma LPS levels were determined using a kit from Abbexa (Abbexa, Cambridge, UK) and following the manufacturer's protocol. TG concentrations were determined using kits

purchased from Wiener Lab Group (Rosario, Argentina) and following the manufacturer's protocol.

2.7. Assessment of monolayer permeability

Monolayer permeability was assessed measuring the transepithelial electrical resistance (TEER) as previously described [39]. Cells were grown on transwell inserts (12 mm, 0.4 μm pore polyester membranes) in 24-well plates (0.3×10^6 cells/transwell), and monolayers were used when TEER values were between 500–800 Ω cm^2. TEER was measured using a Millicell-ERS Resistance System (Millipore, Bedford, MA) that includes a dual electrode volt-ohm meter. Before the measurement, the plate was taken out of the 37°C incubator and equilibrate to room temperature for 20 min for reproducible TEER determination. The initial TEER was determined prior to LPS addition, and the final TEER determination was performed at the end of the corresponding incubations. TEER was calculated as

$$TEER = (R_m - R_i) \times A$$

where R_m is transmembrane resistance; R_i, intrinsic resistance of a cell-free media; and A, the surface area of the membrane in cm^2.

2.8. Cell plasma membrane isolation

Cell plasma membrane fraction was prepared as previously described [15]. Briefly, Caco-2 cells were washed twice with PBS and collected for centrifugation at 800 × g for 8 min at 4 °C. The supernatant was discarded, and the pellet was resuspended and homogenized in Krebs buffer (20 mM HEPES, 119 mM NaCl, 4.7 mM KCl, 1 mM $MgSO_4$, 0.4 mM NaH_2PO_4, 0.15 mM Na_2HPO_4, and 1.25 mM $CaCl_2$) containing 1 mM PMSF and a protease inhibitor

cocktail (Roche, Switzerland), and centrifuged at 800 × g for 8 min at 4 °C. The supernatant was subsequently centrifuged at 100,000 × g for 1 h at 4 °C, and the pellet was collected (membrane fraction) and resuspended in Krebs buffer for subsequent determinations.

2.9. Western blot analysis

Cell total homogenates were prepared as previously described [14]. Protein concentration was measured with Pierce™ BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA) and aliquots containing 25-100 µg protein were separated by reducing 5-18% polyacrylamide gel electrophoresis and electroblotted to PVDF membranes (Biorad Laboratories, Hercules, CA). Colored (Biorad Laboratories, Hercules, CA) and biotinylated (Cell Signaling Technologies, Danvers MA) molecular weight standards were run simultaneously. Membranes were blocked for 1 h in 5% (w/v) non-fat milk, incubated overnight in the presence of the corresponding primary antibodies (1:1,000 dilution) in 5% (w/v) bovine serum albumin in TBS buffer (50 mM Tris, 150 mM NaCl, pH 7.6), containing 0.1% (v/v) Tween-20. After incubation for 90 min at room temperature in the presence of the secondary antibody (HRP-conjugated) (1:10,000 dilution), the conjugates were visualized by chemiluminescence detection using a using a ChemiDoc™ Gel Imaging System (Biorad Laboratories, Hercules, CA).

2.10. Statistical analysis

All values are shown as means ± standard error of the means (SEM). Data were analyzed by One-way analysis of variance (ANOVA) using GraphPad Prism 7.0 (GraphPad Software,

San Diego, CA, USA). Fisher least significance difference test was used to examine differences between group means. A p value < 0.05 was considered statistically significant.

3. Results

3.1. EC, CT, B2, and Hex decreased the basolateral secretion of chylomicrons in Caco-2 monolayer

To test whether OA can promote the formation and secretion of chylomicrons in a Caco-2 cell monolayer transwell model, we incubated for 24 h the monolayer without or with 1.6 mM OA, added to the apical compartment. The basolateral secretion of chylomicron significantly increased between 6-24 h incubation in all groups (Fig. 1A). The ApoB48 levels in OA treated groups are significantly higher between 3-12 h than other groups, while at 24 h ApoB48 levels were similar among treatments (Fig. 1A). The addition of LPS did not affect basolateral chylomicron secretion. We next investigated the effects of 0.1-1 μM EC, CT, B2, and Hex, added to the apical compartment, on ApoB48 secretion. After 12 h incubation, all compounds inhibited, in a dose-dependent manner, chylomicron basolateral secretion (Fig. 1B-1E). All the compounds at 0.1 μM concentration did not have significant effects on the increased chylomicron secretion induced by OA (Fig. 1B-1E). At the concentration of 0.5 μM CT, B2, and Hex, but not EC, significantly diminished OA-increased basolateral secretion of chylomicron (Fig. 1C-1E). At 1 μM concentration, all compounds fully prevented OA-induced ApoB48 increase in the basolateral medium.

3.2. EC, CT, B2, and Hex prevented the chylomicron-dependent LPS transcellular transport

Increased translocation of LPS from the intestinal lumen to the circulation is considered as one potential mechanism underlying HFD-associated postprandial metabolic endotoxemia [20]. We next measured LPS levels in the basolateral media from monolayers incubated without or with OA for up to 24 h. Basolateral LPS concentration was significantly elevated after 12 h incubation with OA, and continued to increase up to 24 h (Fig. 2A). The LPS level of OA treated group at 24 h was significantly higher compared to its level at 3 h as well as higher than those of control and LPS groups at 24 h (Fig. 2A). In the absence of OA, LPS did not affect the level of LPS transported across the monolayer within 24 h compared to controls, but higher levels in LPS groups were detected at 24h compared to those at 3 h (Fig. 2A). We next measured the capacity of 0.1-1 μM EC, CT, B2 or Hex to modulate LPS translocation into the basolateral medium after 12 h incubation with OA. All compounds had complete preventing effects at all tested concentrations on OA-induced LPS transport (Fig. 2B-2E). As shown in Fig. 2F, there were no significant changes between the initial TEER and the final TEER among all the treatment groups, indicating the translocation of cell-associated LPS from the apical to the basolateral side of the Caco-2 monolayer was not determined by paracellular transport but transcellularly associated to chylomicron secretion.

3.3. The JNK pathway is activated by OA in Caco-2 cells: inhibition by EC

It has been proposed that, through the modulation of fatty acid uptake, JNK can regulate basolateral chylomicron release [31]. To assess JNK activation, Caco-2 cells were grown on 60 mm dishes incubated without or with 1.6 mM OA after differentiation and media were collected for ApoB48 analysis at 3, 6, and 12 h. We confirmed that OA-

stimulated chylomicron secretion also occurs in this model (Fig. 3A). OA caused a time-dependent (10 min-12 h) increase in JNK phosphorylation at Thr183/Tyr185 (Fig. 3B). JNK phosphorylation significantly increased (32-49%) within 3-12 h incubation with OA when compared to controls (Fig. 3B). After 12 h incubation, EC (5 μM) partially prevented OA-mediated increase of JNK phosphorylation (Fig. 3C).

3.4. OA regulates proteins involved in fatty acids uptake and chylomicron assembly/secretion in Caco-2 cells: effects of EC

CD36 and MTP are key proteins facilitating fatty acid uptake and chylomicron assembly/secretion, respectively. After 12h incubation, OA did not affect the total CD36 protein levels in the absence or the presence of 5 μM EC (Fig. 4A). Nevertheless, CD36 expression was significantly increased by OA in the plasma membrane fraction after 12 h, but 5 μM EC did not prevent the increase (Fig. 4B). We next followed MTP protein levels after 10 min to 12 h incubation. OA triggered a transient upregulation of MTP expression at 10 min (Fig. 4B), which was prevented by 1 μM EC (Fig. 4C).

3.5. EC ameliorated plasma triglyceride and LPS increases in mice after fat consumption

We next investigated the effects of EC on plasma TG and LPS increases upon gavage with olive oil. In mice injected with tyloxapol to inhibit lipoprotein lipase activity, gavage with olive oil caused an increase in plasma TG levels that continued for 3 h (Fig. 5A). After 3 h, the plasma TG level was 2.5-fold higher in the olive oil-gavaged mice group than in the control mice. Oral supplementation with 10, 20 and 40 mg/kg BW EC decreased plasma TG

concentration by 36%, 35%, and 42%, respectively (Fig. 5B). We next measured plasma LPS concentrations at 3 h. Olive oil consumption caused postprandial metabolic endotoxemia, elevating plasma LPS levels 1.9 times over control values. EC supplementation fully prevented olive oil-induced postprandial endotoxemia at the highest concentration tested (40mg EC/kg BW) (Fig. 5C).

4. Discussion

We previously found that EC supplementation prevents metabolic endotoxemia in mice chronically fed a HFD by preserving intestinal barrier integrity and thus decreasing the paracellular transport of luminal endotoxins [14, 36]. This work supports an additional mechanism underlying the beneficial effects of catechins and PCAs mitigating high fat-induced metabolic endotoxemia, involving the inhibition of the transcellular transport of endotoxins via chylomicrons. OA caused an increase in basolateral chylomicron secretion in Caco-2 monolayers, which was associated with JNK activation and an elevation in LPS transport. The OA-induced upregulation of chylomicron formation and secretion was paralleled with an overexpression of plasma membrane CD36, a key protein involved in fatty acids uptake [25]. OA also stimulated a transient upregulation in MTP protein expression, which is necessary for the lipidation and formation of prechylomicrons in the ER [28]. EC, CT, B2, and Hex prevented OA-mediated increase of chylomicron formation and secretion as well as transcellular transport of chylomicron-associated LPS. *In vivo* experiments, in mice gavaged with olive oil confirmed that high fat intake promoted an increase in circulating LPS concentration which was alleviated by EC supplementation. Together, the capacity of catechins and PCAs to mitigate HFD-induced metabolic

endotoxemia can be in part explained by the inhibition of transcellular transport of luminal endotoxins via chylomicrons.

Endotoxemia is proposed to be a major contributor to obesity-associated comorbidities given its association to the development of chronic inflammation [11]. Diets rich in fat favor endotoxemia originated from gut endotoxins [40]. On the other hand, plant-based diets contain large amounts of polyphenols have been consistently shown to mitigate the adverse consequences of obesity [41]. Among polyphenols, flavonoids have been proposed to exert anti-obesity actions through several mechanisms [42], including preserving the intestinal barrier integrity [36], influencing gut microbiota profiles [43], and modulating lipid metabolism and absorption [44]. While EC and PCAs mitigated secondary bile acid-induced loss of Caco-2 monolayer integrity [14, 45], EC supplementation did not modify HFD-induced microbiota alterations in mice [36]. Alternatively, catechin extract from green tea (7.3% from CT and 5.5% from EC) reduces body fat in humans [46] in part by influencing the formation and secretion of chylomicron in the intestine from both *in vitro* and *in vivo* studies [47, 48]. Red wine polyphenols were able to attenuate the synthesis and secretion of chylomicrons in a Caco-2 cell model [49]. Myricetin, which shares a similar structure with catechin, attenuated dyslipidemia by decreasing plasma chylomicron levels in obese db/db mice [50]. Aligned with these findings, we observed that OA increased the basolateral secretion of chylomicrons and monolayer-associated LPS, which were both prevented by EC, CT, B2 and Hex. This elevated LPS translocation was not due to paracellular transport, as the short-term OA incubation (less than 12 h) did not have a deleterious effect on TJs. Our findings support that LPS increased transport was promoted

through its incorporation into nascent chylomicrons given chylomicrons high affinity for LPS [18]. Stronger effects of EC, CT, B2 and Hex were observed on limiting LPS transcellular transport than reducing ApoB48 level in the basolateral media, suggesting that, besides affecting the chylomicron-dependent way, catechins and PCAs could affect other mechanisms of transcellular transport of LPS such as the suppression of toll like receptor 4 (TLR4)-mediated LPS endocytosis [51, 52].

The absorption of LCFA (e.g. OA) requires the fatty acid transport complex and chylomicrons [53]. JNK is considered as an important player in the regulation of fatty acids uptake and chylomicron biosynthesis and secretion [30, 31]. JNK inhibition was previously found to decrease the synthesis of fatty acid uptake complex proteins and of proteins involved in the vesicular chylomicron excretion, consequently diminishing the basolateral secretion of chylomicron [31]. We observed that EC prevented OA-induced JNK activation, pointing to JNK as a target of EC's action downregulating the chylomicron-dependent transcellular transport of LPS.

LCFA promotes the translocation of CD36, the predominant membrane protein involved in fatty acids uptake [54], from intracellular endosomal stores to the plasma membrane in mouse intestinal epithelial cells [55, 56]. CD36 internalization and ubiquitination also appear to be required for fatty acids uptake and the induction of key proteins participating in chylomicron formation [25, 57]. In agreement with the above findings, we observed that while total CD36 protein levels were not affected, OA promoted the translocation of CD36 to the plasma membrane. This was not affected by EC, suggesting that fatty acid uptake would not be regulated by EC. LCFA also upregulates intestinal MTP

expression in mice gavaged with oil [25]. Under the current experimental conditions, the OA-induced transient (10 min) MTP upregulation was prevented by EC. This effect cannot be attributed to JNK modulation, given that JNK activation by OA occurred later. However, the upregulation of MTP at 10 min was observed to be mediated by ERK1/2 in isolated intestinal segments from $CD36^{+/+}$ mice cultured in the presence of linoleic acid [25]. This also indicates that, besides JNK, ERK1/2 also plays a role in OA-induced chylomicron formation and secretion.

EC was previously shown to lower dyslipidemia and endotoxemia in mice after long-term consumption of a HFD [36, 52]. However, the preventive effects of EC on fat-induced postprandial endotoxemia were never investigated *in vivo*. We investigated this in mice, after LPL inhibition to prevent the plasma clearance of chylomicrons [58]. The capacity of EC to inhibit the postprandial transport of LPS was associated with a simultaneous decrease in TG absorption. Aligning with the results in Caco-2, EC had stronger effects in lowering the levels of postprandial plasma LPS than those of TG. This can be due to the fact that: i) not all fatty acids require incorporation into chylomicrons as TG to be absorbed, and ii) the involvement of multiple mechanisms in the capacity of EC to decrease high fat-induced metabolic endotoxemia.

In summary, our findings support a novel mechanism by which catechins and PCAs can improve high fat-induced metabolic endotoxemia. The protective actions of catechins and PCAs on fat-induced endotoxemia can be in part due to their capacity to mitigate chylomicron-dependent transcellular transport of LPS. With regards to the involved mechanisms, evidence suggest that EC acts in part preventing the activation of the JNK

pathway as well as the upregulation of protein involved in chylomicron formation and secretion. This work provides support to the concept that diets rich in flavonoids can mitigate the adverse effects of high fat diets by decreasing metabolic endotoxemia. Studies are needed to further investigate the mechanisms underlying the described beneficial effects of individual catechins and PCAs.

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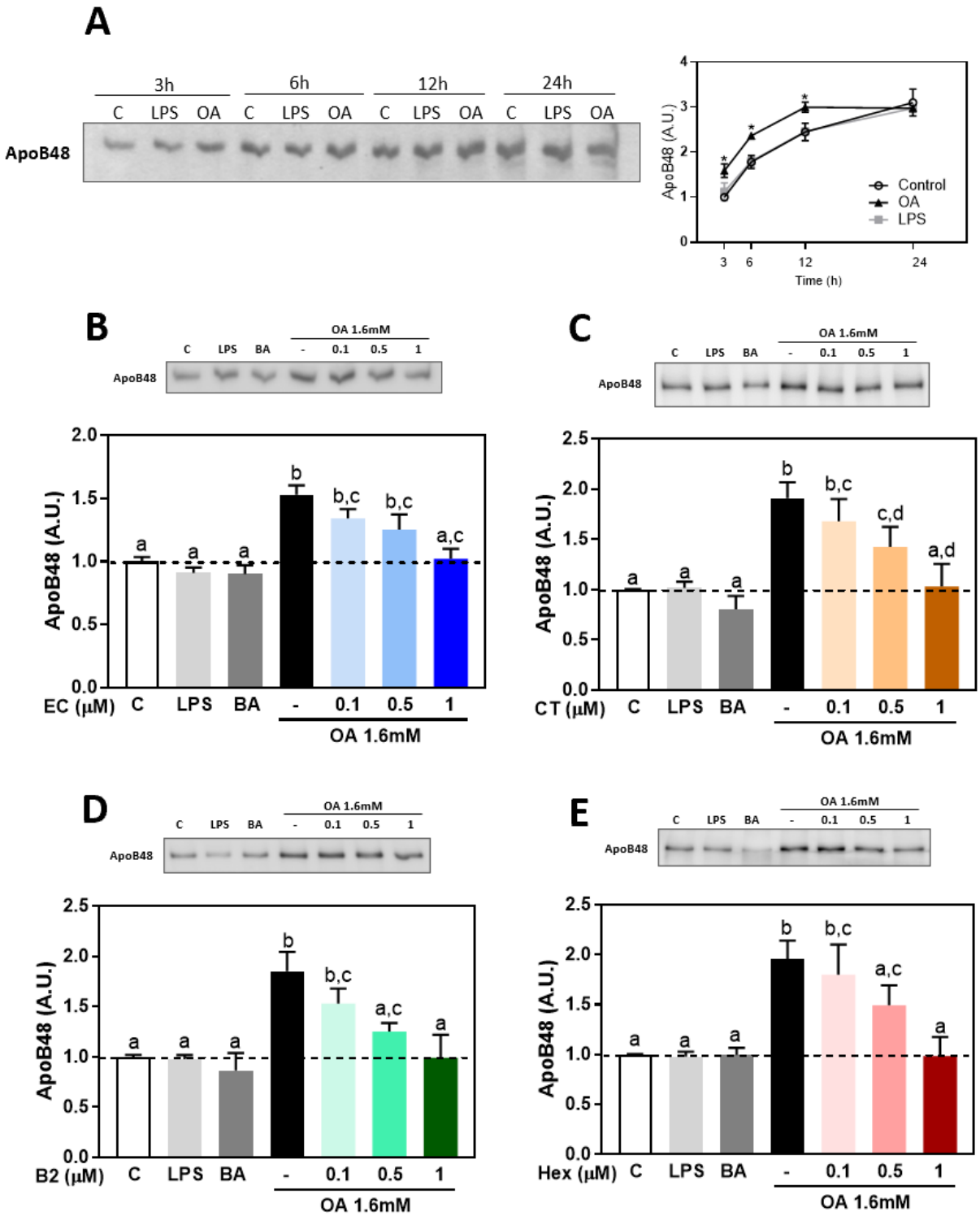
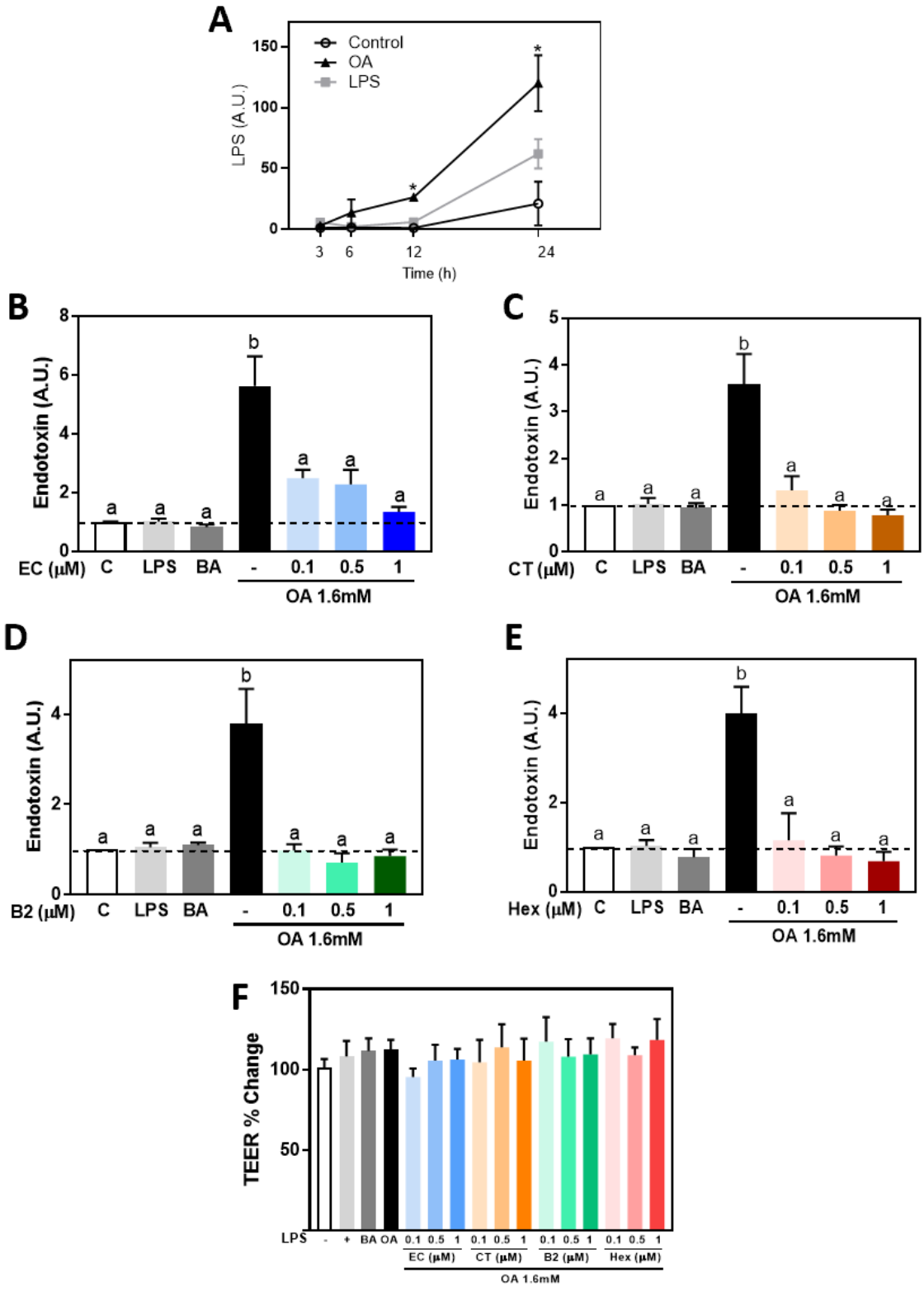
Tables and Figures

Figure 1. OA causes an increase in the basolateral secretion of chylomicrons in Caco-2 monolayer that is prevented by EC, CT, B2, and Hex.


A
3h
6h
12h
24h
C LPS OA C LPS OA C LPS OA C LPS OA
ApoB48
ApoB48 (A.U.)
Time (h)
Control
OA
LPS
B
C
D
E
OA 1.6mM
C LPS BA - 0.1 0.5 1
ApoB48
ApoB48 (A.U.)
EC (μM)
CT (μM)
B2 (μM)
Hex (μM)
a a a b b,c b,c a,c
a a a b b,c c,d a,d
a a a b b,c a,c a
a a a b b,c a,c a

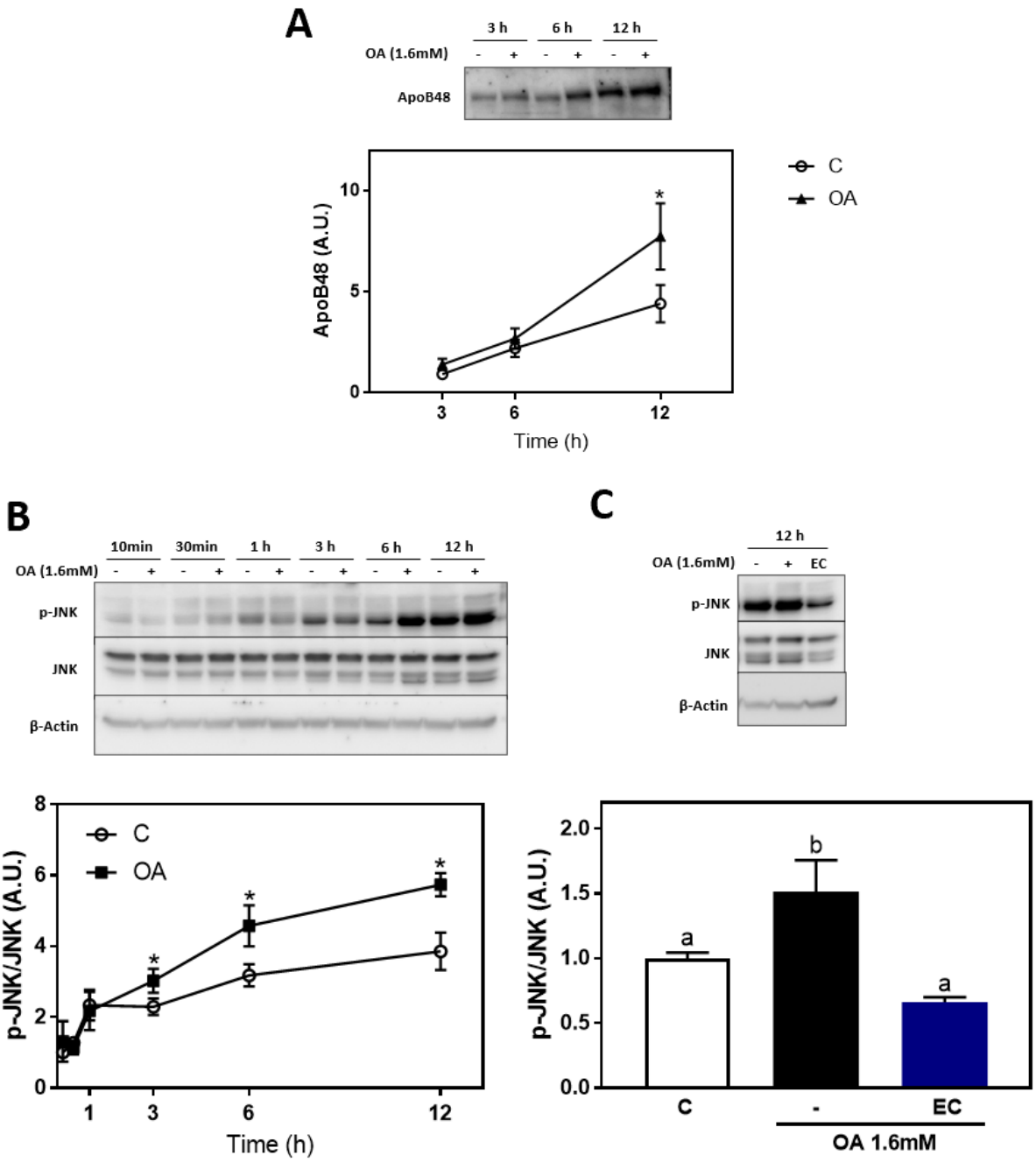
Caco-2 monolayers were incubated in the absence or presence of 0.1 mg/mL LPS and/or 1.6 mM OA with or without the addition of 0.1-1 μM EC, CT, B2, and Hex added to the upper chamber for 0-24 h. Levels of ApoB48 in the basolateral medium were measured by Western blot. **A**- Kinetics of basolateral secretion of chylomicrons in Caco-2 monolayers were evaluated in the absence or presence of LPS and/or OA. **B-E**- Dose-dependent effects of EC (**B**), CT (**C**), B2 (**D**), and Hex (**E**) on OA-induced basolateral secretion of chylomicrons after 12 h incubation. Western blot bands were quantified, and data normalized to control values. Results are shown as mean ± SEM of 6-7 independent experiments. **A**- *Significantly different from control and OA-treated groups or from LPS-treated groups and OA-treated groups at the corresponding time point. **B-E**- Values having different superscripts are significantly different ($p<0.05$, One-way ANOVA).

Figure 2. OA causes an increase in the basolateral secretion of cell-associated LPS in Caco-2 monolayer that is prevented by EC, CT, B2, and Hex.



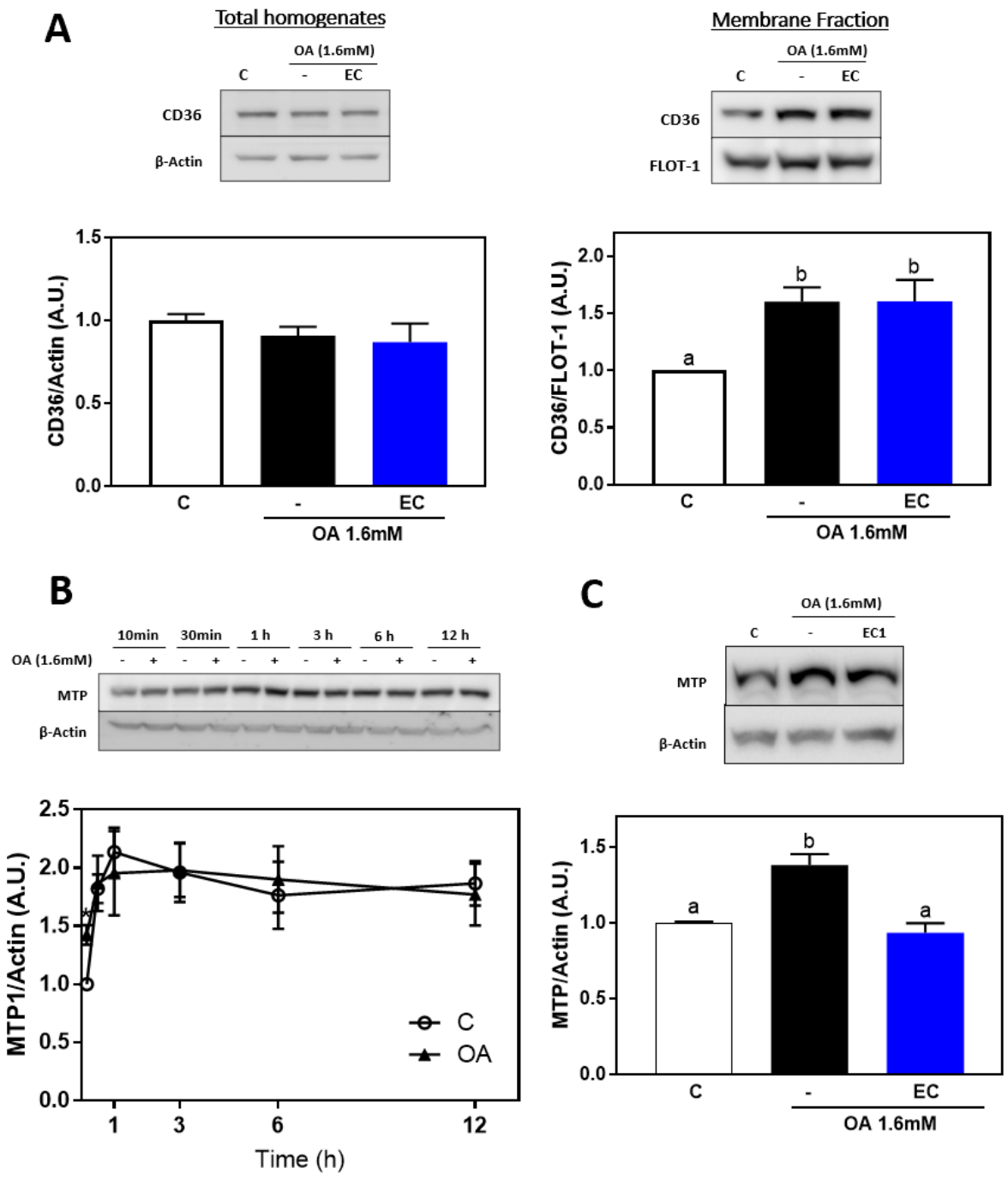
Caco-2 monolayers were incubated in the absence or presence of 0.1 mg/mL LPS and/or 1.6 mM OA with or without the addition of 0.1-1 μM EC, CT, B2, and Hex added to the upper chamber for 0-24 h. **A**- Kinetics (3-24 h) of the basolateral secretion of LPS in Caco-2 monolayers incubated in the absence or presence of LPS and/or OA were evaluated by measuring the concentrations of LPS in the lower chamber medium. **B-E**- Inhibition by EC (**B**), CT (**C**), B2 (**D**), and Hex (**E**) of OA-induced basolateral secretion of LPS after 12 h incubation. **F**- Caco-2 cell monolayer integrity was evaluated by measuring the TEER. Results are shown as mean ± SEM of 6-7 independent experiments. Data were normalized to control values. **A**- *Significantly different from control and OA-treated groups or from LPS-treated groups and OA-treated groups at the corresponding time point. **B-E**- Values having different superscripts are significantly different ($p<0.05$, One-way ANOVA).

Figure 3. JNK activation could be involved in the inhibitory actions of EC on OA-induced chylomicron secretion in Caco-2 cell monolayers.



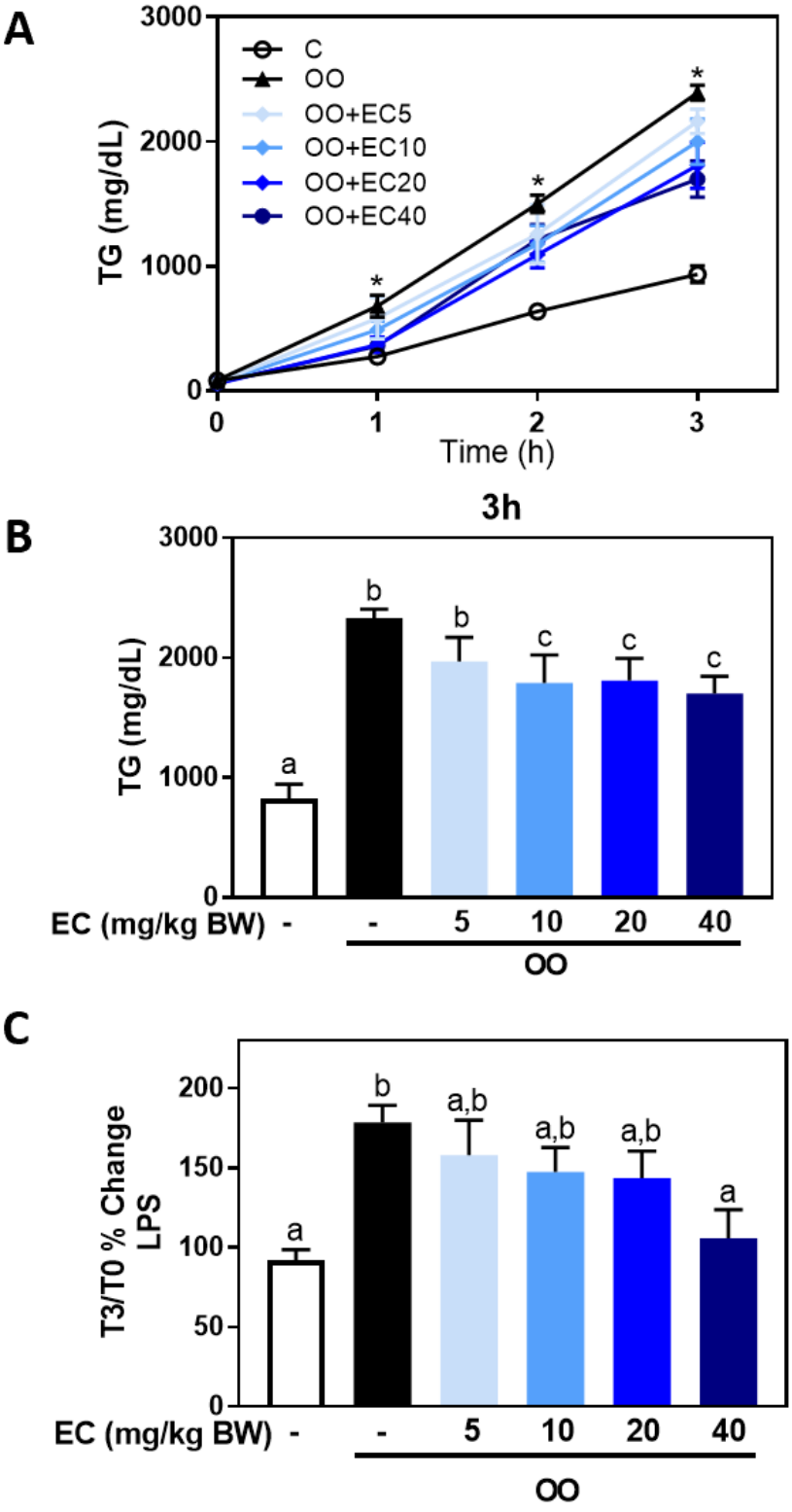
Caco-2 monolayers were incubated without or with 1.6 mM OA in the absence or presence of 5 μM EC for 0-12 h. **A**- The kinetic of chylomicron secretion from Caco-2 monolayers was followed by measuring the levels of ApoB48 in the lower chamber culture medium by Western blot in the absence or presence of OA. **B**- Kinetic (0-12 h) of JNK activation in the absence or presence of OA. Phosphorylation of JNK (Thr183/Tyr185) was evaluated by Western blot. **C**- Effects of 5 μM EC on OA-induced JNK phosphorylation after 12 h incubation. **B,C**- After quantification of bands, values for phosphorylated proteins were referred to the respective total protein content. β-actin were measured as loading controls. Data were normalized to control values. Results are shown as mean ± SEM of 5-6 independent experiments. **A,B**- *Significantly different from control and OA-treated groups at the corresponding time point. **C**- Values having different superscripts are significantly different ($p<0.05$, One-way ANOVA).

Figure 4. Effects of EC on proteins involved in fatty acids uptake and chylomicron assembly/secretion in Caco-2 cells.



Caco-2 monolayers were incubated in the absence or presence of 1.6 mM OA and with or without the addition of 1 or 5 μM EC added for 0-12 h. **A**- Effects of EC (5 μM) on CD36 protein levels in total cell homogenate (left panel) and cell membrane fractions (right panel) were measured by Western blot. After quantification of bands, values were referred to β-actin and flotilin-1 (FLOT-1) for total cell and membrane fractions, respectively. **B**- Kinetic of OA-mediated effects on MTP protein expression. **C**- The effect of EC (1 μM) on MTP protein levels were measured after 12 h incubation with OA by Western blot. **B,C**- Bands were quantified and values referred to β-actin levels. Data were normalized to control values. Results are shown as mean ± SEM of 3-5 independent experiments. **B**- *Significantly different from control and OA-treated groups at the corresponding time point. **A,C**- Values having different superscripts are significantly different ($p<0.05$, One-way ANOVA).

Figure 5. Effects of EC on plasma triglyceride and LPS in mice after an oral fat load.



Mice were fasted for 4 h and blood samples were collected as time 0. Mice were then I.P. injected with tyloxapol, and after 15 min were gavaged with 5% (v/v) Tween-80 saline solution or 5, 10, 20, and 40 mg EC/kg B.W. After 30 min of tyloxapol injection, mice were gavaged with olive oil (OO) (10 uL/g B.W.) or saline solution. Blood samples were subsequently collected at 1 h, 2 h, and 3 h after the second gavage. **A**- Kinetics (0-3 h) of plasma TG concentration. **B**- Mouse plasma TG levels 3 h post-gavage. **C**- Mouse plasma LPS levels 3 h post-gavage. **C**- Values were calculated considering values for the control group (tyloxapol injected and Tween-80/saline-gavaged) as 100%. **A**- *Significantly different in the OA group compared to controls at the corresponding time point. **B,C**- Values having different superscripts are significantly different ($p<0.05$, One-way ANOVA).

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Conclusion

This dissertation work evaluated the capacity of (-)-epicatechin (EC) to alleviate high-fat diet (HFD)-induced obesity-associated metabolic endotoxemia. Obesity represents a critical public health issue as it is linked to chronic inflammation and endotoxemia, which are involved in the development and progression of multiple pathological conditions and diseases [1, 2]. Additionally, an obesity-related increase in medical cost has caused a substantial economic burden universally [3]. Several environmental factors contribute to the increasing global incidence of obesity. Among them, obesogenic Western pattern diets high in dietary fat play a considerable role [4, 5]. The flavan-3-ol EC, a flavonoid abundant in the human diet, can mitigate obesity-related pathologies through several mechanisms [6]. My research identified mechanisms involved in the capacity of EC to mitigate HFD-induced metabolic endotoxemia by preventing the paracellular and transcellular transport of endotoxins at the gastrointestinal (GI) tract.

Chapter one demonstrated that EC supplementation prevented HFD-induced steatosis and insulin resistance partially by preserving the intestinal barrier integrity and mitigating metabolic endotoxemia. A 15-week HFD consumption by male C57BL/6J mice caused obesity, steatosis, and insulin resistance, which was associated with decreased intestinal tight junction (TJ) proteins expression, increased intestinal permeability, and elevated endotoxemia. Mechanistically, HFD consumption induced the upregulation of NOX1 and NOX4 expression, protein oxidation, and the activation of the redox-sensitive NF-κB and ERK1/2 pathways in the mice ileum. The consumption of HFD also decreased the plasma

levels of the gut hormone GLP-2 which has protective effects on the intestinal mucosa. EC supplementation at a dose of 20 mg/kg body weight improved all these adverse effects. In agreement with the *in vivo* study findings, EC and the NADPH oxidase inhibitor apocynin prevented TNF-α-induced NOX1 and NOX4 overexpression, protein oxidation, and permeability in Caco-2 cell monolayer. Mice cecal microbiota profiles were altered by the long-term exposure to a high dietary fat, which was not reverted by EC supplementation.

High concentrations of select bile acids, such as hydrophobic secondary bile acids, are reported to be involved in the pathogenesis of several intestinal disorders having increased intestinal permeability [7]. We observed that the HFD caused an increase in the concentrations of total and individual fecal bile acids in mice, including the secondary bile acid deoxycholic acid (DCA). EC did not prevent these changes. Chapter two investigated the mechanisms underlying bile acid (DCA)-induced Caco-2 monolayer permeabilization and the protective effects of EC. We found that DCA induced Caco-2 monolayer permeabilization by decreasing EGFR activation and NADPH oxidase expression. Downstream, DCA activated ERK1/2, and promoted the phosphorylation of the myosin light chain (MLC) protein by inhibiting MLC phosphatase. This alters TJ structure and function. DCA decreased TJ protein (ZO-1 and occludin) expression partially by upregulating MMP-2 activity. All the above events were mitigated by EC, NADPH oxidase inhibitors (VAS-2870 and apocynin), and a MEK inhibitor (U0126). These results indicated that EC's capacity to mitigate HFD-induced intestinal permeabilization is in part through the inhibition of bile acids-induced NADPH oxidase and ERK1/2 activation.

Increased gastrointestinal permeability allows unwanted luminal substances such as LPS to pass through the intestinal barrier paracellularly, subsequently causing endotoxemia. Additionally, LPS can also be transported transcellularly in newly formed chylomicrons [8]. High dietary fat can upregulate chylomicron secretion due to its role in lipids absorption. The third chapter evaluated the capacity of EC to mitigate fat-induced increased transcellular transport of endotoxins via chylomicrons. We observed that the catechins EC and (+)-catechin (CT), and procyanidins (PCAs) B2 dimer (B2) and hexamer (Hex) decreased the oleic acid (OA)-triggered basolateral secretion of chylomicrons and chylomicron-dependent LPS transcellular transport in Caco-2 monolayer. Mechanistically, JNK could be a target of EC inhibitory capacity. OA also induced a transient overexpression of microsomal triglyceride transfer protein (MTP), a protein that is involved in chylomicron assembly/secretion), which was prevented by EC. Consistent with the *in vitro* study, EC supplementation ameliorated olive oil-induced postprandial plasma triglyceride and LPS increases in mice.

In summary, findings from this dissertation support the view that dietary EC may be of relevance in alleviating the adverse effects of Western pattern diets. Understanding the beneficial actions of individual flavonoids on obesity-associated comorbidities is critical to developing human dietary strategies and possible novel dietary supplements to mitigate the undesirable consequences caused by obesity, both for individuals and societies worldwide.

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(−)-Epicatechin and the comorbidities of obesity

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ABSTRACT

Obesity has major adverse consequences on human health contributing to the development of, among others, insulin resistance and type 2 diabetes, cardiovascular disease, non-alcoholic fatty liver disease, altered behavior and cognition, and cancer. Changes in dietary habits and lifestyle could contribute to mitigate the development and/or progression of these pathologies. This review will discuss current evidence on the beneficial actions of the flavan-3-ol (−)-epicatechin (EC) on obesity-associated comorbidities. These benefits can be in part explained through EC's capacity to mitigate several common events underlying the development of these pathologies, including: i) high circulating levels of glucose, lipids and endotoxins; ii) chronic systemic inflammation; iii) tissue endoplasmic reticulum and oxidative stress; iv) insulin resistance; v) mitochondria dysfunction and vi) dysbiosis. The currently known underlying mechanisms and cellular targets of EC's beneficial effects are discussed. While, there is limited evidence from human studies supplementing with pure EC, other studies involving cocoa supplementation in humans, pure EC in rodents and *in vitro* studies, support a potential beneficial action of EC on obesity-associated comorbidities. This evidence also stresses the need of further research in the field, which would contribute to the development of human dietary strategies to mitigate the adverse consequences of obesity.

1. Introduction

Overweight and obesity are major public health problems worldwide [1]. The "Clinical Guidelines on the Identification, Evaluation, and Treatment of Overweight and Obesity in Adults" defined overweight as a body mass index (BMI) between 25 kg/m^2 and 29.9 kg/m^2 and obesity as a BMI of ≥30 kg/m^2 [2]. According to the World Health Organization (WHO), in 2016 overweight affected 1.9 billion adults (including 650 million obese) aged 18 years and older worldwide. Alarmingly, the prevalence of obesity was almost three times higher in 2016 compared to 1975. Numerous comorbidities and an increased risk of mortality are associated to obesity [3–5]. Among those comorbidities, obesity can increase the risk to develop: i) insulin resistance and type 2 diabetes (T2D); ii) hypertension and cardiovascular disease (CVD); iii) dyslipidemia; iv) non-alcoholic fatty liver disease (NAFLD); v) altered behavior and cognition; vi) cancer, and vii) alterations in the gastrointestinal, musculoskeletal, renal, respiratory and immune systems.

Abbreviations: ADME, absorption, distribution, metabolism and excretion; AMPK, 5′-AMP-activated protein kinase; ApoB, apolipoprotein B; BW, body weight; BDNF, brain-derived neurotrophic factor; BMI, body mass index; CNS, central nervous system; CVD, cardiovascular disease; DCA, deoxycholic acid; EC, (−)-epicatechin; ER, endoplasmic reticulum; ERK1/2, extracellular signal-regulated kinase 1/2; ET-1, endothelin-1; FFA, free fatty acids; GI, gastrointestinal; GLP, glucagon-like peptide; HDL-C, high-density lipoprotein cholesterol; HOMA-IR, Homeostatic Model Assessment of Insulin Resistance; IKK, inhibitor of nuclear factor-κB kinase; IL-6, interleukin 6; IR, insulin resistance; IRS-1, insulin receptor substrate-1; ISI, insulin sensitivity index; JNK, c-Jun N-terminal kinase; LDL-C, low density lipoprotein cholesterol; LPS, lipopolysaccharides; MCP-1, monocyte chemoattractant protein 1; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; NF-κB, nuclear factor-κB; NO, nitric oxide; NOX, NADPH oxidase; PAC, proanthocyanidins; PGC-1α, PPARγ coactivator-1α; PI3K, phosphatidylinositol-3-kinase; PPARγ, peroxisome proliferator-activated receptor-gamma; QUICKI, quantitative insulin sensitivity check index; SCAP, SREBP cleavage-activating protein; SIRT, sirtuin; SREBP, sterol regulatory element-binding protein; SREM, structurally-related EC metabolites; T2D, type 2 diabetes; TG, triglycerides; TJ, tight junction; TLR4, Toll-like receptor 4; TNFα, tumor necrosis factor alpha; UPC-1, uncoupling protein-1; VLDL-C, very low density lipoprotein cholesterol; WAT, white adipose tissue

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It has been proposed that 50% of the main public health concerns, including obesity, T2D and NAFLD, could be mitigated or prevented by changes in dietary habits [6]. Thus, while a worldwide increased consumption of foods rich in sugars and fat can in part explain the current obesity crisis, dietary changes towards a higher intake of fruits, vegetables and derived foods could contribute to mitigate it. In support of this, diets low in fruits have been found to be the third major contributor to mortality and burden of disease after high blood pressure and smoking [6]. In such context, identifying specific components (bioactives) in foods that could provide health benefits in pathologies associated to obesity is of outmost relevance. Furthermore, there is a need to understand bioactives' mechanisms of action, as well as biological targets, metabolism and individual responses due to genetic and biological (e.g. microbiota) modifiers of absorption and biological effects.

Among bioactives, the flavan-3-ol (−)-epicatechin (EC), and its polymers, the proanthocyanidins (PAC) are abundant in the human diet, being present in large amounts in cocoa (*Theobroma cacao*), grapes, apples, blueberries, hazelnuts, pecans and tea (*Camellia sinensis*) [7]. EC has the basic flavonoid chemical structure C6–C3–C6, with two (A and B) aromatic rings linked by a heterocycle formed by an extra three-carbon chain and one oxygen atom (ring C) (Fig. 1A). EC has hydroxyl residues at carbons 5 and 7 (ring A), 3 (ring C), and 3′ and 4' (ring B). EC can polymerize to form PAC (Fig. 1B). In B-type PAC, monomers are linked through a 4β→6 or 4β→8 carbon–carbon bond (Fig. 1B), and in A-type PAC, through both 4β→8 carbon–carbon and 2β→O7 ether bonds (Fig. 1C). As summarized in Fig. 2, EC has been found to have beneficial effects on several of the pathologies associated with obesity, both in human and in rodent models. This review will mostly address studies done with EC. Given the few studies involving EC supplementation in humans, this review will also discuss results from human studies done with cocoa, which contains as polyphenolic compounds EC, its isomer (+)-catechin, and B-type PACs.

2. EC and PAC metabolism

The beneficial health effects of EC and PAC can be mediated by both parent compounds and/or their metabolites. The human absorption, distribution, metabolism and excretion (ADME) of EC has been recently reviewed by Borges et al. [8]. Briefly, EC is extensively metabolized by tissues and by the intestinal microbiota. In humans, EC and mostly structurally-related EC metabolites (SREM) (sulfated, methylated, glucuronidated EC derivatives) appear in plasma after cocoa consumption. EC and SREM reach maximum plasma concentration values 2–3 h post cocoa ingestion [9,10]. Considering urinary SREM, they account for 21% of the ingested EC [11]. EC can also be metabolized to select SREM by intestinal epithelial cells. Thus, sulfated EC formed in the intestinal epithelium not only contributes to the circulating pool but is also excreted into the intestinal lumen [11]. Finally, the microbiota cleaves EC mainly into 5C-ring fission metabolites, i.e. 5-(hydroxyphenyl)-γ-valerolactones and 5-(hydroxyphenyl)–γ-hydroxyvaleric acids. These metabolites can be efficiently absorbed, as measured through their presence in plasma and urine. Based on their content in urine, it was calculated that they correspond to a large proportion (42%) of ingested EC [8].

The absorption of PAC is very limited [12] and restricted to dimers [13,14]. After oral consumption PAC are present throughout the GI tract, as observed in rats and pigs fed a PAC-rich grape seed extract [15,16]. PAC present in the diet do not contribute to the blood pool of flavan-3-ols [13]. On the other hand, PAC can be metabolized by the intestinal microbiota into smaller compounds, e.g. valerolactones and phenolic acids, that can then be transported into the circulation [13,17].

Overall, dietary EC and PAC can exert biological effects both at the gastrointestinal (GI) tract, when not absorbed, and systemically mainly as SREM and smaller absorbable microbiota-derived metabolites.

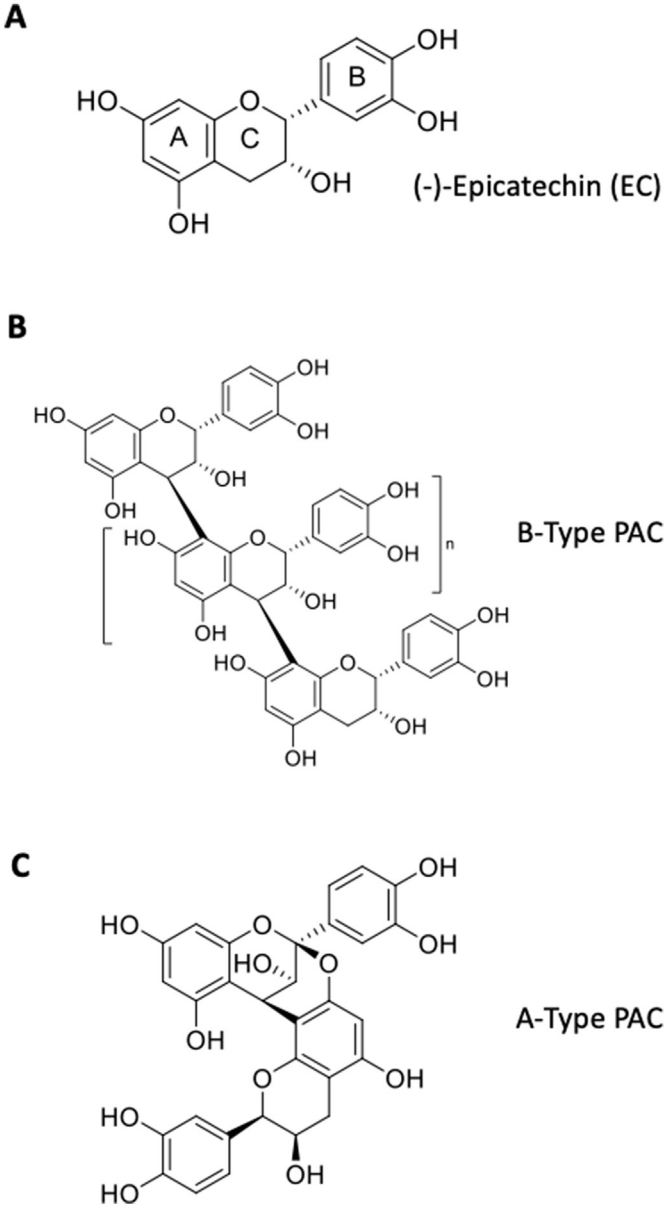

Fig. 1. Chemical structures of A- EC, B– B-type PAC and C- A-type PAC. Chemical structures were drawn using ChemDraw Professional 19.0 software (PerkinElemer Inc, Waltham, MA).

3. EC and the gastrointestinal tract

The gastrointestinal (GI) tract plays a relevant role in the development of the comorbidities of obesity. In turn, obesity can also severely affect GI function [18,19]. The GI tract is central to obesity-triggered pathologies because of its: i) direct exposure to dietary components; ii) function at absorbing macronutrients which ultimately determine energy homeostasis; iii) contribution to satiation, satiety and lipid and glucose homeostasis by regulating the secretion of gut hormones; iv) function as a barrier to prevent the passage of luminal endotoxins; and v) microbiota. On the other hand, obesity is also considered as a direct cause of several GI tract complications including, among several others, gastroesophageal reflux disease, erosive gastritis, diverticulitis and cancer [20,21].

Given the high concentrations that dietary flavonoids reach in the GI lumen, they can have relevant participation in the modulation of GI homeostasis [20]. In this regard, EC and PAC have been shown to have beneficial effects on the GI alterations associated with high fat diet-

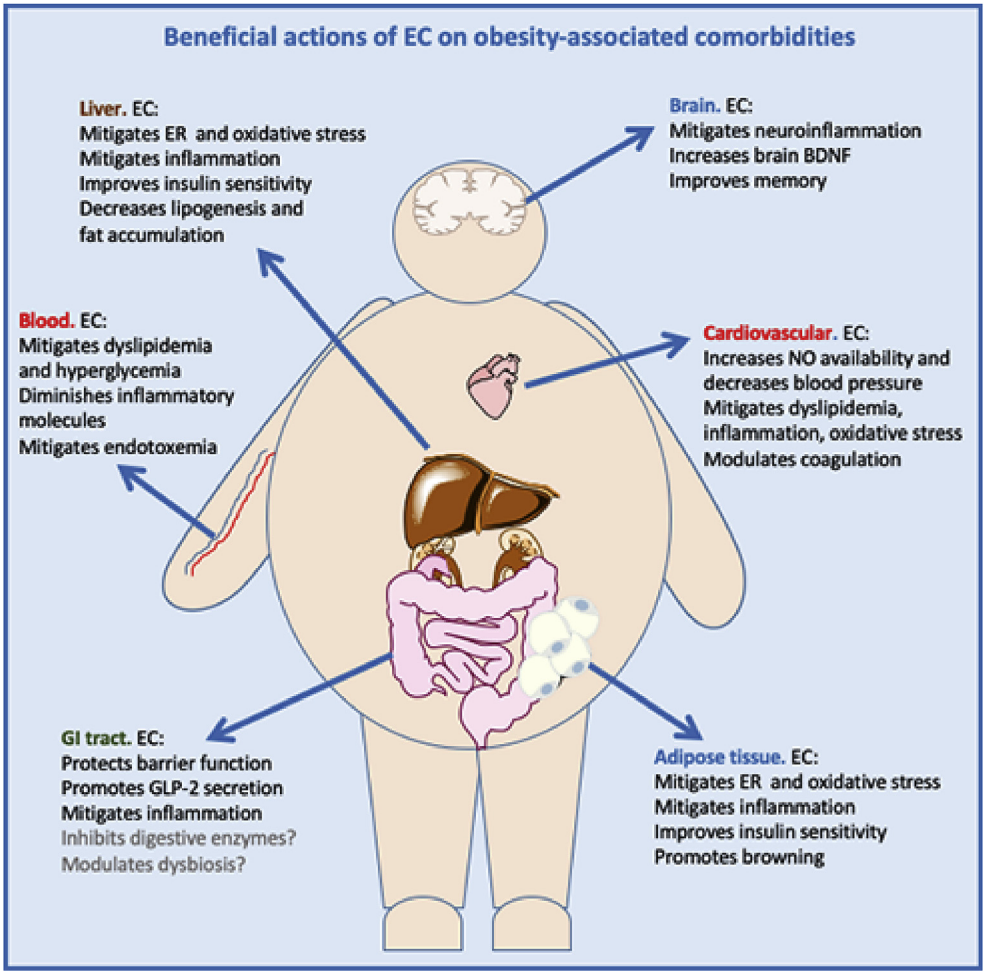

Fig. 2. Obesity comorbidities: potential beneficial actions of EC. Most of the effects described were characterized in rodents. However, while human studies supplementing with EC are scarce, several of the described beneficial actions are supported by studies in humans using EC-rich cocoa. BDNF: brain-derived neurotrophic factor, ER: endoplasmic reticulum, NO: nitric oxide.

induced obesity and on obesity-associated comorbidities linked to GI dysfunction [20–22]. Given the few existing studies on EC effects at the upper GI tract, this section will focus on the actions of EC at the lower GI tract (Fig. 3).

Despite limited evidence in humans, a large body of studies in animal models have shown that the intestinal barrier integrity is impaired in obesity. Increased intestinal permeability can lead to increased paracellular translocation of luminal toxins and microbes. In the case of bacterial lipopolysaccharides (LPS), their translocation from the lumen to the blood stream results in endotoxemia. This metabolic endotoxemia is responsible to a large extent for the chronic systemic low grade inflammation that characterizes obesity, being considered as a key contributor to the development of obesity comorbidities [23,24] (Fig. 3). EC has the capacity to sustain intestinal barrier integrity both *in vitro* [25,26] and in a mouse model of diet-induced obesity [22]. Indeed, consumption of a high fat diet for 15 weeks caused intestinal permeabilization and endotoxemia in mice, which was mitigated by supplementation with EC (2–20 mg/kg body weight (BW)). This can be partially explained by the capacity of EC to prevent high fat diet-mediated alterations in the structure of tight junctions (TJ) [22], which by controlling paracellular transport, are essential to the proper function of the intestinal barrier.

It has been shown that high levels of bile acids and chronic inflammation are two of the main factors involved in obesity-associated increased intestinal permeability. Consumption of high fat diets increases levels of luminal bile acids required for fat absorption. Increased level of select bile acids are associated with intestinal permeabilization both *in vivo* an *in vitro* [27,28]. In Caco-2 cell monolayers, an *in vitro* model of an intestinal epithelium, EC prevented deoxycholic acid (DCA)-induced monolayer permeabilization [26]. The underlying mechanisms involve the attenuation by EC of DCA-mediated NADPH oxidase upregulation, high oxidant production and downstream activation of the mitogen activated kinase ERK1/2 [26]. All these DCA-activated events lead to alterations in TJ structure and function which were preserved by EC. Similar findings were observed for EC oligomers (PAC) isolated from cocoa [29]. GI inflammation triggered by obesity and diet, particularly free fatty acids (FFA), can cause intestinal permeabilization [30,31]. Inflammation leads to the activation of ERK1/2 and transcription factor nuclear factor-κB (NF-κB) which are important regulators of TJ structure and dynamics [32,33]. A large body of evidence support EC's anti-inflammatory actions both *in vitro* and *in vivo* [22,25,34]. In Caco-2 cells EC prevented monolayer permeabilization triggered by exposure to tumor necrosis factor alpha (TNFα) [25]. EC protective effects are in part mediated through the inhibition of TNFα-triggered activation of NADPH oxidase and downstream of the redox-sensitive NF-κB pathway [25]. Similar effects were observed in the ileum of obese mice fed a high fat diet [22].

At the GI tract, EC interacts with nutrients and digestive enzymes. This provides the possibility for EC to mitigate obesity by diminishing the digestion and absorption of calorie-providing nutrients [21]. *In vitro*, EC and cocoa extracts were found to delay carbohydrate digestion and absorption by inhibiting the activity of select enzymes (e.g. α-

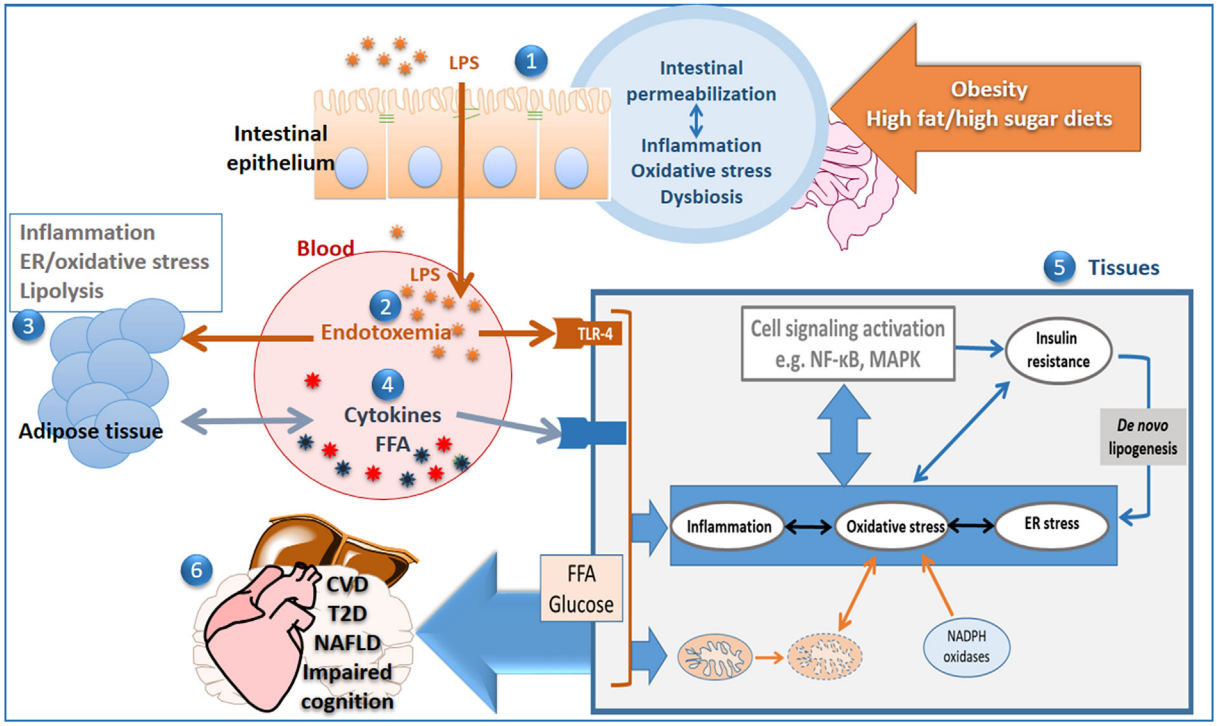

Fig. 3. EC modulates central mechanisms involved in the development of obesity-associated comorbidities: inflammation, oxidative and endoplasmic reticulum (ER) stress. 1- In the intestine, EC mitigates inflammation- and bile acid-induced intestinal permeabilization through the downregulation of NADPH oxidases and redox-sensitive signals (NF-κB, ERK1/2) in rodent models of diet-induced obesity. Dysbiosis could involve an increase in lipopolysaccharide (LPS)-producing bacteria. However, the potential effects of EC on obesity-associated dysbiosis need further characterization. **2-** The prevention of intestinal permeabilization by EC and the associated decreased transcellular transport of proinflammatory LPS, would decrease endotoxemia. The latter effect has major implications in the mitigation of systemic inflammation. **3-** EC mitigates white adipose tissue (WAT) inflammation, endoplasmic reticulum (ER) and oxidative stress. The beneficial effects of EC on WAT inflammation mitigate lipolysis, which contributes to a decreased release of cytokines and free fatty acids (FFA) into the bloodstream (**4**). **5-** In different tissues, EC mitigates inflammation, oxidative and ER stress. This can be in part due to its capacity to decrease blood levels of FFA, proinflammatory cytokines and LPS. Inflammation and excess of FFA and other nutrient load can also cause mitochondria dysfunction increasing oxidant production. Upregulation of NADPH oxidases also contribute to excess oxidant production and oxidative stress. Consequent activation of select signaling cascades (e.g. NF-κB and MAPK) leads to the inhibition of the insulin cascade and tissue insulin resistance. The latter promotes *de novo* lipogenesis leading to tissue lipid deposition. Overall EC mitigates inflammation, oxidative and ER stress, improving tissue insulin sensitivity and inhibiting lipogenesis. **6-** The described mechanism of EC actions can in part explain its capacity to mitigate several of the pathologies associated to obesity.

amylase, glucoamylase) and glucose transporters [35–37]. Although little research has been done to investigate the effects of pure EC on lipid digestion and absorption, cocoa extracts inhibit *in vitro* both pancreatic lipase and phospholipase A_2 activities [37,38]. Overall, studies on the effects of EC or PAC on macronutrient digestion and absorption are scarce. Findings on the capacity of EC to mitigate hyperglycemia and dyslipidemia (discussed in other sections of this review) stress the relevance of further investigating this mechanism.

The GI influences energy homeostasis through the production of hormones secreted by enteroendocrine cells [39]. Among them, L cells synthetize and secrete glucagon-like peptide-1 (GLP-1) and GLP-2. GLP-1 regulates glucose homeostasis, satiety and other metabolic responses, such as lipoprotein secretion and fatty acid metabolism [40]. GLP-2 acts preserving GI physiology and structure [41], also regulating energy balance [42]. Although highly relevant to obesity-associated comorbidities, knowledge on the effects of EC on GLP-1 and GLP-2 homeostasis is scarce. In rats, a grape seed extract, containing ~32% EC, elevated plasma GLP-1 levels after exposure to a glucose challenge [43]. EC supplementation increased plasma GLP-2 levels in mice independently of them consuming control or high fat diets [22].

The involvement of the gut microbiota in obesity was brought to attention by Bäckhed et al., in 2004 [44]. Since then, it has been consistently observed that obese individuals have different gut microbiota profiles compared to lean individuals [45,46]. Mechanisms proposed to be involved in the association of microbial dysbiosis and metabolic disorders include changes in energy harvest and expenditure, elevated level of LPS from Gram-negative bacteria, alterations in bile acid metabolism, and increased gut permeability [45]. A large body of evidence supports a significant impact of flavonoids on the gut microbial community [47]. However, the number of existing studies investigating the influence of pure EC on microbiome composition is limited. In humans, and at a lesser extent than other catechins, EC significantly increased the growth of the *C. coccoides-Eubacterium rectale* group, producers of beneficial short chain fatty acid [48]. On the other hand, EC supplementation (20 mg/kg BW) did not restore high fat diet-induced changes in mouse microbiota profiles [22]. In terms of PAC, a recent study showed that consumption of dark chocolate (70% cocoa) by obese individuals increases the relative abundance of *Lactobacillus*, which might have anti-obesity effects [49]. PACs also exert bacteriostatic effects in humans, that might contribute to the modulation of microbiota composition [50]. Furthermore, several studies in rodents found that PAC from various food sources including apples and grape seeds can improve metabolic homeostasis and obesity-associated alterations of the GI microbiota [51–54].

Given the tight crosstalk between the GI tract and multiple organs, the beneficial effects of EC on obesity-associated comorbidities could be at a significant extent related to its actions within the GI system. For this reason, further research is of outmost importance to better understand the mechanism of actions of EC at the level of the GI tract.

4. EC and the adipose tissue

Adipose tissue dysfunction is at the center of obesity-associated pathologies. Once the capacity of the subcutaneous adipose tissue to store lipids is surpassed, they start accumulating in the visceral adipose tissue and in ectopic lipid deposits, e.g. liver and muscle. Expansion of the white adipose tissue (WAT) involving an increase in the size of adipocytes, causes mitochondrial alterations, secretion of inflammatory adipokines, hypoxia, decreased lipogenesis and increased lipolysis [55]. Furthermore, stressed adipocytes recruit macrophages, which secrete inflammatory cytokines. Overall, adipose tissue dysfunction is a main contributor to circulating molecules, e.g. cytokines, adipokines, FFA, that are recognized contributors to the development of obesity-associated comorbidities.

EC has been found to promote the appearance of "brown-like cells". While brown adipose tissue dissipates energy in the form of heat, WAT accumulates fat. Thus, WAT beiging is considered a beneficial shift from an unhealthy to a metabolically "healthier" tissue. Both in mouse fed a high fat diet and in human adipocytes, EC supplementation upregulated genes involved in thermogenesis and fatty acid oxidation, and mitigated WAT inflammation [56]. In male offspring from obese pregnant rat dams, EC reduced both the viceral fat pads and adipocyte size [57]. In 3T3-L1 adipocytes, EC (1 μM) upregulated the expression of transcritional regulators of "brown-like" adipocyte development, i.e. peroxisome proliferator-activated receptor-gamma (PPARγ), PPARγ coactivator-1α (PGC-1α), PR domain containing 16 (PRDM16) and of uncoupling protein-1 (UCP-1) [58]. UCP-1 mediates the mitochondrial dissipation of energy in the form of heat. The upregulation of these proteins was associated to EC-mediated activation of signaling pathways downstream of the β-adrenergic receptor. Furthermore, EC-mediated increased production of irisin by muscle cells can also contribute to WAT beiging [59]. Irisin is a miokine that after secretion from the muscle promotes browning of WAT by upregulating UCP-1 [60]. Accordingly, in high fat-fed mice, EC supplementation (20 mg/kg BW) upregulated UCP-1, and mitigated the expansion of the adipose tissue surrounding the thoracic aorta [61]. Maintaining the normal phenotype of this fat pad around the thoracic aorta is particularly important because of its capacity to secrete vasocontractile, vasorelaxing, anti-inflammatory and anti-atherogenic molecules.

Adipocyte metabolic stress causes the activation of a series of self-promoting events including endoplasmic reticulum (ER) and oxidative stress, activation of redox sensitive pathways, and inflammation (Fig. 3). Inflammation contributes to adipose tissue lipolysis and release of FFA into the circulation. In a mouse model of high fat diet-induced obesity, EC supplementation mitigated: i) the activation of the three branches of the unfolded protein response; ii) the upregulation of NADPH oxidases; iii) the activation of pro-inflammatory signaling pathways, i.e. transcription factor NF-κB and JNK1/2; iv) overall inflammation; v) macrophage recruitment; and vi) tissue insulin resistence [34,62]. Similar protective effects of EC were observed in WAT from rats fed a high fructose diet [63]. In differentiated 3T3-L1 adipocytes, 1 μM EC and SREM mitigated palmitate-induced increased secretion of the pro-inflammatory cytokines interleukin 6 (IL-6) and TNFα, of the chemokine MCP-1, and a decreased secretion of the anti-inflammatory adipokine adiponectin [34]. The used EC and SREM concentrations are of physiological relevance because after human oral consumption of EC, the maximum concentration of EC and SREM measured in plasma is 1 μM [13].

Given the central role of WAT hypertrophy in the development and progression of obesity comorbidities, EC beneficial actions at this level could explain many of the protective effects described in other sections of this review.

5. EC and insulin resistance

In 2019, about 460 million adults suffered of diabetes. The International Diabetes Federation estimates that approximately 700 million adults will develop diabetes by 2045 [64]. In the past decades, changes in diet and lifestyle had a major impact on the increased prevalence of T2D in both developed and underdeveloped country which goes in parallel with the increases in overweight and obesity, both in men and women [4,65]. The predominant cause of T2D is insulin resistance (IR), which is defined as a complex pathological state in which insulin-dependent organs such as liver, adipose tissue and muscle do not respond to insulin stimulation [66]. IR causes alterations in glucose disposal with consequent impairment of glucose metabolism.

A large body of evidence in both humans and in rodent models of obesity and T2D supports the beneficial effects of flavonoids, especially of EC and EC-rich foods in the improvement of insulin sensitivity and glucose homeostasis [67]. It has been shown that supplementation with pure EC: i) decreased blood glucose and insulin levels; ii) improved insulin sensitivity in liver and adipose tissues; iii) decreased Homeostatic Model Assessment of Insulin Resistance (HOMA-IR) values; and iv) improved glucose metabolism in both rats [63,68] and mice [22,62,69,70] fed high fat and high fructose diets. Two similar studies from Gutierrez-Salmean et al. [68] and Ramirez-Sanchez et al. [70] showed that administration of 1 mg EC/kg BW by gavage for two weeks decreased high fat diet-mediated increases in body weight and glycaemia in rats [68] and mice [70]. In addition, EC (2–20 mg/kg BW) also decreased body weight gain and improved glucose tolerance, insulin sensitivity and lipid profiles in both high fat-fed C57BL/6J mice and high fructose-fed rats [22,62,63,69].

In humans, meta-analysis including randomized, controlled trials, and systematic reviews, have shown beneficial effects of EC-rich cocoa, especially of dark chocolate, on the regulation of glucose homeostasis and insulin sensitivity in humans [71]. Long-term (4 weeks) consumption of cocoa had beneficial effects on glucose metabolism in overweight and obese individuals [72], in postmenopausal women diagnosed with T2D [73], and elderly individuals with mild cognitive impairment [74]. Interestingly, short-term (1–2 weeks) consumption of chocolate bars and cocoa beverages also improved insulin sensitivity and glucose tolerance, when compared to a flavonoid-free chocolate in different populations. These beneficial effects of cocoa were observed in healthy [75], hypertensive and glucose-intolerant [76,77], and overweight and obese [78,79] individuals. In general, greater beneficial responses to the flavonoid-rich chocolate on glucose homeostasis have been observed in overweight and obese compared to normal weight individuals. In most of the human studies described, while blood insulin levels were not affected by consumption of cocoa-rich foods, improvements of insulin sensitivity were observed through the measurement of HOMA-IR, quantitative insulin sensitivity check index (QUICKI), insulin sensitivity index (ISI), and glycaemia (Reviewed in Refs. [67]).

In the majority of clinical trials, EC was provided as a component of cocoa beverages or chocolate bars. However, two studies showed that a 4-weeks consumption period of pure EC (100 mg/day) improves insulin sensitivity by either decreasing HOMA-IR values in healthy men and women [80] or by improving fasting insulin levels and IR (decrease in HOMA-IR and HOMA-β) in adults with high blood pressure [81], compared to a placebo group. *In vitro* studies in hepatocytes (HepG2 cells) [82,83] and adipocytes (3T3-L1) [84] further support the beneficial effects of EC [82,84] and SREM [83] on glucose metabolism. These studies showed that EC and SREM can have direct effects on glucose homeostasis in cells (HepG2 and 3T3-L1) by: i) increasing the activating tyrosine phosphorylation of the insulin receptor and insulin receptor substrate-1 (IRS-1) upon insulin stimulation; ii) mitigating NADPH oxidase upregulation and oxidant production; iii) preventing the oxidant-mediated activation of the redox-sensitive JNK and IKK kinases, which inhibit the insulin cascade by phosphorylating IRS-1 in serine residues; and iv) inhibiting gluconeogenesis via the phosphatidylinositol-3-kinase (PI3K)/AKT and 5′-AMP-activated protein kinase (AMPK) pathways. Fig. 3 summarizes the mechanisms of EC mitigation

of inflammation and nutrient overload leading to improved insulin sensitivity.

Even when the beneficial effects of EC on IR have received support, it should be taken into account that some clinical trials, with short-term [85,86] and long-term [87] consumption of cocoa-based beverages, in both obese and normal weight subjects at risk of IR and T2D, did not observe significant changes in glucose and insulin responses. These controversial results could be due to several limitation factors of the described clinical trials, such as the low number of individuals involved in the studies, the complex composition of extracts/foods provided and the lack of appropriate methods to estimate daily EC intake [73,88]. For instance, the used EC-rich foods, especially dark chocolate, contain other flavan-3-ols, PAC and substances (e.g. caffeine, methylxanthines and theobromine), which have been shown to have *per se* mitigating actions on metabolic disorders and glucose metabolism [89,90]. In terms of assessment of EC intake and bioavailability, a recent study from Ottaviani et al. [91] evaluated the possibility of using EC metabolites in urine as biomarkers of EC intake [91]. This study represents a starting point in the optimization of clinical trials focused on the beneficial health effects of EC.

In conclusion, studies have shown mostly that EC and EC-rich foods can improve insulin sensitivity, further clinical trials involving more individuals and using pure EC, are required to confirm the potential beneficial actions of this flavan-3-ol on glucose homeostasis in humans.

6. EC and dyslipidemia

Dyslipidemia is defined as abnormal levels of lipids in blood (e.g. cholesterol, triglycerides (TG), and phospholipids). This condition can be caused by genetic mutations or by secondary causes, such as obesity and its associated dysmetabolism [92,93]. Prevalence of dyslipidemia positively correlates with increased BMI [94] and it is widely accepted that this undesired lipid alteration could lead to the development of atherosclerosis, CVD, NAFLD and T2D [95–97]. In obese individuals, dyslipidemia is characterized by: i) elevated plasma levels of TG, FFA and very low density lipoprotein cholesterol (VLDL-C); ii) decreased high-density lipoprotein cholesterol (HDL-C) levels; iii) increased plasma apolipoprotein (Apo) B concentration; and iv) increased small dense low density lipoprotein (LDL) levels rather than LDL-C concentration [98,99].

EC modulates dyslipidemia in rodent models of obesity [62,63,100–102]. In C57BL/6J mice, consumption of a high-fat diet for 15 weeks caused dyslipidemia with increased plasma levels of TG, FFA and total cholesterol. EC supplementation (20 mg EC/kg BW) prevented both high fat diet-mediated increases in plasma TG and FFA levels, not affecting those of cholesterol [62]. Rats fed a high fructose diet showed increased plasma levels of TG and LDL-C, decreased HDL-C, and no changes in plasma total cholesterol. An 8 week supplementation with EC (20 mg/kg BW) prevented high fructose-induced increases in TG and LDL-C, improving HDL-C levels [63]. In rats fed a high fat diet for 5 weeks, daily gavage of EC (1 mg/kg BW) for the subsequent 2 weeks improved hypertriglyceridemia. The latter suggests that EC could be effective in treating obesity-associated hypertriglyceridemia [103].

In humans, an oral supplement of EC (1 mg/kg BW) decreased postprandial plasma TG concentration which was proposed to be due to the promotion of fat oxidation both in normal and overweight subjects [104]. Overall, effects were more prominent in overweight subjects [104]. A daily dose of 100 mg of EC for 4 weeks, improved the TG/HDL-C ratio in hypertriglyceridemic subjects [105]. However, daily intake of 25 mg EC for 2 weeks did not significantly affect TG or total cholesterol, LDL-C, HDL-C and oxidized LDL levels in overweight-to-obese adults [106]. Those differences in response to EC highlight that its hypolipidemic efficiency depends on both dose and intervention duration.

In terms of PAC, cocoa-rich foods or beverages were shown to be effective lowering cholesterol absorption, the expression of the LDL-C receptor and, at some extent, plasma TG levels in overweight/obese subjects [107]. Khan et al. investigated the effects of long-term (4 weeks) cocoa consumption in high risk subjects (overweight/obese and/or with family history of premature CVD) [108]. In this study, cocoa consumption (46 mg EC and 426 mg PAC in 40 g cocoa powder) increased HDL-C and decreased oxidized LDL levels [108], having no effects on total cholesterol and TG levels. Consistently, acute cocoa supplementation (40 mg EC and 201 mg PAC in 20 g cocoa powder) increased postprandial HDL-C in obese adults with T2D after consumption of a high fat-containing breakfast [109]. Compared to placebo, cocoa consumption increased postprandial HDL-C levels, while no effects were observed on plasma levels of TG, total cholesterol and LDL-C. Overall, the above studies suggest that consumption of EC and EC/PAC-rich foods could constitute a dietary strategy to mitigate obesity-associated dyslipidemia.

EC and PAC could ameliorate dyslipidemia through different mechanisms [110]. Among them, and as described in a previous section, it has been proposed that EC and PAC could decrease lipid absorption by inhibiting pancreatic lipase activity in the GI lumen. Additionally, cocoa extracts decreased the micellar solubility of cholesterol, which was proposed to underlie their capacity to inhibit cholesterol absorption in rats [111]. Besides the regulation of lipid absorption, EC and PAC could act modulating enzymes and cell signals involved in lipid metabolism. Thus, EC and PAC regulate the expression of transcription factors involved in TG and cholesterol synthesis, including PPARγ, fatty acid binding protein/AP-1, CCAAT/enhancer-binding proteins (C/EBPs) and sterol regulatory element–binding protein (SREBPs) [68,112,113]. Inhibition of WAT inflammation, and consequently of lipolysis, could also mediate EC's capacity to decrease circulating FFA [62] (Fig. 3). In addition, EC and PAC could target miRNAs such as miR-33, miR-122 and miR-483-5p, which have been proposed as regulators of lipid metabolism in adipose tissue and liver [114–116].

Although there is experimental support both *in vivo* and *in vitro* for an action of EC improving dyslipidemia, in human studies the results are variable. Thus, more clinical trials are needed to fully understand the potential beneficial effects of EC on obesity-induced dyslipidemia.

7. EC and cardiovascular disease

Cardiovascular disease (CVD) comprises a group of heart and blood vessels disorders including, among others, coronary heart disease, cerebrovascular disease, heart failure, congenital and rheumatic heart diseases, peripheral arterial disease, deep vein thrombosis and pulmonary embolism. According to the WHO, CVD is the first cause of death globally, with 17.9 million deaths reported in 2016 [117]. In 2017, the Global Burden of Disease Study showed that non-communicable diseases account for ~73% of total deaths worldwide, CVD comprising the largest percentage (43%; 17.8 million deaths) [118].

It is widely recognized that obesity, as well as its associated comorbidities such as hypertension, hyperglycemia, insulin resistance and hyperlipidemia, put individuals at risk of CVD [119,120]. Diet plays a key role in controlling these modifiable risk factors. In this regard, an inverse relationship was observed between flavan-3-ols intake, i.e. cocoa consumption, and the risk for CVD [121,122]. The cardiovascular protective capacity of flavanol-rich foods is at a large extent attributed to the presence of EC [123]. The involved beneficial actions of EC include, among others, the inhibition of inflammation and oxidative stress, the promotion of vasodilation and lowering of blood pressure and the modulation of coagulation mechanisms.

Very relevant to CVD are obesity-associated increased oxidative stress, activation of redox sensitive signals and inflammation, which all contribute to tissue damage. At the concentration of EC and SREM found in tissues other than the GI tract, EC cannot act as a direct antioxidant but can regulate the production of oxidants [124] and modulate redox signaling [125,126]. Inflammation and oxidative stress crosstalk in the promotion and/or perpetuation of CVD. As previously described, it is well stablished that obesity-associated remodeling of the

adipose tissue generates a chronic systemic inflammatory state. Obesity-associated increase in circulating levels of adipocyte- and infiltrated inflammatory cells-derived cytokines, such IL-6, C-reactive protein, MCP-1, TNFα, leptin and resistin, can directly and indirectly affect the cardiovascular system [127,128]. Several human studies showed that regular consumption of cocoa can mitigate systemic inflammation and oxidative stress, reducing plasma concentration of pro-inflammatory cytokines, and consequently protecting against CVD [129–132]. Although evidence is limited, mitigation of obesity-related oxidative stress and systemic inflammation can in part contribute to the capacity of EC and EC/PAC-rich foods to protect against CVD in obesity (Fig. 3).

The obesity-associated chronic inflammatory state could lead to hypertension and endothelial dysfunction. Since the first evidence of an anti-hypertensive effect of cocoa in the Kuna Indians [133], several epidemiological and clinical studies have confirmed the positive effects of EC-containing foods decreasing blood pressure and improving blood flow [77,129,134–136]. Interestingly, the oral intake of pure EC mimics the effect of high-flavanol cocoa consumption on flow-mediated vasodilation, and the peak of plasma SREM matches the maximum effect on endothelial function [123]. Although the mechanisms underlying the hypotensive- and endothelial-protective effects of EC are not fully understood, existing evidence supports the concept that EC acts improving nitric oxide (NO) bioavailability [123,137–140]. In this regard, EC increases endothelial nitric oxide synthase (eNOS) phosphorylation levels at Ser1177 causing the activation of the enzyme. Phosphatidylinositol 3-kinase and Ca^{2+}/calmodulin-dependent kinase II signaling pathways are in part involved in EC-mediated eNOS activation [141]. In addition, EC favors NO bioavailability through the inhibition of NADPH oxidase and consequent reduction of superoxide production [142]. A decreased production of superoxide by NADPH oxidase increases NO availability, given that NO reacts with superoxide to generate peroxynitrite. Additionally, EC could control the vascular tone through the modulation of endothelium-mediated vasodilation. Indeed, EC reduces plasma concentration of the potent vasoconstrictor endothelin-1 (ET-1) in healthy humans [137]. Finally, a relevant factor in the long-term regulation of blood pressure in obesity is the beneficial actions of EC on kidney structure and function. In this regard, EC supplementation reduces kidney inflammation and oxidative stress restoring NO bioavailability and mitigating hypertension in high fructose-fed rats [143,144].

The cardioprotective effects of EC could be also explained by its capacity to modulate hemostasis, coagulation and fibrinolysis [145], all processes dysregulated in obesity. In support of this, acute cocoa beverage consumption (48 mg EC + PAC/g cocoa powder) improves platelet function and primary hemostasis in healthy human subjects [146]. Long-term (4 weeks) supplementation with cocoa tablets providing 234 mg EC + PAC/day also reduced platelet aggregation, activation and degranulation in healthy individuals [147]. In cells and plasma isolated from human blood, EC had anti-platelet aggregation, anti-coagulant and pro-fibrinolytic effects [148], suggesting that this flavonoid might have a mitigating effect on the prothrombotic state favored by obesity.

Cardiovascular disorders are also associated with ectopic fat accumulation in liver, hyperglycemia, insulin resistance and hyperlipidemia. The effects of EC on these CVD risk factors are discussed in separate sections of this review. Despite a large body of evidence suggesting protective effects of EC and EC-rich food on obesity-related cardiovascular disorders, a general conclusion and/or public health recommendation is still missing. Lack of standardization of EC-containing food or dietary supplements used in the different studies, disparities in study design and treatment duration, few human studies using pure EC and additional knowledge on the mechanisms by which EC exerts its cardiovascular protection are aspects that still need to be addressed.

8. EC and non-alcoholic fatty liver disease (NAFLD)

NAFLD is defined as excess of fat accumulation (> 5–10%) in hepatocytes in the absence of secondary contributing factors such as drugs and alcohol consumption. NAFLD is becoming the main cause of chronic liver disease worldwide [149]. NAFLD is characterized by different stages: i) non-alcoholic fatty liver (NAFL or steatosis); ii) non-alcoholic steatohepatitis (NASH); iii) fibrosis; iv) cirrhosis; and ultimately, v) hepatocarcinoma [150]. Together with IR, T2D and hyperlipidemia, obesity is considered among the main risk factors for the development of NAFLD and its subsequent progression up to liver cancer [151]. NAFLD increases in parallel with the global rise in obesity [152]. While the estimation of the prevalence of NAFLD in the general population is 25% [149,153], in obese individuals it is estimated to be up to 90% [154]. Furthermore, the prevalence of liver steatosis and progression to NASH strongly correlates with BMI. In fact, NAFLD affects 65 and 85% of individuals with BMI between 30 and 39 kg/m^2 and 40–59 kg/m^2, respectively [155]. Given this association between obesity and NAFLD, it has been estimated that by 2030 the prevalence of NAFL and NASH will increase worldwide by 21% and 63%, respectively [156].

Changes in dietary habits could mitigate the onset and progression of NAFLD. Very few studies have addressed the effects of EC or EC-rich foods on NAFLD-associated events either *in vitro*, in rodent models or in clinical trials. In a mouse model of high fat diet-induced obesity, EC supplementation (2–20 mg/kg BW) prevented both hypertriglyceridemia and the increased deposition of TG in the liver [22]. This was accompanied by the mitigation of increase in plasma alanine aminotransferase (ALT) activity, a parameter of liver damage, and of liver NAFLD score (NAS). EC also prevented the increase of MCP-1, TNFα, inducible nitric oxide synthase (iNOS), and the macrophage marker F4/80 in the liver [22].

Inflammation, ER stress, increased oxidant production and oxidative stress [157,158] are major players in the development and progression of NAFLD (Fig. 3). The associated insulin resistance also contributes to NAFLD pathogenesis by promoting *de novo* lipogenesis [159]. In rats fed a high fructose diet, EC supplementation (20 mg/kg BW) inhibited the hepatic deposition of TG, mitigated liver insulin resistance, oxidative and ER stress, inflammation and activation of redox-sensitive signaling pathways which amplify these adverse events [63]. Similar results were observed in rats fed a high fat diet and gavaged with 10–40 mg EC/Kg BW [112]. In agreement with *in vivo* findings, studies in HepG2 cells showed that at 0.25–1 μM concentrations, EC and SREM mitigated palmitate-induced upregulation of the NADPH oxidase subunits NOX3, NOX4 and p22phox, excess oxidant production, lipid and protein oxidative modifications (4-hydroxynonenal-protein adducts (4-HNE)), the activation of redox sensitive signaling pathways and insulin resistance [83]. Interestingly, EC protective effects mimic those observed with the NADPH oxidase inhibitors apocynin and VAS2870. These *in vitro* and *in vivo* findings suggest that inhibition and/or downregulation of NADPH oxidase could be a strategy to mitigate fatty acid liver deposition.

In humans, increased levels of oxidants and oxidative stress are also associated to the development and progression of NAFLD from steatosis to NASH [158,160]. Thus, serum levels of soluble NOX2, a proposed marker of NADPH oxidase NOX2 activation, are high in patients diagnosed with NASH compared to healthy individuals [158]. Patients diagnosed with NASH consuming 40 mg/day of dark chocolate (> 85% cocoa) for 14 days, showed lower serum levels of ALT, soluble NOX2 and isoprostanes, the latter a product of lipid oxidation, compared to patients consuming milk chocolate (< 35% cocoa) [160].

Hepatic dysregulation of lipid metabolism is critical to NAFLD pathogenesis. It has been proposed that the activation of SREBP-1c, which regulates transcription of genes implicated in fatty acid synthesis, is involved in NAFLD progression [161]. Two interrelated pathways regulate the activation of SREBP-1c: sirtuins (especially sirtuin 1; SIRT1) and SREBP-SCAP [162–164]. Increased levels of SIRT1 promote

deacetylation of both SREBP-1c and carbohydrate-response element-binding protein (ChREBP) promoters, leading to the inhibition of lipogenic pathways [165]. In parallel, inhibition of SCAP prevents the activation of SREBP-1c which results in the inhibition of *de novo* fatty acid synthesis [164]. Thus, these two pathways could be a target to mitigate NAFLD progression. In this sense, two studies showed that daily gavage of EC (1 mg/kg BW [68] or 10–40 mg EC/kg BW [112]) reduced SIRT-1 expression and upregulation of SREBP-1c and SCAP in liver and adipose tissue of rats fed a high fat diet. These findings emphasize the potential beneficial effect of EC on the regulation of lipogenesis and consequent mitigation of lipid hepatic accumulation.

As discussed in other sections of this review, EC mitigates several obesity-associated events that are involved in NAFLD development. However, the number of studies conducted *in vitro*, in rodents and especially in humans, addressing the potential beneficial effects of EC on the development and progression of NALFD, is very limited.

9. EC, neuroinflammation and behavior

Obesity has been associated with cognitive dysfunction and neurological disorders, such as mild cognitive impairment, dementia, and Alzheimer's disease [166–168]. A higher BMI has been linked to lower cognitive function [169–171] and atrophy in the hippocampus [172], a region of the brain important for learning and memory. The improvement in cognitive function [173,174] and changes in brain structure [175,176] reported after weight loss induced by bariatric surgery further support that obesity might have adverse effects on the brain. Studies in animal models of obesity indicate that obesity-induced inflammation is manifested not only in the periphery but also in the central nervous system (CNS), contributing to neuroinflammation and impairment in cognitive function [177–179].

Epidemiological evidence demonstrates that consumption of EC-rich cocoa flavanols could improve memory and executive function, especially in older adults (reviewed in Refs. [180]). For instance, in a controlled randomized trial, healthy 50-69-year-old men and women who received a high flavanol supplement containing 900 mg cocoa flavanols (138 mg of EC daily) showed a significant improvement in dentate gyrus-associated cognitive performance compared to a matched low-flavanol group [181]. Of note, the improvement in cognition was equivalent to around three decades of life. Moreover, the cerebral blood volume in the body of the hippocampal circuit significantly increased in the high-flavanol group compared to the low-flavanol group [181]. In rodent models, EC improved spatial memory in conjunction with increasing hippocampal angiogenesis and neuronal spine density [182], mitigated doxorubicin-induced brain toxicity by attenuating pro-inflammatory mediators including TNFα, NF-κB, and iNOS [183], and decreased anxiety through the modulation of monoaminergic and neurotrophic signaling pathways [184]. Studies in humans and animal models demonstrate a potential neuroprotective capacity of EC, which appears as a beneficial bioactive that could also mitigate obesity-related neuroinflammation and cognitive decline.

EC could have indirect and direct effects on obesity-associated neuroinflammation. In terms of indirect effects, the capacity of EC to mitigate LPS transcellular or paracellular transport in the intestinal epithelium, can lead to decreased endotoxin-mediated neuroinflammation (Fig. 3). EC can also act improving cerebral blood flow [180], through mechanisms previously discussed. In the case of direct effects, if EC, SREM or smaller microbiota-generated metabolites could reach the brain, they could directly act inhibiting inflammatory cascades. In fact, in the brain of Tg2576 AD transgenic mice consuming a flavan-3-ol-rich grape powder both catechin and EC glucuronidated derivatives were detected [185]. The EC microbial metabolite 5-(hydroxyphenyl)-γ-valerolactone-sulfate was also detected in the brain of mice, rats and pigs after either i.p. injection of the metabolite or upon EC oral consumption [182]. We recently observed that EC supplementation (20 mg EC/kg BW) improves parameters of neuroinflammation and behavior in high fat diet-induced obesity in mice [186]. Consumption of a high fat diet for 13 weeks caused metabolic endotoxemia and upregulated neuroinflammatory markers including Toll-like receptor 4 (TLR4), ionized calcium binding adaptor molecule 1 (Iba-1), and NOX4 in the hippocampus, in conjunction with impaired recognition memory [186]. EC supplementation prevented all these changes, supporting the beneficial effects of an EC-rich diet on obesity-induced neuroinflammation and altered cognition. EC supplementation also upregulated brain-derived neurotrophic factor (BDNF) expression in the hippocampus which in part could explain the observed improved recognition memory in mice. BDNF is a neurotrophin that by regulating hippocampal long-term potentiation is involved in learning and memory processes. In fact, BDNF deletion within the dorsal hippocampus of mice significantly impaired learning and memory [187]. Other studies have shown that BDNF is increased in blood and tissues upon EC or cocoa consumption. In an adult human population (62–75 y of age), consumption of a high flavanol cocoa drink for 12 week resulted in an increase in serum BDNF which was associated with an improvement in global cognitive performance [188]. In mice, long-term (14 weeks) administration of 4 mg EC/day caused an increase in BDNF levels in the hippocampus, which was paralleled by a decrease in anxiety [184].

Currently, most of the work investigating the beneficial effects of EC on human cognition has been attributed to its capacity to modulate cerebral blood flow [180]. However, EC and/or its metabolites could also directly contribute to improvements in cognition, for instance, by mitigating obesity-induced neuroinflammation and increasing hippocampal BDNF [184,186]. Moreover, to the best of our knowledge, no study has been conducted to investigate the effect of pure EC isolated from other flavanols or cocoa products on human cognition. Such studies will need to be carried out to be able to understand singular as well as synergistic effects of EC on cognition. More studies, particularly in obese humans, will also need to be conducted to support the idea that consumption of EC-rich diets could mitigate CNS inflammation and contribute to improve cognition in obesity.

10. Summary

Extensive experimental evidence from rodent studies, and at a lesser extent from human studies, supports a beneficial action of EC and EC/PAC-rich foods to mitigate several comorbidities of obesity. These pathologies have common mechanisms of development and progression that include, among others, exposure of tissues to high levels of glucose and lipids, chronic systemic inflammation, tissue endoplasmic reticulum and oxidative stress, insulin resistance, mitochondria dysfunction and dysbiosis. EC modulates several of these adverse events, which can explain its capacity to mitigate multiple obesity-associated comorbidities (Fig. 3). In particular, the capacity of EC to decrease oxidant production (e.g. by modulating NADPH oxidase expression and activity), and to reduce oxidative stress and the activation of redox-sensitive and pro-inflammatory cascades, emerge as central mechanisms in the beneficial actions of EC in the comorbidities of obesity.

However, understanding of the underlying mechanisms and of the extrapolation of the observed effects to human populations is still limited. When considering human studies, there is a need of a careful characterization of the EC-dietary source used, of using as supplements pure EC instead of complex mixtures, and of standardization in study design. The availability of markers of EC intake, involving the characterization of the presence of EC and its metabolites in human urine, blood, and feces would allow a better evaluation of its beneficial health effects.

Declaration of competing interest

Authors have no conflict of interest to declare.

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