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MODULATION OF OBESITY-RELATED INFLAMMATION BY COCOA

(*THEOBROMA CACAO* L. STERCULIACEAE)

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ABSTRACT

Obesity is associated with many metabolic diseases and it is now recognized as a state of chronic inflammation. Cocoa (*Theobroma cacao* L. Sterculiaceae) is a rich source of polyphenols, in particular oligomeric flavan-3-ols (i.e. procyanidins) which have received considerable attention due to potential health beneficial effects including anti-inflammatory activities. However, the modulation of inflammatory and metabolic effects by cocoa has not been fully elucidated and there are still gaps and inconsistencies in the literature. In my thesis research, I hypothesize that dietary cocoa and cocoa procyanidins (CPs) can exert anti-inflammatory activity and modulate obesity-related pathologies via inhibition of digestive enzymes, suppression of cytokine and eicosanoid production, regulation of nuclear factor-κB (NF-κB) target gene expression and modulation of metabolic endotoxemia. Results from this research would provide a comprehensive body of scientific evidence for the modulatory effects of cocoa on obesity-related inflammation, as well as mechanistic insights into the understanding of its *in vivo* anti-inflammatory actions. This research is significant to public health, and both the pharmaceutical and food industries.

To investigate this hypothesis, I first investigated the *in vitro* inhibitory activities of three cocoa extracts and isolated CPs with degree of polymerization (DP) of 2-10 against three purified digestive enzymes: pancreatic α-amylase (PA), pancreatic lipase (PL) and phospholipase A₂ (PLA₂). These enzymes, all of which play an important role in starch or lipid digestion, represent attractive targets for reducing macronutrient absorption and the prevention of obesity and its co-morbidities. CPs dose-dependently inhibited PA, PL, and PLA₂, and the inhibitory potency increased as a function of DP ($R^2 > 0.93$). Extract of *Lavado* cocoa, which is non-fermented and non-alkalized, was the most potent enzyme inhibitor among the three extracts ($IC_{50} = 8.5-47 \mu g/mL$). The type of inhibition was characterized by kinetic analysis. Results suggest that regular cocoa extract, the CP pentamer (DP=5) and decamer (DP=10) inhibited PL activity in a mixed mode. The pentamer and
decamer non-competitively inhibited PLA₂ activity, whereas the regular cocoa extracts inhibited PLA₂ competitively. This study demonstrates that cocoa rich in procyanidins can inhibit digestive enzymes *in vitro*, which may provide a safe and cost-effective approach for weight management and glycemic control.

Second, given the greater bioactive potential of CPs with higher DP and gaps in the literature regarding their anti-inflammatory activities, I examined the anti-inflammatory effects of high DP CPs on modulation of eicosanoid metabolism and cytokine production *in vitro*. Eicosanoids and pro-inflammatory cytokines are both important mediators in the establishment and maintenance of a state of obesity-related inflammation. In this study, a murine macrophage RAW 264.7 cell line stimulated by bacterial endotoxin (lipopolysaccharide, LPS) was used as a model of inflammation. Results indicate that CPs mixture with DP ≥ 7 dose-dependently inhibited eicosanoid-generating enzymes (PLA₂ and cyclooxygenase-2 (COX-2)) *in vitro*, and significantly decreased the production of their metabolite prostaglandin E₂ (PGE₂) in LPS-stimulated RAW 264.7 macrophages. In addition, CP octamer suppressed pro-inflammatory cytokine production, including tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and monocyte chemoattractant protein-1 (MCP-1). In particular, CP octamer (100 μM) without cytotoxicity significantly decreased the production of IL-6 and MCP-1 by 97% and 70%, respectively in macrophages pre-challenged with LPS. These results suggest that high-DP CPs may counteract the effect of LPS and facilitate the resolution of inflammation. Together, this study demonstrated an anti-inflammatory role and potential therapeutic value of procyanidin-rich cocoa in the diet of patients with inflammatory conditions.

Third, based on the observed modulatory effects of cocoa on digestive enzymes and inflammatory markers *in vitro*, I designed studies to examine the effect of cocoa supplementation on markers of obesity-related inflammation in a mouse model. In this study, male C57BL/6J mice were fed with either a low-fat (LF, 10% kcal from fat) or a high-fat (HF, 60% kcal from fat) diet. After 8 wk, HF-fed mice were randomized into two groups: half were maintained on the HF diet and half were fed with a HF diet supplemented with 8% unsweetened cocoa powder (HF-
HFC group) for 10 wk. At the end of wk 18, blood and tissue samples were collected for biochemical analyses. Results show that cocoa supplementation significantly reduced the rate of body weight gain (15.8%), and this was accompanied by increased fecal lipid content (55.2%) compared to HF-fed control mice. Further, it counteracted obesity-induced insulin resistance and fatty liver disease compared to the HF group. Cocoa supplementation also significantly decreased plasma levels of IL-6 (30.4%) and MCP-1 (25.2%), and increased adiponectin (33.7%) compared to HF-fed mice. Expression of pro-inflammatory genes (Il6, Il12b, Nos2 and Emr1) by adipose tissue macrophages was significantly reduced (37-56%) in the cocoa-supplemented mice. Therefore, in this animal study, I've shown that short-term dietary supplementation with cocoa can ameliorate HF-induced body weight gain, systemic inflammation, insulin resistance and fatty liver disease in mice.

Finally, I conducted another mouse study to examine the effects and mechanisms of cocoa on adipose tissue (AT) inflammation through the alteration of eicosanoid metabolism, regulation of NF-κB target gene expression, and modulation of metabolic endotoxemia in HF-fed mice. In HF-induced obesity, AT has a central role in lipid and glucose metabolism and produces a number of hormones and cytokines that predispose to the development of chronic inflammation. In this study, male C57BL/6J mice were fed a LF diet, a HF diet, and a HFC diet for 18 wk. Results demonstrate that cocoa supplementation significantly decreased pro-inflammatory gene expression (Tnfa, Il6, iNos and Emr1) in AT by 40-60% compared to the HF group, and this was accompanied by decreased protein expression of NF-κB. AT arachidonic acid (AA) levels, an inflammatory eicosanoid precursor, were positively correlated with adiposity (r = 0.57) and cocoa-treated mice had 33% lower levels of AA compared to HF controls. Moreover, protein expression of the eicosanoid-generating enzymes adipose-specific PLA2 (AdPLA) and COX-2 were also reduced in cocoa-treated mice by 53% and 55%, respectively. Finally, plasma levels of endotoxin were found to be 40% lower than that of HF-fed mice. This decrease in metabolic endotoxemia was in parallel with increased a gut barrier function marker (glucagon-like peptide -2, GLP-2). Overall, this study has shown for the first time
that long-term cocoa supplementation reduces AT inflammation in HF-fed mice by down-regulating NF-κB target gene expression and modulating eicosanoid metabolism, which may be partly due to decreased metabolic endotoxemia by cocoa.

In conclusion, cocoa exerted anti-inflammatory activity and modulated obesity-related pathologies \textit{in vitro} and \textit{in vivo}. These effects appear to be mediated in part by inhibition of digestive enzymes and modulation of metabolic endotoxemia. As a result, moderate consumption of cocoa could be part of a healthy diet to prevent and treat obesity-related inflammation and resultant co-morbidities.
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Chapter 1

Introduction and Literature Review: Modulation of Obesity-Related Inflammation by Cocoa
Abstract

Cocoa (*Theobroma cacao* L. Sterculiaceae) is a rich source of polyphenols, in particular oligomeric flavan-3-ols (i.e. procyanidins). Although typically found in high fat, high sugar foods such as chocolate, recent studies have suggested that consumption of cocoa and cocoa products have a positive influence on metabolic risk factors such as blood pressure, cholesterol levels, insulin resistance as well as inflammatory markers. There is increasing evidence that chronic inflammation represents a potential mechanistic link between obesity and its related pathologies: insulin resistance, dyslipidemia and hypertension, which comprise the metabolic syndrome. In this review, the molecular targets by which cocoa and cocoa flavan-3-ols modulate obesity-associated inflammation are discussed. Available data from recent studies regarding the modulation of metabolic syndrome-related inflammation by cocoa and cocoa-derived compounds are reviewed, with emphasis on studies using laboratory animals or human subjects since such studies often represent the strongest available evidence for biological effects. *In vitro* studies are included to provide some mechanistic context, but are critically interpreted. Although the available data seem to support the anti-inflammatory effects of cocoa, continued research, both in model systems and human subjects, is needed to better understand the influence and elucidate the underlying mechanisms of cocoa on metabolic disorders and inflammation.

**Keywords:** cocoa, *Theobroma cacao*, procyanidin, inflammation, obesity
Abbreviations:

5-HpETE, 5-hydroperoxy-6E,8Z,11Z,14Z-eicosa- tetraenoic acid; ALT, alanine aminotrasnferase; AMPK, 5’ AMP-activated protein kinase; ATM, adipose tissue macrophage; BG, blood glucose; BMI, body mass index; BP, blood pressure; BW, body weight; CLPr, cocoa liquor procyanidin extract; CMC, carboxymethyl cellulose; COX, cyclooxygenase; CP, cocoa procyanidin; CPE, cocoa polyphenol extract; CRP, c-reactive protein; CVD, cardiovascular disease; DBP, diastolic blood pressure; DP, degree of polymerization; DSS, dextran sulfate sodium; EC, (-)-epicatechin; FMD, flow-mediated dilation; GLUT4, glucose transporter type 4; HDL, high-density lipoprotein cholesterol; HFD, high-fat diet; ICAM-1, intercellular adhesion molecule-1; IL, interleukin; iNOS, inducible nitric oxide; LDL, low-density lipoprotein cholesterol; LOX, lipoxygenase; LPS, lipopolysaccharides; LT, leukotriene; MAPK, mitogen-activated protein kinases; MCP-1, monocyte chemoattractant protein-1; MDI, isobutylmethylxanthine, dexamethasone and insulin; NAFLD, nonalcoholic fatty liver disease; NF-κB, Nuclear Factor-Kappa B; NO, nitric oxide; n.s., not significant; PBMC, peripheral blood mononuclear cell; PG, prostaglandin; PHA, phytohemagglutinin; PMA, phorbol 12-myristate 13-acetate; ROS, reactive oxygen species; QUICKI, quantitative insulin sensitivity check index; SBP, systolic blood pressure; T2D, type 2 diabetes; TG, triglycerides; TNF-α, tumor necrosis factor-α; TXB2, Thromboxane B2.

Introduction

The prevalence of obesity is increasing and has become a significant public health issue. Today, more than 35% of Americans are obese and if the current trajectory continues, the rate will reach 51% by 2030[1]. Obesity is a multifactorial disorder resulting from improper balances of hormones and gene expression induced by the diet [2]. There is growing evidence that the state of chronic inflammation represents the important link between obesity and its co-morbidities, including type 2 diabetes (T2D), cardiovascular disease (CVD), nonalcoholic fatty liver disease (NAFLD), and certain cancers [3–5]. The obesity-related inflammation is characterized by increased macrophage infiltration into adipose tissue, abnormal cytokine production and eicosanoid metabolism [6–8].

One strategy for the prevention of obesity and obesity-related diseases is the use of agents that inhibit digestive enzymes, including pancreatic α-amylase (PA), pancreatic lipase (PL) and phospholipase A2 (PLA2), which are responsible for macronutrient digestion and absorption. Inhibition of digestive enzymes may provide a mechanism to decrease plasma free fatty acids and glucose, thereby ameliorating obesity and related metabolic disorders. A growing literature suggests that naturally occurring polyphenols, and in particular catechins and procyanidins isolated from teas, berries and other plants, have been shown to inhibit digestive enzymes in vitro and in vivo [9–11].

Another potential strategy to treat obesity and its co-morbidities is the use of anti-inflammatory intervention. Dietary polyphenols have emerged as potentially efficacious and cost-effective alternatives to pharmacological anti-inflammatory treatment (e.g. non-steroidal anti-inflammatory drugs (NSAIDs) and aspirin) [12]. A growing body of evidence has shown that polyphenols from grape seeds, spices and other plants express their anti-inflammatory activity in vitro and in vivo [4,7,13]. Although not fully understood and inconsistency still existed, several mechanisms of action are proposed, including the modulation of pro-inflammatory cytokines,
eicosanoids metabolism, and other major metabolic and immunological pathways [14,15].

Cocoa (*Theobroma cacao* L. Sterculiaceae) is a rich source of polyphenols, such as monomeric (epicatechin and catechin) and oligomeric (procyanidins) flavan-3-ols. Procyanidins are significantly more represented in cocoa when compared to other flavan-3-ol-rich foods such as red wine or green tea [14,16]. Procyanidins with a degree of polymerization (DP) up to decamer have been identified in cocoa [17], and several studies have suggested that DP appears to be an important factor determining the potency of their biological activities. Cocoa polyphenol and flavan-3-ol contents are drastically affected by cocoa processing. Fermentation and “Dutching” (also known as alkalization) have been reported to result in the loss of as much as 90% of the cocoa flavan-3-ols [18].

Over the last decades, a growing number of studies have reported the health benefits of cocoa and cocoa flavan-3-ols, particularly reduced risk of cardiovascular diseases via effects on lowering blood pressure, anti-inflammation, anti-platelet function, elevated HDL and decreased LDL oxidation [19–21]. Recently, a few reports have also demonstrated preventative effects of cocoa and cocoa products on obesity and diabetes in animal models [22–24]. In addition, several *in vitro* studies have shown that certain cocoa flavan-3-ols and procyanidins exert anti-inflammatory activities by modulating the secretion and transcription of pro-inflammatory cytokines in human peripheral blood mononuclear cells, and macrophages [25–28]. However, it should be noted that the evidence for any beneficial effects of cocoa procyanidins in providing a meaningful anti-inflammatory action has been gathered from *in vitro* experiments [29]. The knowledge on the *in vivo* molecular action of anti-inflammatory effects of cocoa is still limited and obscure. Moreover, there is a lack of knowledge and evidence supporting the role of cocoa in inflammation under obese conditions. Therefore, studies are needed to clearly establish the effects of cocoa on obesity-related inflammation, to determine unambiguously if this benefit does indeed exist *in vivo*, and to further elucidate the mechanisms of action.
Given recent animal model studies showing that dietary intake of cocoa might be beneficial in preventing obesity, as well as cocoa being a rich source of anti-inflammatory procyanidins, studies are warranted to determine the inhibition of key digestion enzymes by cocoa and the effect of cocoa on obesity-related inflammation *in vivo*. 
Obesity and Inflammation

Obesity is defined as a phenotypic manifestation of abnormal or excessive fat accumulation that alters health and increases morality [3]. Indeed, obesity is a multifactorial disorder that is a significant risk factor for T2D, CVD, NAFLD, and certain cancers [3–5]. Obesity and the associated metabolic pathologies (i.e. metabolic syndrome) are the most common and detrimental metabolic diseases, affecting over 50% of the adult population [6]. It is becoming more evident that obesity is a chronic inflammatory state, also known as low-grade or systemic inflammation, which represents the important link between obesity and its co-morbidities [3–5].

Inflammation is a biological defense and repair mechanism of the innate immune system in response to harmful stimuli, damaged cells and tissues, toxic chemicals and irritants, and thermal and mechanical stress [13]. Distinct from the acute (classical) inflammation, which is a helpful component of the body’s response to injury or infection, chronic inflammation is a persistent inflammatory process that causes progressive damage to the body that leads to a variety of diseases. This process can be seen in the involved tissues and systemically, in terms of elevated circulating levels of inflammatory markers[30]. Both types of inflammation (acute and chronic) are primarily driven by the production of pro-inflammatory eicosanoids derived from arachidonic acid (AA) [2].

Obesity is accompanied by chronic inflammatory responses, indicated by increased macrophage infiltration into adipose tissue (AT), AT dysfunction, abnormal cytokine production and the activation of inflammatory signaling pathways. A very interesting feature of the obesity-related inflammation is that it appears to be triggered, and to reside predominantly in AT, although other metabolically critical sites may also be involved during the course of the disease [7,79].

Traditionally, AT was considered to passively store triglycerides and release free fatty acids. Now, it is recognized to be an active endocrine organ and
communicates with other organ systems via increases in inflammatory mediators [32]. As a consequence, AT occupies the central position in controlling obesity-induced inflammation by acting as a fat-buffering system [2]. AT can be fractionated into lipid-containing adipocytes and into the stromal-vascular fraction (SVF)[33]. Current literature suggests that the initiation of AT inflammation begins with excessive intake of macronutrients leading to hypertrophy or necrosis of adipocytes, and causing increased recruitment of macrophages into the SVF [2,34]. The enlargement of the AT is also associated with the release of chemoattractant substances (e.g. monocyte chemotactic protein-1, MCP-1), that set off an inflammatory process via activation of macrophages [35,36]. Studies have shown that adipose tissue macrophage (ATM)-specific genes (e.g. Emr1) correlated positively with body mass and adipocyte size[37]. ATMs are highly inflammatory, secrete cytokines such as tumor necrosis factor (TNF-α), interleukins (IL-1, IL-6, IL-12), and contribute to the recruitment of additional macrophages by secreting chemokines including MCP-1. These newly released inflammatory cytokines can interact with their receptors at the surface of nearby adipocytes to signal a further activation of Nuclear factor-κB (NF-κB), the key gene transcription factor that drives the inflammatory responses of the innate immune system[2]. In addition, pro-inflammatory genes such as inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) are activated by NF-κB, which would further contribute to or worsen the progression of inflammation [3]. Thus, this vicious cycle of systemic inflammation forms the pathophysiological foundation of obesity and its comorbidities.
Potential Targets for Modulation of Obesity-Related Inflammation

Digestive Enzymes

One strategy for the prevention of overweight and obesity related disease is the use of agents that interfere with the hydrolysis and absorption of dietary carbohydrates and lipids (Figure 1.1). Pancreatic α-amylase (PA), pancreatic lipase (PL) and phospholipase A₂ (PLA₂), which are delivered into the intestinal lumen as constituents of pancreatic juices, are the major enzymes involved in the hydrolysis of dietary starch and fat. PA is an endoglucosidase that catalyzes the hydrolysis of starch to maltose and maltotriose [38]. PL is a key enzyme for absorption of dietary triglycerides and rapidly converts a triglyceride to a 2-monoglycerol and two free fatty acids [39]. Orlistat (marketed over-the-counter as Alli in U.S.), a potent competitive inhibitor of PL, is available as an anti-obesity drug. It has been reported that Orlistat promoted both short-term and long-term weight loss and minimized weight regain in overweight or obese subjects [40]. PLA₂ serves in the initial digestion of phospholipids to free fatty acids and lysophospholipid, which suppresses hepatic fatty acid oxidation leading to increased VLDL synthesis, decreased glucose tolerance, and promotion of tissue lipid deposition to accentuate diet-induced hyperlipidemia, diabetes, and obesity [41]. Given the key role of these three enzymes play in starch and lipid digestion, they represent attractive targets for prevention of obesity and of obesity-related diseases.
A growing literature has suggested polyphenols from teas, berries and other plants can inhibit some digestive enzymes in vitro and in vivo. For example, Horigome et al. reported that procyanidins from various plants (i.e. black locust, bush clover, wistaria and Japanese knotgrass) have inhibitory effects on lipase, α-amylase, and trypsin [42]. Studies have shown green tea catechins can inhibit the intestinal absorption of lipids in vivo [9,11]. This was associated with in vitro inhibitory activities of tea catechins against PLA2. Among the green tea catechins, EGCG is the most potent inhibitor, and it inhibited PLA2 in vitro by 64.9 % at 2 mM. Harach et al. reported rosemary leaf extract, containing 5-10% phenolic compounds, induced a significant reduction of weight and fat mass gain associated with an increase of fecal lipid excretion in high fat –fed mice, and this effect was related to the inhibition of PL activity by the extract [43]. Sugiyama et al. reported that the oligomeric procyanidins in apples significantly decreased the plasma triglyceride levels in both mice and humans, and inhibited PL activity in vitro. They also suggested that degree of polymerization (DP) of procyanidinds was an important factor in determining the inhibitory potency and a strong inverse correlation was observed [44]. Another study found that procyanidins from persimmon peel showed strong inhibitory activity against α-amylase in vitro (IC50 < 100 µg/ml), and the inhibition of α-amylase activity was dependent on the DP as well [45]. However, no
systematic study has been currently made of the inhibitory potential of the polyphenols in cocoa on digestive enzymes.

**Cytokine Production**

The production by AT and ATMs of pro-inflammatory cytokines is a critical step in the establishment and maintenance of a state of obesity-related inflammation (Figure 1.2), and is therefore, a primary target for anti-obesity and anti-inflammatory interventions [14].

**Figure 1-2** Adipose tissue macrophage-induced cytokine production

Among inflammatory cytokines, MCP-1 is a chemoattractive protein that is responsible for the infiltration of macrophages into AT [8]. Two independent studies convincingly showed that the adipocyte-specific over-expression of MCP-1 in mice was sufficient to increase macrophage recruitment to AT and cause systemic insulin resistance [46,47].

IL-6 is a pro-inflammatory cytokine expressed in both adipocytes and macrophages of the AT [48]. IL-6 decreases lipoprotein lipase activity, which results in increased macrophage uptake of lipids [7]. In addition, IL-6 is directly correlated with body mass index, visceral adiposity and insulin resistance in obese subjects [7,49]. IL-6 has been shown to directly impair insulin signaling in mouse
hepatocytes and 3T3 adipocytes via decreased activation of insulin receptor substrate 1 (IRS-1) and PI3 kinase as well as impaired insulin-induced glycogenesis in liver cells [50].

TNF-α, a pro-inflammatory cytokine, is expressed mainly by macrophages of the AT. TNF-α seems to act locally at the site of the AT, rather than acting systemically, through autocrine or paracrine mechanisms or both, inducing IL-6 secretion and expression of adhesion molecules [51]. TNF-α is also a critical mediator of insulin resistance, activating pro-inflammatory pathways, such as NF-κB and c-jun N-terminal kinase (JNK) [52,53].

In contrast to other cytokines, adiponectin is a pleiotropic anti-inflammatory cytokine, and its receptors are widely distributed in many tissues, including the liver [54]. Adiponectin is highly abundant in human serum but its levels are reduced in obesity and are even lower in patients with hepatic steatosis. Adiponectin antagonizes excess lipid storage in the liver and protects from inflammation and fibrosis [55]. Adiponectin, blocks NF-κB activation, and inhibits release of TNF-α, IL-6 and MCP-1 [53].

During past years, cocoa and other flavan-3-ol-rich plants have expressed their anti-inflammatory activities by modulation of the secretion and expression pro-inflammatory cytokines [14,15]. For example, a flavonoid-rich bamboo leave extract for 12 wk suppressed body weight gain and fat deposition induced by a high fat diet in C57BL/6J mice, and theses effects are accompanied by the modulation of pro-inflammatory cytokines, TNF-α and IL-6 secretion, which resulted in improved insulin resistance [56]. Recently, Kanamoto et al. have found that administration of procyanidin-rich black soybean seed coat extract for 14 wk remarkably decreased TNF-α, MCP-1 and IL-6 expression in mesenteric AT in high-fat (HF) diet-fed mice[8]. Similarly, Terra et al. have found that treatment of procyanidins from grape seed for 19 wk resulted in a decrease in the expression of TNF-α and IL-6 in the mesenteric AT in rats fed a HF diet, but the plasma IL-6 level was not affected[7].

However, it should be noted that the evidence for the anti-inflammatory effects of cocoa flavan-3-ols are gathered predominantly from in vitro experiments,
and the results are ambiguous [29]. For example, Ramiro et al. reported that cocoa flavan-3-ols can down-regulate the secretion of pro-inflammatory cytokines TNF-α and MCP-1 in stimulated macrophages [28]. They also pointed out that the inhibitory effect on cytokine secretion is produced, in part, at the transcriptional level as observed in decreased level of TNF-α, IL-1α and IL-6 mRNA expression. However, given the wide spectrum of cocoa flavan-3-ols and their complex interactions, results are mixed. For example, Mao et al. reported that isolated procyanidins from cocoa promoted TNF-α secretion in stimulated human peripheral blood mononuclear cells [57]. Moreover, the DP of procyanidins were also shown to be critical in the case of TNF-α and IL-1β [58].

**Eicosanoid Metabolism**

Eicosanoids, including prostaglandins (PGs), leukotrienes (LTs) and lipoxins (LXs), are lipid products derived from the metabolism of arachidonic acid (AA), which play a significant role as mediators of the inflammatory cascade (Figure 1.3). Both types of inflammation (acute and chronic) are ultimately mediated by eicosanoids. This hormonal system is intricately balanced to consist of both pro-inflammatory eicosanoids that drive the inflammatory process, as well as equally powerful anti-inflammatory eicosanoids that reverse the inflammatory process. Eicosanoids are important in regulating lipolysis, lipogenesis and adipogenesis [12].
Figure 1-3 Biosynthesis of eicosanoids from arachidonic acid

AA is an ω-6 fatty acid whose levels are entirely controlled by the diet. Anti-inflammatory drugs interact with molecular targets that are downstream from AA, primarily by either inhibiting the enzymes that convert AA into pro-inflammatory eicosanoids or inhibiting the release of AA from phospholipids in the membrane [2]. In AT, the production of AA can be catalyzed by adipose-specific phospholipase A2 (AdPLA), or by other types of PLA2 released from macrophages and other inflammatory cells. AdPLA is the first member of Group XVI PLA2, which has been identified and characterized in white AT in mice [59]. This membrane-associated, calcium-dependent AdPLA is highly expressed in AT and is associated with adipocyte differentiation and lipolysis [60]. Knockout of the gene that encodes AdPLA in mice increases the rate of lipolysis by markedly reducing PGE2 levels. These mice also show reduced tissue mass, triglyceride levels, insulin resistance and increased fatty acid oxidation in adipocytes in response to a HF diet [12,60].

AA can be enzymatically metabolized by three main pathways: cytochrome
P450s, cyclooxygenases (COXs) and lipoxygenases (LOXs) (Figure 1.3). The COX pathway metabolizes AA to form prostanoids, including the PGs. The LOXs are more numerous and convert AA into diverse eicosanoids including LTs. PGs and LTs are eicosanoid metabolites that play important parts in obesity-related inflammatory responses. Brunetti et al. have shown that PGE$_2$ levels were significantly increased in obese rat and it could drive an inflammatory process leading to increased incidence of prostate, kidney and testicular cancer [61]. LTB$_4$ has notable roles in lipid metabolism as well as mitochondrial beta-oxidation, and it has also been shown to strongly induce MCP-1 production in human monocytes, and has emerged as a pathogenic factor in obesity-induced NAFLD [62,63].

Although the effect of cocoa flavan-3-ols on the arachidonic acid metabolism remains to be fully elucidated, certain cocoa flavan-3-ols have been shown to effectively inhibit both isoforms of COXs [64]. A human study has reported that high-dose of cocoa flavan-3-ols are associated with a lower level of plasma LT metabolites starting 2 hr after consumption, possibly through inhibition of LOX [14]. In a study with human recombinant 5-LOX, only the dimeric through pentameric fractions of cocoa procyanidins revealed inhibitory potencies, whereas the larger procyanidin fractions produced little effects [65]. It was reported that intake of flavonoid-rich chocolate by volunteers caused a significant decrease in the ratio of the concentrations of the plasma pro-inflammatory cysteinyl-LTs and anti-inflammatory prostacyclin (PGI$_2$) [66]. These changes were observed along with the significant increases in the plasma concentrations of EC and its metabolites, which suggested that the observed beneficial effects of chocolate on eicosanoid metabolism were attributable to its high concentrations of EC and its oligomers, the procyanidins [67].

**NF-κB Pathway**

The transcription factor Nuclear Factor-κB (NF-κB) (Figure 1.4) is one of the most important inducible transcription factors in mammals; in particular, it regulates the expression of various genes encoding pro-inflammatory cytokines,
adhesion molecules, chemokines, growth factors, and chemoattractants such as MCP-1, and inducible enzymes including COX-2 and inducible nitric oxide synthase (iNOS) [13].

NF-κB consists of different combinations of Rel proteins (i.e., p65 or RelA, RelB, c-Rel, p50, p52) in various heterodimers and homodimers, with p50/p65 representing the most abundant activated form of NF-κB [68]. In resting cells, the inactive form of NF-κB resides in the cytoplasm bound to an inhibitory protein known as IκB. Upon cellular activation by extracellular stimuli, it gets phosphorylated by activated IκB kinases (IKK). Phosphorylated IκB, upon ubiquitination, is directed to proteasomes for degradation. The degradation of IκB allows NF-κB to translocate to the nucleus, and bind to κB motifs found in the promoter. As a transcription factor of many inflammatory genes, including pro-inflammatory cytokines, COX-2 and iNOS, the modulation of the NF-κB signaling pathway is a potential key target for modulating inflammation [69].
Several dietary polyphenols, including curcumin, epigallocatechin gallate (EGCG), resveratrol have been shown to inhibit COX-2 and iNOS expression by preventing NF-κB activation [70]. Similarly, comprehensive experimental evidence has demonstrated that cocoa-derived monomeric ((-)epicatechin and (+)-catechin) and dimeric flavan-3-ols reduce NF-κB activation thus resulting in reduced IL-2 production and oxidative stress [71,72]. While further studies are awaited, it has been hypothesized that impact on NF-κB activation might constitute a common trait to all cocoa anti-inflammatory effects [14].

**Metabolic Endotoxemia**

One of the prominent, emerging theories regarding the etiology of obesity-related inflammation is “metabolic endotoxemia”, which is defined as a subclinical elevation in circulating endotoxin levels [73] (Figure 1.5). Endotoxin, also known as lipopolysaccharides (LPS), is a unique glycolipid in the cell wall of gram-negative bacteria [74]. The LPS molecule is structurally divided into three parts: lipid A, the oligosaccharide core and the O-antigen. Lipid A is the portion of the LPS molecule that is responsible for its endotoxicity [75]. Studies have targeted LPS as a potential mediator of the underlying inflammatory conditions which give rise to obesity and other metabolic diseases [73].
Recent studies have hypothesized that endogenous LPS is continuously produced in the gut by the death of Gram-negative bacteria and physiologically translocate into intestinal capillaries through a toll-like receptor 4 (TLR4)-dependent mechanism. Moreover, LPS is transported from the gut toward target tissues by a mechanism facilitated by lipoproteins, notably chylomicrons freshly synthesized from epithelial intestinal cells in response to a HF diet. Finally, the endotoxin LPS triggers the secretion of pro-inflammatory cytokines when it binds to the complex of membrane-bound CD14 (mCD14, a co-receptor along with TLR4 for the detection of LPS) and the TLR4, which is upstream of the NF-κB pathway, to induce the transcription of pro-inflammatory genes innate immune cells including ATMs [76]. Therefore, endotoxin may serve as an early factor in the triggering of HF diet-induced metabolic diseases [77].

Consumption of a HF diet is believed to facilitate gut absorption of endotoxin, cause changes of gut microbiota composition as well as increase intestinal permeability that allow for excess endotoxin to enter systemic circulation (i.e.
metabolic endotoxemia). Several animal studies have investigated the relationship between HF diet and plasma endotoxin levels, as well as shifts in gut microbiota [78–80]. A study by Cani et al in 2007 was the first of its kind to demonstrate that mice fed a HF diet for 4 wk caused the plasma LPS levels to rise 2-3 times higher compared to mice fed a regular diet [77]. Findings from recent studies suggest that the gut microbiota contribute towards the pathophysiological regulation of endotoxemia and set the tone of inflammation for occurrence of obesity[81]. Thus, in order to prevent or treat HF-diet induced metabolic diseases, it would be useful to develop specific strategies to modulate metabolic endotoxemia through alteration of gut microbiota and intestinal permeability.

Although there is little research has been done to support the role of cocoa in modulation of metabolic endotoxemia, a recent clinical trial has demonstrates its prebiotic effects [82]. In this double-blinded, randomized study, researchers compared the outcomes of consuming a high-cocoa flavan-3-ol (HCF) vs. a low-cocoa flavan-3-ol (LCF) drinks in healthy adults. Compared with the LCF drink (23 mg cocoa flavan-3-ols/d), the daily consumption of the HCF drink (494 mg cocoa flavan-3-ols/d) for 4 wk significantly increased the Bifidobacterial and Lactobacilli populations but significantly decreased Clostridia counts. These microbial changes were also paralleled by significant reductions in plasma triglycerides and C-reactive protein concentrations.
Cocoa

History of Use

Cocoa is derived from the beans of *Theobroma cacao*, a tree native to Mesoamerica. Cocoa has a rich history of human use. The Olmec, Maya, Aztec and Inca considered cocoa to have strong medicinal properties, including the treatment or prevention of infection, inflammation, heart palpitations, and angina[83]. Indeed, they believed that cocoa is “the food of gods”, an association that gave rise to the scientific name of the cocoa tree, *Theobroma cacao*, from the Greek words *Theos* (god) and *broma* (food)[83]. Cocoa was introduced to Europe after the 16th century conquest of Central America by Spain, where it was typically viewed as a healthy and nutritious beverage. Between the 16th and 20th centuries, well over 100 uses for cocoa, as a medical treatment, have been documented [15]. Today, although cocoa and chocolate are still widely consumed as beverages, they are most commonly consumed as confections with high fat and sugar content. Concern regarding the fat content of chocolate often overshadows its potential value as a source of beneficial nutrients and non-nutrient phytochemicals [84].

Production, Consumption and Processing

Cocoa was introduced to other tropical regions of the world as a result of colonial activity. Today, nearly 75% percent of the world’s cocoa bean production occurs in West Africa [85]. Cocoa is now a dietary ingredient with worldwide popularity. Consumption of cocoa is often associated with the high-calorie confection, chocolate, but is also consumed as an ingredient in diverse foods and beverages. In 2011-2012, worldwide production of cocoa was 3.98 million metric tonnes [1]. According to the National Health and Nutrition Examination Survey, 12.9% of adults in the United States could be classified as chocolate consumers [2], and an average American consumes about 5.3 kg of chocolate per year [85].

The cocoa bean is the seed of the fruit of the cocoa tree. Cocoa liquor is the paste made by grinding the fermented, roasted and shelled cocoa beans, also called
cocoa nibs. It contains both nonfat cocoa solids and cocoa butter. Cocoa powder is made by removing some of the cocoa butter from the liquor, and chocolate is made by combining cocoa liquor with additional cocoa butter and sugar. The proportion of cocoa liquor in the final product determines the level of darkness in the chocolate. Polyphenol content varies by the proportion of cocoa liquor present in different types of chocolate (white, milk, and dark), but even within categories there may be significant variation due to differences in cocoa bean processing [15].

Evidence from the literature indicates that total amount of polyphenols and flavan-3-ols present in cocoa and chocolate foods depends largely on post-harvest handling and processing techniques [88]. As cocoa beans processed on the farm, they are often fermented for two to greater than six days. Unfermented cocoa is referred to as *Lavado* (meaning “washed” in Spanish) cocoa and contains the highest amount of polyphenols. Dutching (also known as Alkalization) is a process in which cocoa powder is treated with a base, typically sodium or potassium carbonate, at elevated temperature. Dutching is used to change the color, develop the flavor, and improve the dispersibility of cocoa powders. Fermentation and Dutching have been reported to result in the loss of as much as 90% of the cocoa flavan-3-ols [18].

**Chemical Composition**

Cocoa is a rich source of polyphenols with levels reaching 12–18% by dry weight [89]. The polyphenol profile in cocoa beans varies for different cultivars and can be quite diverse, but the most abundant are the flavan-3-ols. Flavan-3-ols belong to a subclass of flavonoids, which is very commonly found in most of the plants. Flavan-3-ols are defined by the presence of a hydroxyl group at position 3 (ring C, the middle ring) and constitute the most complex subfamily of flavonoids, ranging from simple monomers to the oligomeric and polymeric forms, called procyanidins [90]. Studies on the composition of cocoa products have shown that non-esterified monomers of flavan-3-ols and procyanidins are the quantitatively most important types. Those chemical species have been identified as (-)-epicatechin (EC) (Figure 1.6 A), (+)-catechin (Figure 1.6 B), and mostly oligomeric and polymeric B-type
procyanidins (Figure 1.6 C) with EC-(4β-8)-EC linkage repeating units. The monomers account for only about 10% of the total with the procyanidins accounting for about 90% of the flavan-3-ol content [91]. Procyanidins with a degree of polymerization (DP) up to decamer have been identified in cocoa [17], and they are at significantly higher levels than in other flavan-3-ol-rich foods such as red wine or green tea [14,16].

Figure 1-6 Major non-nutritive phytochemicals in cocoa: (A) (-)-Epicatechin, (B) (+)-Catechin, (C) Procyanidin oligomers with C4β-C8 linkage repeating units, (D) Theobromine.

In addition to polyphenols, cocoa contains methylxanthine compounds—predominantly theobromine—about 2% to 3% by weight (Figure 1.6 D). Caffeine is also present in small amounts (0.2%) [15]. Cocoa also contains important minerals, including iron, magnesium, phosphorus, potassium and copper [85]. The fiber present within the cocoa bean also lies within the cocoa powder portion, providing 2g of dietary fiber for every tbsp (about 5~6 g) of cocoa powder.
Absorption and Bioavailability of Cocoa Polyphenols

The spectrum of potential biological activities of cocoa flavan-3-ols is dictated in part by their absorption and bioavailability. Plasma concentrations of cocoa polyphenols are often in the nanomolar or low micromolar range depending on the specific chemical properties of the polyphenol of interest following oral administration [92–95].

Among those three major phenolic compounds in cocoa, EC is relatively best absorbed [92,93], with a maximum plasma concentration at around 2 h, and about 20 % of consumed epicatechin is excreted in the urine [96]. Studies have shown that EC is better absorbed than (+)-catechin possibly due to differences in hydrophobicity [97]. Donovan et al. [94] and Holt et al. [92] reported that doses of 35 or 160 mg (+)-catechin (in red wine or chocolate) yielded plasma concentrations of 0.1–0.2 µM, and a similar dose of EC yielded about 0.2 µM in plasma reported by Yang et al. [95].

Although the bioavailability of the polyphenol monomers has been well-studied, there is still limited information concerning procyanidin absorption and bioavailability. Procyanidin absorption depends on its DP. Dimers and trimers are absorbed in the small intestine but less efficiently (under 0.5%) than EC and (+)-catechin monomers, whose absorption level is in the 22–55% range [98–101]. Holt et al. reported that cocoa procyanidin dimer B2 [EC-(4β-8)-EC] can be detected in the plasma of humans within 30 min of consuming a cocoa beverage, reaching a maximum concentration in the plasma approximately 2 hr after consumption [92]. There is no data available regarding the bioavailability of the larger procyanidin oligomers.

Some researchers believe that many of the large oligomers may be broken down to monomers and dimers in the acidic environment of the stomach [102]. Other oligomers may travel to the colon intact and be metabolized by microflora possibly by various mechanisms including hydrolysis, ring-cleavage, reduction, decarboxylation and demethylation [103]. Rios et al. [104] have reported that cocoa
procyanidins are stable during gastric transit in humans and reach the small intestine intact. Therefore, some of the studies suggested that because of their rather poor absorption in an intact form, ingested cocoa polyphenols are present in the intestinal lumen at higher concentrations and are able to affect the digestive and absorptive process [94,105]. It has also been suggested that even if the larger procyanidins are not absorbed, they could exert significant biologic effects within the gut by protecting nutrients from oxidative damage during digestion, protecting the endothelial cells lining the gut as well as preventing the leakage of endotoxin (i.e. LPS) from the gut [88,106,107].
Effect of Cocoa on Obesity-Related Inflammation

In vitro Studies

In vitro as well as cell culture studies have identified several cellular and molecular targets by which cocoa polyphenols may exhibit anti-obesity, antioxidant and anti-inflammatory, as well as anti-atherogenic activity (Table 1.1).

Given the key role of α-glucosidase in glucose uptake, inhibition of intestinal α-glucosidase activity represents a well-documented approach to controlling blood glucose and serum insulin levels in individuals with type 2 diabetes. Yamashita et al. have reported that cocoa liquor procyanidin extract (0.01-0.15%) significantly inhibited intestinal α-glucosidase (maltase and sucrose-isomaltase) activity in a dose-dependent manner in vitro, which may lead to prevention of hyperglycaemia in vivo [108].

Currently available in vitro data indicates that cocoa flavan-3-ols and their related procyanidins might modulate the eicosanoid metabolism through inhibition of eicosanoid-generating enzymes. The inhibitory effect of cocoa procyanidins appears to be related to DP. Fractions of procyanidins from cocoa caused a dose-dependent inhibition of rabbit 15-LOX-1 with the larger oligomers being more active; the decamer revealed an IC$_{50}$ of 0.8 µM [109]. On the contrary, the procyanidin decamer was less potent than EC (IC$_{50}$=15 µM) in inhibiting the recombinant human platelet 12-LOX in vitro. Moreover, EC and low-DP procyanidins (dimer-pentamer) inhibited both dioxygenase and LTA$_4$ synthase activities of human 5-LOX, whereas the larger procyanidins (hexamer- nonamer) were almost inactive [65]. Thus, cocoa polyphenols may be expected to control the formation of eicosanoid levels in inflammatory cells through inhibition of LOX activity. For example, a significant lowered leukotriene/prostacyclin ratio was reported upon treatment of human aortic endothelial cells with cocoa extract in vitro [66].

Several studies in cultured cells suggest that cocoa polyphenols may exhibit anti-inflammatory activity by modulation the production of inflammatory cytokines including IL-1β, IL-2, IL-4, IL-6, TNF-α and MCP-1. However, the results are
inconsistent, and opposing effects on inflammatory cytokine production *in vitro* of higher-DP flavan-3-ols have been reported. Mao *et al.* have shown that co-incubation of human peripheral blood mononuclear cell (PBMC) with phytohemagglutinin (PHA) and smaller fractions of cocoa procyanidins (monomer-tetramer, 25 µg/mL) consistently reduced IL-1β expression by up to 15%, while the larger oligomers (pentamer-decamer) increased expression by 4-52% [26]. These data, observed at the transcription level, were also reflected in protein levels in PHA-induced PBMC. Similarly, the same group also examined the TNF-α secretion in PHA-stimulated human PBMC [57]. They found that the intermediate-sized cocoa procyanidins (tetramer-octamer) were the most active, enhancing TNF-α secretion in the range of the range of 48–128%, while monomers and dimers were slightly inhibitory[57]. In LPS-stimulated macrophages, treatment of cocoa extract (10-50 µg/mL) and EC (1-200µM) caused a significant reduction in both secretion and expression of TNF-α, IL-1α/β, IL-6 and MCP-1[28]. The regulatory effects of cocoa and cocoa flavan-3-ols on NF-κB activation have also been studied. Cocoa extract, as well as EC, (+)-catechin, and their dimers were found to inhibit the activation and translocation of NF-κB in several cell models including inflammatory cells [110] and adipocytes [111], which also accompanied by decreases in NF-κB regulated cytokine productions. In a complementary study, Zhang *et al.* demonstrated that pretreatment of differentiated human monocytic THP cells with procyanidin B2 (50 µM) reduced the LPS-induced transcription and protein expression of COX-2. This effect was correlated to the decreased activation of extracellular-signal-regulated kinase (ERK), Jun-terminal kinase (JNK), and p38 mitogen-activated protein kinase (MAPK), as well as to the suppression of NF-κB activation through stabilization of IκB proteins. However, the enzyme activity of COX-2 was unaffected [112].

Cocoa extract (100, 200 µg/mL) inhibits isobutylmethylxanthine, dexamethasone and insulin (MDI)-induced adipogenesis in 3T3-L1 preadipocytes by reducing the expressions of adipogenesis-mediated proteins PPARγ and CEBPα, and blocked mitotic clonal expansion (MCE) of preadipocytes by reducing proliferating signaling pathways [113]. This in turn attenuates lipid accumulation during the
differentiation of 3T3-L1 preadipocytes. Moreover, cocoa extract significantly inhibits the activity of insulin-receptor (IR) kinase via direct binding, although the protein expression level of IR was unaffected.
**Table 1-1** *In vitro* studies of the effects of cocoa on obesity-related inflammation

<table>
<thead>
<tr>
<th>Enzymes/Cells</th>
<th>Treatment</th>
<th>Outcomes</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Glucosidase (maltase and sucrose-isomaltase)</td>
<td>CLPr (0.01-0.15%)</td>
<td>Enzyme activity (0.01-0.15% CLPr) ↓</td>
<td>[108]</td>
</tr>
<tr>
<td>Rabbit 15-LOX-1</td>
<td>Cocoa EC</td>
<td>Enzyme activity ↓ (IC_{50} = 60 μM)</td>
<td>[109]</td>
</tr>
<tr>
<td>Recombinant human platelet 12-LOX</td>
<td>Cocoa procyanidin (CP) decamer</td>
<td>Enzyme activity ↓ (IC_{50} = 0.8 μM)</td>
<td>[109]</td>
</tr>
<tr>
<td>Recombinant human 5-LOX</td>
<td>Cocoa EC</td>
<td>Enzyme activity ↓ (IC_{50} = 15 μM)</td>
<td>[109]</td>
</tr>
<tr>
<td>PHA-stimulated human PBMC</td>
<td>CPs (monomer-decamer, 25 μg/mL)</td>
<td>IL-2 expression (pentamer-heptamer) ↓; IL-1β expression (pentamer-decamer) ↑; IL-4 secretion &amp; expression (hexamer-decamer) ↓</td>
<td>[25]</td>
</tr>
<tr>
<td>LPS-stimulated human PBMC</td>
<td>Flavan-3-ols fractions (hexamer-decamer, 20 μg/mL)</td>
<td>IL-1β, IL-6, TNF-α, IL-10 secretion ↑</td>
<td>[114]</td>
</tr>
<tr>
<td>LPS-stimulated human monocyteic THP-1 cells</td>
<td>CP dimer B2 (50 μM)</td>
<td>COX-2 transcription and protein expression, NF-kB activation and translocation ↓</td>
<td>[112]</td>
</tr>
<tr>
<td>PMA-stimulated human monocytes</td>
<td>Cocoa extract (10 μg/mL)</td>
<td>NF-κB translocation ↓; TNF-α, IL-6 secretion ↓</td>
<td>[115]</td>
</tr>
<tr>
<td>PMA-stimulated Jurkat T cells</td>
<td>Cocoa catechin, EC &amp; CP dimer (1.7-12.2 μM)</td>
<td>NF-κB activation, IL-2 expression &amp; secretion ↓</td>
<td>[71]</td>
</tr>
<tr>
<td>LPS-stimulated rat NR8383 macrophages</td>
<td>Cocoa extract (50 μg/mL), EC (100, 200 μM)</td>
<td>MCP-1 secretion ↓</td>
<td>[28]</td>
</tr>
<tr>
<td>LPS-stimulated murine RAW 264.7 macrophages</td>
<td>Cocoa extract (5-100 μg/mL) and EC (200-400 μM)</td>
<td>NO production ↓</td>
<td>[28]</td>
</tr>
<tr>
<td>LPS- and INF-γ-stimulated murine J774.1 macrophages</td>
<td>Cocoa extract (0.05%, 0.25%)</td>
<td>NO production ↓</td>
<td>[117]</td>
</tr>
<tr>
<td>LPS-induced mouse peritoneal macrophages</td>
<td>Cocoa extract (25 μg/mL)</td>
<td>TNF-α, IL-1β, IL-6 secretion ↓</td>
<td>[110]</td>
</tr>
<tr>
<td>TNF-α-induced 3T3-L1 adipocytes</td>
<td>EC (1, 10 μM)</td>
<td>NF-κB activation, TNF-α, MCP-1, IL-6 expression ↓</td>
<td>[116]</td>
</tr>
<tr>
<td>MDI-induced 3T3-L1 preadipocytes</td>
<td>Cocoa extract (100, 200 μg/mL)</td>
<td>Adipogenesis, lipid accumulation ↓; Cell differentiation, cell cycle progression ↓; Insulin-receptor (IR) kinase activity ↓</td>
<td>[113]</td>
</tr>
<tr>
<td>Human coronary artery endothelial cells</td>
<td>Cocoa EC (1 μM)</td>
<td>NO production ↑; eNOS activity ↑</td>
<td>[118]</td>
</tr>
<tr>
<td>Human endothelial cells from umbilical veins</td>
<td>Cocoa extract (6.25-100 μg/mL)</td>
<td>Angiotensin-converting enzyme (ACE) activity ↓; NO production (100 μg/mL) ↑</td>
<td>[119]</td>
</tr>
<tr>
<td>Human aortic endothelial cells</td>
<td>Cocoa Extract (2 mg/L)</td>
<td>Leukotriene-prostacyclin ratios ↓</td>
<td>[66]</td>
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</tbody>
</table>

1. 5-HpETE, S-hydroperoxy-5,6,8,11,12,14-eicosatetraenoic acid; CP, cocoa procyanidin; LPS, lipopolysaccharides; MDI, isobutylmethylxanthine, dexamethasone and insulin; PBMC, peripheral blood mononuclear cells; PHA, phytohemagglutinin; PMA, phorbol 12-myristate 13-acetate.
Animal Model Studies

There is a growing body of data derived from animal models demonstrating the anti-inflammatory effects of cocoa and cocoa polyphenols in prevention of metabolic syndrome (Table 1.2). Beneficial effects have been observed with regard to inflammatory biomarkers, serum lipid profile and insulin resistance. However, the underlying physiologic and molecular mechanisms are not yet fully understood, and it is unclear whether the anti-inflammatory effects observed are due to direct inhibition of inflammatory signaling or the result of modulation of weight gain, dyslipidemia, hyperglycemia, etc.

Yamashita et al. found that treatment of HF diet-fed C57BL/6] mice with cocoa liquor procyanidin extract (CLPr, 0.5 and 2%) for 13 wk promoted glucose transporter type 4 (GLUT4) translocation in skeletal muscle and brown adipose tissue, significantly reduced body weight (BW), blood glucose (BG), insulin and total cholesterol levels in obese mice [120]. In addition, phosphorylation of AMP-activated protein kinase α (AMPKα) was enhanced by CLPr (2%) in skeletal muscle, AT and liver. Plasma levels of anti-inflammatory adipokines leptin and adiponectin were decreased as well. Taken together, these findings may suggest a preventative role of procyanidin-rich cocoa in obesity, hyperglycemia and insulin resistance, principally by activating the key metabolic regulator AMPKα and modulation of adipokines.

Tomaru et al. found that diabetic obese mice fed a standard diet with 1% CLPr extract for 3 wk demonstrated lower BG levels, but no significant changes in BW were observed [121]. In another recent study, Min et al. found that C57BL/6 mice fed a HF diet with cocoa polyphenol extract (200 mg per kg BW) for 5 wk significantly reduced BW and epididymal fat mass, and plasma triglyceride (TG) levels, whereas BG and total cholesterol were unaffected [113].

Cocoa ingestion has also shown to prevent obesity by regulating the expression of genes for fatty acid metabolism. Matsui et al. reported that cocoa powder supplementation (12.5%) for 3 wk significantly decreased weight gain in HF-fed rats comparing to HF-fed controls [122]. DNA microarray analysis showed
that cocoa powder ingestion suppressed the expression of genes for enzymes involved in fatty acid synthesis in liver and white AT, as well as decreased the expression of genes related to fatty acid transport.

The metabolic effects of cocoa dependent on the composition of test compounds. In a Japanese study, male Wistar rats were fed a high-cholesterol diet or a high-cholesterol diet containing 1% polyphenol extract from cocoa powder (PE group) or a mixture of catechin and EC (CE group, equivalent to the amount present in PE) for 4 wk [123]. The PE group had significantly lower plasma cholesterol concentrations, and had significantly greater fecal cholesterol and total bile acids excretion than the high-cholesterol control group, possibly due to decreased micellar solubility of cholesterol by oligomeric procyanidins. However, the CE group diet did not influence any of these parameters. These results suggest that oligomeric procyanidins from cocoa might be the principal active components responsible for the hypocholesterolemic effect, and inhibit the intestinal absorption of cholesterol and bile acids by decreasing micellar cholesterol solubility.

In addition to polyphenols, other bioactive substances in cocoa such as methylxanthines and dietary fibers have also contributed to the metabolic benefits in animal models. Administration of methylxanthine-rich cocoa extract for 4 wk significantly reduced plasma free fatty acid, total cholesterol, TG and oxidative stress biomarker (8-isoprostane) in obese-diabetic (Ob-db) rats [22,27]. Oral glucose tolerance test revealed that cocoa administration in Ob-db rats significantly reduced plasma glucose at 60 and 90 min compared to control Ob-db rats [22].

In spontaneously hypertensive rats (SHR), a single oral administration of cocoa powder (50-600 mg/ kg body weight) containing 16 mg/g theobromine and 43 mg/g total flavan-3-ols was studies for its anti-hypertensive effect [125]. Results have shown that decrease in systolic blood pressure (SBP) was dose-dependent up to the dose of 300 mg/kg, and the maximum decrease in the diastolic blood pressure (DBP) was achieved 4 h post-administration of 50 or 100 mg/kg of cocoa powder. The effects of a soluble cocoa fiber (SCF) have been studied in Zucker fatty rats [126]. Zucker fatty rats were fed either standard diet or 5% SCF-enriched diet for 7
wk. The SCF group showed less body weight gain and food intake than the standard group. Lower values of the total cholesterol/HDL ratio, index of insulin resistance and plasma triglyceride levels were observed in those fed cocoa fiber-enriched diet.
Table 1-2 Animal studies of the effects of cocoa on obesity-related inflammation

<table>
<thead>
<tr>
<th>Species</th>
<th>No.</th>
<th>wk</th>
<th>Control</th>
<th>Treatment</th>
<th>Outcomes</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6 mice</td>
<td>36</td>
<td>13</td>
<td>Standard diet; HFD</td>
<td>Standard diet or HFD with 0.5%, 2% CLPr</td>
<td>BW, retropitoneal WAT (HFD with 2% CLPr) ↓ GLUT4 translocation (HFD with 2% CLPr)↑ BG, insulin, HOMA-IR, total cholesterol (HFD with 0.5% and 2% CLPr) ↓ Plasma leptin, adiponectin (HFD with 0.5% and 2% CLPr) ↑</td>
<td>[120]</td>
</tr>
<tr>
<td></td>
<td>44</td>
<td>5</td>
<td>Standard diet; HFD</td>
<td>HFD with either 40 mg/kg, 200 mg/kg BW of cocoa polyphenol extract (CPE)</td>
<td>Food intake, BW, TG (200 mg/kg CPE) ↓ Epididymal WAT, liver weight (40 and 200 mg/kg CPE) ↓ n.s. BG, total cholesterol level</td>
<td>[113]</td>
</tr>
<tr>
<td>C57BL/6KsJ diabetic obese mice</td>
<td>45</td>
<td>3</td>
<td>Standard diet</td>
<td>Standard diet with either 0.5% or 1% CLPr</td>
<td>BG ↓ Fructosamine, creatinine, kidney weights (1% CLPr) ↓ n.s. BW or plasma ALT</td>
<td>[121]</td>
</tr>
<tr>
<td>DSS-induced Balb/C mice</td>
<td>30</td>
<td>1</td>
<td>Water; 5% DSS (w/v) in water</td>
<td>5% DSS and 500 mg/kg of CPE</td>
<td>Leukocyte infiltration in colon ↓ NO production in colon ↓</td>
<td>[110]</td>
</tr>
<tr>
<td>Wistar rats</td>
<td>20</td>
<td>3</td>
<td>HFD with mimic cocoa</td>
<td>HFD with 12.5% (w/v) cocoa powder</td>
<td>BW and mesenteric WAT ↓ Total cholesterol, HDL ↑ Expression of genes involved in fatty acid synthesis in liver and WAT ↓</td>
<td>[122]</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>4</td>
<td>High-cholesterol diet</td>
<td>High-cholesterol diet with either 1% CPE, or a mixture of 0.024% catechin and 0.058% EC</td>
<td>Liver weight (CPE) ↓ Fecal weight, cholesterol and steroids excretion (CPE) ↑ n.s. BW, liver TG or cholesterol</td>
<td>[123]</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>7</td>
<td>High-cholesterol diet and 600 g/kg fructose solution</td>
<td>High-cholesterol diet and 600 g/kg fructose solution with cocoa (100 mg/ kg BW) by intragastric gavage</td>
<td>Serum TG, BG, HDL and BP ↓ n.s. total cholesterol or LDL</td>
<td>[127]</td>
</tr>
<tr>
<td>Obese-diabetic (Ob-dh) rats</td>
<td>40</td>
<td>4</td>
<td>3 mL of 0.03% (w/v) CMC by oral gavage</td>
<td>Cocoa extract (600 mg/kg BW/d) in 3 mL of 0.03% (w/v) CMC by oral gavage</td>
<td>BG in oral glucose tolerance test ↓ Plasma free fatty acids and 8-iso-prostanet ↓</td>
<td>[22]</td>
</tr>
<tr>
<td>Streptozotocin-diabetic rats</td>
<td>80</td>
<td>4</td>
<td>Standard diet</td>
<td>Standard diet with 1, 2, 3% of CPE</td>
<td>Serum total cholesterol and BG (1, 3% CPE) ↓ n.s. insulin or insulin sensitivity</td>
<td>[23]</td>
</tr>
<tr>
<td>Zucker fatty rats</td>
<td>20</td>
<td>7</td>
<td>Standard diet</td>
<td>5% soluble cocoa fiber-enriched diet</td>
<td>BW and food intake ↓ Plasma total cholesterol and TG ↓ Plasma insulin and HOMA-IR ↓</td>
<td>[126]</td>
</tr>
<tr>
<td>Spontaneously hypertensive rats</td>
<td>52</td>
<td>-</td>
<td>Oral administration of water</td>
<td>Oral administration of a flavan-3-ol-rich cocoa powder (50, 100, 300, and 600 mg/kg BW)</td>
<td>SBP, DBP ↓</td>
<td>[125]</td>
</tr>
<tr>
<td>Hamsters</td>
<td>18</td>
<td>10</td>
<td>High saturated fat and cholesterol diet</td>
<td>High saturated fat and cholesterol diet with 0.1%, 1% cocoa powder</td>
<td>TG, % atherosclerosis (0.1% cocoa powder) ↓ Plasma total cholesterol (0.1% and 1% cocoa powder) ↓</td>
<td>[128]</td>
</tr>
</tbody>
</table>

1. ALT, alanine aminotransferase; CLPr, cocoa liquor proanthocyanidin extract; CMC, carboxymethyl cellulose; CPE, cocoa polyphenol extract; DSS, dextransulfate sodium; HFD, high-fat diet; HOMA-IR, homeostasis model assessment of insulin resistance; WAT, white adipose tissue.
**Human Intervention and Epidemiological Studies**

There is growing evidence from both observational and experimental studies that consumption of cocoa and cocoa products may reduce inflammation as observed in decreased level of serum C-reactive protein (CRP) and pro-inflammatory cytokines. In an Italian study of over 10 000 people, of whom 4849 subjects were free of any chronic disease, 1317 people reported having eaten any chocolate during the past year and 824 ate chocolate regularly in the form of dark chocolate only [129]. After adjustment for multiple confounders, regular consumption of dark chocolate associated with lower levels of serum CRP concentration ($P = 0.038$). A J-shaped relationship between dark chocolate consumption and serum CRP was observed; consumers of up to 1 serving (20 g) of dark chocolate every 3 d had significantly lower serum CRP concentrations than non-consumers or those consuming more than 20 g chocolate per d. In another cross-sectional study, the investigators found that total flavonoid intake was inversely associated with serum CRP concentration after adjusting for multiple covariates ($P < 0.05$) in U.S. adults ($n = 8335$) using the data from the NHANES 1999-2002 [130].

Human intervention studies that have been conducted with cocoa and chocolate products are summarized in **Table 1.3**. In a randomized, crossover trial, 20 obese healthy subjects consumed either a control beverage or three cocoa beverages containing 180–900 mg flavan-3-ol per day for 5 d. Cocoa consumption dose-dependently decreased the circulating biomarkers of inflammation and oxidative stress (CRP, IL-6 and 8-isoprostane) [131]. Schramm et al. have reported that treatment with high dose of cocoa procyanidins (148 mg/d) associated with a 29% decrease in plasma LTs starting 2 hr after consumption, possibly through inhibition of LOX [66]. In a recent clinical trial of 18 healthy individuals, acute consumption of 40 g of cocoa powder mixed with water significantly reduced phosphorylation of p65, the transcriptional active subunit of NF-κB, in peripheral blood lymphocytes [111]. This result suggested a decreased activation of NF-κB induced by cocoa treatment. Interestingly, when the cocoa was mixed with milk, the
effect was ablated. The authors proposed that protein in the milk may reduce the bioavailability of active components in the cocoa.

The clinical trial data regarding the anti-inflammatory effects of cocoa consumption is not universally positive. For example, in a small uncontrolled intervention study, healthy subjects consumed dark chocolate (36.9 g/d) and a cocoa powder drink (30.95 g powder/d) for 6 wk; there was no change in plasma CRP, TNF-α, IL-1β, IL-6 or soluble P-selectin with either treatment [132].

Several studies have failed to find an anti-inflammatory effect in unhealthy patients. A study of 20 hypertension patients, consumption of flavan-3-ol-rich cocoa drink (900 mg flavan-3-ols/d) for 2 wk failed to decrease the plasma levels of TNF-α, IL-1β or IL-6, although the average brachial artery diameter was increased compared to placebo group [133]. Monagas et al. reported the effect of a 4-wk randomized cross-over trial of 40 g cocoa powder in skimmed milk daily versus skimmed milk in 42 patients at high risk of CVD [134]. Plasma levels of MCP-1, IL-6 or CRP were unchanged; however, concentrations of P-selectin and ICAM-1 were lower after the cocoa powder intervention. Thus, there is some evidence that cocoa and cocoa-rich foods might reduce low-grade systemic inflammation.
Table 1-3 Human studies of the effects of cocoa on obesity-related inflammation

<table>
<thead>
<tr>
<th>Population</th>
<th>No.</th>
<th>Duration</th>
<th>Design</th>
<th>Control</th>
<th>Treatment</th>
<th>Outcomes</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>18</td>
<td>Acute</td>
<td>Randomized, blind,</td>
<td>250 mL Milk</td>
<td>40 g of cocoa with 250 mL milk (CM) or with 250 mL water (CW)</td>
<td>NF-kB activation (CW), n.s. BW or lipid profile</td>
<td>[111 ]</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>Acute</td>
<td>Randomized, blind,</td>
<td>37 g low-CP chocolate (0.09 mg/g)</td>
<td>37 g high-CP chocolate (4.0 mg/g)</td>
<td>Plasma leukotriene-prostacyclin ratios ↓</td>
<td>[66]</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>2 wk</td>
<td>Randomized, double-blind</td>
<td>46 g low-flavonoid dark chocolate bars</td>
<td>46 g high-flavonoid dark chocolate bars (213 mg/g CPs, 46 mg/g EC)</td>
<td>FMD ↑, n.s. LDL oxidation, 8-isoprostanes, BP, lipid profile or BW</td>
<td>[136]</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>15 d</td>
<td>Randomized, blind,</td>
<td>90 g white chocolate bars</td>
<td>100 g dark chocolate bars (500 mg polyphenols/d)</td>
<td>HOMA-IR, BP ↓, QUICKI ↑</td>
<td>[137]</td>
</tr>
<tr>
<td>Healthy subjects</td>
<td>45</td>
<td>3 wk</td>
<td>Controlled</td>
<td>White chocolate (75g/d)</td>
<td>Dark chocolate, or polyphenol-enriched dark chocolate (75 g/d)</td>
<td>HDL ↑, n.s. plasma, isoprostanes or hydroxy fatty acids</td>
<td>[138]</td>
</tr>
<tr>
<td></td>
<td>160</td>
<td>4 wk</td>
<td>Double-blind</td>
<td>low-CP cocoa powder</td>
<td>13, 19.5, or 26 g high-CP cocoa powder</td>
<td>HDL ↑, n.s. TG, total cholesterol or LDL</td>
<td>[139]</td>
</tr>
<tr>
<td></td>
<td>23</td>
<td>4 wk</td>
<td>Randomized, crossover</td>
<td>An average American diet (AAD)</td>
<td>An AAD with 22 g cocoa powder and 16 g dark chocolate (466 mg CPs/d)</td>
<td>LDL oxidation lag time, HDL ↑, n.s. urinary excretion of TXB2 or 6-keto-PG F1α.</td>
<td>[140]</td>
</tr>
<tr>
<td></td>
<td>32</td>
<td>4 wk</td>
<td>Randomized, double-blind</td>
<td>Placebo tablets (≤ 6 mg cocoa flavan-3-ols/d)</td>
<td>Active tablets (234 mg cocoa flavan-3-ols/d)</td>
<td>Platelet aggregation, activation ↓, n.s. SBP or DBP</td>
<td>[141]</td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>4 wk</td>
<td>Randomized, double-blind</td>
<td>Low-cocoa flavan-3-ols drink (29 mg/flavan-3-ols/d)</td>
<td>High-cocoa flavan-3-ols drink (494 mg/flavan-3-ols/d)</td>
<td>Improve gut microflora, <em>Bifidobacteri</em> and <em>Lactobacilli</em> populations ↑, clostridia counts ↓ Plasmatotal cholesterol, TG, CRP ↓, n.s. HDL, LDL, or BG</td>
<td>[82]</td>
</tr>
</tbody>
</table>
Table 1.3 (Cont’d) Human studies of the effects of cocoa on obesity-related inflammation

<table>
<thead>
<tr>
<th>Population</th>
<th>No.</th>
<th>Duration</th>
<th>Design</th>
<th>Control</th>
<th>Treatment</th>
<th>Outcomes</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Obese healthy subjects</td>
<td>20</td>
<td>5 d</td>
<td>Randomized, crossover</td>
<td>Control beverage (30 mg flavan-3-ol)</td>
<td>Cocoa beverages (180-900mg flavan-3-ol)</td>
<td>isoprostone, CRP, IL-6↓, n.s. TG, BG, insulin, HOMA-IR</td>
<td>[131]</td>
</tr>
<tr>
<td>Overweight and obese subjects</td>
<td>14</td>
<td>2 wk</td>
<td>Randomized, single-blind, crossover</td>
<td>-</td>
<td>20 g dark chocolate with 500mg or 1000mg CP</td>
<td>BG, BP ↓, n.s. BMI, total cholesterol</td>
<td>[142]</td>
</tr>
<tr>
<td>Overweight and obese subjects</td>
<td>49</td>
<td>12 wk</td>
<td>Randomized, double-blind, parallel</td>
<td>Low-flavan-3-ol (36mg) cocoa</td>
<td>High-flavan-3-ol (902 mg) cocoa</td>
<td>Insulin Resistance↓, FMD↑</td>
<td>[143]</td>
</tr>
<tr>
<td>Subjects with pre-hypertension</td>
<td>44</td>
<td>18 wk</td>
<td>Randomized, single-blind, parallel</td>
<td>63 g CP-free white chocolate</td>
<td>6.3 g dark chocolate (30 mg CP)</td>
<td>SBP, DBP ↓, NO↑, n.s. BW, plasma TG, BG or B↓, isoprostone.</td>
<td>[144]</td>
</tr>
<tr>
<td>Subjects with pre-hypertension</td>
<td>32</td>
<td>15 d</td>
<td>Randomized, parallel</td>
<td>25 g white chocolate</td>
<td>30 g dark chocolate</td>
<td>SBP↓, serum NO↑, n.s. DBP</td>
<td>[145]</td>
</tr>
<tr>
<td>Hypertension patients</td>
<td>20</td>
<td>15 d</td>
<td>Randomized, blind, crossover</td>
<td>90 g white chocolate bars</td>
<td>100 g dark chocolate bars (88 mg flavan-3-ol)</td>
<td>Serum total cholesterol, LDL↑, BP↑, HOMA-IR↓, QUICKI↑, FMD↑</td>
<td>[146]</td>
</tr>
<tr>
<td>Hypertension patients</td>
<td>20</td>
<td>2 wk</td>
<td>Randomized, double-blind, crossover</td>
<td>Flavan-3-ol-poor (28 mg) placebo</td>
<td>Flavan-3-ol-rich (900 mg) cocoa</td>
<td>Brachial artery diameter↑, n.s. BP, IR, plasma TNF-α, MCP-1 or IL-6</td>
<td>[133]</td>
</tr>
<tr>
<td>Subjects with hypertension and glucose intolerance</td>
<td>19</td>
<td>15 d</td>
<td>Randomized, single-blind, cross-over</td>
<td>100 g flavan-3-ol-free white chocolate</td>
<td>100 g flavan-3-ol-rich dark chocolate (1008 mg CP)</td>
<td>HOMA-IR, SBP, DBP, total cholesterol, LDL↓, QUICKI↑, FMD↑, n.s. HDL, TG, CRP</td>
<td>[147]</td>
</tr>
<tr>
<td>Subjects with T2D</td>
<td>12</td>
<td>8 wk</td>
<td>Randomized, double-blind crossover</td>
<td>45 g chocolate (&lt; 2 mg EC/d)</td>
<td>45 g chocolate (16.6 mg EC/d)</td>
<td>HDL↑, n.s. BP, BG, insulin, TG, total cholesterol or LDL</td>
<td>[148]</td>
</tr>
<tr>
<td>Patients at high risk of CVD disease</td>
<td>42</td>
<td>4 wk</td>
<td>Randomized, blind, crossover</td>
<td>500 mL skim milk</td>
<td>500 mL skim milk with 40 g cocoa powder</td>
<td>P-selectin, ICAM-1↓, n.s. plasma MCP-1, IL-6 or CRP</td>
<td>[134]</td>
</tr>
</tbody>
</table>

1. BMI, body mass index; BP, blood pressure; FMD, flow-mediated dilation; ICAM-1, intercellular adhesion molecule-1; n.s., not significant; QUICKI, quantitative insulin sensitivity check index; TXB₂, Thromboxane B₂;
Conclusions

Obesity is a multi-factorial disorder, which is often associated with many other significant diseases such as diabetes, hypertension and CVD. Inflammation represents the characteristic feature of the pathologies that contribute to obesity and its co-morbidities. The development of dietary bioactive agents, alone or in combination with lifestyle changes and pharmaceutical agents, could represent a cost-effective and safe approach to the problem [149].

A growing body of scientific evidence is becoming available to support a beneficial role of cocoa and cocoa products that could lead to the prevention and treatment of obesity-related inflammation. Several mechanisms are proposed to explain their beneficial effects. In addition to anti-oxidative activity, they regulate cytokine secretion and expression, modulate eicosanoid metabolism, inhibit NF-κB activation, as well as possibly modulate metabolic endotoxemia (Figure 1.7). Although existing evidence indicates that cocoa flavan-3-ols potentially display a multi-targeting anti-inflammatory action in vitro and in vivo, a clear conclusion on their effects in human subjects cannot be drawn.

![Diagram](image)

**Figure 1-7** Molecular targets of cocoa to prevent and treat obesity and obesity-related inflammation that predispose to the development of metabolic syndrome.
Although several animal studies demonstrated a preventive role of cocoa in obesity and diabetes, results on inflammatory markers in the context of obesity are limited. It is worthy of note that body weight, as one of the most important physiological outcomes in obesity, was not affected in most of the cocoa-feeding studies. The dose levels, animal models, as well as the duration of treatment might be the contributing factors. In addition, the effects on plasma total cholesterol levels are ambiguous, possibly due to the mixed effects of cocoa on HDL and LDL. Moreover, most of the cocoa intervention studies in animals investigated anti-obesity and/or anti-diabetic effects of cocoa in a preventive model [23,122,123,128], where cocoa is administrated before the establishment of disease conditions. However, a corrective model which focuses on a counteracting effect of cocoa supplementation would very well embrace the current status of prevalence of obesity, as the majority people who are seeking for treatment are individuals at risk. Overall, studies on anti-obesity and anti-diabetic effects of cocoa in animal models are in the nascent stages and results on associated inflammatory markers are lacking. Further research using a physiologically achievable dose and well-designed animal models is needed.

So far, combining the multiple lines of evidence from experimental and observational studies on the health benefits of cocoa and cocoa products, there is rather strong evidence supporting that cocoa consumption improves several important metabolic factors (e.g. insulin resistance, endothelial function) and likely ameliorates the progression of obesity-related inflammation, although the anti-inflammatory effects of cocoa in human studies are ambiguous. Positive data of cocoa intake on central obesity are missing, which possibly due to the fact that cocoa is commonly consumed as chocolate, an energy-dense product that might have negative effects on weight management. However, with daily chocolate consumption, no adverse changes of body weight were observed in human trials, supporting the concept that moderate consumption of flavan-3-ol-rich chocolate and the combination with a balanced diet may help to attenuate obesity-related inflammation without adverse effects on body weight. In conclusion, in order to
elucidate the potential anti-inflammatory benefits of cocoa in humans, more laboratory-based evidences about mechanism and molecular targets are needed. Continued research, both in model systems and human subjects, is needed to better understand the influence of cocoa on metabolic disorders and inflammation.
References


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Chapter 2

Hypothesis & Specific Aims, Significance and Innovation
Hypothesis & Specific Aims

Recent experimental and observational studies have suggested that consumption of cocoa and cocoa products has a positive influence on human health with anti-oxidant, anti-hypertensive, anti-inflammatory, and anti-atherogenic properties, as well as positively influencing insulin sensitivity and vascular endothelial functions [1–14]. These beneficial effects have been supported by recent meta-analyses, supporting a potential role for cocoa and cocoa products in ameliorating metabolic risk factors such as blood pressure, cholesterol levels, insulin resistance, and inflammatory markers [8,15–22]. Based on these previous studies, I hypothesize that dietary cocoa and cocoa procyanidins can exert anti-inflammatory activities and modulate obesity-related pathologies in vitro and in vivo.

In order to test this hypothesis, I propose the following specific aims:

1. To determine the inhibitory activity of cocoa extracts and individual cocoa procyanidins against pancreatic α-amylase (PA), pancreatic lipase (PL), and phospholipase A2 (PLA2) in vitro. (Chapter 3)

2. To investigate the anti-inflammatory effects of CPs with high degrees of polymerization (DP) on the modulation of eicosanoid metabolism and production of pro-inflammatory cytokines in a cell culture model of inflammation. (Chapter 4)

3. To study the corrective effect of short-term dietary cocoa powder supplementation on body weight, systemic inflammation, insulin resistance, and obesity-related fatty liver disease in high-fat (HF) diet-fed mice. (Chapter 5)

4. To explore the preventive effect and mechanism of long-term dietary cocoa powder supplementation on adipose tissue inflammation through the regulation of NF-κB target gene expression and eicosanoid metabolism, as well as metabolic endotoxemia in HF diet-fed mice. (Chapter 6)
Significance

Currently, data concerning the efficacy and molecular mechanisms of action for the prevention of obesity and obesity-related inflammation by cocoa are still limited. Given the growing incidence of obesity in the United States and the role of chronic inflammation in the development of obesity-related pathologies, there is a growing need for dietary approaches for the prevention and treatment of these conditions. My thesis research is significant to public health because, if successful, it will provide insight into the efficacy and the mechanism of action of cocoa and cocoa procyandins against obesity and obesity-related pathologies. Based on an understanding of the impact of these dietary phytonutrients on various molecular targets, it may become possible to make rational dietary recommendations, to identify additional dietary compounds and potential synthetic derivatives as possible anti-inflammatory agents, and to identify additional molecular targets for the modulation of obesity-related inflammation. As cocoa is predominantly consumed as energy-dense chocolate, potential detrimental effects of overconsumption exist. However, this proposed study may suggest the benefits of moderate cocoa or dark chocolate consumption likely outweigh the possible risks. Consequently, insights generated by the proposed work will give rise to a new niche in the functional food industry for cocoa and other procyanidin-rich foods to be considered as value-added products that appeal to consumer interests and dietary needs.
Innovation

To my knowledge, this is the first investigation of the inhibition of key digestive enzymes by cocoa extracts and cocoa procyanidins. Moreover, the studies in this report will also provide the first scientific evidence and mechanistic insight into the modulation of dietary cocoa on obesity-related inflammation and metabolic risk factors in obese mice, as well as *in vivo* anti-inflammatory actions including the modulation of eicosanoid metabolism and metabolic endotoxemia.
References


Chapter 3

Inhibition of Key Digestive Enzymes by Cocoa Extracts and Procyanidins
Abstract

This study determined the in vitro inhibitory effects of cocoa extracts and procyanidins against pancreatic α-amylase (PA), pancreatic lipase (PL) and secreted phospholipase A₂ (PLA₂), and characterized the kinetics of such inhibition. Lavado, regular and dutch-processed cocoa extracts as well as cocoa procyanidins (degree of polymerization (DP) = 2-10) were examined. Cocoa extracts and procyanidins dose-dependently inhibited PA, PL, and PLA₂. Lavado cocoa extract was the most potent inhibitor (IC₅₀ = 8.5-47 µg/mL). An inverse correlation between Log IC₅₀ and DP (R² > 0.93) was observed. Kinetic analysis suggested that regular cocoa extract, the pentamer and decamer inhibited PL activity in a mixed mode. The pentamer and decamer non-competitively inhibited PLA₂ activity, whereas the regular cocoa extracts inhibited PLA₂ competitively. This study demonstrates that cocoa polyphenols can inhibit digestive enzymes in vitro and may, in conjunction with a low-calorie diet, play a role in body weight management.

Keywords: cocoa, Theobroma cacao, procyanidins, phospholipase A₂, pancreatic lipase, α-amylase
Abbreviations:

BMI, body mass index; DP, degree of polymerization; EC, (-)-epicatechin; IC₅₀, median inhibitory concentration; Kₘ, Michaelis-Menten constant; PA, pancreatic α-amylase; PL, pancreatic lipase; PLA₂, phospholipase A₂; Vₘₐₓ, maximum velocity.

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Introduction

Chronic imbalance between energy intake and energy expenditure is the major cause of weight gain and development of obesity (body mass index (BMI) $\geq 30$) [1]. Today, $>60\%$ of Americans are overweight, and if the current trajectory continues, the rate will reach $86\%$ by 2030 [2]. Elevated BMI is attributable to a global shift in diet towards increased intake of energy-dense foods that are high in fat and carbohydrates but low in vitamins, minerals, and other micronutrients as well as the increased sedentary lifestyle [3]. Elevated BMI is also a major risk factor in the development of heart disease, fatty liver disease, cancer, and Type II diabetes [4]. One strategy for the prevention of overweight and obesity related disease is the use of agents that interfere with the hydrolysis and absorption of dietary carbohydrates and lipids. Pancreatic $\alpha$-amylase (PA), pancreatic lipase (PL) and pancreatic phospholipase A$_2$ (PLA$_2$), which are delivered into the intestinal lumen as constituents of pancreatic juices, are the major enzymes involved in the hydrolysis of dietary starch and fat [5]. PA is an endoglucosidase that catalyzes the hydrolysis of starch to maltose and maltotriose [6]. PL is a key enzyme for absorption of dietary triglycerides and rapidly converts a triglyceride to a 2-monoglycerol and two free fatty acids [5]. Orlistat (marketed over-the-counter as Alli in U.S.), a potent competitive inhibitor of PL, is available as an anti-obesity drug. It has been reported that Orlistat promoted both short-term and long-term weight loss and minimized weight regain in overweight or obese subjects [7]. PLA$_2$ serves in the initial digestion of phospholipids to free fatty acids and lysolipids. Considerable evidence from cell and animal studies suggest the importance of PLA$_2$ in facilitating the digestion and absorption of lipids [8]. Given the key role these three enzymes play in starch and lipid digestion, they represent attractive targets for prevention of excessive body weight gain and of obesity-related diseases including diabetes.

A growing literature has suggested polyphenols from teas, berries and other plants can inhibit some digestive enzymes in vitro and in vivo. For example, Horigome et al. reported that procyanidins from various plants (i.e. black locust,
bush clover, wistaria and Japanese knotgrass) have inhibitory effects on lipase, α-amylase, and trypsin[9]. Studies have shown green tea catechins can inhibit the intestinal absorption of lipids in vivo [8,10]. This was associated with in vitro inhibitory activities of tea catechins against PLA2. Among the green tea catechins, EGCG is the most potent inhibitor, and it inhibited PLA2 in vitro by 64.9 % at 2 mM. Harach et al. reported rosemary leaf extract, containing 5-10% phenolic compounds, induced a significant reduction of weight and fat mass gain associated with an increase of fecal lipid excretion in high fat–fed mice, and this effect was related to the inhibition of PL activity by the extract [11].

Cocoa (Theobroma cacao) is a rich source of polyphenols with levels reaching 12–18% by dry weight[12]. Cocoa polyphenols are primarily composed of monomeric flavan-3-ols ((-)epicatechin and to a lesser degree (+)-catechin) and oligomeric and polymeric C4β-C8 linked B-2 type procyanidins (Figure 3.1). The monomers account for only about 10 % of the total with the oligomeric and polymeric procyanidins accounting for about 90% of the flavan-3-ol content[13]. Procyanidins with a degree of polymerization (DP) up to decamer have been identified in cocoa [14]. Evidence from the literature indicates that cocoa processing dramatically affects the polyphenol and flavan-3-ol content. As cocoa beans are processed on the farm, they are often fermented for 2 to 6 days. Unfermented cocoa that has been immediately water washed and dried is referred to as “lavado” cocoa and it contains the highest amount of polyphenols. Once fermented and dried, the nib of the cocoa bean is roasted and ground, resulting in the cocoa liquor or separated into cocoa powder and cocoa butter, which are the basis for chocolate manufacture. Dutch-processing (or Alkalization) can also be applied to change the color and develop the flavor of cocoa products. Fermentation and dutch-processing have been reported to result in the loss of as much as 90% of the cocoa flavan-3-ols [15].
Studies on the health benefits of cocoa have primarily focused on the effects on the risk of cardiovascular disease [16]. Recently, a few studies on the antiobesity and antidiabetic potential of cocoa have been conducted in animal models. Matsui et al. showed that cocoa supplementation for 3 wk significantly decreased weight gain in high fat-fed rats comparing to high fat-fed controls [17]. Another study by Ruzaidi et al. also showed that dietary cocoa extract (1-3% w/w) dose-dependently reduced body weight gain, serum glucose levels, and total triglycerides in diabetic rats compared to control-fed animals [18]. Tomaru et al. reported that a diet containing 0.5 or 1.0% cocoa procyanidins decreased the levels of blood glucose and fructosamine in diabetic obese mice compared with the control treatment [19]. Jalil et al. found that short-term (acute) supplementation of cocoa extracts significantly reduced the plasma glucose level in obese-diabetic rats at 60 min and 90 min compared with untreated[20]. Given recent animal model studies showing that dietary intake of cocoa might be beneficial in preventing the onset of obesity and type II diabetes, as well as cocoa being a rich source of procyanidins, studies of inhibition of key digestion enzymes by cocoa polyphenols were warranted.

The purpose of the present study was to determine the in vitro inhibitory effects of a series of cocoa extracts, ranging from high total flavan-3-ols (lavado) to
low flavan-3-ols (dutch-processed) and isolated cocoa procyanidins against PA, PL and PLA₂, and to characterize the kinetics of such inhibition.
Materials and Methods

Materials

Cocoa procyanidins (DP = 2–10, B type) and three cocoa extracts (from regular, lavado and dutch-processed cocoa powder) were provided by The Hershey Co. (Hershey, PA). The purity of all cocoa procyanidins was >85% by HPLC-MS. The polyphenol levels in the three extracts were assessed using the Folin-Ciocalteu reagent (Sigma-Aldrich, St Louis, MO). (-)-Epicatechin (EC), orlistat and Lipase from porcine pancreas (Type II) and 4-nitrophenyl butyrate (4-NPB, 98%) were purchased from Sigma-Aldrich (St. Louis, MO). Stock solutions were prepared in dimethyl sulfoxide (EMD Chemicals Inc., Gibbstown, NJ) and stored at -80°C. α-Amylase from porcine pancreas and Red-starch were purchased from Megazyme (Wicklow, Ireland). An EnzChek Phospholipase A2 Assay Kit was purchased from Invitrogen (Carlsbad, CA). All the other reagents were of the highest grade commercially available.

Pancreatic α-Amylase Inhibition Assay in vitro

Inhibition of PA by cocoa extracts and procyanidins was examined using a modification of the chromogenic Red-starch method (Megazyme, Wicklow, Ireland). PA (0.3 U/mL) in 20 mM phosphate buffer (pH 6.9) containing 6.7 mM sodium chloride and Red-starch (7 mg/mL in 0.5 M potassium chloride) were combined with cocoa procyanidins (0–100 µM) or cocoa extracts (0–200 µg/mL). After incubation at 37°C for 10 min, the reaction was stopped by addition of 95% ethanol. After equilibration to room temperature, the solution was centrifuged at 1000g for 10 min, and the absorbance of the supernatant was measured at 510nm using a BECKMAN DU 650 spectrophotometer.

Pancreatic Lipase Inhibition Assay in vitro

Inhibition of PL by cocoa polyphenols was tested by monitoring the cleavage of 4-NPB to release 4-nitrophenol. Cocoa procyanidins (0-20 µM) or cocoa extracts
(0-200 µg/mL) were combined with PL (100 µg/mL) in 0.1 M Tris-HCl buffer (pH 8), and 4-NPB (0.2 mM) was added to start the reaction. Following incubation at room temperature for 10 min, absorbance was read at 400 nm. Orlistat was used as a positive control.

**Phospholipase A<sub>2</sub> Inhibition Assay in vitro**

Inhibition of PLA<sub>2</sub> was examined using a commercially available fluorometric method (Invitrogen, Carlsbad, CA). Buffered PLA<sub>2</sub> solution (1 U/mL, pH 8.9) and cocoa procyanidins (0-100 µM) or cocoa extracts (0-200 µg/mL) were combined in a 96 well plate. A fluorogenic PLA<sub>2</sub> substrate (Red/Green BODIPY PC-A2, 1.67µM) was dispensed to each well to start the reaction. After incubation at room temperature in the dark for 10 min, fluorescence was determined at λ<sub>ex</sub> = 485 nm and λ<sub>em</sub> = 538 nm (Fluoroskan Ascent FL, ThermoFisher Scientific Inc., Waltham, MA).

**Kinetic Analysis**

Cocoa procyanidin pentamer and decamer as well as regular cocoa extract were selected for kinetic analysis of inhibition against PL and PLA<sub>2</sub>. Reaction conditions were analogous to those above with the following modification. Cocoa procyanidins or cocoa extracts were held at constant concentrations and incubated in the presence of increasing concentrations of substrates (50 – 400 µM, PL substrate; 0.5 – 4 µM, PLA<sub>2</sub> substrate) together with enzymes and buffer solutions.

**Data Analysis**

The median inhibitory concentration (IC₅₀) of each cocoa procyanidin and extract was determined by interpolation or extrapolation of a dose-response curve using GraphPad Prism software (San Diego, CA). For kinetic analysis, Michaelis-Menten plots were generated using GraphPad Prism, and the maximum velocity (V<sub>max</sub>), Michaelis-Menten constant (Kₘ) and mode of inhibition were determined from those plots.
Data are expressed as mean ± standard deviation (SD) of the mean of at least three independent experiments. $V_{\text{max}}$ and $K_m$ values were compared by one-way ANOVA or Student’s t test as appropriate. $P < 0.05$ were considered as statistically significant.
Results

Inhibition of Digestive Enzymes by Cocoa Extracts *in vitro*

The inhibitory effects of cocoa extracts against PA, PL and PLA₂ were dose-dependent (*Figure 3.2*). Lavado, regular, and dutch-processed cocoa extracts inhibited PA by 25, 20 and 10\%, respectively, at 200 μg/mL. PL was more sensitive to all three of the cocoa extracts with IC₅₀ = 47.0, 57.7, and 172.4 μg/mL for lavado, regular and dutch-processed cocoa extract, respectively. PLA₂ was the most sensitive to cocoa extract with the lavado and regular cocoa extract showing IC₅₀ = 8.5 and 19.7 μg/mL, respectively. The dutch-processed cocoa extract was not as potent and inhibited PLA₂ only by 30\% at 200 μg/mL. To determine if the inhibition of digestive enzymes correlated with the phenol content of the extracts, we examined the levels of these compounds in the cocoa extracts using the Folin–Ciocalteu reagent (*Table 3.1*). We found, as expected, that the lavado extract had the highest levels of phenols, followed by regular cocoa, and last dutch-processed cocoa.
Figure 3-2 Inhibition of (A) PA, (B) PL, and (C) PLA<sub>2</sub> activity by cocoa extracts (regular, lavado and dutch-processed)

Values are normalized to vehicle-treated controls and expressed as the mean ± SD of at least three independent experiments.
Table 3-1 Phenol contents of the lavado, regular and dutch-processed cocoa extracts

<table>
<thead>
<tr>
<th>Cocoa Extracts</th>
<th>Phenolic Content (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regular</td>
<td>271.0 ± 5.8 a</td>
</tr>
<tr>
<td>Lavado</td>
<td>481.4 ± 9.2 b</td>
</tr>
<tr>
<td>Dutch-processed</td>
<td>128.4 ± 4.0 c</td>
</tr>
</tbody>
</table>

1 For each inhibitor, values in the same row not sharing a common superscript are, significantly different ($P<0.05$).

Inhibition of Digestive Enzymes by Cocoa Procyanidins in vitro

Dose-response curves shown in Figure 3.3 summarize the inhibitory activity of cocoa procyanidins (DP=2-10) as well as (-)-epicatechin (EC) against PA, PL and PLA$_2$. Lower molecular weight compounds with DP< 5 showed < 15% inhibition against PA at a concentration of 100 µM, whereas the higher molecular weight procyanidins (DP=5–10) inhibited PA by 17 - 45.5% at 100 µM. Cocoa procyanidins generally showed much stronger inhibitory activity against PL, EC and the dimer showed only 15% inhibition at 20 µM, whereas the trimer and tetramer caused 25% inhibition at 20 µM. Compounds with DP ≥ 5 inhibited PA by 37 - 53% at 20 µM. Orlistat was used as a positive control and inhibited PL by 72% at 10 µM. Though EC only inhibited PLA$_2$ by 4.5% at a concentration up to 100 µM, the cocoa procyanidins were particularly effective in inhibition of PLA$_2$. Cocoa procyanidins with DP=2–5 inhibited PLA$_2$ by 46 - 74% at 100 µM. For higher DP procyanidins (DP=6–10), approximately 90% of total enzyme activity was inhibited at 50 µM, and the IC$_{50}$ values for these compounds were < 5 µM.
Figure 3-3 Inhibition of (A) PA, (B) PL, and (C) PLA₂ activity by EC and cocoa procyanidins (DP=2 – 10)

Values are normalized to vehicle-treated controls and expressed as the mean ± SD of at least three independent experiments.
Correlations between DP of Cocoa Procyanidins and Their IC_{50} Values

On the basis of the dose-response curves for the pure procyanidins against PL and PLA_{2}, DP appears to be an important factor determining the potency of compound. By regression analysis, I observed a strong inverse relationship between Log IC_{50} and DP (R^2 > 0.93, Figure 3.4). Similar analysis comparing Log IC_{50} and hydrophobicity (Log P) showed no significant correlation (data not shown). Because the procyanidins did not approach the IC_{50} of PA, a similar analysis was conducted using estimated IC_{50} values.

Figure 3-4 Relationship between degree of polymerization (DP= 2 to 10) of cocoa procyanidins and the IC_{50} against PA, PL and PLA_{2}
Regression analysis was performed using Graph Pad Prism software (San Diego, CA). R^2 values are shown in the figure key.

Kinetic Analysis of PL and PLA_{2} inhibition

Because PL and PLA_{2} were more sensitive to inhibition by cocoa extracts and procyanidins, we selected these enzymes for further kinetic analysis to determine the mode of inhibition. The procyanidin pentamer and decamer as well as the regular cocoa extract were selected as test inhibitors. All three test substances reduced the V_{max} and increased K_{m} of PL (Figure 3.5, Table 3.2). These results suggest a mixed-type inhibition with respect to substrate concentration.
Figure 3-5 Inhibitory kinetics of (A) cocoa procyanidin pentamer, (B) decamer and (C) regular cocoa extract on PL

Inhibition kinetics were determined using Michaelis-Menten analysis. Values are expressed as the mean ± SD of at least three independent experiments.
Table 3-2 Effects of cocoa procyanidin pentamer, decamer and regular cocoa extract on \( V_{\text{max}} \) and \( K_m \) values of PL\(^1\)

<table>
<thead>
<tr>
<th></th>
<th>Pentamer (µM)</th>
<th>Decamer (µM)</th>
<th>Regular Cocoa Extract (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>( V_{\text{max}} )</td>
<td>142.8(^b)</td>
<td>123.8(^b)</td>
<td>142.8(^b)</td>
</tr>
<tr>
<td>( K_m )</td>
<td>77.91(^b)</td>
<td>168.3(^b)</td>
<td>77.91(^b)</td>
</tr>
</tbody>
</table>

| Inhibition Type | Mixed | Mixed | Mixed |

1 For each inhibitor, values in the same row not sharing a common superscript are significantly different (\( P < 0.05 \)).

On the other hand, Michealis-Menten plots of PLA\(_2\) inhibition by the procyanidin pentamer and decamer showed decreased \( V_{\text{max}} \) values but no effect on \( K_m \), indicating a noncompetitive inhibition with respect to substrate concentration (Figure 3.6, Table 3.3). By contrast, the regular cocoa extract increased \( K_m \) but had no effect on \( V_{\text{max}} \). These results suggest a competitive mode of inhibition against PLA\(_2\) with respect to substrate concentration (Figure 3.6, Table 3.3).
**Figure 3-6** Inhibitory kinetics of (A) cocoa procyanidin pentamer, (B) decamer and (C) regular cocoa extract on PLA$_2$

Inhibition kinetics were determined using Michaelis-Menten analysis. Values are expressed as the mean ± SD of at least three independent experiments.
Table 3-3 Effects of cocoa procyanidin pentamer, decamer and regular cocoa extract on $V_{\text{max}}$ and $K_m$ values of PLA$_2$

<table>
<thead>
<tr>
<th>Inhibition Type</th>
<th>Pentamer (µM)</th>
<th>Decamer (µM)</th>
<th>Regular Cocoa Extract (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>5</td>
<td>20</td>
</tr>
<tr>
<td>$V_{\text{max}}$</td>
<td>74.66$^a$</td>
<td>69.97$^a$</td>
<td>25.53$^a$</td>
</tr>
<tr>
<td>$K_m$</td>
<td>1.981$^a$</td>
<td>2.691$^a$</td>
<td>1.71$^a$</td>
</tr>
</tbody>
</table>

1 For each inhibitor, values in the same row not sharing a common superscript are significantly different ($P < 0.05$).
Discussion

In this study, the *in vitro* inhibitory effects of cocoa extracts and cocoa procyanidins against PA, PL and PLA$_2$ were investigated. Kinetic analysis was performed to determine the mode of inhibition by regular cocoa extracts and the procyanidin pentamer and decamer with respect to substrate concentration. To my knowledge, this is the first detailed study to report the *in vitro* inhibition of key digestive enzymes by cocoa extracts and cocoa procyanidins. Additionally, this is the first report on the kinetics of inhibition of PL and PLA$_2$ by procyanidins from any source. This study extends previous work by Goncalves *et al.*, showing that these compounds can affect digestive proteases [21,22].

It is increasingly recognized that polyphenols can regulate carbohydrate and lipid metabolism by affecting the activity of digestive enzyme. Inhibition of α-amylase *in vitro* by cocoa phenolic extracts has been noted earlier in Quesada *et al.* [20]. In the present study, all three cocoa extracts demonstrated inhibitory activities *in vitro*. Among three cocoa extracts, lavado (meaning “washed” in Spanish) cocoa undergoes the least processing (without fermentation or dutch-processing), and this extract exerted the highest inhibitory activity against all of three digestive enzymes. By contrast, the dutch-processed or alkali treated cocoa, which is the most highly processed, showed the least inhibitory effect against the enzymes tested. Because it is expected that the lavado cocoa extract is the highest in polyphenols and flavan-3-ols, followed by the regular cocoa extract and the least would be found in the dutch-processed cocoa extract, these results suggest that the inhibitory effects of cocoa extracts are related to their polyphenol content. In this study, the lavado cocoa extract was a potent enzyme inhibitor, and the results are comparable or superior to some recent studies with other polyphenol-rich extracts. Moreno *et al.* found that grape seeds extract at a concentration of 1 mg/mL resulted in 80% inhibition against PL, and they also suggested that the inhibitory effect may be caused by a synergistic action of several phenolic compounds including procyanidins within the extracts [23]. Polyphenol-rich berry fruits such as strawberry and raspberry have
been shown to inhibit PA, but the effects were relatively weak (e.g. 25 mg/mL strawberry extract inhibited PA by 14.7 % in vitro) [24,25].

In general, the cocoa procyanidins (DP=2-10) showed greater inhibitory activity against PLA2 than PL and PA. These results mirror the results of cocoa extracts and suggest that procyanidins are the components in cocoa responsible for inhibition of these digestive enzymes. The inhibitory potency of the cocoa procyanidins is increased as a function of DP. These results are in agreement with some recent studies. Sugiyama et al. reported that the oligomeric procyanidins in apples significantly decreased the plasma triglyceride levels in both mice and humans, and inhibited PL activity in vitro. They also suggested that DP was an important factor in determining the inhibitory potency, and a strong inverse correlation was observed [24]. Another study found that procyanidins from persimmon peel showed strong inhibitory activity against α-amylase in vitro (IC_{50} < 100 µg/mL), and the inhibition of α-amylase activity was dependent on the DP [26].

The results of kinetic analysis suggested that regular cocoa extracts, the procyanidin pentamer and decamer inhibited PL activity in a mixed mode. By contrast, the procyanidin pentamer and decamer noncompetitively inhibited PLA2 activity, whereas the regular cocoa extracts inhibited PLA2 in a competitive fashion. These results demonstrate the diversity of potential interactions between the procyanidins, the enzyme surface and/or the substrate, and such interactions need further study by in silico or crystallographic methods. These results suggest that other compounds in cocoa beyond the procyanidins might also contribute to the inhibitory potency of the extract. In addition to the flavan-3-ols, cocoa is also rich in methylxanthines (caffeine, theobromine and theophylline), which have been shown to have thermogenic, diuretic and appetite-suppressing properties that may aid in obesity and diabetes prevention [27]. However, scientific data in relation to the in vitro inhibition of digestive enzymes by methylxanthines are still limited.

Biological properties of cocoa polyphenols are modulated by their bioavailability. One proposed limitation of cocoa procyanidins is their low systemic bioavailability. Studies have shown that monomers and dimers in cocoa can be
absorbed, and they began to appear in plasma within 30 – 60 min post consumption [28,29]. Despite their presence in cocoa in high amounts, procyanidin oligomers larger than dimers have not been detected in human plasma following the consumption of cocoa products [30]. However, because this study is focused on the small intestine lumen as the site of action, I believe the bioavailability is not a limiting factor. Previous studies have shown that these compounds are stable in the stomach and small intestinal milieu and are expected to be present in the small intestinal lumen at relatively high concentrations following consumption of cocoa products, particularly those with high polyphenol content (e.g., dark chocolate) [31,32]. I believe that the effective concentrations in the enzyme inhibition assays are physiologically achievable in this situation, although further studies are needed to confirm the \textit{in vivo} activity and small intestinal bioavailability of these compounds.

In summary, the present study provides the first evidence that cocoa extracts and cocoa procyanidins are potent inhibitors of key enzymes in digestion of carbohydrates and lipids \textit{in vitro}, and these inhibitory activities are related to polyphenol content in cocoa extracts and the degree of polymerization of cocoa procyanidins. Further \textit{in vivo} studies are needed to examine whether cocoa extracts and/or cocoa procyanidins can inhibit digestive enzymes \textit{in vivo} and related downstream pathways at dose levels achievable in the diet.
References


Chapter 4
Cocoa Procyanidins Modulate Eicosanoid Metabolism and Cytokine Production in RAW 264.7 Macrophages
**Abstract**

Inflammation is a normal host defense response that is triggered by harmful stimuli such as infection, but unresolved chronic inflammation also contributes to the pathophysiology of many chronic diseases. Cocoa (*Theobroma cacao*) is a rich source of polyphenols, particularly oligomeric flavan-3-ols (i.e. procyanidins), which have received considerable attention due to potential anti-inflammatory activities. However, the underlying mechanisms of action of cocoa procyanidins (CPs) are not well-understood and there are still gaps and inconsistencies in the literature. The objective of the present study is to investigate the anti-inflammatory effects of CPs with high degrees of polymerization (DP) on the modulation of eicosanoid metabolism and cytokine production *in vitro*. The results indicate that a mixture of CPs with DP ≥ 7 dose-dependently inhibited purified phospholipase A$_2$ (PLA$_2$) (IC$_{50}$=3.2 µg/ml) and cyclooxygenase-2 (COX-2) (IC$_{50}$= 57.7 µg/ml) *in vitro*, while dose-dependently decreasing prostaglandin E$_2$ (PGE$_2$) production by lipopolysaccharide (LPS)-stimulated RAW 264.7 murine macrophages. In addition, CP octamers (DP=8) suppressed pro-inflammatory cytokine production, including tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and monocyte chemoattractant protein-1 (MCP-1) by LPS-stimulated RAW 264.7 cells without significant cytotoxicity. Together, this study demonstrates an anti-inflammatory role and potential therapeutic value of procyanidin-rich cocoa in the diet of patients with inflammatory conditions and may lead to a natural approach to treating and preventing chronic diseases.

**Key Words:** Cocoa, *Theobroma cacao*, procyanidin, inflammation, eicosanoid, cytokine
**Abbreviations:**

CPs, cocoa procyanidins; **COX-2**, cyclooxygenase-2; **DP**, degree of polymerization; **IC**$_{50}$, the median inhibitory concentration; **IL-6**, interleukin-6; **MCP-1**, monocyte chemoattractant protein-1; **PGE$_2$**, prostaglandin E$_2$; **PLA$_2$**, phospholipase A$_2$; **TNF-α**, tumor necrosis factor-α; **LPS**, lipopolysaccharide.
Introduction

Inflammation is a biological defense and repair mechanism of the innate immune system in response to harmful stimuli [1]. Distinct from acute (classical) inflammation, which is a helpful component of the body's response to injury or infection, chronic inflammation is a persistent inflammatory process that causes progressive damage to the body that leads to a variety of diseases. This process can be seen in the involved tissues and systemically by elevated circulating levels of inflammatory markers [2]. Both types of inflammation (acute and chronic) are primarily driven by the production of pro-inflammatory eicosanoids [3]. There is growing evidence that obesity is a state of chronic inflammation, and this inflammation has been proposed as one of the mediating processes in metabolic disease development such as diabetes and cardiovascular diseases [4]. Obesity-related inflammation is characterized by the increased production of a number of cell-signaling proteins, including cytokines such as tumor necrosis factor (TNF-α) and interleukin-6 (IL-6), and chemokines (i.e. chemotactic cytokine) such as monocyte chemoattractant protein-1 (MCP-1) [5]. In obese individuals, there is increased macrophage infiltration into adipose tissue, and the infiltrated macrophages are the main source of these inflammation-related cytokines [6].

Cocoa (Theobroma cacao) and cocoa-based products contain high concentrations of polyphenols. The primary polyphenols present in cocoa are monomeric ((-)epicatechin and (+) catechin) and C4β-C8 linked B-type oligomeric and polymeric (procyanidins) flavan-3-ols (Figure 4.1). The monomers account for only about 10% of the total flavan-3-ol content while the oligomeric and polymeric procyanidins account for about 90% [7]. Procyanidins with a degree of polymerization (DP) up to decamer have been identified in cocoa [8], and they are present at significantly higher concentrations in cocoa than in other procyanidin-rich foods such as red wine or green tea [9,10].

One of the anti-inflammatory mechanisms of cocoa flavan-3-ols and CPs is through the inhibition of eicosanoid-generating enzymes including phospholipase
$A_2$ (PLA$_2$) and cyclooxygenase-2 (COX-2), thereby reducing the concentrations of pro-inflammatory eicosanoids [10–12]. PLA$_2$ catalyzes the hydrolysis of the acyl ester bond at the sn-2 position of membrane phospholipids. This forms the first step in the synthesis of eicosanoids. COX-2 is a pivotal enzyme isoform that has long been recognized as a chemopreventive target as well as an anti-inflammatory drug target. Prostaglandin E$_2$ (PGE$_2$) is one of its eicosanoid metabolites that plays an important role in obesity-related inflammatory responses.

![Figure 4-1 Structure of B type cocoa procyanidins with C4β-C8 linkage](image)

Recent studies also suggest that cocoa procyanidins (CPs) may exhibit anti-inflammatory activity by suppressing the production of inflammatory cytokines including IL-1β, IL-2, IL-4, IL-6, TNF-α and MCP-1 [13,14]. However, given the wide spectrum of molecules and their complex interactions, the results are limited and inconsistent and opposing effects on inflammatory cytokine production of higher-DP procyanidins have been reported [15,16].

The objective of the present study is to investigate the anti-inflammatory activities of CPs, particularly those with high DP, through the inhibition of eicosanoid-generating enzymes in vitro, the modulation of inflammatory cytokines, and eicosanoid metabolism in a lipopolysaccharide (LPS)-stimulated macrophage cell line. The present study will allow us to develop a clearer mechanistic
understanding of the anti-inflammatory action of CPs, and provide mechanistic biomarkers that can be tested in future animal studies of obesity-induced inflammation.
**Materials and Methods**

**Chemicals**

CPs mixture, an extract of procyanidins with DP ≥ 7 from cocoa, and CP octamers (DP=8) with purity >85% were generously provided by The Hershey Company (Hershey, PA). CPs mixture and CP octamers were dissolved in DMSO and stored at -80°C. All other chemicals were of the highest grade commercially-available.

**Cell Culture and Viability Assay**

RAW 264.7 murine macrophage cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) containing 10% (v/v) fetal bovine serum (Atlanta Biologicals, Norcross, GA) and 1% (v/v) penicillin/streptomycin solution (Cellgro, Hemdon, VA) at 37 °C under a humidified atmosphere of 5% carbon dioxide/95% air. The medium was changed every 48 hrs. The viability of cells was assessed by the 3-(4,5-dimethylthiazoly-2)-2,5-diphenyltetrazolium bromide (MTT) assay in 96-well plates. In brief, following treatment with the test agent of interest, the medium was aspirated and cells were washed with fresh medium. MTT (Sigma-Aldrich, St. Louis, MO) in PBS (1 mg/mL) was added to each well. After incubation for 30 min at 37 °C, the medium was removed and the resulting formazan precipitate was solubilized in 100 µL of dimethyl sulfoxide (DMSO, EMD Chemicals, Inc., San Diego, CA). The absorbance was then measured at 540 nm.

**PLA₂ Assay**

Inhibition of PLA₂ was examined using a commercially-available fluorometric method (Invitrogen, Carlsbad, CA) as described previously [17]. Briefly, PLA₂ solution (1 U/mL, pH 8.9) and CPs mixture (DP≥ 7, 0-100 µg/ml) were combined in a 96-well plate. A fluorogenic PLA₂ substrate (Red/Green BODIPY® PC-A2, 1.67µM) was dispensed to each well to start the reaction. After incubation at room temperature in the dark for 10 min, fluorescence was determined at λ_ex = 485 nm.
and $\lambda_{em} = 538$ nm (Fluoroskan Ascent FL, ThermoFisher Scientific Inc., Waltham, MA).

**COX-2 Assay**

Inhibition of purified human recombinant COX-2 (Cayman Chemical Co., Ann Arbor, MI) was determined by monitoring oxygen consumption using an oxygraph equipped with an oxygen electrode (Hansatech, Norfolk, UK). The reaction mixture consisted of 0.1M Tris-Cl buffer (pH 8.0), 1 µM hematin, 5mM EDTA, 0.5mM L-tryptophan, 5 µg enzyme, and 100 µM arachidonic acid. CPs mixture (0-200 µg/mL) was dissolved in DMSO. The reaction was initiated with the addition of substrate (i.e. arachidonic acid), the rate of oxygen consumption was measured at 37°C for 20 seconds in the oxygraph chamber, and the results were charted on a computer.

**PGE$_2$ Production by LPS-stimulated RAW 264.7 Macrophage Cells**

Cells were plated at a density of $1 \times 10^5$ cells/mL in 96-well plates. Three treatment schemes were applied to the cells: 1) cells were pre-treated with CPs mixture for 6 hr and then stimulated with 1 µg/mL LPS for additional 6 hr (Pre-CPs); 2) cells were treated with CPs mixture and 1 µg/mL LPS for 12 hr (Co-treat); 3) cells were challenged with 1 µg/mL LPS for 6 hr and then treated with CPs mixture for another 6 hr. A wide range of concentrations of the CPs mixture (0-100 µg/mL) was used in all three conditions of treatments. After 12 hr of incubation, cell supernatants were collected, and the production of PGE$_2$ was measured by a commercially-available ELISA kit (Cayman Chemical Co., Ann Arbor, MI).

**Cytokine Production in LPS-stimulated RAW 264.7 Macrophage Cells**

To study the inhibition of LPS-stimulated cytokine production by CP octomer (0-200 µM), the treatment schemes analogous to those employed for PGE$_2$ production were used. In the Pre-CP treatment, cells were treated with CP octamer for 12 hr and then stimulated with 1 µg/mL LPS for 10 hr. In the Co-treat, cells are treated with CP octamer and 1 µg/mL LPS for a total of 22 hr. Finally, in the Pre-LPS
treatment, cells were challenged with 1.5 μg/mL LPS for 1 hr and then treated with CP octamer for 8 hr. In all three experiments, at the end of the prescribed incubation time, the cell supernatants were collected and the levels of pro-inflammatory cytokines (TNF-α, IL-6 and MCP-1) were determined using commercially available ELISAs (R&D Systems, Minneapolis, MN).

**Data and Statistical Analysis**

The median inhibitory concentration (IC\(_{50}\)) of CPs mixture and CP octamer was determined by a fitted dose-response curve using GraphPad Prism software (San Diego, CA). Data are expressed as mean ± standard deviation (SD) of the mean of at least three independent experiments and were compared by one-way ANOVA with Dunnett’s post hoc test. \(P < 0.05\) was considered statistically significant.
Results

Inhibition of Purified PLA₂ and COX-2 by Cocoa Procyanidins

CPs mixture (DP ≥ 7) demonstrated potent inhibitory activity both against PLA₂ (Group III secreted) and COX-2 in vitro (Figure 4.2). CPs mixture concentrations ranging from 1.6 to 100 μg/mL exerted an increasing inhibition of PLA₂ enzyme activity, which reached a plateau (90% inhibition) at around 25 μg/mL and the IC₅₀ was 3.2 μg/mL. CPs mixture also inhibited COX-2 enzyme activity in a dose-dependent manner but less potently than inhibition of PLA₂ with an IC₅₀ = 57.7 μg/mL.

Figure 4-2 Inhibition of cocoa procyanindin (CPs) mixture (DP ≥ 7) against (A) PLA₂ and (B) COX-2 in vitro

Values are expressed as mean ± SD of at least three independent experiments.

PGE₂ Production by LPS-Simulated RAW 264.7 Macrophages

To determine the effects of CPs mixture on the production of PGE₂ in LPS-stimulated (1 μg/mL) RAW 264.7 macrophages, cells received three treatment schemes: pre-treated before stimulation (Pre-CPs), co-treated (Co-treat) or treated after stimulation with varying concentrations of CP mixture (Pre-LPS). In order to ensure that inhibition of PGE₂ production was not secondary to inhibition of cell viability, the cytotoxicity of the CPs mixture was examined at concentrations up to
100 µg/mL (Figure 4.3 A). Whereas Pre-CPs and Co-treatment displayed a dose-dependent and moderate cytotoxicity (near 40% reduction in cell viability at 100 µg/mL), the Pre-LPS treatment showed no significant cytotoxicity.

The production of PGE2 was measured in all three aforementioned treatments (Figure 4.3 B). Overall, the CPs mixture significantly decreased PGE2 production in LPS-stimulated RAW264.7 cells at all concentrations compared to control. Among the three treatment regimens, Pre-CPs was the most potent treatment (IC50 = 6.2 µg/mL). Co-treat was the next most potent regimen (IC50 = 10.3 µg/mL). Cells pre-challenged with LPS were less responsive to treatment, but inhibitory effects were still dose-dependent (IC50= 33.1 µg/mL).

Figure 4-3 Impact of CP mixture (DP > 7) on the (A) cell viability and (B) PGE2 production of LPS-stimulated RAW 264.7 macrophages
Cells were pre-treated for 6 hr, co-treated for 12 hr, or treated for the last 6 hr with varying concentrations of cocoa procyanindin (CPs) mixture (DP ≥ 7). Values are expressed as mean
± SD of at least three independent experiments. Cell viability was compared by One-way ANOVA with Dunnet's post-test (* P<0.05, *** P<0.001).

Cytokine Production by LPS-Simulated RAW 264.7 Macrophages

Elevated production of pro-inflammatory cytokines is a characteristic of many inflammatory processes. I examined the effect of CP octamer (DP=8) on cytokine production in LPS-stimulated RAW 264.7 macrophages. Similar to the results of CPs mixture, Pre-CP and Co-treat with CP octamer both exerted a moderate cytotoxic effect against RAW 264.7 cells with IC₅₀s of 89.4 μM and 41.4 μM, respectively (Figure 4.4 A & B), whereas Pre-LPS has little effect on cell viability (Figure 4.4 C).

Production of TNF-α, IL-6 and MCP-1 were compared at varying concentrations of CP octamer. Similar to the results on PGE₂, Pre-CP was a potent treatment scheme: at 50 μM, TNF-α, IL-6, and MCP-1 production was reduced by 72.4, 100, and 47.5%, respectively compared to LPS-stimulated controls (Figure 4.4 D). Co-treat also resulted in significantly reduced cytokine levels, but the effects were less potent (Figure 4.4 E). By contrast, TNF-α production was not observed in the Pre-LPS group (with very low signals below the limit of detection); however, it significantly reduced IL-6 production at all tested concentrations (P< 0.001) and MCP-1 production at concentrations higher than 3.1 μM (P< 0.01, Figure 4.4 F). Among the three pro-inflammatory cytokines, IL-6 was the most sensitive in response to CP octamer treatments. The IC₅₀ values of CP octamer against IL-6 production were 3.76 μM (Pre-CP), 26.25 μM (Co-treat), and 2.66 μM (Pre-LPS).
**Figure 4** Impact of CP octamer (DP=8) on the (A-C) cell viability and (D-F) cytokine production of LPS-stimulated RAW 264.7 macrophages

Cells were either pre-treated with CP octamer before LPS stimulation (Pre-CP, A & D), co-treated with LPS (co-treat, B & E) or pre-challenged with LPS before CP treatment (Pre-LPS, C & F). Values are expressed as mean ± SD of at least three independent experiments, and were compared by One-way ANOVA with Dunnet’s post-test (* P<0.05, ** P< 0.01, *** P<0.001).
Discussion

The inflammatory process is defined as a sequence of events that occurs in response to noxious stimuli and is orchestrated by a highly regulated interplay of molecular events [18,19]. As a hallmark characteristic of innate immunity, inflammation has gained recognition as an underlying contributor to many chronic diseases including obesity and the associated metabolic disease cluster. Eicosanoids and cytokines are two groups of multifunctional mediators that are extensively involved in many steps of inflammatory responses [19,20]. In the present study, I investigated the in vitro modulatory effects of CPs on eicosanoid metabolism and cytokine production. CPs with high DP demonstrated inhibitory activities against the eicosanoid-generating enzymes, PGE$_2$ production, and pro-inflammatory cytokine production including TNF-α, IL-6, and MCP-1.

PLA$_2$-catalyzed hydrolysis of membrane phospholipids results in the stoichiometric production of a free fatty acid, most importantly arachidonic acid, which serves as a precursor for inflammatory mediators such as eicosanoids [21]. PLA$_2$s are found both in the intracellular and secretory forms, among which, group III secreted PLA$_2$ is a known mediator of inflammation, atherosclerosis, and cancer in mammals [22,23]. Analyses of group III secreted PLA$_2$ in transgenic mice showed potential participation of this enzyme in plasma lipoprotein modification and macrophage foam cell formation [24]. In the present study, in vitro enzyme activity of PLA$_2$ was almost completely inhibited by CPs mixture at a relatively low concentration (25 μg/mL). Moreover, modeling studies have revealed that the CPs were able to occupy the active site-containing tunnel of PLA$_2$ and sterically block access to the active site for the substrate resulting in a non-competitive inhibition [25].

Inhibition of prostaglandin (PG) synthesis is at the center of current anti-inflammatory therapies, and COX-2 is the key enzyme for prostaglandin biosynthesis. There is accumulating evidence indicating that COX-2 and its product, PGE$_2$, are involved in obesity-related inflammatory processes. Ghoshal et al.
reported that mice deficient in COX-2 had significantly reduced final body weight and percent body fat as well as decreased adipose tissue differentiation and inflammation [26]. Brunetti et al. have shown that PGE\(_2\) levels were significantly increased in obese rats and that PGE\(_2\) could drive an inflammatory process leading to increased incidence of prostate, kidney, and testicular cancer [27]. Direct inhibition of the enzyme activities of COX-2 has also been actively pursued as a pharmacological approach to treat inflammation resulting in the development of drugs such as celecoxib [28]. My results show that CPs mixture inhibited purified COX-2 enzyme and reduced the production of PGE\(_2\) by LPS-stimulated RAW264.7 cells. Together, these results suggest that CPs mixture may be a potential anti-inflammatory agent and an emerging alternative to traditional anti-inflammatory drugs such as aspirin and other NSAIDs (non-steroidal anti-inflammatory drugs).

RAW 264.7 macrophage cell line is established in vitro model of inflammation and, in response to treatment with bacterial LPS, up-regulate the expression of inflammatory mediators including cytokines and eicosanoids [29]. The production of pro-inflammatory cytokines is a critical step in the establishment and maintenance of a state of chronic inflammation, and is therefore a primary anti-inflammatory target for treatment of chronic metabolic diseases [10]. Although the underlying mechanisms by which CPs modulate cytokine production have not yet been clarified, my results demonstrate the inhibitory effects of CP octamer on the production of TNF-\(\alpha\), IL-6, and MCP-1 in a cell culture model of inflammation. The results also suggest modulatory effects of CP octamer were dependent on the cytokine targets as well as the treatment schemes. TNF-\(\alpha\) and IL-6 are both pro-inflammatory cytokines which may directly impair insulin signaling and are critical mediators of insulin resistance [30–33]. Compared to TNF-\(\alpha\), IL-6 was much more sensitive to CP treatments. In the case of TNF-\(\alpha\), the DP of individual CPs seemed to be an important factor in determining its inhibitory activity. For example, Mao et al. [34] examined the TNF-\(\alpha\) secretion in PHA-stimulated human PBMC and found that the intermediate-sized CPs (tetramer-octamer) enhanced TNF-\(\alpha\) secretion in the range of 48–128%, while monomers and dimers were slightly inhibitory. MCP-1 is a
chemotactic cytokine (chemokine) that recruits immune cells to sites of inflammation, and it is responsible for the infiltration of macrophages into adipose tissue in obese subjects [14]. Ramiro et al. have reported that treatment of cocoa extract (50 µg/mL) and (-)-epicatechin (200µM) caused a significant reduction in the production of MCP-1 in LPS-stimulated NR8383 macrophages [13]. The results in the present study indicate that CP octamer is an effective inhibitor of MCP-1 with IC50 values of 50 -100 µM regardless of whether LPS stimulation was added before, after, or during the CP treatment. Moreover, the two pretreatment methods (Pre-CP and Pre-LPS) were overall more potent than Co-treat, which is likely due to an interaction between LPS and CP as well as the added toxicity by co-treatment on cell growth.

It is noted that the CPs mixture and CP octamer concentrations are not physiologically achievable, which is similar to previous cell culture studies, Bioavailability studies indicate that plasma concentrations of cocoa flavan-3-ols following dietary intake are low and mostly in the nanomolar range. Therefore, the underlying mechanisms involved in the cellular uptake of CPs as well as its cellular concentrations and subcellular distribution need to be studied in the future. At the same time, studies have reported that CPs are stable during gastric transit in humans and can reach the small intestine intact [35]. Therefore, it is suggested that because of their rather poor absorption in an intact form, larger CPs are present in the intestinal lumen at higher concentrations and are able to modulate the inflammatory responses of intestinal-resident macrophages, especially those in Peyer’s patches. Peyer’s patches are aggregations of lymphoid tissue that are usually found in the lowest portion of the small intestine. Macrophages in Peyer's patches are part of the first-line defense mechanisms (i.e. innate immune system) and are important in the initiation of gastrointestinal immune responses to enteric pathogens [36]. Additionally, I noticed from the MTT results that CPs demonstrated a moderate to strong cytotoxicity with LPS stimulation in RAW 264.7 cells, especially in the condition of Pre-CP and Co-treat. Therefore, the observed inhibitory effects on PGE2 as well as cytokine production may be partly related to
the decreased cell viability by CP treatments. However, the Pre-LPS treatment, which had little to no cytotoxicity, showed the most potent inhibition against cytokine IL-6 and MCP-1 production of the three treatments. Therefore, the observed anti-inflammatory activity of CPs is not likely to be secondary to the decreased cell viability. Furthermore, the addition of CP after LPS challenge may exert a cytoprotective activity counteracting the effect of LPS and facilitating its clearance and resolving inflammation. Therefore, CP treatment seems to be particularly effective in correcting a disorder or disease in subjects at risk. Moreover, since the interaction and cross-talk between macrophages and adipocytes play a central role in obesity-associated inflammation, future studies will need to elucidate the bioactivities of CP in an adipocyte-macrophage co-culture system.

In conclusion, CPs exert anti-inflammatory activities by modulating eicosanoid metabolism and the production of pro-inflammatory cytokines TNF-α, IL-6, and MCP-1 in vitro. These findings demonstrate the potential value of cocoa and CPs in the diet of patients with inflammatory conditions and may lead to a natural approach to treat and prevent chronic diseases.
References


Chapter 5

Dietary Cocoa Ameliorates Obesity-Related Inflammation in High-Fat Fed Mice
Abstract

The purpose of the present study is to investigate the effect of cocoa powder supplementation on obesity-related inflammation in high-fat (HF)-fed obese mice. Male C57BL/6J (n=126) mice were fed with either low-fat (LF, 10% kcal from fat) or HF (60% kcal from fat) diet for 18 wk. At wk 9, mice from HF group were randomized to HF diet or HF diet supplemented with 8% unsweetened cocoa powder (HF-HFC group) for 10 wk. Blood and tissue samples were collected for biochemical analyses. Results have shown that cocoa powder supplementation significantly reduced the rate of body weight gain (15.8%) and increased fecal lipid content (55.2%) compared to HF-fed control mice. Further, cocoa supplementation attenuated insulin resistance, as indicated by improved HOMA-IR, and reduced the severity of obesity-related fatty liver disease (decreased plasma alanine aminotransferase (ALT) and liver triglyceride) compared to HF group. Cocoa supplementation also significantly decreased plasma levels of the pro-inflammatory mediators interleukin-6 (IL-6, 30.4%), monocyte chemoattractant protein-1 (MCP-1, 25.2%), and increased adiponectin (33.7%) compared to HF-fed mice. Expression of pro-inflammatory genes (Il6, Il12b, Nos2 and Emr1) in the stromal vascular fraction (SVF) of the epididymal adipose tissue (AT) was significantly reduced (37-56%) in the cocoa-supplemented mice. In conclusion, short-term dietary supplementation with cocoa ameliorates HF-induced body weight gain, systemic inflammation, insulin resistance and fatty liver disease in mice. These effects appear to be mediated in part by a modulation of dietary fat absorption and inhibition of macrophage infiltration in AT.

Key words: cocoa, *Theobroma cacao*, polyphenols, inflammation, obesity
Abbreviations:
ALT, alanine aminotransferase; AT, adipose tissue; ATM, adipose tissue-associated macrophage; DP, degree of polymerization; HF, high-fat; HFC, high-fat diet supplemented with 8% cocoa powder; HOMA-IR, Homeostasis model assessment of insulin resistance; IL, interleukin; iNOS, inducible nitric oxide synthase; LF, low-fat; MCP-1, monocyte chemoattractant protein-1; NO, nitric oxide; ORFLD, obesity-related fatty liver disease; SVF, stromal vascular fraction; TNF-α, tumor necrosis factor-α.

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Introduction

Obesity is defined as a phenotypic manifestation of abnormal or excessive fat accumulation that alters health and increases mortality [1]. Today, more than 35% of Americans are obese and if the current trajectory continues, the rate will reach 51% by 2030 [2]. Indeed, obesity is a multifactorial disorder that is a significant risk factor for type 2 diabetes, cardiovascular disease, obesity-related fatty liver disease (ORFLD), and certain cancers [1, 3, 4]. Obesity and the associated metabolic pathologies are the most common and detrimental metabolic diseases, affecting over 50% of the adult population [5]. It is becoming more evident that obesity is a chronic inflammatory state, also known as low-grade or systemic inflammation, which represents the important link between obesity and its co-morbidities [1, 3, 4].

Obesity-related inflammation is characterized by macrophage infiltration into adipose tissue (AT), abnormal cytokine production, and activation of inflammatory signaling pathways [5–7]. Compared with AT from lean individuals, AT from obese individuals expresses increased amounts of pro-inflammatory mediators including tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), inducible nitric oxide synthase (iNOS, also known as NOS2), and monocyte chemoattractant protein-1 (MCP-1), as well as decreased levels of adiponectin [8, 9]. Adipose tissue-associated macrophages (ATMs) are responsible for nearly all TNF-α released by AT and approximately 50% of AT-derived IL-6 in obese mice [8, 10]. Most cytokines produced by AT are closely associated with obesity-induced insulin resistance and hepatic steatosis (fatty liver), the excess accumulation of triglycerides in hepatocytes [11], which is commonly reflected by abnormal circulating concentrations of hepatic enzymes (e.g. alanine aminotransferase (ALT)).

Recently, a growing number of studies have reported the beneficial health effects of cocoa (*Theobroma Cacao*) and cocoa polyphenols, including modulation of atherosclerosis and hypertension [12, 13]. A number of potential mechanisms have been proposed for these effects, including inhibition of platelet aggregation, as well as antioxidant and anti-inflammatory effects [14, 15]. Relatively few studies have
investigated the preventive or therapeutic effects of cocoa and cocoa constituents against obesity and metabolic syndrome [16–20]. For example, treatment of high-fat fed rats with 12.5% cocoa powder for 3 wk significantly decreased final body weights, mesenteric AT weights, and modulated the expression of genes related to fatty acid metabolism [17]. Tomaru et al. [18] and Yamashita et al. [20] have both reported that dietary supplementation with cocoa liquor procyanidins suppressed high-fat diet-induced hyperglycemia, glucose intolerance and fat accumulation in AT in diabetic obese mice.

Several in vitro studies have shown that certain isolated flavan-3-ols and procyanidins from cocoa exert anti-inflammatory activities by modulating the transcription and secretion of pro-inflammatory cytokines in human peripheral blood mononuclear cells and macrophages [21–25]. For example, epicatechin and cocoa procyanidins have been found to reduce secretion of TNF-α, MCP-1, and nitric oxide (NO) in macrophages in vitro [24]. Previous studies in my thesis research have reported that cocoa-derived procyanidins potently inhibited the activity of secreted phospholipase A$_2$ in a cell-free system [26] (Chapter 3) and suppressed the production of inflammatory cytokines by macrophages (Chapter 4). Inhibitory potency increased as a function of degree of polymerization (DP) with higher molecular weight compounds (DP>7) having the most potent activity. These studies would suggest that cocoa has anti-inflammatory effects in vivo but further experimental evidence is needed to establish effective dose levels, demonstrate efficacy and establish the underlying mechanisms of action.

In the present study, I investigated the effects of cocoa powder supplementation on markers of obesity-related inflammation and co-pathologies in high fat-fed obese C57BL/6J mice.
Materials and Methods

Diets and Chemicals

Unsweetened cocoa powder was generously provided by Blommer Chocolate Co. (Chicago, IL). The composition of the cocoa powder including polyphenol content is shown in Table 5.1. Total polyphenols in cocoa powder were quantified by Folin-ciocalteu method with gallic acid as standard, and the contents of flavan-3-ols (from monomers to decamer) were determined by diol HPLC, described by previous method [27]. All other chemicals were of the highest grade commercially-available. Low-fat (LF, 10% kcal from fat, D12450B), high-fat (HF, 60% kcal from fat, D12492) and HF diet supplemented with 80 g/kg cocoa (HFC, D10052503) diet were prepared by Research Diets (New Brunswick, NJ). The composition of the diets is given in Table 5.2. Fatty acid profile of each diet is also given in Table 5.3., it was determined by gas chromatography (GC). Briefly, about 200 mg of each diet sample was weighed before lipid extraction and methylation. A one-step lipid extraction and fatty acid methyl ester (FAME) preparation was carried out using a modified method from Garces and Mancha [28]. AT was heated with a reagent containing methanol: heptane: toluene: 2,2-dimethoxypropane: H₂SO₄. During heating, the simultaneous digestion and lipid transmethylolation took place in a single phase. After cooling two phases were formed with the upper phase containing the FAMEs used for GC analysis. FAMEs were quantified using a GC (Agilent 6890 series, Atlanta, GA) equipped with a silica-fused capillary column and a flame ionization detector. Percentage of fatty acid peak areas were compared.
Table 5-1 Composition of unsweetened cocoa powder

<table>
<thead>
<tr>
<th></th>
<th>Unsweetened Cocoa Powder</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Energy (kcal/g)</strong></td>
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</tr>
<tr>
<td><strong>Macronutrients (mg/g)</strong></td>
<td></td>
</tr>
<tr>
<td>Total Fat</td>
<td>100</td>
</tr>
<tr>
<td>Total Carbohydrate</td>
<td>600</td>
</tr>
<tr>
<td>Dietary Fiber</td>
<td>400</td>
</tr>
<tr>
<td>Sugar</td>
<td>0</td>
</tr>
<tr>
<td>Total Protein</td>
<td>200</td>
</tr>
<tr>
<td><strong>Polyphenols (mg/g)</strong></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>71.0</td>
</tr>
<tr>
<td>Monomers</td>
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</tr>
<tr>
<td>Epicatechin</td>
<td>4.6</td>
</tr>
<tr>
<td>Catechin</td>
<td>2.2</td>
</tr>
<tr>
<td>Procyanidins (DP=2-10)</td>
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</tr>
<tr>
<td>Dimer</td>
<td>3.3</td>
</tr>
<tr>
<td>Trimer</td>
<td>3.1</td>
</tr>
<tr>
<td>Tetramer</td>
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</tr>
<tr>
<td>Pentamer</td>
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<tr>
<td>Hexamer</td>
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</tr>
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<td>Heptamer</td>
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</tr>
<tr>
<td>Octamer</td>
<td>2.6</td>
</tr>
<tr>
<td>Nonamer</td>
<td>1.5</td>
</tr>
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<td>Decamer</td>
<td>1.7</td>
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Table 5-2 Composition of mouse diets

<table>
<thead>
<tr>
<th>Macronutrient composition</th>
<th>LF</th>
<th>HF</th>
<th>HFC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein, % of energy</td>
<td>20.0</td>
<td>20.0</td>
<td>21.0</td>
</tr>
<tr>
<td>Carbohydrate, % of energy</td>
<td>70.0</td>
<td>20.0</td>
<td>21.0</td>
</tr>
<tr>
<td>Fat, % of energy</td>
<td>10.0</td>
<td>60.0</td>
<td>59.0</td>
</tr>
<tr>
<td>Energy (MJ/kg)</td>
<td>15.9</td>
<td>21.8</td>
<td>21.4</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Ingredient (g/kg)</th>
<th>LF</th>
<th>HF</th>
<th>HFC</th>
</tr>
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<tbody>
<tr>
<td>Casein</td>
<td>189.6</td>
<td>258.4</td>
<td>237.8</td>
</tr>
<tr>
<td>L-Cystine</td>
<td>2.8</td>
<td>3.9</td>
<td>3.8</td>
</tr>
<tr>
<td>Corn starch</td>
<td>298.6</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Maltodextrin</td>
<td>33.2</td>
<td>161.5</td>
<td>148.6</td>
</tr>
<tr>
<td>Sucrose</td>
<td>331.7</td>
<td>88.9</td>
<td>81.8</td>
</tr>
<tr>
<td>Cellulose</td>
<td>47.4</td>
<td>64.6</td>
<td>59.4</td>
</tr>
<tr>
<td>Soybean oil</td>
<td>23.7</td>
<td>32.3</td>
<td>29.7</td>
</tr>
<tr>
<td>Lard</td>
<td>19.0</td>
<td>318.6</td>
<td>291.3</td>
</tr>
<tr>
<td>Mineral mix&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9.5</td>
<td>12.9</td>
<td>11.9</td>
</tr>
<tr>
<td>Vitamin mix&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9.5</td>
<td>12.9</td>
<td>11.9</td>
</tr>
<tr>
<td>Choline bitartrate</td>
<td>1.9</td>
<td>2.6</td>
<td>2.4</td>
</tr>
<tr>
<td>Cocoa</td>
<td>0.0</td>
<td>0.0</td>
<td>80.0</td>
</tr>
</tbody>
</table>

<sup>a</sup>Mineral mix adds the following components (per g mineral mix): sodium chloride, 259 mg; magnesium oxide, 41.9 mg; magnesium sulfate, 257.6 mg; chromium K sulfate, 1.925 mg; cupric carbonate, 1.05 mg; sodium fluoride, 0.2 mg; potassium iodate, 0.035 mg; ferric citrate, 21 mg; manganous carbonate, 12.25 mg; ammonium molybdate, 0.3 mg; sodium selenite, 0.035 mg; zinc carbonate, 5.6 mg.

<sup>b</sup>Vitamin mix adds the following components (per g vitamin mix): vitamin A palmitate, 400 IU; vitamin D<sub>3</sub>, 100 IU; vitamin E acetate, 5 IU; menadione sodium bisulfite, 0.05 mg; biotin, 0.02 mg; cyanocobalamin, 1 μg; folic acid, 0.2 mg; nicotinic acid 3 mg; calcium pantothenate, 1.6 mg; pyridoxine-HCl, 0.7 mg; riboflavin, 0.6 mg; thiamin HCl, 0.6 mg.
Table 5-3 Fatty acid profile of mouse diets

<table>
<thead>
<tr>
<th>Fatty acid profile (%)</th>
<th>LF</th>
<th>HF</th>
<th>HFC</th>
</tr>
</thead>
<tbody>
<tr>
<td>C14:0</td>
<td>0.8660**</td>
<td>1.107</td>
<td>1.095</td>
</tr>
<tr>
<td>C16:0</td>
<td>14.91***</td>
<td>18.88</td>
<td>19.27</td>
</tr>
<tr>
<td>C16:1</td>
<td>0.7969***</td>
<td>1.421</td>
<td>1.398</td>
</tr>
<tr>
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<td>1.574</td>
</tr>
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<td>0.1989</td>
<td>0.2182*</td>
</tr>
<tr>
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<td>0.5964**</td>
</tr>
<tr>
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<td>0.6702</td>
</tr>
<tr>
<td>C20:3</td>
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<tr>
<td>C22:0</td>
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<td>0.1155</td>
<td>0.1038</td>
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<td>0.1652**</td>
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</table>

* Values were compared with One-way ANOVA with Dunnet’s post-test (HF as control). * P<0.05, ** P<0.01, *** P<0.001.

Animals and Treatment

All animal experiments were conducted in accordance with a protocol (IACUC# 28962 and 37115) approved by the Institutional Animal Care and Use committee at the Pennsylvania State University (University Park, PA). Male C57BL/6J mice (4 wk old) were purchased from Jackson Laboratories (Bar Harbor, ME) and maintained on 12 h light/dark with access to food and water ad libitum. After a two-wk acclimatization period, mice were randomized to low-fat diet (LF, n=12) or high-fat diet (HF, n=46) treatments. After 8 wk, HF-fed mice were randomized again into two groups based on body weight: half were maintained on the HF diet (HF group, n=23) and half were fed with HFC diet (HF-HFC group, n=23) for 10 wk. Body weight and food intake were recorded weekly. At the end of wk 18,
mice were food-deprived for 7 h (7 am – 2 pm), anesthetized and sacrificed by exsanguination via cardiac puncture. Hearts, livers, spleens, kidneys and visceral fat depots (epididymal, retroperitoneal, and mesenteric) were harvested, rinsed and weighed. Plasma samples were isolated by centrifugation at 3200 g for 15 min. All samples were snap-frozen and stored at -80°C until further analysis. The study was repeated (Exp 2) a year after the original study (Exp 1) using the same experimental design, where n=23 for LF, and n=21 for HF, and n=24 for HF-HFC.

**Glycemic Markers**

Fasting blood glucose measurements were recorded on weeks 0, 4, 8, 10, 12, 14, 16 and 18 for each treatment group using a hand-held Contour glucose monitor (Bayer Healthcare, Tarrytown, NY). Mice were food-deprived for 7 h after the cage bedding was changed (to prevent coprophagy) and blood was sampled from the tail vein. Fasting plasma insulin was determined at the end of the experiment using an ELISA kit (Crystal Chem, Downers Grove, IL) according to the manufacturer’s protocol. Insulin resistance was estimated based on the final blood glucose and insulin values using the Homeostasis model assessment of insulin resistance (HOMA-IR) [28].

**Fecal lipid Content**

Samples were weighed, pulverized in liquid nitrogen and then extracted twice with an equal volume of methanol: chloroform (2:1, v:v). The organic phase was filtered through 0.45 μm PTFE membrane and dried under vacuum. The residue was weighed and normalized to fecal wet weight.

**Biochemical Analysis of Plasma Samples**

Plasma ALT levels were measured using a spectrophotometric method ($\lambda_{max}$ = 340 nm) from Catachem (Bridgeport, CT) according to the manufacture’s protocol. Plasma levels of TNF-α, IL-6, MCP-1 and adiponectin were determined using
commercially-available ELISAs for mice from R&D Systems (Minneapolis, MN) according to the manufacturer’s protocols.

**Liver Triglycerides**

Liver triglycerides were determined by homogenizing liver tissue (50 - 100 mg) in 2 mL isopropanol. The homogenate is centrifuged at 2000 g for 10 min and the supernatant was analyzed with L-Type Triglycerides kit (Wako Diagnostics, Richmond, VA). Lipid concentrations are normalized to tissue wet weight.

**Stromal Vascular Fraction (SVF) Isolation**

Epididymal AT was excised and minced into small (<10 mg) pieces and placed into digestion media consisting of Dulbecco’s Modified Eagle’s Medium (DMEM, Mediatech, Manassas, VA) supplemented with 2.5% HEPES, 10 mg/mL bovine serum albumin, and Collagenase Type II (0.3%, Sigma-Aldrich, St. Louis, MO). Following incubation in a shaking 37°C water bath for 45 min, samples were filtered through a 70 µm cell strainer to remove debris and centrifuged at 4°C at 300g for 8 min. The pellet, consisting of the stromal vascular fraction (SVF), was washed with DMEM and centrifuged at 4 oC at 300 g for 8 min. The supernatant was discarded and erythrocytes were lysed by incubation in 1 mL ACK lysis buffer (NH₄Cl 150 mM, KHCO₃ 10 mM, EDTA-Na₂·2H₂O 10 µM) for 1 min on ice before addition of 4 mL of DMEM to stop the reaction. Samples were then centrifuged at 4°C at 300 g for 10 min. The pellet was frozen at -80 °C for RNA isolation.

**Real-time PCR**

Total RNA was extracted and genomic DNA contamination was removed using the RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer’s protocol. Total RNA was quantified with a Nanodrop 2000 spectrophotometer and reverse-transcribed to cDNA using a RT² HT First Strand Kit (SA Biosciences, Valencia, CA). Real-time PCR was performed using an Applied Biosystems 7900HT Fast Real-Time PCR System (San Francisco, CA). The reactions included 5 µL
perfeCTa® qPCR SuperMix, ROX™ (Quanta BioSciences, Gaithersburg, MD), 0.5 μL TaqMan® probe (Applied Biosystems, Foster City, CA, Table 5.4), and 4.5 μL diluted cDNA. PCR reactions were incubated in a 384-well plate at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 sec and annealing/extension at 60 °C for 1 min. Data were recorded and analyzed with Sequence Detector Software (Applied Biosystems, Foster City, CA). Relative quantification or fold change in gene expression were determined using the \(2^{-\Delta\Delta C_T}\) method, where \(\Delta\Delta C_T = (C_T,\text{target} - C_T,\text{reference})_{HF} - (C_T,\text{target} - C_T,\text{reference})_{LF}\), with Gapdh as the reference gene.

**Table 5-4** Taqman® probes used in this study

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Gene Name</th>
<th>Accession ID</th>
<th>Manufacture’s No.</th>
<th>Length</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gapdh1</td>
<td>glyceraldehyde-3-phosphate dehydrogenase</td>
<td>NM_008084.2</td>
<td>Mm99999915_g1</td>
<td>107</td>
</tr>
<tr>
<td>IL6</td>
<td>interleukin 6</td>
<td>NM_031168.1</td>
<td>Mm00446190_m1</td>
<td>78</td>
</tr>
<tr>
<td>IL12b</td>
<td>interleukin 12b</td>
<td>NM_008352.2</td>
<td>Mm00434174_m1</td>
<td>75</td>
</tr>
<tr>
<td>iNOS (Nos2)</td>
<td>nitric oxide synthase 2, inducible</td>
<td>NM_010927.3</td>
<td>Mm00440502_m1</td>
<td>66</td>
</tr>
<tr>
<td>TNF (TNFa)</td>
<td>tumor necrosis factor</td>
<td>NM_013693.2</td>
<td>Mm00443260_g1</td>
<td>61</td>
</tr>
<tr>
<td>Ccl2 (MCP1)</td>
<td>chemokine (C-C motif) ligand 2</td>
<td>NM_011333.3</td>
<td>Mm00441242_m1</td>
<td>74</td>
</tr>
<tr>
<td>Emr1</td>
<td>EGF-like module containing, mucin-like, hormone receptor - like sequence 1</td>
<td>NM_010130.4</td>
<td>Mm00802529_m1</td>
<td>92</td>
</tr>
</tbody>
</table>

1Gapdh was used as the reference gene.

**Statistical Analysis**

Data are presented as the mean ± standard error of the mean (SEM). Two-way ANOVA with Bonferroni’s post-test was used for body weight, food intake and blood glucose comparisons over the course of the study. One-way ANOVA with Dunnet’s post-test was used for all other data comparisons. A \(P< 0.05\) was considered statistically significant. All analyses were performed using GraphPad Prism 5.0 (San Diego, CA).
Results

Effect of Cocoa on Body Weight, Body Fat Mass and Organ Weights

After 8 wk of treatment, the average body weight of the HF-treated mice was 1.3-fold higher ($P < 0.001$) than that of the LF-treated mice (Figure 5.1 A). Cocoa supplementation for 10 wk significantly decreased final body weight by 4.7% ($P < 0.05$) compared to HF-fed controls, and significantly decreased the rate of body weight gain by 15.8% ($P < 0.001$, Figure 5.1 B) without affecting the food and energy intake (Table 5.5). Cocoa-supplemented mice also displayed lower gross heart, liver and retroperitoneal AT weights compared to HF-fed mice ($P < 0.05$, Figure 5.1 C&D). The experiment was repeated twice, and the body weight, tissue weight and food intake data were not significantly different for the two studies. I therefore combined the data from the two experiments.
**Figure 5-1** Effect of cocoa supplementation on (A) body weight, (B) body weight gain, (C) body fat mass, and (D) organ weight

(A) Body weights were determined over the course of 18 wk. (B) The rate of body weight gain was calculated after cocoa supplementation (wk 9 to 18). (C) Visceral fat mass and (D) gross organ weights were determined at the end of the experiment. Data were pooled from two identical studies (Exp 1 and Exp 2). LF, n=35; HF, n=44; and HF-HFC, n=47. Values are expressed as mean ± SEM. Body weights were compared by two-way ANOVA with Bonferroni’s post-test (an asterisk indicates $P < 0.05$ compared to HF group). Mice body weights in LF group were significantly lower than HF mice since wk 1. All other parameters were compared by one-way ANOVA with Dunnett’s post-test (HF as control). * $P<0.05$, ** $P<0.01$, *** $P<0.001$. 
Table 5-5 Effect of cocoa treatment on food and energy intake, and fecal lipid excretion by high-fat-fed obese mice

<table>
<thead>
<tr>
<th></th>
<th>LF</th>
<th>HF</th>
<th>HF-HFC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Food intake (g/mouse/wk)</td>
<td>20±0.2</td>
<td>20.5±1.0</td>
<td>20.7±1.0</td>
</tr>
<tr>
<td>Energy intake (kcal/wk)</td>
<td>75.7±0.8***</td>
<td>106.3±1.3</td>
<td>105.2±1.3</td>
</tr>
<tr>
<td>Fecal lipid content (% wet weight)</td>
<td>8.4±1.3</td>
<td>9.2±0.7</td>
<td>14.3±1.9*</td>
</tr>
</tbody>
</table>

1 Values are expressed as mean ± SEM. Data on food and energy intake were pooled from both Exp 1 and Exp 2. Fecal samples were collected from each cage at wk 15 in Exp1 in duplicate. Statistical significance was determined using the One-way ANOVA with Dunnet’s post-test (HF as control). * P<0.05, *** P<0.001.

Effect of Cocoa on Food Intake and Fecal Lipid Content

Fecal samples were taken after 7 wk of treatment with cocoa-supplemented diet. Cocoa treatment increased the fecal lipid content by 55.2% (P < 0.05) compared to the HF obese mice without affecting food and energy intake (Table 5.5).

Effect of Cocoa on Glycemic Markers and Insulin Resistance

No significant differences were found between mean fasting blood glucose (data not shown) and final fasting blood glucose (Table 5.6) in cocoa-supplemented mice and HF-fed controls. Fasting plasma insulin was determined at the completion of the experiment, and cocoa-supplemented mice had 26.7% lower (P < 0.001) plasma insulin levels than HF-treated mice (Table 5.6). Moreover, HF-fed obese mice had increased HOMA-IR scores (P < 0.001) compared to LF-fed lean mice (Table 5.6). This increase was ameliorated in cocoa-supplemented mice.
Table 5-6 Effect of cocoa treatment on glycemic markers in high fat-fed obese mice

<table>
<thead>
<tr>
<th></th>
<th>LF</th>
<th>HF</th>
<th>HF-HFC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Final fasting glucose (mmol/L)</td>
<td>7.29±0.18***</td>
<td>9.96±0.17</td>
<td>9.53±0.25</td>
</tr>
<tr>
<td>Fasting plasma insulin (pmol/L)</td>
<td>165.47±15.49***</td>
<td>1176.40±64.11</td>
<td>862.58±81.42***</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>7.74±0.83***</td>
<td>73.76±4.32</td>
<td>50.32±4.17***</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM. Data on fasting blood glucose was pooled from both Exp 1 and Exp 2. Plasma insulin was from Exp 2 and HOMA-IR was calculated from Exp 2. Statistical significance was determined using the One-way ANOVA with Dunnet’s post-test (HF as control). *** P<0.001.

Effect of Cocoa on Markers of ORFLD

HF diet increased the plasma ALT levels by 2.2-fold (P < 0.01) compared to LF-fed controls. Cocoa supplementation attenuated this increase, resulting in 41.1% lower (P < 0.01) plasma ALT levels than HF-fed mice (Figure 5.2A). Liver triglyceride levels were also elevated in HF-fed mice (2.1-fold increase, P < 0.001) compared to LF-fed mice. Cocoa supplementation reduced the liver triglyceride levels by 32.3% (P < 0.001) compared to HF-fed mice (Figure 5.2B).

Figure 5-2 Effect of cocoa supplementation on markers of ORFLD: (A) Plasma alanine aminotransferase (ALT) and (B) liver triglyceride levels

Data were determined at the end of the experiment. Values are expressed as mean ± SEM. Plasma samples were taken from Exp 1. LF, n=12; HF, n=23 and HF-HFC, n=23. Lipid was extracted from liver samples from Exp 2. LF, n=23; HF, n=21 and HF-HFC, n=24. Means
were compared by one-way ANOVA with Dunnet’s post-test (HF as control). ** P<0.01, *** P<0.001.

**Effect of Cocoa on Plasma Cytokine Levels**

Plasma levels of MCP-1 and IL-6 were significantly elevated (P < 0.05) in HF-fed obese mice compared to the LF-fed lean mice. Supplementation with cocoa reduced plasma MCP-1 and IL-6 production by 25.3% (P < 0.01) and 30.4% (P < 0.01) respectively, compared to HF-fed mice (Figure 5.3 A & B). There was no significant difference in TNF-α level among the three groups (P = 0.27), although the cocoa-supplemented mice tended to have lower mean levels than the HF-fed controls (Figure 5.3 C). Conversely, plasma adiponectin levels were significantly lower (P < 0.05) in HF group compared to LF-fed controls, and cocoa treatment increased adiponectin levels by 33.7% (P < 0.05) compared to HF-fed mice (Figure 5.3 D).
Figure 5-3 Effect of cocoa supplementation on systemic circulating levels of (A) MCP-1, (B) IL-6, (C) TNF-α, and (D) adiponectin

Plasma samples were taken from Exp 1. LF, n=12; HF, n=23; and HF-HFC, n=23. Values are expressed as mean ± SEM. Means were compared by one-way ANOVA with Dunnett’s post-test (HF as control). * P<0.05,  ** P<0.01, *** P<0.001.

Effect of Cocoa on Pro-Inflammatory Gene Expression

The SVF of AT is enriched in macrophages, which are related to inflammatory processes in obesity. The mRNA levels of Il6, Il12b, Nos2 and Emr1 in the epididymal AT of the HF-fed mice were increased by 2.4- (P < 0.05), 2.9- (P < 0.05), 4.8- (P < 0.01), and 8.5-fold (P < 0.001) respectively, compared to LF-fed lean mice. Cocoa supplementation reduced the expression of these genes by 37 – 56% (P < 0.05, Fig. 4). However, there was no significant difference in the expression of Tnfa and Ccl2 (Mcp1) among the three groups (Figure 5.4).
Figure 5-4 Effect of cocoa supplementation on the expression of pro-inflammatory genes (A) Il6, (B) Il12b, (C) Nos2, (D) Emr1, (E) Tnfa, (F) Ccl2 in SVF of AT

Expression of pro-inflammatory genes was determined at the end of the experiment using RNA isolated from the epididymal SVF from a set of representative mice from Exp 2. LF, n=10; HF, n=18; and HF-HFC, n=18. Values are expressed as mean ± SEM. Means were compared by one-way ANOVA with Dunnett’s post-test (HF as control). * P<0.05, ** P<0.01, *** P<0.001.
Discussion

Obesity is accompanied by systemic inflammatory responses, and factors believed to contribute to this process include increased levels of circulating cytokines, as well as tissue-specific derangements, such as hepatic inflammation and the accumulation of inflammatory macrophages in adipose tissue [8, 29, 30]. In this study, I report for the first time the therapeutic effect of dietary supplementation with cocoa on obesity-related inflammation, insulin resistance, and fatty liver disease in HF-fed C57BL/6J mice.

In the present study, dietary supplementation of HF-fed, obese C57BL/6J mice with 8% cocoa powder for 10 wk significantly reduced the rate of body weight gain (16% decrease), as well as final body weight (5% decrease) and retroperitoneal AT weight (11% decrease) compared to HF-fed controls. This dose of cocoa showed no signs of toxicity and did not affect food intake. Dietary supplementation with 8% cocoa powder supplementation in mice is equivalent to an approximate daily dose of 54 g of cocoa powder consumption in humans based on a 2000 kcal daily energy intake. This amount of cocoa powder is enough to make 4 cups of hot cocoa according to typical preparation methods (15 g in 250 mL). These results are similar to those reported previously in HF-fed rats supplemented with 12.5% cocoa for 3 wk [17]. I found that these effects on body weight and body fat were related to a 55% increase in fecal lipid output by cocoa supplemented mice. This result suggests that the effect of cocoa powder on the rate of body weight gain is related to inhibition of lipid absorption. My previous study (Chapter 3) indicates that cocoa extracts and their component polyphenols can inhibit the activity of pancreatic lipase and secreted phospholipase A2 in vitro suggesting a potential mechanism for inhibition of dietary fat absorption [26].

Increasing evidence suggests that chronic inflammation is a key mediator of obesity-related pathologies including insulin resistances and fatty liver disease. There is growing evidence that obesity-related inflammation results from increased macrophage infiltration in AT, as well as increased expression, production, and
release of a number of pro-inflammatory cytokines [31]. Adipose tissue macrophages (ATMs) are highly inflammatory, which have been identified as the primary source of many of the circulating cytokines that are detected in the obese state, such as tumor necrosis factors (e.g. TNF-α), interleukins (e.g. IL-1, IL-6, IL-12), and contribute to the recruitment of additional macrophages by secreting chemokines including MCP-1.

Here, I found that plasma pro-inflammatory cytokine levels are dramatically elevated in obese HF mice compared to the lean LF mice, and that these increases were ablated by cocoa supplementation. Furthermore, the expression of pro-inflammatory genes (Il6, Il12b and Nos2) was elevated in HF-fed obese mice in SVF of AT, where macrophages reside. Cocoa-supplementation suppressed their expression levels by nearly 50%. However, there was no significant difference of levels of Tnfa and Ccl2 among three groups. Interestingly, although, I observed a dramatic decrease in the expression of several pro-inflammatory genes in the epididymal fat depot of cocoa-treated obese mice, there was no decrease in the mass of that depot. These results suggest that the effects on gene expression are not secondary to decreased adipose tissue mass, but may be due to some more direct mechanism. I also found that Emr1 (macrophage F4/80 specific gene) was increased in the HF group but decreased in the HF-HFC group, which suggests a possible anti-inflammatory mechanism of cocoa through the inhibition of macrophage infiltration. Although this is the first study to examine the effect of cocoa on obesity-induced increases in plasma inflammatory cytokines, Kanamoto et al. [7] have reported that administration of a procyanidin-rich black soybean seed coat extract for 14 wk remarkably decreased plasma leptin level, as well as Tnfa, Ccl2 and Il6 expression in mesenteric AT in HF diet-fed mice. Similar results were reported for a study of HF-fed rats supplemented with a procyanidin-rich grape seed preparation [6].

In the present study, I found that cocoa supplementation significantly decreased the fasting plasma insulin level in obese mice and improved HOMA-IR score, but did not affect fasting blood glucose levels, compared to HF-fed obese mice. Previous studies have shown that cocoa and cocoa products can exert hypoglycemic
properties and improve insulin resistance. Yamashita et al. [20] have showed that a procyanidin-rich cocoa liquor extract suppressed HF diet-induced hyperglycemia through activation of AMP-activated protein kinase, translocation of glucose transporter 4 in HF diet-fed obese mice. Grassi et al. [32] reported that the short-term administration of dark chocolate improved insulin resistance in terms of improved HOMA-IR and QUICKI in healthy subjects. Increasing evidence from human population studies and animal research has established correlative as well as causative links between obesity-induced chronic inflammation and insulin resistance [29, 33]. Circulating levels of pro-inflammatory cytokines are correlated with insulin resistance through indirect inhibition of insulin signal transduction [7, 10, 33, 34], and possibly via activation of Jun N-terminal kinase and inhibitor of κB kinase β [35]. Based on this I speculate that the effects of cocoa supplementation on insulin-resistance may, in part, be secondary to the observed anti-inflammatory effects in terms of suppression of cytokine production and pro-inflammatory gene expression.

Finally, in addition to the effects on insulin resistance, cocoa supplementation ameliorated the symptoms of ORFLD compared to HF-fed obese mice. One of the consequences of insulin resistance is elevated adipose lipolysis, which results in enhanced free fatty acid flux to the liver that lead to excess esterification to triglycerides and hepatic lipid accumulation [11]. Hepatic steatosis is the hallmark of ORFLD, characterized by elevated concentrations of markers of liver injury, including ALT, aspartate aminotransferase (AST) and γ-glutamyltransferase (GGT) [36]. Of these liver enzymes, ALT is most closely related to liver fat accumulation, and is often used in epidemiological studies as a surrogate marker for ORFLD [37]. In the present study, I observed a decrease in plasma ALT levels, hepatic triglyceride levels, and gross liver weight in cocoa-treated mice compared to HF group. Recent studies also demonstrated a key hepatoprotective role for adiponectin, an adipokine with known anti-inflammatory activities. Buecher et al. [38] reported that adiponectin antagonizes excess lipid storage in the liver and protects from inflammation and fibrosis. Interestingly, cocoa supplementation
significantly reduced systemic adiponectin levels in cocoa-supplemented mice compared to HF-fed controls, with its mean level (18.3 µg/mL) even lower than LF group (19.1 µg/mL). As mentioned earlier, I observed that cocoa supplementation can increase fecal lipid content, perhaps by modifying lipid digestion. These two activities may work together to produce the observed liver protective effect of cocoa.

In summary, I observed that dietary supplementation with cocoa ameliorates obesity-related inflammation, insulin resistance and fatty liver disease in HF-fed obese mice, principally mediated by down-regulation of pro-inflammatory gene expression in AT. These effects appear to be due in part to modulation of dietary fat absorption and inhibition macrophage infiltration in adipose tissue. These results provide support for future human intervention studies on the anti-inflammatory effects of cocoa at nutritionally-relevant doses of cocoa powder. Future studies are needed to identify the active chemical components in cocoa, and to more clearly delineate the mechanistic relationship between modulation of dietary fat absorption and the observed anti-inflammatory effects.
References


Chapter 6

Dietary Cocoa Reduces Adipose Tissue Inflammation in High-Fat Fed Mice
Abstract

In diet-induced obesity, adipose tissue (AT) is in a chronic state of inflammation predisposing the development of metabolic syndrome. Cocoa (Theobroma cacao) is a polyphenol-rich food with putative anti-inflammatory activities; however, little research has been done to support the role of cocoa in modulating AT inflammation and the underlying mechanisms remain to be fully elucidated. The objective of this study is to determine the impact and underlying mechanisms of action of cocoa on AT inflammation in high-fat (HF) fed obese mice. In the present study, male C57BL/6J mice were fed a HF diet (HF, n=21), a HF diet with 8% (w/w) unsweetened cocoa powder (HFC, n=24), or a low-fat diet (LF, n=23) for 18 wk. Results showed that cocoa supplementation decreased pro-inflammatory gene expression (Tnfa, Il6, iNos, and Emr1) by 40–60% in AT compared to the HF group, and this was accompanied by decreased NF-κB p65 protein expression in the nucleus. Levels of AT arachidonic acid (AA), an inflammatory eicosanoid precursor, were positively correlated with adiposity (r = 0.57), with and cocoa-treated mice having 33% lower levels of AA compared to HF controls. Moreover, protein expression of the eicosanoid-generating enzymes, adipose-specific phospholipase A2 (AdPLA) and cyclooxygenase-2 (COX-2), were reduced in cocoa-treated mice by 53 and 55%, respectively. Finally, plasma levels of bacterial endotoxins were found to be 40% lower in cocoa-treated mice compared to HF-fed mice. This decrease in metabolic endotoxemia was in parallel with increased expression of the gut barrier function marker, glucagon-like peptide 2 (GLP-2). In conclusion, the present study has shown for the first time that long-term cocoa supplementation reduces AT inflammation in HF-fed obese mice by down-regulating NF-κB target gene expression and modulating eicosanoid metabolism, which may be partly due to decreased metabolic endotoxemia by cocoa.

Key words: cocoa, Theobroma cacao, polyphenol, obesity, inflammation, adipose tissue
Abbreviations:
AA, arachidonic acid; AdPLA, adipose-specific phospholipase A2; AT, adipose tissue; ATMs, Adipose tissue macrophages; COX-2, cyclooxygenase-2; DP, degree of polymerization; FAME, fatty acid methyl ester; GC, Gas Chromatography; GLP-2, glucagon-like peptide-2; HF, high-fat; HFC, high-fat diet with 8% (w/w) unsweetened cocoa powder; iNos, inducible nitric oxide synthase; LF, low-fat diet; LOX, lipoxygenase; LPS, lipopolysaccharide; LTs, leukotrienes; MCP-1, monocyte chemoattractant protein-1; NF-κB, Nuclear factor-κB; PGs, prostaglandins; PUFA, polyunsaturated fatty acid; SVF, stromal vascular fraction; TLR4, toll-like receptor 4; TNF-α, tumor necrosis factor-α.
Introduction

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

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

In addition to inflammatory cytokines, recent data have implicated bioactive inflammatory lipid mediators in the development of obesity-induced AT inflammation [4,9]. Eicosanoids, a large family of compounds generated from the ω-6-polyunsaturated fatty acid (PUFA) arachidonic acid (AA), represent one of the most potent classes of endogenous inflammatory mediators. In AT, upon activation of adipose-specific phospholipase A2 (AdPLA), AA is released from membrane
phospholipids and becomes available as a substrate for the intracellular biosynthesis of eicosanoids through two major enzymatic routes: the cyclooxygenase (COX) and lipoxygenase (LOX) pathways [9]. Studies have shown that mice that are deficient in key eicosanoid-generating enzymes (i.e. COX-2, 5-LOX and 12/15 LOX) exhibit decreases in adipocyte differentiation and macrophage infiltration and are protected from high-fat (HF) diet-induced elevation of inflammatory cytokines [10–12]. Thus, the metabolism of eicosanoids represents a novel target in the prevention or treatment of obesity-associated inflammation.

In addition, an increasing number of studies have suggested that metabolic endotoxemia, characterized by an excess of circulating bacterial wall lipopolysaccharide (LPS), is also associated with obesity and systemic inflammation [13,14]. Studies have shown that the consumption of a HF diet can alter the composition of gut microbiota, increase the incorporation of LPS into chylomicrons, and affect the intestinal permeability which allows excess endotoxin to enter systemic circulation [15,16]. Metabolic endotoxemia is believed to trigger the AT inflammation via CD14 and toll-like receptor 4 (TLR4) signaling [14,17].

One potential strategy to reduce obesity-related inflammation is through the consumption of polyphenol-rich foods, which have been reported to have anti-inflammatory properties in a number of model systems [1]. Cocoa (Theobroma cacao) and cocoa-based products are among the richest food sources of polyphenols. Cocoa polyphenols are primarily composed of monomeric (epicatechin and catechin) and oligomeric (procyanidins) flavan-3-ols. In addition to polyphenols, cocoa also contains a significant amount of dietary fiber (approximately 40%) as well as theobromine (2-3%) and a small amount of caffeine (0.2%) [18]. Over the last decade, a growing number of studies have reported the health benefits of cocoa and cocoa flavan-3-ols, particularly reducing the risk of cardiometabolic diseases via increased nitric oxide bioavailability, as well as antioxidant, anti-inflammatory, and anti-platelet activities [19–23]. However, it should be noted that the evidence for the anti-inflammatory action of cocoa has been gathered predominantly from in vitro experiments [23,24]. Relatively few
A previous study from my thesis research (Chapter 5) has demonstrated that dietary cocoa supplementation (8% w/w) for 10 wk can significantly ameliorate the body weight gain, nonalcoholic fatty liver disease, and systemic inflammation in HF-fed obese mice, principally through modulation of cytokine secretion and inhibition of dietary fat absorption [25]. In addition, my previous study (Chapter 3) has also shown that cocoa extracts can dose-dependently inhibit activity of digestive enzymes including secretory phospholipase A2 (PLA2, IC_{50} < 20 \mu g/mL) in vitro [26].

In the present study, I investigated the preventative effects of a long-term dietary cocoa powder supplementation on AT inflammation though the regulation of pro-inflammatory gene expression, eicosanoid metabolism, and metabolic endotoxemia in HF-fed C57BL/6J mice.
Materials and Methods

Diets and Chemicals

Compositions of the low-fat (LF, 10% kcal from fat), high-fat (HF, 60% kcal from fat), and HF supplemented with 80 g/kg unsweetened cocoa powder (HFC) diets were described previously [25] and are shown in Chapter 5 (Table 5.1, 5.2 & 5.3). The unsweetened cocoa powder used in this study was provided by Blommer Chocolate Co. (Chicago, IL). All other chemicals were of the highest grade commercially-available.

Animals and Treatments

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

Glycemic Markers

Fasting blood glucose, plasma insulin levels were assessed as previously described [25]. Briefly, fasting blood glucose were measured using a hand-held Contour glucose monitor (Bayer Healthcare, Tarrytown, NY) after 7 hr of fasting. Fasting plasma insulin was determined at the end of the experiment using an ELISA
kit (Crystal Chem, Downers Grove, IL) according to the manufacturer’s protocol. Homeostasis model assessment of insulin resistance (HOMA-IR) was estimated based on the final blood glucose and insulin values [27].

**Fasting Plasma Triglycerides and Free Fatty Acids**

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

**Gene Expression Analysis in Stromal Vascular Fraction (SVF) Of AT**

Isolation of SVF of epididymal AT and gene expression analysis by quantitative reverse transcriptase polymerase chain reaction (qPCR) were conducted as previously described [25]. Briefly, epididymal AT were minced and filtered through a cell strainer and lysed, and the SVF was collected after centrifugation. Total RNA of SVF was extracted and genomic DNA contamination was removed using the RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer’s protocol. Gene expression of *Tnfa, Il6, iNos,* and *Emr1* was analyzed by reverse-transcriptase qPCR using commercial TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA) and normalized to *Gapdh* as an endogenous control. Information of the Taqman® probes used in this study was provided previously [25] and are shown in Chapter 5 (Table 5.4). Gene expression was analyzed according to the delta delta Ct ($\Delta\Delta C_T$) method normalized to *Gapdh*, and expression levels were calculated as $2^{-\Delta\Delta C_T}$.

**Adipocyte Cell Size Image Analysis**

AT samples from epididymal AT were fixed in paraformaldehyde, embedded in paraffin, cut into 5 μm sections, and stained with hematoxylin and eosin. The sections were viewed at 100X magnification and images were obtained with a DV-130 digital camera (Hawking Technology, Irvine, CA). A measure scale was added to
each image using LissView (Hawking Technology, Irvine, CA). The images were pre-edited using Picasa 3 (Google Inc., Mountain View, CA). The images were conditioned by changing “Invert Color,” “Fill Light,” “Highlights,” and “Shadows” and minor adjustments were made by using “Sharpen” to make the image crisper and less fuzzy. After pre-editing the images, Adobe PhotoShop CS 8.0 (Adobe systems, San Joes, CA) was used to optimize the images for adipocyte measurement. The following commands were used for the conversion: “Bilevel Thresholding,” “Erode,” “Dilate,” and “Watershed Segmentation.” The binary black and white images were compared with the original images to ensure an accurate conversion and minor adjustments were made using following commands: “Fill Holes” and “Paintbrush.” The cell diameter of adipocytes was calculated with the command “Measure All” after measure scale calibration. Results were directly loaded into Excel (Microsoft Inc., Redmond, WA) for analysis. Cells in each sample were first ranked based on cell diameters and the top 25 cells were selected for cell size distribution analysis.

**Arachidonic Acid (AA) Detection by Gas Chromatography (GC)**

AA levels were detected in retroperitoneal AT. Each AT sample was weighed and kept in liquid nitrogen before lipid extraction and methylation. A one-step lipid extraction and fatty acid methyl ester (FAME) preparation was carried out using a modified method from Garces and Mancha [28]. AT was heated with a reagent containing methanol:heptane:toluene:2,2-dimethoxypropane:H$_2$SO$_4$. During heating, the simultaneous digestion and lipid transmethylation took place in a single phase. After cooling two phases were formed with the upper phase containing the FAMEs used for GC analysis. FAMEs were quantified using a GC (Agilent 6890 series, Atlanta, GA) equipped with a silica-fused capillary column and a flame ionization detector. Fatty acid peaks were identified in the GC analysis using pure FAME standards (C13 and C19).

**Protein Expression by Western Blots**

Frozen retroperitoneal AT was homogenized in tissue protein extraction
buffer containing a cocktail of protease inhibitors and phosphatase inhibitors (Sigma-Aldrich, St. Louis, MO). The nuclear fraction of AT was prepared using the Nuclear Extract Kit (Active Motif, Rixensart, Belgium). After centrifugation, the protein concentration in supernatant was determined by the Bradford assay (Sigma-Aldrich, St. Louis, MO). Whole cell lysates and nuclear lysates were then combined with an equal volume of laemmli sample buffer (Bio-Rad, Hercules, CA) and denatured at 90°C for 5 min. Proteins (60 µg) were separated by polyacrylamide (15%) gel electrophoresis, transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA), and probed with primary antibodies (Cell Signaling Technology, Danvers, MA) for AdPLA (1:200 dilution, Cayman Chemical, Ann Arbor, MI), COX-2 (1:200 dilution, Cayman Chemical), 5-LOX (1:1000 dilution, Cayman Chemical, Ann Arbor, MI), NF-κB p65 (1:100 dilution, Biotechnology, Santa Cruz, CA), GAPDH (1:1000 dilution, Cell Signaling Technology, Danvers, MA), and Histone H3 (1:500 dilution, Cell Signaling Technology, Danvers, MA) overnight at 4°C. After incubation with a fluorescently-labeled secondary antibody (LI-COR Biosciences, Lincoln, NE), proteins were imaged with an Odyssey imaging system (LI-COR, Lincoln, NE). Protein loading was normalized to GAPDH or Histone H3. Band density was quantified using Odyssey Application Software version 3.0.

**Plasma Endotoxin**

Plasma endotoxin (i.e. lipopolysaccharide [LPS]) determinations were performed using a *Limulus amebocyte* lysate kit (LAL kit, Lonza, Walkersville, MD). Samples were diluted 1:5 to 1:10 with endotoxin-free LAL reagent water and heated at 70°C for 10 min before analysis.

**Plasma Glucagon-Like Peptide-2 (GLP-2)**

Plasma GLP-2 levels were determined by ELISA (MyBioSource, Inc. San Diego, CA) following the manufacturer's instructions.
Statistical Analysis

Data are presented as the mean ± standard error of the mean (SEM). Two-way ANOVA with Bonferroni’s post-test was used for body weight, food intake, and blood glucose comparisons over the course of the study. One-way ANOVA with Dunnett’s post-test was used for all other data comparisons. Pearson’s correlation coefficient (r) was used as a measure of the strength of the association between two variables. Analyses were performed using GraphPad Prism 5.0 (San Diego, CA). A P < 0.05 was considered statistically significant. Multivariate Analysis of Variance (MANOVA) by SAS version 9.3 (SAS Institute, Cary, NC) were used to test the overall difference between the HF and HFC diet-fed groups, considering their effects on physiological parameters including final body weight, final blood glucose, epididymal fat depot, retroperitoneal fat depot, mesenteric fat depot, total visceral fat depot, plasma insulin, plasma triglycerides, and plasma free fatty acids. The SAS code of the MANOVA test is shown below.

```
Proc import out= WORK.MiceIIcocoa
datafile= "C:\Documents and Settings\yug114\Desktop\MiceIIcocoa Manova.xlsx"
dbms=EXCEL REPLACE;
RANGE="Sheet1$";
run;
proc univariate normal plot;
var BW BG Efat Rfat Mfat Tfat Insulin TG FFA;
run;
Proc glm data=MiceIIcocoa order=internal;
class Group;
model BW BG Efat Rfat Mfat Tfat Insulin TG FFA= Group/ ss3;
means Group;
manova h= _all_;
contrast 'HF vs HFC' Group -1 1 0;
manova h= Group;
run;
```
Results

Effect of Cocoa on Physiological Parameters

A significant increase in body weight of mice fed the HF diet was observed at the end of experiment compared to that of the LF-treated mice ($P < 0.001$, Table 6.1). Dietary cocoa supplementation (HFC group) for 18 wk did not significantly change the final body weight compared to HF-fed control. Food and energy intake was also unaffected by cocoa treatment (Table 6.1). Although no significant differences were found between mean fasting blood glucose (data not shown) and final fasting blood glucose (Table 6.1) in cocoa-supplemented mice and HF-fed controls, fasting plasma insulin levels determined at the completion of the experiment were decreased by 14.8% in HFC group ($P < 0.05$). In addition, HF-fed obese mice had increased HOMA-IR scores ($P < 0.001$) compared to LF-fed lean mice (Table 6.1), and this increase was attenuated in cocoa-supplemented mice ($P < 0.05$). In addition, cocoa-supplemented mice also displayed lower fasting plasma triglycerides ($P < 0.05$) and fasting plasma free fatty acids ($P < 0.001$) than HF group. Spleen and kidney weight was decreased in HFC mice compared to HF-fed mice ($P < 0.05$, Table 6.1).
Table 6-1 Physiological parameters of mice fed with LF, HF and HFC diet

<table>
<thead>
<tr>
<th>Parameter</th>
<th>LF</th>
<th>HF</th>
<th>HFC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Final body weight (g)</td>
<td>34.83±0.69***</td>
<td>49.54±0.99</td>
<td>47.05±0.43</td>
</tr>
<tr>
<td>Food Intake (g/wk/mouse)</td>
<td>20.33±0.26</td>
<td>21.44±0.45</td>
<td>20.60±0.34</td>
</tr>
<tr>
<td>Energy Intake (kcal/wk/mouse)</td>
<td>77.20±0.97***</td>
<td>111.14±2.30</td>
<td>104.92±1.69</td>
</tr>
<tr>
<td>Final fasted blood glucose (mmol/l)</td>
<td>7.31±0.27***</td>
<td>9.86±0.28</td>
<td>9.78±0.21</td>
</tr>
<tr>
<td>Fasted plasma triglycerides (mmol/l)</td>
<td>0.33±0.01***</td>
<td>0.45±0.02</td>
<td>0.36±0.02**</td>
</tr>
<tr>
<td>Fasted plasma free fatty acids (mmol/l)</td>
<td>0.98±0.05</td>
<td>0.89±0.04</td>
<td>0.67±0.02***</td>
</tr>
<tr>
<td>Fasted plasma insulin (pmol/l)</td>
<td>165.47±15.49***</td>
<td>1176.40±64.61</td>
<td>1001.93±55.56*</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>7.33±0.84***</td>
<td>71.51±4.18</td>
<td>60.64±3.26*</td>
</tr>
<tr>
<td>Heart (g)</td>
<td>0.14±0.004**</td>
<td>0.17±0.005</td>
<td>0.16±0.004</td>
</tr>
<tr>
<td>Liver (g)</td>
<td>1.20±0.01***</td>
<td>2.14±0.01</td>
<td>2.08±0.01</td>
</tr>
<tr>
<td>Spleen (g)</td>
<td>0.07±0.002***</td>
<td>0.10±0.003</td>
<td>0.09±0.003*</td>
</tr>
<tr>
<td>Kidney (g)</td>
<td>0.31±0.01***</td>
<td>0.37±0.01</td>
<td>0.34±0.01*</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM with LF, n=23; HF, n=21; and HFC, n=24. Data were compared by one-way ANOVA with Dunnett’s post-test (HF as control). * P<0.05, ** P<0.01, *** P<0.001.

Effect of Cocoa on Inflammatory Gene Expression in SVF of AT

The mRNA levels of Tnfa, Il6, iNos (or Nos2), and Emr1 in the SVF of epididymal AT were determined. Gene expression levels of Il6, iNos, and Emr1 were significantly elevated (P < 0.05) in HF-fed obese mice compared to the LF-fed lean mice (Figure 6.1 B-D). Supplementation with dietary cocoa remarkably reduced the expression of Tnfa, Il6, iNos, and Emr1 by 40.0–60.8% (P < 0.05) compared to HF-fed mice (Figure 6.1 A-D).
Figure 6-1 Effect of cocoa supplementation on the inflammatory gene expression of (A) Tnfa, (B) Il6, (C) iNos, and (D) Emr1 in the SVF of C57BL/6J mice

Expression of pro-inflammatory genes was determined at the end of the experiment using RNA isolated from the epididymal SVF from a set of representative mice from each group. LF, n=10; HF, n=18; and HFC, n=18. Values are expressed as mean ± SEM. Means were compared by one-way ANOVA with Dunnett’s post-test (HF as control). * P<0.05, ** P<0.01, *** P<0.001.

Effect of Cocoa on Adiposity and Adipocyte Cell Size Distribution

Visceral adiposity of HF-fed obese mice was significantly elevated compared to LF-fed mice (P < 0.05, Figure 6.2 A). Cocoa supplementation reduced the mass of retroperitoneal AT, but did not affect total visceral adiposity. In spite of the lack of effect on total visceral adipose tissue mass, cocoa treatment did influence the adipocyte cell size distribution. HFC mice had a significantly larger number of smaller adipocytes (0–20 μm, P <0.001) and a smaller number of larger cells (40–60 μm, P <0.01) in epididymal AT (Figure 6.2 B). These results indicate that cocoa treatment can reduce adipocyte hypertrophy.
**Figure 6-2** Effect of cocoa supplementation on (A) visceral adiposity and (B) adipocyte cell size distribution in C57BL/6J mice

Visceral fat depots were weighed at the end of experiment with LF, n=23; HF, n=21; and HFC, n=24. Adipocyte cell size distribution was analyzed from a set of representative histological section samples of epididymal AT with n=8 for each group. Values are expressed as mean ± SEM. Means were compared by one-way ANOVA with Dunnett’s post-test (HF as control). * P<0.05, ** P<0.01, *** P<0.001.

**Effect of Cocoa on AA Levels in AT and Its Correlation with Adiposity**

Eicosanoids represent a group of inflammatory lipid mediators derived from AT. AA is an ω-6 fatty acid that is the precursor of various eicosanoids. Cocoa-supplemented mice had a 32.8% reduction in AA in adipose tissue compared to HF
group ($P<0.001$, **Figure 6.3 A**). Moreover, AA levels in AT is positively correlated with adiposity (Pearson $r=0.57$, $P=0.02$) as shown in **Figure 6.3 B**.

**Figure 6-3** Effect of cocoa supplementation on (A) arachidonic acid (AA) levels in AT and (B) its correlation with adiposity

AA levels were determined in duplicate from a set of representative retroperitoneal AT samples with LF, n=6; HF, n=5; and HFC, n=6. Values are expressed as mean ± SEM. Means were compared by one-way ANOVA with Dunnett's post-test (HF as control). *** $P<0.001$. The correlation between arachidonic acid levels and the adiposity was assessed by GraphPad Prism 5.0 (San Diego, CA).
Effect of Cocoa on Protein Expressions of Eicosanoid-Generating Enzymes and NF-κB in AT

In AT, AA is mainly released from the membrane phospholipids by the action of AdPLA, and the released AA can be further converted to eicosanoids by COX enzymes and LOX enzymes. Western blot results (Figure 6.4 A-C) show that the protein expression of AdPLA and COX-2 were reduced by nearly 50% in cocoa treatment group ($P < 0.01$). By contrast, there is no significant effect of cocoa on the expression of 5-LOX among the three groups. Furthermore, the expression of NF-κB (p65 subunit) in the nucleus was also determined (Figure 6.4 D). Cocoa supplementation significantly decreased NF-κB p65 expression in the nucleus compared to HF control group ($P < 0.05$), which may reduce NF-κB activation resulting in down-regulation of inflammatory gene expression.

![Western blot images](image)

**Figure 6-4** Effect of cocoa supplementation on the protein expression of (A) AdPLA, (B) COX-2, (C) 5-LOX, and (D) nucleus NF-κB p65 in adipose tissue of C57BL/6J mice.
Protein expression of eicosanoid-generating enzymes (AdPLA, COX-2, and 5-LOX) was determined in whole cell lysate from a set of representative mouse retroperitoneal adipose tissue samples with n=6 for each group. Protein expression of NF-κB p65 was measured in the nuclear fraction from a set of representative mouse retroperitoneal adipose tissue samples with n=6 for each group. Values are expressed as mean ± SEM. Means were compared by one-way ANOVA with Dunnett’s post-test (HF as control). * P<0.05, ** P<0.01, *** P<0.001.

Effect of Cocoa on Plasma Endotoxin Levels

The HF diet induced a 1.8-fold increase in plasma endotoxin levels (P < 0.001) compared to LF-fed controls (Figure 6.5). Cocoa supplementation reduced this elevation, resulting in 40.8% lower (P < 0.001) plasma endotoxin levels than HF-fed mice.

![Figure 6-5](image)

**Figure 6-5** Effect of cocoa supplementation on the plasma endotoxin levels in C57BL/6J mice

Plasma endotoxin levels were determined at the end of experiment with LF, n=23; HF, n=21; and HFC, n=24. Values are expressed as mean ± SEM. Means were compared by one-way ANOVA with Dunnett’s post-test (HF as control). *** P<0.001.

Effect of Cocoa on Plasma Glucagon-like peptide-2 (GLP-2) Levels and Its Correlation with Plasma Endotoxin Levels

GLP-2 is a gastrointestinal hormone that has a number of actions in the intestine including the stimulation of mucosal growth, improvement of gut barrier function, and subsequently the reduction of intestinal permeability. Compared to
LF-fed mice, obese mice fed with a HF diet had lower levels of plasma GLP-2 \((P < 0.01)\); cocoa-treated mice had increased GLP-2 levels by 36.1% \((P < 0.01)\) compared to HF-fed mice (Figure 6.6 A). Moreover, plasma GLP-2 levels had a strong negative correlation between the plasma endotoxin levels (Pearson \(r = -0.52, P = 0.001\)), which suggests a role of GLP-2 in metabolic endotoxemia (Figure 6.6 B).

**Figure 6-6** Effect of cocoa supplementation on (A) plasma GLP-2 levels and (B) its correlation with plasma endotoxin levels in C57BL/6J mice

Plasma GLP-2 levels were determined at the end of the experiment from a set of representative plasma samples with \(n=12\) for each group. Values are expressed as mean ± SEM. Means were compared by one-way ANOVA with Dunnett's post-test (HF as control).
*** $P<0.001$. The correlation between plasma GLP-2 levels and plasma endotoxin levels was assessed by GraphPad Prism 5.0 (San Diego, CA).

**Overall Difference between the Effects of HF and HFC Diet in Mice**

To test the overall difference between the effects of HF and HFC diet in mice, a multivariate analysis of variance (MANOVA) was used. MANOVA is a statistical test procedure for comparing multivariate means of several groups. The variables that were considered in the MANOVA test were all physiological parameters that are related to central obesity and insulin resistance. The variables are final body weight, final blood glucose, epididymal fat depot, retroperitoneal fat depot, mesenteric fat depot, total visceral fat depot, plasma insulin, plasma triglycerides, and plasma free fatty acids. The SAS result output of the MANOVA test for the “hypothesis of no overall HF vs. HFC effect” is shown in Table 6.2. The results suggest that there is an overall difference between the effects of HF and HFC diet in mice ($P=0.0002$).

**Table 6-2** MANOVA test results for the “hypothesis of no overall HF vs. HFC effect”

<table>
<thead>
<tr>
<th>Statistic</th>
<th>Value</th>
<th>F Value</th>
<th>Num DF</th>
<th>Den DF</th>
<th>Pr &gt;F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wilks' Lambda</td>
<td>0.59322875</td>
<td>4.34</td>
<td>9</td>
<td>57</td>
<td>0.0002</td>
</tr>
<tr>
<td>Pillai's Trace</td>
<td>0.40677125</td>
<td>4.34</td>
<td>9</td>
<td>57</td>
<td>0.0002</td>
</tr>
<tr>
<td>Hotelling-Lawley Trace</td>
<td>0.68569038</td>
<td>4.34</td>
<td>9</td>
<td>57</td>
<td>0.0002</td>
</tr>
<tr>
<td>Roys Greatest Root</td>
<td>0.68569038</td>
<td>4.34</td>
<td>9</td>
<td>57</td>
<td>0.0002</td>
</tr>
</tbody>
</table>
Discussion

AT has an important endocrine function in the regulation of whole-body metabolism. Obesity leads to a chronic inflammation of the AT, which disrupts this endocrine function and results in metabolic derangements such as type-2 diabetes and cardiovascular diseases [29]. Bioactive food components, such as polyphenols, have been shown to suppress both systemic and AT inflammation and have the potential to improve these obesity-associated metabolic disorders [29–31]. The present study provided the first scientific evidence for the preventive effects of long-term supplementation with dietary cocoa on AT inflammation in C57BL/6J obese mice.

In this study, although dietary cocoa supplementation for 18 wk did not decrease the final body weight in HF-fed mice, markers of hyperinsulinemia and hyperlipidemia were significantly improved by cocoa treatment. Dietary supplementation with 8% (w/w) cocoa powder attributes approximately 0.6% cocoa polyphenols diet or about 50 mg polyphenols/kg body weight (calculated based on the information in the present study that a HFC-fed mouse consumes about 3 g per day and weighs about 35 g on average over the course of 18 wk). Previous studies using a similar dose level of cocoa polyphenols have also reported the beneficial effects of cocoa in obese mice. For example, in a 2012 study, Min et al. found that C57BL/6 mice fed a HF diet with cocoa polyphenol extract (200 mg per kg body weight) for 5 wk had a significantly reduced body weight and epididymal fat mass, as well as decreased plasma triglycerides levels, whereas blood glucose was unaffected [32]. In another study, Tomaru et al. found that diabetic obese mice fed a standard diet with 1% cocoa liquor procyanidin extract for 3 wk demonstrated lower blood glucose levels, but no significant changes in body weight were observed [33]. In addition, my previous studies have reported that cocoa extracts demonstrated potent inhibitory activities against key digestive enzymes in vitro [26] and cocoa supplementation can significantly increase fecal lipids, modulate systemic circulation of inflammatory cytokines (e.g. IL-6), and modulate adiponectin in HF-
fed obese mice [25]. Thus, the effects of dietary cocoa on insulin resistance and plasma lipids in the present study may be due to inhibition of lipid absorption and modulation of cytokine secretion.

Chronic inflammation in obese AT is characterized by increased macrophage infiltration and abnormal cytokine production. I found that mRNA expression of pro-inflammatory cytokines (Tnfa and Il6), iNos, and macrophage surface marker (Emr1) was elevated in HF-fed obese mice in ATMs of epididymal fat; however, cocoa supplementation reduced their expression by 40.0-60.8% compared to HF controls. Interestingly, the decreased expression of Emr1 (also known as F4/80 in mice) indicates that there was decreased macrophage infiltration into AT by cocoa treatment. Studies have also shown that Emr1 was significantly and positively correlated with both adipocyte size and body mass [6].

Fat mass expansion in obesity occurs via adipocyte hyperplasia (increased adipocyte number) and/or adipocyte hypertrophy (increased size of adipocytes) [29]. The latter is frequently seen in obese AT as a result of excessive lipid storage and is often associated with AT dysfunction and inflammation [34]. Although there was no change in the mass of the epididymal fat depot induced by cocoa treatment, the epididymal adipocyte cell size distribution was markedly changed with an increase in the number of smaller cells and a decrease in the number of larger cells. Skurk et al. have reported that adipocyte size is an important determinant of the production of cytokines including IL-6, IL-8, and MCP-1 in AT [35].

Eicosanoids are lipid products derived from the metabolism of AA that play a significant role as mediators of the inflammatory cascade and have important activities for maintaining homeostasis [36]. AA is an ω-6-PUFA and is the primary source of fatty acids that mediate inflammatory responses [9]. Elevated tissue levels of AA have been associated with a number of disease states, including coronary heart disease, breast cancer, obesity, and diabetes [37]. A recent study has associated increased AA levels in retroperitoneal AT with a high prevalence of metabolic syndrome in human subjects [37]. As a result, the AA levels in mouse AT
were determined. I found that cocoa-supplemented mice had 32.8% lower levels of AA compared to HF group.

In AT, the production of AA can be catalyzed by AdPLA or by other types of PLA2 released from macrophages [9]. AdPLA is highly and exclusively expressed in AT and is associated with adipocyte differentiation and lipolysis [38]. Ablation of AdPLA in mice caused a reduction in plasma triglyceride levels and insulin resistance in response to a HF diet [9,38,39]. In the present study, the protein expression of AdPLA in AT was significantly suppressed by cocoa treatment. Together with reduced AA levels in AT, these results suggest a decreased availability of enzymatic substrates for the synthesis of eicosanoids. The COX pathway metabolizes AA to form prostanoids, including the prostaglandins (PGs). Ghoshal et al. have reported that COX-2 deficiency attenuated AT differentiation and macrophage-dependent inflammation in mice [10]. The LOX pathway metabolizes AA into many bioactive eicosanoids including leukotrienes (LTs). Recent studies have demonstrated a role of the LOX pathway in signaling the AT dysfunction and inflammation [11,12,40]. Here, I found that cocoa supplementation significantly reduced COX-2 protein expression in AT compared to the HF group, while the expression of 5-LOX was unaffected. Although the effect of dietary cocoa on downstream eicosanoid products including PGs and LTs in AT requires further investigation, this study reported for the first time the modulatory effects of cocoa on eicosanoid metabolism in AT in vivo, whereas the current available data is limited in vitro. For example, Zhang et al. [41] have reported that pretreatment with cocoa procyanidin dimers (50 μM) for 30 min significantly suppressed COX-2 expression in LPS-stimulated human monocyte cells.

NF-κB is a ubiquitous and well-characterized transcription factor controlling the expression of genes encoding the pro-inflammatory cytokines, chemokines, adhesion molecules, inducible enzymes (iNOS and COX-2), etc., all of which play a critical role in inflammatory cell signaling [42]. In the present study, the protein levels of NF-κB p65 subunit in the nucleus of adipocytes were compared and the cocoa supplementation group exhibited significantly lower levels of nuclear p65
levels than that of HF group. Together with decreased pro-inflammatory gene expression by cocoa treatment, these results suggest that dietary cocoa may prevent the nuclear translocation and activation of NF-κB pathway which results in down-regulation of inflammatory gene expression. Terra et al. [43] have reported that the administration of a grape-seed procyanidin extract (30 mg/kg per day) for 14 wk in HF fed rats significantly decreased Tnfa, Il6, and Emr1 expression in mesenteric AT and a reduced NF-κB activity in liver was also observed which can be related to low expression rates of hepatic inflammatory markers. Additionally, in vitro studies have shown that cocoa extracts and cocoa polyphenols inhibited the translocation and activation of NF-κB in several cell models including immune cells and adipocytes; this inhibition was accompanied by decreases in NF-κB-regulated cytokine production, however, this is the first report of this effect in vivo [44–47].

A growing number of studies suggests that a HF diet can induce “metabolic endotoxemia” through the alteration of gut microbiota and gut barrier functions, which promotes leakage of endotoxins from the gut lumen into circulation, resulting in inflammatory changes in tissues including AT [14–16]. Therefore, modulation of metabolic endotoxemia may represent a promising mechanism for the anti-inflammatory activities of cocoa. Because cocoa contains dietary fiber and high molecular weight, poorly-bioavailable polyphenols, and studies have shown that they might be metabolized by gut microbiota and directly influence the intestinal ecosystem [48,49]. In the present study, the HF diet significantly elevated plasma endotoxin (LPS) levels compared to LF-fed controls. On the contrary, cocoa supplementation significantly reduced this elevation with levels similar to that of LF group. Moreover, the plasma levels of GLP-2 were also tested. GLP-2 can increase intestinal crypt cell proliferation and mucosal thickness resulting in reduced intestinal permeability in animal models [50]. Results in my study demonstrated that cocoa treatment significantly increased plasma GLP-2 levels compared to HF-fed mice. With all groups, a strong negative correlation was also observed between plasma GLP-2 and endotoxin levels. Similar correlation was reported previously by Cani et al. [51], who demonstrated that GLP-2 administration to ob/ob mice
dramatically decreased plasma LPS by about 50%, and this was accompanied by a lower level of plasma inflammatory cytokines.

It is noteworthy that decreased endotoxemia may also be associated with reduced plasma triglycerides by cocoa treatment based on the hypothesis that endotoxin or LPS is transported from the intestine toward target tissues by a mechanism facilitated by triglyceride-rich lipoproteins, notably chylomicrons synthesized from epithelial intestinal cells in response to a HF diet [14]. Since I previously observed that cocoa procyanidins inhibited the activity of PL in vitro and increased fecal lipid content, it is possible that cocoa reduces metabolic endotoxemia by this alternative mechanism.

Therefore, the modulatory effects of cocoa on metabolic endotoxemia may be partly due to its decreased incorporation of LPS into chylomicrons and alteration of intestinal permeability. A recent study by Tzounis et al. [52] also showed a direct effect of cocoa consumption on gut microbiota population. In this double-blind, randomized clinical trial, consumption of cocoa flavanols significantly affected the growth of select gut microbiota in humans and it was accompanied by changes in plasma triglyceride and C-reactive protein concentration, which suggests the potential prebiotic benefits of dietary cocoa.

In conclusion, the present study has shown for the first time that long-term cocoa supplementation can reduce AT inflammation in HF-fed mice by down-regulating NF-κB target gene expression and modulating eicosanoid metabolism. These anti-inflammatory effects are likely mediated via decreased metabolic endotoxemia. Although more mechanistic studies are needed in order to determine which bioactive component(s) or metabolite(s) coming from the gut microbiota are responsible for these effects and their pathways, results in the present study suggest that dietary cocoa powder can confer health benefits and may lead to a natural alternative in the prevention of obesity-related diseases.
References


Chapter 7

Conclusions and Recommendations for Future Work
Conclusions

Results from my enzyme, cell culture and animal studies suggest that dietary cocoa and cocoa procyanidins exert anti-inflammatory activities and modulate obesity-related pathologies through inhibition of digestive enzymes, suppression of pro-inflammatory cytokine production systemically, alteration of eicosanoid metabolism in adipose tissue (AT), down-regulation of NF-κB signaling, and modulation of metabolic endotoxemia. The overall effects are summarized in Figure 7.1.

Figure 7-1 Summarized effects of cocoa on obesity and obesity-related inflammation
In the small intestine, cocoa extract and cocoa procyanidins (CPs) acted as potent digestive enzyme inhibitor against pancreatic lipase (PL), pancreatic α-amylase (PA) and phospholipase A2 (PLA2), which may reduce absorption of macronutrients including carbohydrates, lipids and phospholipids. Lipid and carbohydrate digestive enzyme inhibitors have been recently recognized as a treatment for obesity and diabetes [1]. Thus, cocoa rich in CPs that inhibit digestive enzymes may provide a safe, natural and cost-effective approach for weight management and glycemic control. Moreover, the inhibition of lipid absorption by cocoa treatment was confirmed by increased fecal lipid excretion in vivo. In addition, since the AT is the major storage site for fat in the form of triglycerides, inhibition of digestive enzyme and macronutrient absorption might also play a role in modulation of adipocyte hypertrophy and macrophage infiltration, which are key factors for the initiation of AT inflammation in obesity.

In AT, as the accumulation of lipid and development of obesity, the adipocyte cell size is increasing. The enlarged AT would start recruiting macrophages and releasing a range of inflammatory cytokines that predispose to the systemic inflammation. In my cell culture study, CPs significantly suppressed the production of pro-inflammatory cytokines including TNF-α, IL-6 and MCP-1 in LPS-stimulated macrophages. In addition, short-term cocoa supplementation also significantly reduced systemic levels of these cytokines in high-fat (HF) fed obese mice. Moreover, results from my long-term cocoa supplementation study suggest that dietary cocoa can decrease adipocyte cell size and inhibit macrophage infiltration through down-regulation of macrophage-specific gene (Emr1). Furthermore, the expression pro-inflammatory genes encoding cytokines (Tnfa, Il6, Il12), inducible enzyme (iNos) were also reduced by cocoa treatment, and this decrease was accompanied by reduced protein expression of NF-κB in nucleus. Cocoa and CPs were also able to modulate eicosanoid metabolism by inhibiting eicosanoid-generating enzymes (PLA2 and COX-2). In AT specifically, protein expression of AdPLA (adipose-specific PLA2) was significantly lowered by cocoa treatment.
compared to HF control group, and its metabolites, arachidonic acid levels were also decreased in cocoa-supplemented mice.

In gut (mostly large intestine), HF diet-induced leakage of bacterial wall component lipopolysaccharides (LPS or endotoxin) from the lumen into the systemic circulation induces metabolic endotoxemia, which dysregulates the inflammatory tone and triggers AT dysfunction and insulin resistance. My animal study showed that cocoa supplementation significantly decreased plasma endotoxin (i.e. LPS) levels, which may account for the reduction in HF diet-induced AT inflammation.

Overall, results from my thesis research have shown that cocoa and CPs are bioactive and modulate obesity-related inflammation both in vitro and in vivo. Moderate consumption of cocoa and CPs, incorporated into a healthy diet, may be useful for the prevention of chronic inflammation and metabolic syndrome.
Future Work

*In Silico* Modeling of the Inhibition of Cocoa Procyanidins against Pancreatic Lipase

In Chapter 3 (Inhibition of Key Digestive Enzymes by cocoa extracts and procyanidins), the results of kinetic analysis suggested that the procyanidin pentamer and decamer inhibited PL activity in a mixed mode. By contrast, the procyanidin pentamer and decamer noncompetitively inhibited PLA₂ activity. These results demonstrate the diversity of potential interactions between the procyanidins, the enzyme surface, and/or the substrate, and such interactions need further study by *in silico* modeling methods. Moreover, previous modeling studies from our laboratory have revealed that the cocoa procyanidins (B type) were able to occupy the active site-containing tunnel of PLA₂, and sterically blocked access to the active site for the substrate and thus resulted in non-competitive inhibition against PLA₂ [2]. Therefore, future work is needed to develop an understanding of the complex chemical interactions that govern the inhibitory activity of cocoa procyanidins of various degree of polymerization against PL. Hopefully, using the *in silico* modeling method, we can develop an understanding of the binding characteristics of the cocoa procyanidins to the enzyme that lead to a mixed inhibition. Furthermore, understanding the chemical interactions between cocoa procyanidins and digestive enzymes would also provide a structural basis to screen additional plant-derived compounds, and synthetic compounds, with similar chemical structures for their inhibitory activities against digestive enzymes. Based on the knowledge of the impact of these dietary phytochemicals on digestive enzymes, it becomes possible to develop a general outline of an anti-obesity/anti-nutritional diet that can offer a unique synergism for treatment of obesity and its associated co-morbidities.
Effects of Cocoa Procyanidins in an LPS-Induced Adipocyte-Macrophage Co-Culture System

In Chapter 4 (Cocoa Procyanidins Modulate Eicosanoid Metabolism and Cytokine Production in RAW 264.7 Macrophages), I’ve shown that cocoa procyanidins with high degree of polymerization (DP) can suppress the prostaglandin E\textsubscript{2} (PG E\textsubscript{2}) and pro-inflammatory cytokine productions in LPS-stimulated RAW 264.7 macrophages. Recently, macrophages have been suggested to infiltrate into adipose tissue, to interact with adipocytes, and their interaction with low endotoxin (i.e. LPS) stimulation appear to amplify and maintain the LPS-induced low-grade inflammation, producing higher amounts of inflammatory cytokines [3]. Thus the interaction and cross-talk between adipocytes and macrophages play the central role in obesity-related inflammation, and further studies should focus on elucidating the bioactivities of cocoa procyanidins, and the underlying mechanisms, in an adipocyte-macrophage co-culture system.

In brief, co-culture of adipocytes and macrophages can be performed either in the contact system, where two types of cells are in direct contact, or in the transwell system, where the adipocyte (bottom layer) are separated from macrophages (top layer) by a porous membrane to allow signaling proteins to pass through [4]. In the co-culture model, cells are stimulated with a very low concentration of LPS (1 ng/mL) to mimic the microenvironment of metabolic endotoxemia. First of all, the modulatory effects of cocoa treatment on LPS induced-cytokine production will be determined. Second, the regulatory effects of cocoa on Toll-like receptor (TLR4) signaling in co-culture of adipocyte and macrophage will be investigated. A growing number of studies have suggested that bacterial endotoxin induces AT inflammation and stimulates adipocyte lipolysis via TLR4 signaling pathway [5,6]. The recognition of an LPS molecule by TLR4 is mediated by the LPS-binding protein (LBP), the CD14 co-receptor of TLR4 and the myeloid differentiation protein-2. TLR4 is present on the membrane surface of both macrophages and adipocytes. Upon recognition of LPS, TLR4 undergoes oligomerization and recruits its downstream adaptor molecules into lipid rafts of
the membrane, leading to the activation of downstream signaling pathways, such as NF-kB and mitogen-activated protein kinase (MAPK), which can lead to inflammation[7]. Therefore, studies of the effects of cocoa procyanidins in an LPS-induced adipocyte-macrophage co-culture system would help us better understand the in vivo anti-inflammatory actives of cocoa, and this co-culture condition has the advantage of evaluating cell-cell interactions of a metabolic endotoxemia microenvironment in obesity.

Determinant of the Active Components in Cocoa Powder Responsible for Its Anti-Inflammatory Effects in High-Fat Fed Mice and Their Synergism: Potential Application in Food Industry

In Chapter 5 (Dietary Cocoa Ameliorates Obesity-Related Inflammation in High-Fat Fed Mice), cocoa supplementation for 10 wk significantly attenuated the body weight gain, insulin resistance, and obesity-related fatty liver disease markers as well as systemic inflammation in high-fat (HF) fed mice. However, the active components in cocoa powder responsible for its anti-inflammatory effects under obese conditions in vivo should be determined.

Cocoa is a highly complex food source including minerals, fiber, antioxidants, vasoactive, and even psychoactive compounds [8]. Many polyphenols found in cocoa, including (-)-epicatechin, (+)-catechin and procyanidins, have been found to modulate obesity and inflammation both in vitro and in vivo [9,10]. In addition to polyphenols, theobromine from cocoa has also shown a putative effect on weight changes and lipid profile of Wistar rats [11]. Moreover, unsweetened cocoa powder contains almost 2 g of fiber per tablespoon (1tbsp = 5~6 g cocoa powder), and the majority of fiber in cocoa is insoluble, which has been associated with improved insulin resistance and reduced risk of type 2 diabetes [8,12]. Therefore, we might speculate that there are multiple active compounds in the cocoa powder responsible for the observed anti-obesity and anti-inflammatory effects, and there might be a synergism that contributes to the overall health effects of cocoa powders.
The same HF-induced obesity models in C57BL/6J mice as in Chapter 5 could be used to determine the bioefficacy of cocoa procyanidins (~0.2% of diet) and dietary fiber (~3% of diet) from cocoa powder, as well as their combination, respectively. The dose levels are equivalent to those present in an 8% (w/w) cocoa supplemented HF diet. Briefly, after 8 wk of HF diet to develop obesity, mice will be switched to and maintained a HF-diet either supplemented with 0.2% cocoa procyanidins or 3% of cocoa fiber or the combination for 10 wk. Biomarkers related to obesity, insulin resistance and systemic inflammation would be measured and compared. Results from this feeding study with cocoa procyanidins and fiber, respectively, will provide scientific evidence for identifying the major active components of cocoa powder in obesity-related inflammation. In addition, by comparing these results with the data from the combination group and the short-term cocoa powder supplementation study, it is possible to determine if the isolated specific components act synergistically to impart health benefits and if they do, to what extent.

Although the individual compounds that may confer health benefits to a comparable extent as the whole foods, implication from the results of the synergistic study will help develop a diet or functional food formulation that may reduce obesity-related inflammation to a greater degree rather than encourage dietary supplements. In addition to cocoa powder, fruits such as apple, blueberry and cranberry are also rich in procyanidins and fiber; as a result, they might exhibit similar biological activities as cocoa. Food scientists can utilize the unique synergy of these dietary phytochemicals, and develop a formulation that would offer a powerful health benefits and creating value-added products in the functional food industry.

Changes of Gut Microbiota and Intestinal Permeability by Cocoa Supplement in High-Fat Fed Mice

In Chapter 6 (Dietary Cocoa Reduces Adipose Tissue Inflammation in High-Fat Fed Mice), I hypothesized that long-term cocoa supplementation reduces AT
inflammation through decreased metabolic endotoxemia. The plasma endotoxin levels were significantly reduced in cocoa-supplemented mice compare to the HF control mice. Moreover, the plasma levels of glucagon-like peptide 2 (GLP-2), which is a negative indicator of intestinal permeability, were also significantly increased by cocoa treatment. Although the underlying mechanisms of HF diet-induced metabolic endotoxemia require further study to clarify, there is increasing evidence that chronic HF diets can affect the composition of gut microbiota, increase the incorporation of LPS into chylomicrons and compromise gut mucosal integrity, which can result in the entry of bacterial endotoxin from the intestinal lumen into the blood stream. Therefore, future studies are needed to elucidate the direct effects of long-term cocoa supplementation on changes of gut microbiota and intestinal permeability in HF-fed mice.

Changes of gut microbiota composition can be determined by real-time quantitative PCR. In brief, the DNA was extracted from the cecal content of mice, and the 16S ribosomal RNA (rRNA) genes are amplified by PCR. Candidate primers designed to target total bacteria and specific for Bifidobacteria, Lactobacilli or Clostridium was used in the PCR reaction mixture to study the quantitative effect of the treatment on the population of these bacteria. 16S rRNA gene is a section of prokaryotic DNA found in all bacteria, and it provides tractable combination of conserved sites for PCR primers and variable regions that act as evolutionary chronometers, and it is therefore, usually used for phylogenetic identification [13].

Intestinal permeability can be measured either directly in vivo using a fluorescence-based method as described previously [14] or indirectly by determination of expression of tight junction (TJ) proteins. In the fluorescence-based method, briefly, after fasting mice will be given a fluorescence-labeled compound by gavage, and the fluorescence of plasma samples collected from different time points will be measured as an indicator of intestinal permeability. Moreover, TJ protein expression, determined either by Western blots or qPCR, is also a factor related to the leakage of endotoxin. TJs represent the major barrier of gut by sealing the spaces between intestinal epithelia cells that that line the
digestion tract [15]. Proteins of TJ are composed of claudins, occludins, zonula occludens (ZO), etc. It has recently been shown changes in gut microbiota and increase of intestinal permeability by reducing the expression of two TJ proteins ZO-1 and occludin in obese mice [14]. Thus, results obtained from both gut microbiota composition and intestinal permeability analysis would help to reveal the prebiotic effects of long-term cocoa supplementation in HF-fed mice. Together with the observed anti-inflammatory activities in AT and the role of LPS signaling in mediation of AT inflammation, we may identify the gut as the primary and initial site of action in obese mice where cocoa exerts its anti-inflammatory activities.
Closing Words

Although cocoa and cocoa products have been consumed for hundreds of years, we are just beginning to understand the potential health benefits of these high-flavan-3-ol foods. Clearly, there is a great deal of promising areas related to my thesis research that could be explored further. In the mean time, data from my research suggest that we may be able to judiciously incorporate cocoa and cocoa products as part of a healthy diet rather than contemplate their consumption with twinges of guilt.
References


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PUBLICATIONS


