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REVIEWS: CURRENT TOPICS

Mechanisms by which cocoa flavanols improve metabolic syndrome and related disorders☆

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Abstract

Dietary administration of cocoa flavanols may be an effective complementary strategy for alleviation or prevention of metabolic syndrome, particularly glucose intolerance. The complex flavanol composition of cocoa provides the ability to interact with a variety of molecules, thus allowing numerous opportunities to ameliorate metabolic diseases. These interactions likely occur primarily in the gastrointestinal tract, where native cocoa flavanol concentration is high. Flavanols may antagonize digestive enzymes and glucose transporters, causing a reduction in glucose excursion, which helps patients with metabolic disorders maintain glucose homeostasis. Unabsorbed flavanols, and ones that undergo enterohepatic recycling, will proceed to the colon where they can exert prebiotic effects on the gut microbiota. Interactions with the gut microbiota may improve gut barrier function, resulting in attenuated endotoxin absorption. Cocoa may also positively influence insulin signaling, possibly by relieving insulin-signaling pathways from oxidative stress and inflammation and/or *via* a heightened incretin response. The purpose of this review is to explore the mechanisms that underlie these outcomes, critically review the current body of literature related to those mechanisms, explore the implications of these mechanisms for therapeutic utility, and identify emerging or needed areas of research that could advance our understanding of the mechanisms of action and therapeutic potential of cocoa flavanols.

Keywords: Epicatechin; Procyanidins; Diabetes; β-Cells; GLP-1; Endotoxin

1. Introduction

1.1. Metabolic syndrome

Metabolic syndrome is a cluster of related conditions that increases an individual's risk for developing cardiovascular disease and Type 2 diabetes mellitus (T2DM) [1,2]. The components of metabolic syndrome include abdominal obesity, dyslipidemia, elevated blood pressure, insulin resistance, glucose intolerance, β-cell loss, low-grade chronic inflammation and a prothombotic state [1–3]. The prevalence of obesity, cardiovascular disease and diabetes has been increasing in the United States and worldwide for the past several decades. Approximately one in ten adults in the United States has diabetes, one in three has a cardiovascular disease and one in three is obese [4,5]. Many individuals with metabolic syndrome will progress to the full expression of these diseases. The prevalence of metabolic syndrome is now greater than 34% in the U.S. [6]. Increasing attention has been directed toward finding novel strategies to prevent, slow the onset and/or progression of and potentially reverse metabolic syndrome [7].

1.2. Flavanols and metabolic syndrome

Dietary flavanols offer an interesting potential complementary strategy that may improve this complex, multifaceted syndrome. First, flavanols may help reduce glucose excursion by slowing digestion and enhancing the incretin response. Second, flavanols may help reduce systemic endotoxin exposure *via* improvement in gut barrier function. While flavanols from a variety of dietary sources appear promising, cocoa flavanols represent an emerging approach for intervention in metabolic syndrome. Following an overview of polyphenols, this review will focus on flavanols found in cocoa. Cocoa bioavailability will be briefly reviewed, followed by a summary of the primary research utilizing cocoa, and lastly, the hypothesized mechanisms by which cocoa flavanols improve metabolic syndrome will be discussed.

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2. Cocoa flavanols

2.1. Flavanols

Polyphenols are secondary metabolites found ubiquitously in plants. One prominent subclass of polyphenols is the flavonoids. The basic flavonoid skeleton consists of two benzene rings linked by a 3 carbon heterocyclic (*O*-containing) ring (Fig. 1A). Flavonoids are further divided into subclasses based on the nature of the heterocyclic ring and substituents: flavanols, flavonols, flavones, flavanones, isoflavones and anthocyanins [8]. Flavanols are hydroxylated at C3 in the heterocyclic ring (Fig. 1B) and are thus sometimes referred to as *flavan-3-ols*. This hydroxyl group may be modified by an addition of a gallate group. Flavanols may exist as monomers, or as oligomers/polymers [with various degrees of polymerization (DP)] comprised of flavanol monomer residues (known as *proanthocyanidins*). Major dietary flavanol monomers include (+)-catechin (+C), (−)-catechin (−C), (−)-epicatechin (EC) (Fig. 1C) and others. Cocoa is unique in that it is the only significant dietary source of −C. Procyanidins (PCs, as opposed to prodelphinidins) specifically refer to proanthocyanidins with predominantly catechin and epicatechin monomer residues [9]. A representative cocoa procyanidin dimer is shown in Fig. 2. Although largely beyond the scope of this review, PCs may also contain either A- or-B-type linkages [10]. Cocoa, the focus of this review, contains PCs with B-type linkages.

2.2. Dietary sources of flavanols

Significant levels of flavanols are found in a variety of dietary plants including tea, apples, grapes, cocoa, berries, plums, apricots and nuts [9,11–13]. The flavanol content is higher in certain foods such as grapes, tea and cocoa, compared to other plants, and thus the body of literature focuses on these products. Cocoa is generally regarded as the most concentrated dietary source of flavanols with the strongest antioxidant potential [7,14].

Although many potentially bioactive compounds are found in cocoa, many of the health benefits associated with its consumption are likely due to its high flavanol content. Cocoa is composed of flavanol monomers, oligomers, and polymers [15]. The most common monomers found in cocoa are epicatechin (up to 35% of polyphenol content) [16,17], as well as (±)-catechin. It is important to note that cocoa is one of the few foods with appreciable levels of (−)-catechin, which is produced by epimerization of (+)-catechin during fermentation. Cocoa contains PCs composed of up to 12 monomeric residues [18], although larger species likely exist but are not easily measured by common chromatographic methods. There can be great variability in cocoa phenol content from *Theobroma cacao* plants of different origins [16] and the polyphenol content of cocoa powder is largely dependent on processing methods.

The impacts of tea and grape seed on metabolic syndrome have been extensively reviewed and analyzed [19–22]. Furthermore, there is a large body of literature regarding the effects of cocoa on cardiovascular disease [23–25]. However, the potential link between cocoa and improvements to metabolic syndrome and, specifically, glucose homeostasis and diabetes is a newer, less-studied area and warrants further investigation and a review of the current literature. Therefore, this review focuses specifically on the potential mechanisms by which cocoa flavanols improve metabolic syndrome, particularly glucose homeostasis and diabetes.

2.3. Bioavailability of cocoa flavanols

Understanding flavanol bioavailability is critical for identifying flavanol bioactivities [13]. Bioavailability of cocoa flavanols from food is a multistep process including digestion and release of flavanol from its food matrix, solubilization and absorption into enterocytes, xenobiotic metabolism in the enterocytes, liver and colon and, lastly, elimination [26]. While an exhaustive discussion of flavanol bioavailability is beyond the scope of this review, unique aspects of cocoa flavanol bioavailability warrant mention as they pertain to mechanism.

Potential PC instability during gastric transit has been suggested as a factor limiting bioavailability of orally administered flavanols. PCs could be hydrolyzed to form monomers (or partially hydrolyzed to form monomers and smaller PCs) in the low pH conditions of gastric juice. Spencer *et al.* [27] reported that PC oligomers (up to DP 6) were degraded to monomeric flavanol residues when incubated in an acidic solution (pH~2.0) for up to 3.5 h. However, there are conflicting reports on this phenomenon in both animals and humans [28–33]. Tsang *et al.* [30] found that polyphenols from grape seed extract (catechin, epicatechin PC dimers, trimers and tetramers) were intact in the GI tract after an oral gavage in Sprague–Dawley rats. They concluded that there was neither a sizeable increase in monomers nor a concomitant decrease in oligomers, suggesting that the oligomers were stable through gastric transit [30]. Rios *et al.* [28] reported that PCs were intact after being ingested with a meal in humans. After participants drank a 500-ml cocoa beverage, the pH of the stomach was elevated, keeping the cocoa powder protected from an extremely acidic environment (such as the environment utilized in the study conducted by Spencer *et al.* [27]). Further, the *in vivo* study showed that the 500-ml beverage was emptied from the stomach in about 50 min, whereas the incubation study lasted up to 3.5 h [28]. Therefore, it appears that PCs, as well as monomeric flavanols, remain intact during gastric transit. Some depolymerization may occur, but the amount is so small that any increase in monomer concentration would be negligible [9,30]. Therefore, gastric degradation is unlikely to limit flavanol bioavailability and bioactivity.

Bioavailability is thought to reduce potential flavanol bioactivity *in vivo*. Monomers (catechin and epicatechin) are relatively well absorbed compared to PCs [28,34,35]. They first appear in the circulation 30–60 min after ingestion [36] and reach peak plasma concentrations at 2–3 h [28]. Epicatechin appears in greater concentrations in human plasma than catechin. Holt *et al.* [37] reported that there is a preferential absorption of epicatechin. When catechin and epicatechin were given to participants in equal concentrations, there was 5.92-μM epicatechin but only 0.16-μM catechin in the plasma 2 h after ingestion [37]. Furthermore, the (+)-catechin is more bioavailable than (−)-catechin, which predominates in fermented cocoa [38]. Dimeric, trimeric and tetrameric PCs are also absorbed in their intact form but at a much lower rate compared to the monomers [9]. Interestingly, Deprez *et al.* [39] showed that (+)-catechin and PC dimers and trimers had similar permeability coefficients as mannitol (an indicator of paracellular transport) in Caco-2 monolayers. Therefore, these smaller flavanols are likely entering the bloodstream *via* paracellular diffusion [39,40]. Polymers larger than tetramers are generally not absorbed intact [9] and proceed to the colon, along with unabsorbed fractions of monomers and smaller PCs. Approximately 5–10% of polyphenols can be absorbed in the small intestine while the remaining 90–95% proceed to the colon [41]. Poor PC bioavailability therefore is likely a main factor that limits bioactivity in peripheral tissues, particularly for larger PCs. Their relatively low bioavailability indicates that the gut may be the primary location of action for cocoa PCs due to the high concentrations present there compared to levels in circulation [9,42]. Concentrations of flavanols in the blood and tissues are typically less than 5 μM [37,43–45], which are at the lower end of concentrations typically used *in vitro* to asses bioactivity in cell models [46]. However, when the intestinal lumen or epithelial surface is the site of action (such as inhibition of digestive enzymes or absorption transporters, modulation of gut barrier integrity, *etc.*), bioavailability is not a limiting factor.

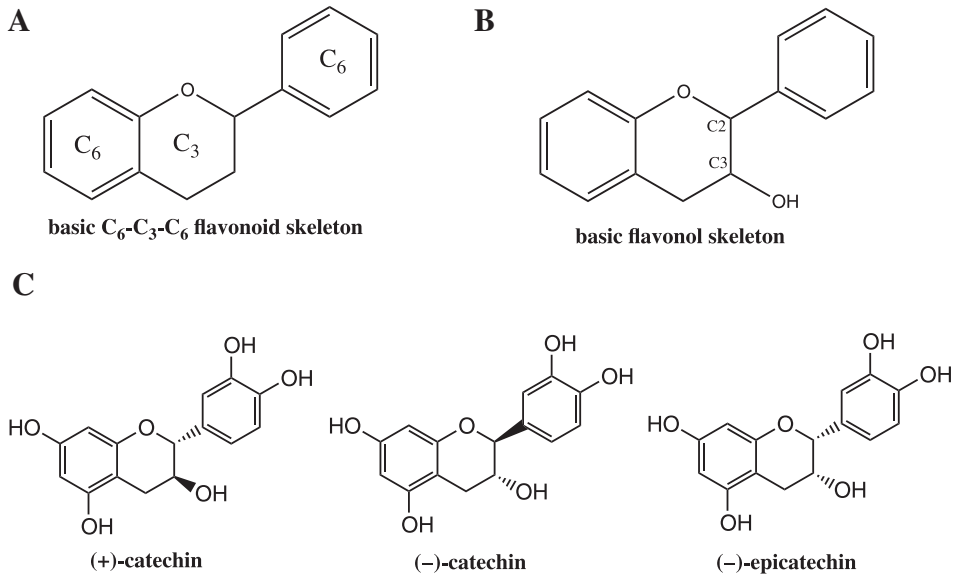

Fig. 1. The basic 3-ring flavonoid skeleton (A), the C_3-hydroxylated flavanol skeleton (B) and structures of predominant flavanol monomers in cocoa (+) catechin, (−)-catechin and (−)-epicatechin (C).

Flavanols are degraded in the colon by the gut microbiota, and some of the resulting metabolites can then be absorbed into the circulation. The conversion of (+)-catechin to (+)-epicatechin is a prerequisite step for microbial metabolism [47]. These monomers are typically metabolized to form 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone, 5-phenyl-γ-valerolactone and phenylpropionic acid [47]. The majority of cocoa PCs are degraded into many metabolites, including phenolic acids and phenylvalerolactones [9,18,48,49], and possibly others that have not been identified. As PCs increase in size, the ability of bacteria to metabolize them decreases [50]. Gonthier *et al.* [51] found that the yield of phenolic acids from monomers and PC dimers (10% and 7%) was much greater than those from PC trimers and polymers (0.7% and 0.5%).

Microbial metabolites of flavanols should be considered as potential contributors to the health effects of these compounds observed following oral administration [41,52], as they are extensively produced and comparatively more bioavailable [53,54] than the native compounds themselves (particularly the PCs). Despite general recognition that these microbial metabolites are likely to contribute extensively to the activities observed during consumption of flavanols (and polyphenols in general) [55–57], very little is known about the bioactivities of these compounds. In terms of glucose homeostasis, Fernandez-Millán *et al.* [58] showed that 3,4-dihydroxyphenylacetic acid, 2,3-dihydroxybenzoic acid and 3-hydroxyphenylpropionic acid potentially improve glucose-stimulated insulin secretion and resistance to oxidative stress in β-cells and rat islets. Carrasco-Pozo *et al.* [59] recently demonstrated that 3,4 dihydroxyphenylacetic acid protected β-cells against impaired insulin secretion, mitochondrial dysfunction and increased apoptosis induced by cholesterol. These metabolites are also known to have antiinflammatory effects [52,60]. Therefore, these microbial metabolites appear to have significant activities related to improving glucose homeostasis, but only a few of the dozens of compounds have been investigated, and the impact of these metabolites in most tissues critical to glucose homeostasis remains unstudied. To the best of our knowledge, no published data exist regarding the potential impacts of these metabolites on skeletal muscle, adipose tissue or liver physiology and metabolism. *In vitro* tissue culture experiments are needed in order to determine the impacts of microbial metabolites on pathways related to glucose homeostasis in these tissues.

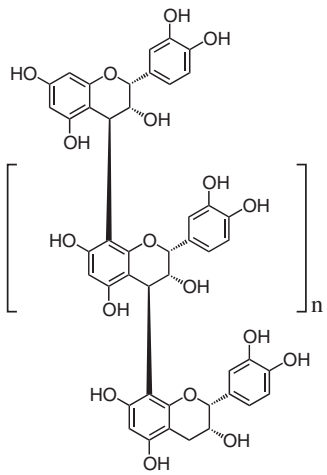

Fig. 2. Representative structure of cocoa B-type procyanidins.

The majority of research has focused on characterizing the formation, bioavailability and pharmacokinetics of these metabolites. Few studies have examined the activities of the microbial metabolites directly, likely due to several reasons. First, not all microbial metabolites are commercially available [49,61]. Second, in order to test compounds that are not commercially available, *in vitro* or *in vivo* fecal fermentations must be performed and the desired product(s) extracted, isolated and purified from a complex mixture of several dozen native and metabolite compounds and then characterized analytically. The complexity, time, cost and low yields associated with this process can be prohibitive. Third, some microbial metabolites are highly transient [49], particularly the intermediate products which are subsequently converted into smaller products. Thus, these compounds are even more difficult to isolate. Fourth, the large number of metabolites makes screening of these compounds for biological activity laborious. Finally, *in vivo* testing of these compounds is

difficult, as they are formed only in the lower gut. Therefore, studies involving direct oral administration of these metabolites are problematic, as activities in the stomach and small intestine (as well as absorption from those regions) are likely to be observed despite being irrelevant to activities resulting from colonic formation of the metabolites. One solution is to observe the activities of metabolites by eliminating them: native flavanols could be fed to both normal animals and either germ-free or antibiotic-fed animals, and the differences in activities are likely associated with the microbial metabolites. Due to this issue, *in vitro* cell culture studies are currently the most promising and urgently needed aspect of understanding how these metabolites contribute to the health benefits of flavanol consumption. Specifically, studies are needed which examine the activities in β-cells (insulin secretion, proliferation, apoptosis, resistance to oxidative stress), intestinal L-cells (incretin hormone secretion), hepatocytes (gluconeogenesis, lipid accumulation), skeletal muscle (insulin sensitivity, metabolic flexibility, mitochondrial function, lipid accumulation) and adipocytes (differentiation, lipid accumulation, hormone secretion). These activities may represent major mechanisms by which orally consumed cocoa flavanols exert their activities. Of all the mechanisms described in this review, this is the least investigated area and the area in which relevant data are most urgently needed. Therefore it is possible that the potential activities of microbial metabolites are the area in which the greatest advances in knowledge stand to be gained.

3. Animal and clinical studies

Prior to reviewing mechanisms of action, we summarize outcomes of relevant animal and human studies to identify the impact of cocoa and cocoa flavanols on metabolic syndrome.

3.1. Animal studies

Many animal studies have been conducted to examine whether cocoa may reduce circulating endotoxin, oxidative stress and inflammation and, thus, improve glucose control and other outcomes related to metabolic syndrome. These studies are summarized in Table 1. The studies listed are mostly chronic studies, lasting anywhere from 1 to 18 weeks [62,63], and there were only two acute studies [64,65]. Many rodent models mimicking diabetes or prediabetes were utilized, and many of the studies utilized high-fat diets. Many of these studies reported improvements in glucose-related outcomes (fasting glucose levels as well as glucose tolerance) [17,64,66–72], while three studies reported no changes [63,73–75]. One study reported changes in gut microbiome [76], and two studies reported attenuated endotoxin levels [17,63].

When evaluating these studies, it is important to note the experimental procedures by which cocoa was given to the animals. There was a wide range of doses used as well as a variety of dosing methods (discussed in more detail below). The dosing method may impact the mechanisms by which cocoa flavanols act *in vivo*. Some cocoa was available *ad libitum* by adding it into the chow or the drinking water. In this case, flavanols were co-consumed with macronutrients, thereby facilitating flavanol-mediated alteration of nutrient digestion. This cocoa supplement was often reported as a percentage of food (w/w) or water (w/v). Other studies supplemented the cocoa by means of an oral gavage, and these doses were often reported as a dose in mg/kg body weight. Oral gavage is often done in the fasted state, in which case flavanols would not be co-consumed with macronutrients, thereby precluding the opportunity for flavanol-mediated alteration of nutrient digestion. While each procedure had its advantages, it is important to note the differences between the two. When cocoa was provided *ad libitum*, the dose was dependent on food intake, which was sometimes not reported. Cocoa is extremely bitter, and high percentages of cocoa may have been unpalatable and therefore led to a reduced food intake, possibly contributing to the observed positive outcomes. This is a potential mechanism of action in animal studies that is not likely translatable to humans. Further, studies comparing high-fat diets to normal diets (each with cocoa supplements) [69] had significantly different food intakes, meaning different doses of cocoa were being ingested. Studies comparing normal animals to diabetic animals [66,67] also had the same dilemma. In studies using diabetic rats, there were also significant differences in food intake, where the diabetic animals ate more and therefore were consuming more polyphenols.

Another aspect of study design to consider when evaluating the effective dose and potentially bioactive constituents of cocoa is the different types of cocoa product utilized. Animal studies have used cocoa liquor (liquefied cocoa mass), chocolate (cocoa liquor + sugar and possibly other ingredients), cocoa powder (cocoa liquor with most of the cocoa butter removed), cocoa extracts (prepared by distinct extraction procedures, containing various profiles of phenolic acids, flavanols, *etc.*) and pure compounds (catechins, epicatechin, *etc.*). These all have different amounts of fiber, lipids and polyphenols, all of which may possess beneficial activities that may have synergistic, or even antagonistic, effects with flavanols. While the majority of studies show efficacy, these confounding components make interpretation of the effective dose problematic. These non-flavanol components may act by mechanisms distinct from the flavanols. On the other hand, purified compounds alone are not representative of the complexity of cocoa products. While most studies show some efficacy of these various cocoa products, studies are still needed to isolate the activities of individual components. For example, the effect of flavanols *versus* nonflavanol components could be elucidated by comparing the impact of cocoa *versus* an equivalent dose of heavily Dutched cocoa. Furthermore, cocoa could be deconstructed by sequentially extracted cocoa lipids (with hexane) and then flavanols (with acetone:water:acetic acid), leaving fiber and other insoluble components. The various fractions could then be compared against whole cocoa, or cocoa minus specific components, to elucidate the role of each component. When evaluating the potential translational benefits to humans, it should be understood that humans generally consume chocolate, cocoa powder and cocoa liquor (in solid form) and generally do not consume cocoa extracts or pure compounds (although cocoa extracts or products with added cocoa extracts can be obtained in supplement form).

In summary, animal studies of the impacts of cocoa, chocolate, cocoa extracts or cocoa monomers on metabolic syndrome have been highly descriptive. These studies have suggested potential mechanisms but do not definitively isolate or interrogate the proposed mechanisms.

3.2. Clinical studies

There have been a variety of clinical trials assessing the effects of habitual cocoa intake on glycemic and insulinemic outcomes. These are summarized in Table 2. Many of the studies found cocoa to be beneficial for glucose control [77–83]. Cocoa treatments were often provided in the form of chocolate bars [78–82] or beverages [83–87]. Chronic studies lasted from 5 days to 3 months [83,86,87], but most lasted about 2 weeks.

In select studies, cocoa and cocoa flavanols improved insulin sensitivity and reduced blood glucose, insulin, and HbA1c in subjects with varying degrees of glucose homeostasis (normoglycemic, prediabetic or T2DM) within 2–4 weeks [78–83,85,88]. However, other studies showed no effect [84,86,89,90]. Despite its promising effects *in vitro* and in animal models, only five chronic studies of cocoa and glucose control have been performed in subjects with prediabetes or diabetes [78,80,81,86,89], as the majority of studies were focused

Table 1
Animal studies related to the effects of dietary cocoa or cocoa flavanols on metabolic outcomes

Author, year	Animal model	Treatment/Delivery	Animal dose (mg/kg body weight)	Human equivalent dose[a] (mg/day)	Acute/Chronic design, diet	Cocoa treatment outcomes
Matsui, 2005 [225]	Male Wistar rats	12.5% (w/w) cocoa powder, in food	7,040[b]	79,913[c]	Chronic, 3 weeks, high-fat diet	↓final body weights, ↓fatty acid synthesis
Ruzaidi, 2005 [66]	Male diabetic Wistar rats, (STZ[d] induced)	1, 2, 3% (w/w) cocoa extract[e], in food	Diabetic rats[f]: 1% = 868 2% = 1,776 3% = 2,580 Normal Rats: 1% = 433 2% = 860 3% = 1,200	Diabetic rats: 1% = 9,853 2% = 20,160 3% = 29,286 Normal rats: 1% = 4,919 2% = 9,762 3% = 13,622	Chronic, 4 weeks, normal diet	↓glycemia, ↓hypercholesteremia
Tomaru, 2007 [67]	Female, *db/db* mice (obese, diabetic)	0.5%, 1.0% (w/w) cacao liquor proanthocyanidin, in food	0.5% = 1,107[g] 1.0% = 2,044	0.5% = 5,771 1.0% = 11,602	Chronic, 3 weeks, normal diet	↓blood glucose in a dose dependent manner
Jalil, 2008 [158]	Male *ob/db* Sprague–Dawley rats (STZ induced)	Cocoa extract, by oral gavage	600	6,811	Chronic, 4 weeks, high-fat diet	↓oxidative stress (8-isopostane)
Jalil, 2009 [68]	Male *ob/db* Sprague–Dawley rats (STZ induced)	Cocoa extract, by oral gavage	600	6,811	Chronic, 4 weeks, high-fat diet.	↑ glucose tolerance (OGTT- AUC), ↓total cholesterol, ↓triglycerides. No changes in insulin sensitivity
Perez-Berezo, 2011 [242]	Female Wistar rats	2%, 5%, or 10% (w/w) cocoa powder, in food	Unknown (food intake data not reported).	Unknown (food intake data not reported).	Chronic, 3 weeks, normal diet	↓immune response (IgG1, IgG2, S-IgA) (5 and 10% treatments)
Si, 2011 [73]	Male *db/db* mice	0.25% epicatechin, in drinking water	150	851	Chronic, 15 weeks, normal diet.	↓inflammatory markers (CRP, IL1B), oxidative stress (GSH, SOD), ↑ lifespan. no change in glycemia
Massot-Cladera, 2012 [76]	Female Wistar rats	10% (w/w) cocoa powder[h], in food	Unknown (food intake data not reported).	Unknown (food intake data not reported).	Chronic, 6 weeks, normal diet.	Altered gut microbiome (↓Bacteroides, Staphylococcus, Clostridium)
Yamashita, 2012 [243]	Male C57BL/6 mice	0.5, 2.0% (w/w) cacao liquor procyanidins[i], in food	Normal diet[j]: 0.5% = 588 2.0% = 2,344 High-fat diet: 0.5% = 310 2.0% = 1,532	Normal diet: 0.5% = 3,337 2.0% = 13,304 High-fat diet: 0.5% = 1,759 2.0% = 8,695	Chronic, 13 weeks, control or high-fat diet	↓fasting glucose (2.0% treatment); ↑glucose tolerance (OGTT AUC). ↑translocation of GLUT4, AMPK phosphorylation, UCP expression
Yamashita, 2012 [62]	Male C57BL/6 mice	0.5%, 1% (w/w) cocoa liquor procyanidins, in food	Unknown (food intake data not reported).	Unknown (food intake data not reported).	Chronic, 1 week, normal diet	↑glucose tolerance in a dose dependent manner (OGTT-AUC)
Yamashita, 2012 [62]	Male ICR[k] mice	Cocoa liquor procyanidins, by oral gavage	50 or 250	283 or 1,418	Acute	↑glucose tolerance (OGTT-AUC) (250-mg/kg dose)
de Oliveira, 2013 [74]	Male Wistar STZ-induced diabetic rats	Cocoa liquor[l], by oral gavage	3,600 or 7,200	1,157 or 2,317	Chronic, 40 days, normal diet	↑ antioxidant capacity (ORAC, FRAP), no change in blood glucose levels
Yamashita, 2013 [64]	Male ICR mice	Procyanidins, by oral gavage	0.01	0.06	Acute	↑plasma insulin; ↑ GLP-1 levels
Dorenkott, 2014 [17]	Male C57L/l6 mice	Monomeric, oligomeric and polymeric cocoa extract fractions, in food	25	142	Chronic, 12 weeks, high-fat diet	Oligomeric fraction ↓ fasting blood glucose, ↑glucose tolerance; ↑ insulin tolerance (OGTT); ↓endotoxin
Gu, 2014 [75]	High fat-fed obese male C57BL/6 J mice	8% (w/w) cocoa powder, in food	11,828[m]	67,135	Chronic, 10 weeks, high-fat diet	↓weight gain, ↑fecal lipid content, ↑insulin sensitivity (HOMA-IR), ↓inflammatory markers (IL-6,MCP-1), no change in blood glucose
Gu, 2014 [63]	Male C57BL/6 J mice	8% (w/w) cocoa powder, in food	4,998[n]	28,367	Chronic, 18 weeks, high-fat diet	↓inflammation (adipose tissue NF-κB expression), ↑insulin sensitivity

(continued on next page)

Table 1 (*continued*)

Author, year	Animal model	Treatment/Delivery	Animal dose (mg/kg body weight)	Human equivalent dose[a] (mg/day)	Acute/Chronic design, diet	Cocoa treatment outcomes
						(HOMA-IR), ↑gut barrier function (plasma GLP-2), ↓plasma endotoxin
Gutierrez-Salmean, 2014 [190]	High fat-fed, obese, male Wistar rats	(−)-epicatechin, by gavage	1	11	Chronic, 2 weeks, low fat or high-fat diet	↓blood glucose, ↓ triglyceride levels, ↑mitochondrial function (TFAM, mitofilin expression)
Gutierrez-Salmean, 2014 [72]	Male Wistar rats	(−)-epicatechin, by gavage	1	11	Chronic, 2 weeks, low fat or high-fat diet	↓fasting glucose; ↑glucose tolerance.
Matsumura, 2014 [65]	Male ICR mice	Flavanol fraction or (−)-epicatechin, by gavage	10	57	Acute	Flavanol fraction ↑energy expenditure (REE), ↑blood catecholamines
Osakabe, 2014 [3]	Male Wistar rats	0.2% (w/w) flavanols, in food	78	890	Chronic, 4 weeks, high-fat diet	↓thermogenesis, ↓lipolysis
Papadimitrou, 2014 [227]	Male SHR[o] rats, diabetic (STZ induced)	Cocoa powder, by gavage	24	272	Chronic, 16 weeks, normal diet	AMPK, ↓NOX4 signaling
Watanabe, 2014 [70]	Male C57BL/J mice	Cocoa flavanols[p] by gavage	50	284	Chronic, 2 weeks, normal diet	↓plasma glucose. ↓resting energy requirements, mitogenesis
Fernandez- Millan, 2015 [71]	Male Zucker diabetic fatty rat	10% (w/w) cocoa powder, in food	8,311[q]	94,345	Chronic, 9 weeks, normal diet	Prevented B cell mass loss, ↑glucose tolerance (OGTT) insulin sensitivity (HOMA-IR), ↑β cell function (HOMA-B), ↓oxidative stress (carbonyl groups, TBARs)

Human equivalent doses were calculated by the equation provided by Reagan-Shaw *et al.* [241] using food intake and body weight data, if provided. Assumptions made for calculation are indicated in the footnotes.

[a] Based on a 70 kg human.
[b] Used reported final body weight to calculate animal and human equivalent doses.
[c] Author reported 50,000 mg/70 kg/day human equivalent dose.
[d] Streptozotocin.
[e] 285.6 mg polyphenols/g extract.
[f] Assumed body weights of rats were 0.30 kg for normal rats and 0.25 kg for diabetic rats, based on reported body weights, to calculate animal and human equivalent doses.
[g] Average food intake and body weights during weeks 4–6 were used to calculate animal and human equivalent doses.
[h] Cocoa powder contains 10.62-mg/g polyphenols.
[i] Cocoa liquor procyanidin contained 69.8% polyphenols.
[j] Based on body weights at the end of the experiment and total food intake averaged over the entire experiment.
[k] Institute of Cancer Research/Imprinting Control Region mouse.
[l] Total phenolics 2845-mg/100 g dry weight.
[m] Based on average weight at the start of the experiment (0.020 kg mouse) and does not account for weight gained during the experiment, since final weights not provided (only displayed in graph).
[n] Based on average final weights (0.0471-kg mouse).
[o] Spontaneously hypertensive rat.
[p] Flavanol fraction was 72.4% w/w total polyphenols.
[q] Based on final weight (0.2335-kg rat) and average food intake (19-g food/day) over 10 weeks.

on cardiovascular or cardio-metabolic outcomes [78–81,87]. In terms of diabetes biomarkers, most of these studies focused on insulin resistance/sensitivity; few focused on overall blood glucose control [81,89], which is a critical clinical outcome. Furthermore, only two of these five studies in prediabetic or diabetic subjects studies lasted >15 d [81,89]. Neither of these two longer studies examined prediabetes (both used subjects with existing T1/2DM) [81,89]. Therefore, the potential for cocoa to improve long-term glucose control has not been sufficiently studied. Additional studies lasting 1–3 months (or potentially longer) are needed. Furthermore, the potential impact of cocoa in individuals with prediabetes has not yet been evaluated. Clinical trials in individuals with prediabetes are thus needed in order to determine the potential utility of cocoa for improvement of long-term blood glucose control and prevention of T2DM in this population, where early prevention may significantly reduce or delay progression to T2DM. Furthermore, additional studies are needed in individuals with T2DM in order to evaluate the potential for cocoa to ameliorate T2DM and slow progression to β-cell exhaustion and failure.

Interestingly, no significant glycemic improvements were observed in the two studies that utilized epicatechin only [87,89]. This supports the idea that the larger PCs may be important, despite their relatively low bioavailability [17]. However, these studies [87,89] only examined patients with prediabetes or T2DM, so health status may be an important mediator for interventions with epicatechin; these interventions may be more effective in healthier individuals.

Overall, the existing clinical trials support the premise that cocoa can improve glycemic outcomes in healthy, overweight or hypertensive adults. While many of these findings seem promising, these studies do not provide insight into the mechanisms responsible.

Furthermore, many of these studies (and the selected primary outcomes) were related to cardiovascular disease (generally hypertension), not glucose homeostasis/diabetes. Further, as stated above, there have been no long-term studies examining the effects of cocoa consumption in an at-risk (prediabetic) population, and only 5 studies in individuals with diabetes (4 examined T2DM, while one study did not specify whether subjects were diagnosed with T1 or T2DM) [81].

Table 2
Clinical trials assessing the effect of dietary cocoa on metabolic outcomes, in chronological order

Author, year	Subjects	Health status	Treatment (daily dose)	Acute/Chronic (duration)	Outcomes
Nguyen, 1994 [244]	$N=10$	Healthy	100-g chocolate bar, (45-g cocoa)	Acute	Lesser but prolonged increase in glucose and insulin.
Brand Miller, 2003 [77]	$N=10$	Healthy	6 food pairs, one flavored with cocoa[a]	Acute	↑insulin response (insulin index) but not glycemic differences with chocolate flavored products.
Basu, 2015 [88]	$N=14$	Obese, Type 2 diabetic	Cocoa beverage (960-mg polyphenols, 480-mg flavanols[b])	Acute	↑ postprandial insulin secretion, no improvements in blood glucose or insulin resistance (except 4-h postmeal)
Grassi, 2005 [78]	$N=15$	Healthy	100-g chocolate bar, (500-mg polyphenols)	Chronic (15 days), crossover design	↑insulin sensitivity (HOMA-IR, QUICKI), ↑glucose tolerance (OGTT).
Grassi, 2005 [79]	$N=20$	Hypertensive	100-g chocolate bar, (88-mg flavanols[c])	Chronic (15 days), crossover design	↑insulin sensitivity (HOMA-IR, QUICKI, ISI).
Muniyappa, 2008 [84]	$N=20$	Hypertensive	150-ml beverage, 2×/day. (900-mg flavanols[d])	Chronic (2 weeks), crossover design	No effects on insulin sensitivity (QUICKI and clamp).
Grassi, 2008 [80]	$N=19$	Hypertensive, impaired glucose tolerance	100-g chocolate bar, (1008-mg phenols)	Chronic (15 days), crossover design	↑insulin sensitivity (HOMA-IR, QUICKI, SI), ↑β cell function.
Davison, 2008 [83]	$N=49$	Overweight and obese (BMI>25 kg/m^2)	150-ml cocoa beverage (2×/day), high flavanol (902 mg) and low flavanol (36 mg)	Chronic (12 weeks), randomized arm	↑insulin sensitivity (HOMA2-IR) at 6 and 12 weeks.
Mellor, 2010 [89]	$N=12$	Type 2 diabetic	45-g chocolate (3 bars/day), (16.6-mg epicatechin[e])	Chronic (8 weeks), crossover design	No change in glycemic control (HOMA-IR, HbA1c, fasting glucose). ↑HDL cholesterol.
Almoosawi, 2012 [82]	$N=42$	Healthy (BMI<25 kg/m^2) compared to overweight (BMI>25 kg/m^2)	20-g dark chocolate, (500-mg polyphenols)	Chronic (4 weeks), crossover design	Treatment prevented unfavorable changes in insulin sensitivity (QUICKI, HOMA-IR) seen in the placebo treatment.
Desideri, 2012 [85]	$N=90$	Mild cognitive impairment	Cocoa beverage, (990-mg, 520-mg or 45-mg flavanols).	Chronic (8 weeks), randomized arm	High flavanol and intermediate flavanol treatments ↓fasting glucose, ↑insulin sensitivity (HOMA-IR) but not fasting insulin compared to the low flavanol group.
Stote, 2012 [86]	$N=19$	Adults at risk for insulin resistance	Cocoa beverage (2×/day), (30-, 180-, 400- or 900-mg flavanols)	Chronic (5 days), crossover design	No effects on glycemia (OGTT) or insulinemia (HOMA, QUICKI, ISI)
Stellingwerff, 2013 [90]	$N=16$	Trained cyclists	Dark chocolate, (240-mg polyphenols[f])	Acute, crossover design	↑ Blood glucose and ↑insulin
Haghighat, 2013 [81] (abstract only)	$N=69$	Hypertensive diabetic adults	25-g dark chocolate, (450-mg polyphenols)	Chronic (8 weeks), randomized arm	↓Fasting glucose, ↓HbA1c
Ramirez-Sanchez, 2013 [87]	$N=5$	T2D/Stage II and Stage III heart failure patients (compared with healthy controls)	18-g cocoa powder in a beverage (2×/day), (100-mg epicatechin)	Chronic (3 months), parallel arm	No effects on glycemia/insulinemia. ↓oxidative stress in mitochondria.

[a] Foods used include Coco Pops (Kellogg's cereal), Betty Crocker chocolate fudge super moist cake and creamy deluxe Dark fudge frosting. Plain chocolate block (classic full cream milk chocolate from Nestle), Ultra chocolate classic ice cream from Sara Lee and chocolate instant pudding (White Wings Foods).
[b] Placebo contained 110-mg polyphenols, <0.1-mg flavanols.
[c] Consists of the flavanols: catechin, epicatechin, quercetin, kaempferol and isorhamnetin.
[d] Placebo contained 14-mg flavanols.
[e] Placebo contained <2-mg epicatechin.
[f] Polyphenols included epicatechin, catechin, procyanidin B2, procyanidin B5, trimer C and tetramer D.

Additional studies of the mechanisms specifically related to glucose homeostasis in these populations are greatly needed moving forward.

As with reported animal studies, human clinical studies of cocoa or chocolate have been largely descriptive. While it is considerably more difficult to perform elegant mechanistic studies in humans due to feasibility or ethical concerns, opportunities to move toward mechanistic studies in humans will be discussed later in this review.

4. Potential molecular mechanisms of action

There are numerous potential primary molecular mechanisms by which cocoa flavanols appear to prevent or ameliorate metabolic syndrome. It is critical to clearly define the *primary molecular mechanism of action* and differentiate it from downstream effects. The primary molecular mechanism of action is the initial biological effect caused directly by the bioactive compound of interest. In other words, the primary molecular mechanism of action is the most "upstream" activity induced by the compound of interest that results in the observed effects. The primary molecular mechanism of action may then have numerous downstream consequences in various pathways. As discussed below, most research on cocoa flavanols and other dietary bioactive compounds in animals or humans (including studies from our lab [17,91]) has been primarily "descriptive" in nature: a compound or food is administered, and biomarkers or outcomes are observed. These descriptive studies demonstrate the effects of the intervention and suggest, but do not definitively identify, primary molecular mechanisms of action by which these effects are achieved [92]. Such studies are extremely valuable for hypothesis generation regarding the primary molecular mechanism of action. However, mechanism-oriented research (beyond measuring biomarkers of disease) is needed to isolate and identify the primary molecular mechanisms of action [93–97]. One additional limitation of descriptive studies is that the relationship between observed effects typically remains unclear. Dietary interventions may result in modulation of several pathways or systems that all likely contribute to improvements to glucose homeostasis. However, the order in which these improvements occur, the importance of each observed effect in the overall improvement in glucose homeostasis and the degree to which pathways influence one another are often not clear in descriptive studies. To elucidate primary molecular mechanisms,

studies that isolate and probe specific molecular interactions and biological pathways (such as knockout or "knock-in" mouse models, use of receptor agonists/antagonists, use of pathway inhibitors in cell assays, gene silencing by siRNA, *etc.*) are needed.

Caution should be used when interpreting descriptive biomarker studies in search of primary mechanism, as many different primary molecular mechanisms can have similar effects, and each unique primary molecular mechanism can have pleiotropic effects. Numerous studies have demonstrated the positive effects of cocoa and cocoa flavanols, including improved glucose homeostasis, body composition and others. However, the key initial events in the cascades of biological processes regulating these outcomes remain to be identified. Several possibilities include inhibition of digestive enzymes, inhibition of glucose transporters, reduced metabolic endotoxemia and stimulation of the incretin response. Possible mechanisms are illustrated in Fig. 3. In all probability, flavanols act through various mechanisms simultaneously. The most well-studied and promising potential mechanisms and their implications will be reviewed here.

4.1. Carbohydrate digestion

Perhaps the most direct mechanisms by which flavanols may improve glucose homeostasis is by slowing carbohydrate digestion and absorption in the gut, as explained below.

4.1.1. Glucose homeostasis

One component of metabolic syndrome is derangement of glucose homeostasis, resulting in hyperglycemia and glucose intolerance. Glucose levels are primarily controlled by the hormones insulin and glucagon. These two hormones are under tight regulation in order to maintain blood glucose levels between 4 and 7 mM in normal individuals (glucose homeostasis) [98,99]. Failure to maintain glucose homeostasis can lead to a wide variety of conditions, including adiposity, dyslipidemia, vascular damage, vision loss, kidney disease, neuropathy, atherosclerosis and myocardial infarction [100,101]. When the insulin signaling pathway is impaired, as for example due to chronic inflammation [1], a cyclical effect occurs where blood glucose levels become elevated and β-cells are constantly stimulated. This causes β-cells to deteriorate and lose their ability to produce insulin, leading to prediabetes, T2DM and then frank diabetes with β-cell failure. Inadequate insulin secretion can then lead to hyperglycemia and ketoacidosis.

4.1.2. Inhibition of digestive enzymes

Cocoa can slow the rate and extent of macronutrient digestion by noncovalently binding to and antagonizing digestive enzymes. The complex ring structure with abundant hydroxyl groups allows cocoa to bind to proteins, particularly digestive enzymes. Cocoa flavanols interact with digestive enzymes by a variety of primary inhibition mechanisms [102].

Cocoa may inhibit α-amylase [36], an enzyme that breaks down starch into glucose oligomers. There is evidence to suggest that polyphenols bind to this enzyme, reducing its activity [103]. Yilmazer-Musa *et al.* [103] found that grape seed extract (GSE) (including catechin, epicatechin and PCs) with 86% total phenolics by weight was just as efficient as the drug acarbose at inhibiting α-amylase. Acarbose, the positive control, had a median inhibitory concentration (IC_{50}) of 6.9 μg/ml compared to GSE with an IC_{50} of 8.7 μg/ml. On the other hand, white tea, which contains predominantly monomeric flavanols and only 34% total phenolics by weight, had an IC_{50} of 378 μg/ml. While total flavanol concentration plays a role in the observed IC_{50} values, it also appears that the more complex the structure, the greater its ability to inhibit digestive enzymes. Thus, flavanols may reduce digestion of starches, thereby lowering glucose absorption via inhibiting this enzyme in the diabetic population. Interestingly, α-amylase expression is higher in individuals with T2DM than healthy individuals [46,104].

Glucosidase inhibitors are well studied and commercially available, but unwanted side effects such as diarrhea, gas and cramping have been reported for these drugs [103,105]. Acarbose is one such synthetic glucosidase inhibitor. Acarbose has reportedly been effective in reducing weight gain and comorbidities related to metabolic syndrome, such as diabetes and cardiovascular disease [46]. Flavanols may also inhibit α-glucosidase, which cleaves small oligosaccharides at the 1,4 linked alpha glucose residues, resulting in monomeric sugars that are ready for absorption. This is another key enzyme involved in

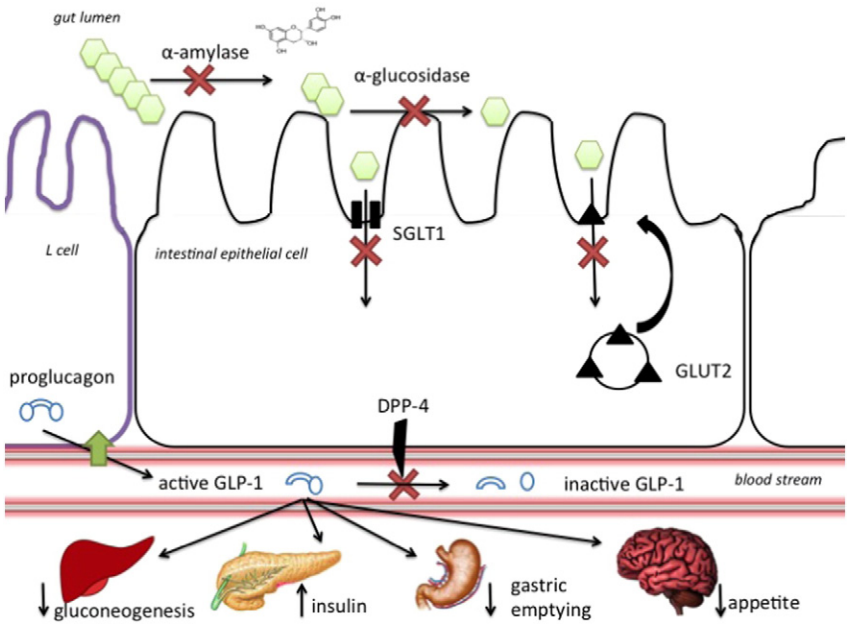

Fig. 3. Hypothetical mechanisms by which cocoa flavanols may affect carbohydrate digestion. Mechanisms include inhibiting digestive enzymes α-amylase and α-glucosidase, inhibiting glucose transporters SGLT1 and GLUT2, promoting GLP-1 secretion and inhibiting DPP-4.

carbohydrate digestion. When these enzymes are inhibited, the breakdown of carbohydrates is slowed, resulting in an attenuated elevation of blood glucose after a meal [106]. Yamashita *et al.* [62] found that a 0.01% cocoa liquor procyanidin extract inhibited α-glucosidase activity *in vitro*; however, this result was not observed in an *in vivo* model using 250-mg/kg cocoa liquor PCs. In the study conducted by Yilmazer-Musa *et al.* [103], acarbose also inhibited α-glucosidase, but the IC_{50} values were 13 times lower compared to acarbose's inhibitory effect on a-amylase. Notably, both GSE (IC_{50} = 1.2 μg/ml) and white tea extract (IC_{50} = 2.5 μg/ml) were more potent α-glucosidase inhibitors than acarbose (IC_{50} = 90 μg/ml).

The structure of flavanols affects the affinity to which they can bind to these proteins. A study by Barrett *et al.* [106] compared flavanols from grape, cranberry, pomegranate and cocoa to determine how well each can inhibit α-amylase. It should be noted that the cocoa used in this study primarily consisted of monomers and dimers, a composition that may not be reflective of most cocoa powders. It was found that all compounds had an effect, but cocoa flavanols (containing the smallest mean degree of polymerization used in the experiment) had the least inhibitory effect on either enzyme. More complex polyphenols, such as ones found in cranberries and pomegranates, were more successful at inhibiting the breakdown of carbohydrates [106]. Andujar and Gu state that the greater the degree of polymerization, the more potently the polyphenol can inhibit digestive enzymes [16,36]. In addition, a study conducted by Gu *et al.* [36] found that cocoa potently inhibits pancreatic amylase, pancreatic lipase and phospholipase A2. This group also examined the effects of processing methods on inhibitory capability. They found that the least processed cocoa, termed *lavado* (an unfermented cocoa which has the greatest concentration and largest cocoa PCs), had the strongest inhibitory effect on these pancreatic enzymes. Inhibition of lipases will be reviewed in Section 4.6.

It has been established that cocoa flavanols can inhibit digestive enzymes, but the extent to which this inhibition affects postprandial glucose excursions is unclear. It is also unclear if these effects are observable *in vivo*. Reducing rapid increases in blood glucose after a meal is important for patients with metabolic disorders, since it helps them maintain glucose homeostasis. Cocoa flavanols may be as effective at inhibiting digestive enzymes as some pharmaceuticals and therefore deserve further consideration.

4.1.3. Inhibition of glucose transporters

Cocoa polyphenols not only inhibit certain digestive enzymes, but they may also inhibit glucose transporters. Similar to digestive enzyme inhibition, the primary molecular mechanism of action may be nonspecific flavanol–protein interactions or competitive inhibition at the transport active site. Inhibiting glucose transporters in the intestine could attenuate glucose excursion after a meal. Intestinal transporters that may be inhibited include glucose transporter 2 (GLUT2) and sodium/glucose cotransporter 1 (SGLT1) [107,108].

GLUT2 is found on both the apical and basolateral surfaces of enterocytes. GLUT2 vesicles store the transporters within the cell and fuse with the cell membrane and facilitate transport of glucose (similar to insulin-stimulated GLUT4) upon increased glucose load. In diabetic patients, the control of this vesicle is lost, and increased amounts of GLUT2 transporters are always found on the cell surface, contributing to elevated blood glucose levels. Kwon *et al.* [107] found that *in vitro* GLUT2-mediated glucose transport was inhibited by quercetin (IC_{50} = 12.7 μM), but not by epicatechin (no inhibition) or catechins (no inhibition). Further studies examining the effects of PCs with varying degrees of polymerization are necessary to understand whether or not inhibition of transporters occurs in response to cocoa consumption.

SGLT1 is a Na^+/glucose cotransporter, which permanently resides on the apical membrane of intestinal epithelial cells. T2DM patients exhibit increased expression of SGLT1 compared to healthy individuals, leading to decreased glucose control [46]. Monomeric (+)-catechin (0.5 mM) inhibited SGLT1 in a competitive mechanism in an *in vitro* study using *Xenopus* oocytes [108]. Polyphenols found in tea [(−)-epicatechin gallate and (−)-epigallocatechin gallate] also inhibited expression of SGLT1. The extent to which cocoa flavanols with large degrees of polymerization can inhibit this transporter is unknown.

Flavanol metabolites that reach circulation may exert an inhibitory effect on glucose transporters in peripheral tissues. However, the concentration of metabolites in circulation is relatively low (<3–5 μM) and is fleeting [37,43–45,107]. Therefore, given the low bioavailability of cocoa flavanols and short half-lives of flavanol metabolites, inhibition of glucose transporters is likely a mechanism occurring exclusively in the gut. Again, this mechanism would be helpful for patients with metabolic disorders because it may reduce rapid glucose excursions after a meal, therefore promoting glucose homeostasis.

4.2. Hormonal response to meals

Cocoa flavanols also appear to modulate the secretion and activities of hormones critical for maintenance of glucose homeostasis, as explained below.

4.2.1. Stimulating the incretin response

The incretin response may be a key mechanism enhanced by cocoa. Incretins (GLP-1, GIP) are secreted from enteroendocrine cells after a meal. One of the roles of these hormones is to stimulate insulin secretion for glucose disposal [109]. Incretin hormones have other effects on the pancreas, including increasing somatostatin secretion, decreasing glucagon secretion and stimulating β-cell growth and neogenesis. Incretin hormones are not limited to stimulating the pancreas; incretin receptors are found in many tissues throughout the body, including the brain, liver, adipose and skeletal muscle. Other incretin functions include suppressing appetite, delaying gastric emptying and increasing glycogen synthesis [110,111]. The incretin response is impaired in noninsulin T2DM, possibly due to a lack of incretin secretion [110,112]. The incretin response is greatly reduced when a glucose load is administered intraperitoneally compared to an oral glucose load [113]. This suggests that the gut is an important location for interventions targeting incretin levels and, therefore, an interesting potential target for cocoa flavanols with poor bioavailability. It is possible that cocoa may enhance the incretin response by either stimulating incretin release or extending the half-life of incretin hormones.

4.2.2. Incretin hormones

The incretin hormone glucagon-like peptide 1 (GLP-1) is released from epithelial endocrine L-cells found in the distal small intestine and colon. In response to either glucose or a mixed meal, proglucagon is cleaved and GLP-1 is released into the circulation [109]. The half-life of GLP-1 is about 2 min. GLP-1 exerts biological actions via its receptors, which are found on islet α- and β-cells in the pancreas, in the brain and on vagal afferents [110,114]. GLP-1 receptor agonists have been developed (*i.e.*, Liraglutide, Novo Nordisk) and promote weight loss by suppressing hunger, reducing the duration of eating and delaying gastric emptying [114,115].

Gonzalez-Albuin *et al.* [116,117] showed an increase in GLP-1 concentration in healthy rats fed an oral glucose load (2 g/kg bw) 40 min after oral gavage of grape seed procyanidin extract (1 g/kg bw) compared to control. The increased concentration was not significantly different from the positive control treatment, 1-mg/kg bw of Vildagliptin (a DDP-4 inhibitor). Yamashita *et al.* [64] also demonstrated increased GLP-1 secretion in mice 60 min after oral gavage of

10-μg/kg bw Cinnamtannin A2, a tetrameric cocoa procyanidin. This study was novel because it was performed in the absence of any macronutrients. Not only did it increase GLP-1 secretion, but insulin secretion and insulin action [measured by phosphorylation of insulin receptor substrate 1 (IRS-1) and insulin receptor (IRβ)] was increased as well [64]. However, the impact of cocoa flavanols on incretin response in the presence of glucose is not yet known.

Gastric inhibitory peptide (also referred to as glucose-dependent insulinotropic polypeptide) (GIP) is secreted from K cells in the proximal small intestine. The release of GIP is stimulated by the presence of nutrients, primarily fats, in the small intestine [118]. The *in vivo* half-life of GIP is approximately 5–7 min. When studying this peptide, it is important to distinguish between the cleaved, non-insulinotropic metabolite [GIP (3–42)] *versus* the active hormone [GIP (1–42)] [118]. Gonzalez-Abuin *et al.* [117] found that GIP concentration was significantly reduced after a gavage of grapeseed procyanidin extract (1 g/kg bw) prior to an oral glucose load (2 g/kg bw). This response was similar to that of the positive control, Vildagliptin. However, clinical studies using solely pharmaceuticals (*i.e.* sitagliptin) find that GIP concentration and area under the curve typically increases in healthy, nondiabetic males [119]. It is unclear why GLP-1 and GIP seem to respond differently in response to grape seed PCs. This is an area that warrants additional investigation, as research on flavanols has focused on GLP-1.

The primary molecular mechanism by which cocoa flavanols stimulate GLP-1 and GIP secretion likely occurs in the secretory cells but remains unknown. It seems likely that consumption of cocoa polyphenols stimulates the release of GLP-1, but the effects of cocoa on GIP are less understood. It would be interesting to utilize a GLP-1 receptor knock-out model to see if cocoa can stimulate an incretin response via GIP. Further, a double incretin receptor knock-out (DIRKO) model could be used to assess if an incretin response is an important mechanism utilized by cocoa to reduce glucose excursion in an acute fashion. Stimulating an incretin response is beneficial for patients with metabolic disorders because it assists in glucose disposal, slows gastric emptying and reduces appetite.

4.2.3. DPP-4

Dipeptidyl peptidase IV (DPP-4) cleaves the penultimate proline or alanine residue in proteins [120,121]. It is a transmembrane glycoprotein [122] found in nearly all human tissues and fluids [120]. Two DPP-4 targets are GLP-1 and GIP [109,118,121]. These hormones are cleaved, and therefore inactivated, by DPP-4 almost immediately after they are secreted from their respective endocrine cells; consequently, the incretin hormones have short half-lives. DPP-4 levels in patients with Type 2 diabetes, impaired glucose tolerance and/or obesity are not different than normal controls [123,124]. DPP-4 inhibitors have been considered potential treatments for T2DM because extending the active lifespan of these hormones could prolong the beneficial effects that incretin hormones have on glucose control [120]. Indeed, DPP-4 inhibition has been shown to improve glycemic outcomes in diabetic models and delay the onset of diabetes in Zucker diabetic fatty rats [125]. DPP-4 inhibitor drugs (commonly named gliptins) mimic many of the same actions as GLP-1 receptor agonists (stimulating insulin secretion, inhibiting glucagon secretion, *etc.*) but they do not exhibit the same improvements in weight loss [110]. This is likely because the resulting increase in incretin hormones is much less compared to activating the GLP-1 receptor directly [110]. Gliptins are currently employed as a second-line therapy for T2DM poorly controlled by metformin alone [126–128].

It appears that inhibition of DPP-4 may be another primary molecular mechanism of action of cocoa flavanols. Gonzalez-Albuin *et al.* [120] examined the effects of grape seed procyandin extract on DPP-4 using several methods. First, they determined that the extract is able to achieve 70% inhibition of commercial DPP-4 at the highest dose reported, 200 mg/L. Next, using cultured Caco-2 cell epithelial monolayers, they found that 100 mg/L of grape seed extract incubated for 3 days resulted in 20% inhibition of DPP-4 (shorter incubation periods did not show significant changes in inhibition). This was associated with a significant reduction in DPP-4 gene expression, as well. The same group examined the effects of grape seed extract on DPP-4 in *in vivo* models [116,117]. They found that an acute grape seed extract (1 g/kg bw) inhibits intestinal DPP-4 activity [117].

Ultimately, it appears that while plasma DPP-4 inhibition is possible, it is likely not the main mechanism that would result in improved glucose homeostasis [120]; gut DPP-4 inhibition is a more plausible mechanism. DPP-4 inhibition has not been studied using cocoa extract or cocoa powder and remains an area in need of further investigation.

4.3. Metabolic endotoxemia and inflammation

Endotoxin, or lipopolysaccharide (LPS), is derived from the outer membrane of Gram-negative (−) bacteria. If the bacteria lyse, LPS can separate from the membrane and, if gut barrier function is poor, the LPS can enter the circulation via paracellular diffusion and activate proinflammatory pathways through molecular pattern recognition receptors in systemic circulation and in tissues. Several factors appear to modulate the concentration of LPS in circulation, including the gut microbial environment, high-fat diet and intestinal permeability [129]. Chronic, low-grade, inflammation may contribute to the pathogenesis of obesity and metabolic syndrome. Circulating endotoxin binds to toll-like receptor 4 (TLR4), a molecular pattern recognition receptor, and initiates an inflammatory response [129]. This chronic, endotoxin-derived inflammation can disrupt energy homeostasis and insulin signaling, leading to elevated blood glucose levels (Fig. 4). If the bacteria lyse, LPS can separate from the membrane, and if gut barrier function is poor, the LPS can enter the circulation via paracellular diffusion.

Recent evidence has suggested that cocoa flavanols can aid in the attenuation of this metabolic endotoxemia [17,63]; however, the underlying mechanisms are less explored. These changes are primarily attributed to the chronic consumption of cocoa. Possible intermediate mechanisms responsible for this effect of cocoa are modulation of the gut microbiome composition and function, improvements of the gut barrier function and improved insulin signaling.

4.3.1. Gut microbiota

Recently, the gut microbiome has become a very popular field of research. While once considered a “black box,” the commensal microbial communities of the human gastrointestinal tract are now known to be diverse and complex and to have significant impacts on human health. It is believed that one's diet plays a large role in the development and maintenance of the microbial community [129,130]. Further, links have been drawn between the composition of one's microbiome and their likelihood to present with obesity or metabolic disease [130]. Certain species are associated with harvesting nutrients and producing short-chain fatty acids, improving the mucosa in the colon and improving gut barrier function, among many other outcomes [131,132]. It is possible that cocoa may modulate levels and activities of certain species in the gut microbiome, although the primary mechanisms of action by which this is achieved remain poorly understood. Mechanistic studies are needed to understand the molecular interactions between flavanols and commensal bacteria, both on an individual cell and community level.

A large proportion of cocoa flavanols proceed to the colon where they interact with the gut microbiota. As discussed previously, the gut microbiota metabolize polyphenols. However, polyphenols also modulate the gut microbiome and exert prebiotic effects. A *prebiotic* is defined as a “non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host

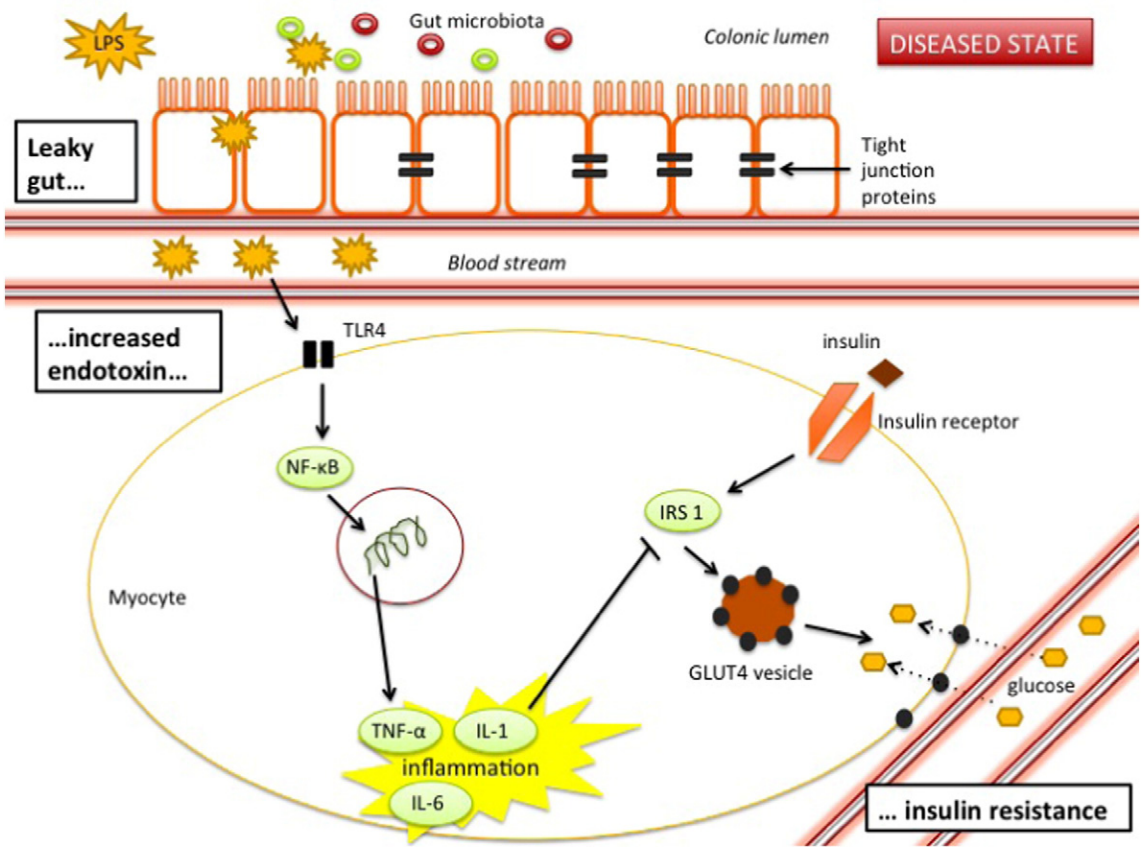

Fig. 4. Suggested mechanism by which increased gut permeability and endotoxin levels lead to insulin resistance.

health" [133]. While prebiotics are commonly thought to be indigestible carbohydrates that are fermented by gut microbiota, flavanols can also fulfill this definition. Cocoa flavanols have shown prebiotic activity *in vitro* [47], in rodents [76] and in humans [134].

Tzounis *et al.* [47] found that incubation of (+)-catechin with fecal samples from healthy volunteers significantly increased the growth of *Clostridium coccoides-Eubacterium rectale* group, *Bifidobacterium spp.* and *Escherichia coli*, as well as a significant inhibitory effect on the growth of the *Clostridium histolyticum* group. A rodent study showed decreases in *Bacteroides*, *Staphylococcus* and *Clostridium* genera after a 6-week cocoa treatment (100-g cocoa/kg chow) compared to a reference group; this study utilized healthy animals and a normal chow diet [76]. It is important to note that the dietary fiber in cocoa could potentially elicit many of these observed benefits, and this study's control group did not have matched soluble fiber content. Cocoa flavanols were also found to modulate human gut microbiota. After a 4-week cocoa treatment (494-mg flavanols/day), healthy volunteers had an increase in *Bifidobacterium* and *Lactobacillus*, and a decrease in *Clostrum* compared to a low flavanol treatment (29 mg flavanols/day) group [134]. Both treatments had equal amounts of dietary fiber.

It is evident that cocoa can exert prebiotic effects in both animals and humans and improve gut barrier function. Both of these properties would be beneficial for patients with metabolic disorders. However, more research is necessary to understand if cocoa can exert prebiotic effects in an unhealthy or at-risk population and to what degree the naturally occurring fiber found in cocoa powder affects these results.

4.3.2. Tight junction proteins

The purpose of tight junction proteins is to ensure the integrity of epithelial tissues and act as a barrier to limit paracellular diffusion of water, ions and other molecules. Occludin, claudin and junction adhesion molecules (JAM) are important proteins found in tight junctions between epithelial cells [135]. The transmembrane proteins occludin and claudin attach to actin filaments within the cell via intracellular plaque proteins, such as zonula occludens (ZO) [136].

Gut barrier function is important for human health. A high-fat diet [129], alcohol [137] and exercise [138] can increase gut permeability. Certain diseases such as Crohn's disease, inflammatory bowel disease and Celiac's disease are associated with compromised integrity of the gut barrier [139]. In the perspective of metabolic syndrome and metabolic endotoxemia, a leaky gut allows endotoxin to enter the circulation via paracellular diffusion. Systemic endotoxin causes an inflammatory response that can disrupt insulin signaling and contribute to atherosclerosis and obesity [140]. Therefore, improving gut barrier function is an important target for preventing and/or resolving metabolic endotoxemia.

There has been evidence that the gut microbiome can affect gut barrier integrity [141]. The mucus layer in the GI tract is important for gut health [142], and certain bacterial species, such as *Akkermansia*, reside in this layer [143]. Increased mucus production by goblet cells is a prime environment for mucus-eating bacterium, such as *Akkermansia*, which has been shown to protect against metabolic syndrome [131]. Everard *et al.* [143] showed that *Akkermansia* is beneficial to gut barrier function and normalizes metabolic endotoxemia. This species also improved glucose tolerance and decreased hepatic glucose production in mice with diet-induced obesity [143]. Interestingly, *Akkermansia* is a Gram (−) bacterium.

It is unclear if cocoa flavanols can increase *Akkermansia* populations, but flavanols have been shown to improve gut barrier function [91,144]. While the mechanism by which this occurs remains

unknown, this protective effect against gut permeability and therefore inflammation could be the mechanism for health-promoting effects of cocoa flavanols. This is a very intriguing research area that should be further explored.

There has been limited evidence to suggest that flavanol consumption has been correlated with improvements in tight junction protein expression and gut permeability. Goodrich *et al.* [91] found that 0.1% GSE in drinking water (100 mg/kg/day) increased occludin expression in the proximal colon in healthy rats compared to the control group. Another group found that GSE in a standard chow diet (250 mg/kg/day) increased ZO-1 and occludin expression and decreased intestinal permeability in the small intestine in healthy rats [144].

The primary molecular mechanisms behind the increased expression of tight junction proteins are unclear, but it may be related to prebiotic-induced changes in gut microbiota. In addition, flavanols may interact directly with the epithelium to induce these changes. Future research is needed to determine if cocoa can protect against derangements in gut barrier function and inflammation caused by a high-fat diet and if these changes are associated with bacterial species such as *Akkermansia*.

4.3.3. Endotoxin-derived inflammation

LPS is the primary ligand for TLR4, which is found on the cell surface of immune cells, skeletal muscle and many other tissues [145]. LPS binding to TLR4 initiates an inflammatory cascade that leads to nuclear translocation of nuclear factor kappa B (NF-κB), resulting in production of inflammatory cytokines [145]. Poor gut barrier function will lead to elevated plasma endotoxin levels and metabolic disease [129]. Endotoxin-induced inflammation has been shown to disrupt energy homeostasis associated with metabolic syndrome [146]. Inflammation can hinder the normal processes of many tissues, including skeletal muscle, liver, adipose, brain, pancreas and the endothelium of arteries. Inflammation can also disrupt insulin and leptin signaling; both of these hormones are involved with perceptions of satiety and fuel handling [147].

Several studies have explored the effects of cocoa on inflammation and its contribution to diseases [73,75,86,148]. There are also studies investigating the effects of cocoa on metabolic endotoxemia [17,75]. A study conducted by Gu *et al.* [63] examined the effects of an 18-week cocoa treatment (8% w/w cocoa powder in a high-fat diet) in male mice on cytokine and endotoxin levels. It was found that the cocoa treatment was effective in reducing plasma LPS, TNFα and IL-6 compared to the control high-fat diet group. This study also showed that the cocoa treatment improved gut barrier function, resulting in 40.8% lower plasma endotoxin levels compared to the high-fat diet group. Dorenkott *et al.* [17] also saw reductions in serum endotoxin levels, along with improvements in glycemic outcomes, in a similar study using a lower dose (25-mg/kg bw of cocoa extract monomeric and oligomeric fractions) for 12 weeks. Both studies utilized a C57Bl/6 mouse model on a high-fat diet.

Overall, cocoa and other flavanols have the potential to improve gut barrier function, which may, in turn, alleviate metabolic endotoxemia. Further research is needed to confirm these results, and a clinical study is warranted. It is unknown if reduced endotoxemia is due solely to alterations to gut microbiota and barrier function, or if flavanols can directly bind and inactivate LPS in the gut or blood, or modulate LPS-TLR4 binding and downstream signaling at the levels of skeletal muscle cells.

4.4. β-cells

Deterioration of functional β-cell mass is observed during T2DM and metabolic syndrome disease progression. *Functional β-cell mass* is defined as the β-cell insulin secretion rate, and the total β-cell mass is a factor of cellular proliferation and cellular death [149]. Decreased functional β-cell mass critically impinges on the ability to maintain normoglycemia. There are various studies that suggest that cocoa polyphenols may protect β-cells against death-inducing damaging factors, enhance glucose stimulated insulin secretion and induce β-cell replication. The primary molecular interactions by which flavanols induce improved β-cell function, proliferation and survival remain unknown and, therefore, warrant investigation.

T2DM and other metabolic diseases are associated with chronic, low-grade inflammation and excess reactive oxygen species, which can damage β-cells, thereby further exacerbating metabolic instability. Individuals with metabolic disorders can also present with a decrease in antioxidant potential (*i.e.*, glutathione levels), so a dietary antioxidant may be beneficial for the health of these patients. Cocoa polyphenols have antioxidant properties and may help protect β-cells from oxidative damage. Further, Martín *et al.* [150] showed that cocoa flavanols protected against oxidative stress in INS-1 cells, a rat insulin-secreting cell line. Similarly, Youl *et al.* [151] demonstrated that quercetin (which has also been found in cocoa [152] although not a flavanol) is able to protect INS-1 cells from oxidative damage, supporting the findings that cocoa flavanols protect against oxidative stress. Most recently, in a rodent model using Zucker diabetic fatty rats, a 9-week treatment with cocoa-enriched diet (10% (w/w) cocoa powder) prevented β-cell apoptosis by reducing oxidative stress [71]. These data are supported by studies showing that quercetin prevents streptozotocin-induced oxidative stress and damage [153,154]. These data strongly demonstrate that in animal models of β-cell destruction, there is appreciable protection given to the β-cell mass from flavanol compounds, in particular from cocoa. However, it is unclear if the cocoa flavanols act as reducing agents in the gut (possibly on acrylamide), in the circulation or in the pancreas directly. Further studies are needed to identify the exact location that these antioxidant effects are taking place in an *in vivo* model and at doses more comparable to human intake.

Cocoa may exert protective effects on β-cells by inhibiting lipid accumulation in the cells. While peripheral insulin resistance is common during obesity and aging in mice and people, its progression to T2DM is largely due to insulin secretory dysfunction and significant apoptosis of functional β-cells. Accumulating evidence suggests that chronic hyperlipidemia (lipotoxicity) causes β-cell apoptosis and impairs its function, thereby contributing to the pathogenesis of T2DM [155]. In a study with cafeteria-fed rats, treatment with grape seed procyanidin extract for 30 days significantly reduced triglyceride levels in the pancreas, resulting in improved insulin secretion [156]. Further studies are needed to examine the impacts of cocoa flavanols with differing degrees of PCs on β-cell health and function.

Cocoa flavanols, in particular epicatechins, have been shown to enhance glucose-stimulated insulin secretion [88]. Early studies demonstrated that epicatechins are sufficient to increase insulin secretion from rat islets [157]. More recent studies using *ob*/*ob* rats fed cocoa extracts demonstrated that in addition to decreasing oxidative stress, the treatment enhanced insulin secretion [158]. Using quercetin treatment of INS-1 cells, it was shown that the flavonol-mediated potentiation of insulin secretion was dependent on MEK-regulated phosphorylation of Erk1/2 [151]. These data clearly demonstrated that the flavonols, and similar compounds such as flavanols, were able to protect against oxidative stress and enhance insulin secretion. Preliminary studies suggest that similar effects are seen in human populations; consumption of high polyphenol dark chocolate for a 15-day period increased the 2-h corrected insulin response, which suggests improved β-cell function in these individuals [80]. Taken together, these data suggest that cocoa flavanols have the capacity to enhance glucose-stimulated insulin secretion and thereby enhance functional β-cell mass.

Finally, a number of studies have demonstrated that cocoa-derived compounds can induce β-cell proliferation. β-cell proliferation is a

highly regulated process, and in the majority of individuals, less than 1% of β-cells can be measured replicating after adolescence [159]. Therefore, compounds and factors that enhance proliferation would have a direct therapeutic effect with patients suffering from T2DM or metabolic syndrome. Early studies demonstrated enhanced DNA replication and regeneration of β-cells when rats were treated with epicatechins [160–162]. Studies using quercetin (a flavonol, also found in cocoa) in the STZ-treated rat diabetes model demonstrated maintenance of β-cell mass, which could indicate decreased cell death or enhanced proliferation [153,154]. These data were validated in the NOD (nonobese diabetic) T1DM model where epicatechin treatment increased β-cell mass [163]. Finally, rats fed a cocoa-rich diet had increased small islet size and maintenance of total islet mass, suggesting induction of β-cell proliferation [71]. Taken together, these data demonstrate that cocoa flavanols have beneficial effects on β-cells: enhanced survival, insulin secretion and proliferation. The molecular mechanisms by which these effects are induced are yet to be elucidated.

4.5. Insulin signaling

It is increasingly recognized that chronic inflammation is associated with defective insulin signaling and insulin resistance. It has been shown that proinflammatory molecules inhibit the insulin-signaling pathway. For example, tumor necrosis factor-alpha (TNF-α) can induce the phosphorylation of the serine residues on IRS-1, which subsequently inhibits tyrosine auto-phosphorylation of the insulin receptor [164], thereby impairing glucose disposal. Chronic hyperglycemia is toxic to pancreatic β-cells, causing impairments in insulin secretion and cell apoptosis, therefore further exacerbating elevated glucose levels.

Cordero-Herrera *et al.* [165,166] studied the effects of epicatechin and cocoa extract at physiologically relevant doses on insulin-signaling mechanisms in HepG2 cells. Both treatments successfully enhanced the activities of IR, IRS-1, IRS-2, PI3K/AKT pathway and AMPK. However, it is unclear if the Phase-II and/or colonic metabolites would produce the same effects as the native polyphenols. Future studies may want to examine these outcomes with conjugated metabolites of epicatechin and other flavanols, since the metabolites would be most prevalent in circulation compared to native flavanols.

Yamashita *et al.* [62] showed that cocoa liquor extract provoked the translocation of GLUT4 to the plasma membrane in absence of insulin in L6 myotubes. This is an interesting finding for several reasons. Individuals with T2DM have a blunted GLUT4 translocation in response to insulin, despite the fact that they typically have normal amounts of GLUT4 expression in skeletal muscle [19,23,167]. If cocoa can promote the translocation of GLUT4, glucose disposal will be enhanced and blood glucose levels will normalize. Since this is an insulin-independent mechanism, this is especially useful for diabetic patients who may have deficits in insulin production. Future studies are warranted to see if these outcomes are reproducible *in vivo*.

Cocoa polyphenol extract was shown to inhibit insulin receptor kinase by direct binding, resulting in reduced lipid accumulation and differentiation in preadipocytes *in vitro* [168]. This is thought to be one mechanism by which cocoa flavanols may inhibit the onset of obesity.

In conclusion, cocoa may modulate insulin signaling in several ways. First, a heightened incretin response, discussed in Section 4.2.2, will promote insulin secretion. Second, if cocoa can improve gut barrier function, it will lend to a reduction in LPS and chronic inflammation, resulting in improved insulin signaling. Third, cocoa flavanols reduce insulin resistance by both insulin-dependent and insulin-independent mechanisms (including activation of the insulin-signaling cascade in the absence of insulin). Glucose intolerance and insulin resistance are characteristic of metabolic syndrome. Dietary components aiding in either insulin secretion or insulin action would prove beneficial for patients with metabolic syndrome. However, the cellular mechanisms by which cocoa flavanols achieve these effects in glucose-disposing tissues remain unknown. Further research with pathway inhibitors, overexpression and gene-silencing experiments is needed to move beyond identification of up-regulated/stimulated pathways and pinpoint the mechanistic targets that produce those effects (such as AMPK signaling, CAMK signaling, PI3K/Akt signaling, *etc.*). This, in turn, will enable therapeutic targeting of those primary mechanisms. In summary, potential mechanisms by which cocoa flavanols may improve glucose homeostasis are shown in Fig. 5.

4.6. Other potential mechanisms

The mechanisms addressed in this review are only a portion of the proposed mechanisms that are reported in the literature. Other mechanisms by which cocoa may affect health outcomes are important to acknowledge in order to fully understand the potential effects that cocoa flavanols may have on glucose homeostasis.

This includes an antioxidant potential of cocoa flavanols that can be very beneficial to cardiovascular health and has been extensively studied and reviewed elsewhere [169]. Cocoa can impact nitric oxide production, endothelial function and, ultimately, atherosclerosis. Cardiovascular health is an important facet of metabolic syndrome and must not be overlooked when developing drugs or designing studies to alleviate or assess this metabolic disorder.

Oxidative stress is present in obesity and metabolic syndrome. Reactive oxygen species can accumulate in metabolically active tissues and cause lipid peroxidation, damage β-cells, modulate the gut microbiota and hinder cardiovascular function, insulin signaling and mitochondrial function. Flavanols may protect against the effects of oxidative stress [28].

Gu *et al.* [75] suggests that inflammation can be reduced by cocoa flavanols via reducing lipid absorption. Along with the digestive enzymes already discussed, flavanols also inhibit digestive lipases, which results in increased lipid content in fecal matter. Further, this will reduce macrophage infiltration into adipocytes, lowering inflammatory tone [75].

Dyslipidemia is an important facet of metabolic syndrome. Many studies have examined the effects of chronic cocoa treatments on LDL cholesterol, HDL cholesterol and triglycerides [78,79,84,156,170–173]. Cocoa may be able to beneficially modulate cholesterol and triglyceride levels in metabolically unhealthy individuals [174]. Cocoa flavanols may improve blood glucose control indirectly, by modulating lipid digestion and thus reducing hyperlipidemia and its subsequent deleterious effects on glucose homeostasis. PCs are potent lipase inhibitors *in vitro* [36,175]; they also reduce acute postprandial [175] and fasting plasma triglycerides [63] and increase fecal lipid excretion [75] in animals and humans. It has been well established that cocoa and PCs reduce blood triglycerides and lipid accumulation in viscera, liver and β-cells in animal models [18,75,158,176,177]. Prevention of lipid accumulation by cocoa PCs may indirectly improve glucose homeostasis by preserving metabolic flexibility and insulin sensitivity in skeletal muscle [178,179], insulin sensitivity in liver [180,181] and β-cell viability and function [71,150,177,182]. Clinical studies have shown that inhibition of lipid absorption and associated hyperlipidemia and fat accumulation can improve blood glucose control and insulin sensitivity in humans [183–185]. Cocoa flavanols have not been evaluated for inhibition of lipid digestion and absorption in humans.

Finally, cocoa flavanols have been associated with an increase in lipolysis, fatty acid oxidation and energy expenditure in animal models [65,69,70,186–189]. Other suggested mechanisms involve the endocannabinoid system [146], mitochondrial function [190], anti-carcinogenic properties [155] and modulating immune function [24].

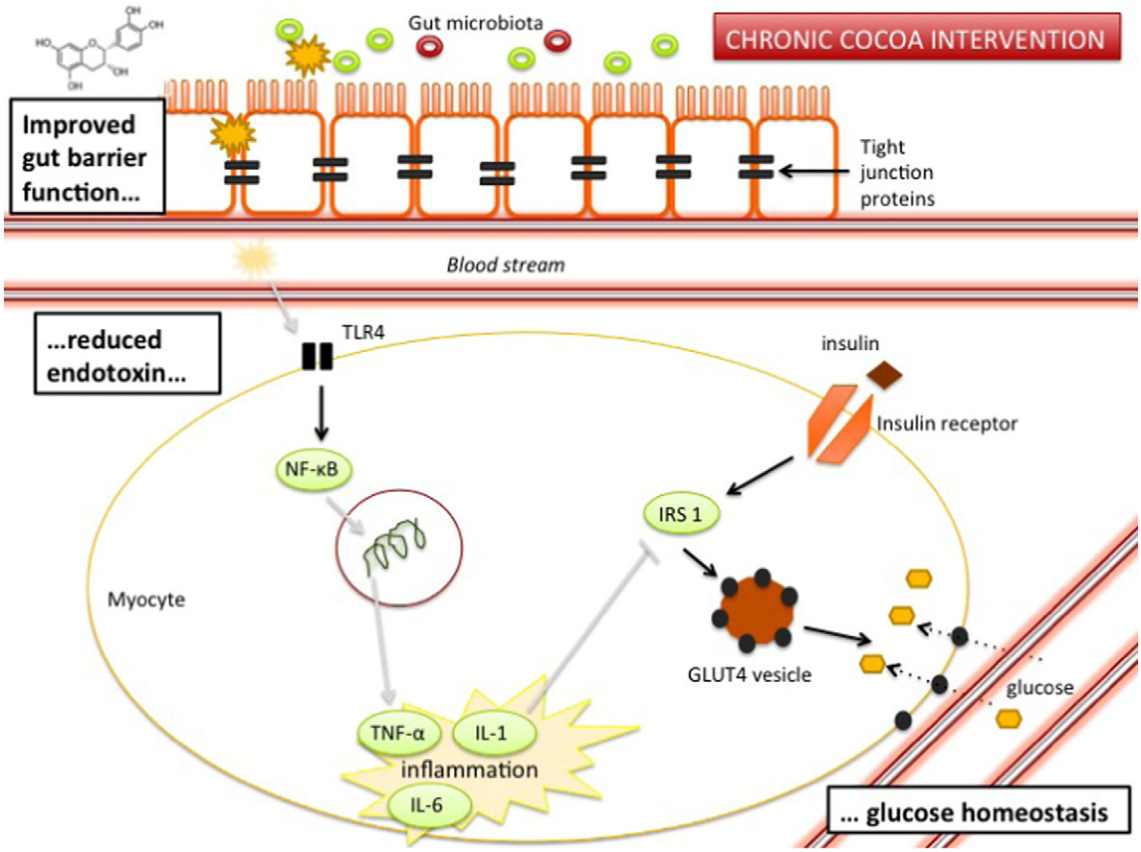

Fig. 5. One mechanism by which a chronic cocoa supplement may improve glucose homeostasis. Cocoa may improve gut barrier function, leading to a reduction in serum endotoxin, minimizing inflammation, allowing for normalized glucose control.

In summary, there are many possible primary and intermediate mechanisms that are outside the scope of this review, but they are still important to consider when evaluating the effects of cocoa on metabolic syndrome. It is likely that cocoa and cocoa flavanols exert pleiotropic effects on metabolism, which likely act synergistically to prevent or slow prediabetes and T2DM. However, it remains unknown which mechanisms and pathways are affected directly by cocoa and which are modulated indirectly as downstream effects of improvements in the primary targets. In some cases, definitive identification of the primary molecular mechanism of action may be unnecessary. However, when moving forward to expensive, time-consuming clinical trials, knowledge of the most upstream targets will facilitate improved study design, identification of appropriate biomarkers to evaluate efficacy and, perhaps most importantly, define the biological contexts in which cocoa flavanols are likely to be effective.

5. Implications of potential mechanisms

As detailed above, cocoa flavanols appear to possess important antidiabetic activities. In some cases, these activities are similar to current pharmaceuticals for control of diabetes and obesity [191], such as acarbose [192,193], gliptins [126,194,195] and orlistat [183–185]. Increased intake of cocoa flavanols may represent a viable dietary strategy to obtain the glucose-lowering benefits of these pharmaceuticals without the deleterious side effects (oily stool, diarrhea, gas, bloating, *etc.*). However, the clinical utility of cocoa in preventing and ameliorating prediabetes and/or T2DM by exploiting these mechanisms remains largely unknown.

5.1. Importance of understanding mechanism

Strategies that maximize the efficacy of flavanol interventions are desirable. However, as discussed above, the primary mechanism(s) by which flavanols act *in vivo* remain poorly understood. This mechanistic uncertainty limits our ability to focus on modulating specific mechanistic targets. Furthermore, the impact of flavanols on various substates of diabetes (prediabetes, early T2DM with hyperinsulinemia, late T2DM with β-cell exhaustion/failure, *etc.*) remains poorly understood. This precludes targeting of specific substates (such as impaired fasting glucose *versus* impaired glucose tolerance, which typically present exclusively of each other in prediabetes [196–198] and primarily represent derangements of gluconeogenesis *versus* insulin sensitivity, respectively) as opposed to a "shotgun" approach that does not require mechanistic knowledge and does not finely target specific physiological conditions.

Similarly, understanding the location of activity is key for targeting. If the primary mechanism is located in the gut, strategies to maximize gut levels and activity should enhance efficacy. Conversely, if the primary molecular mechanism is located in peripheral tissues, strategies to enhance flavanol bioavailability would be most likely to improve efficacy [199–201]. Furthermore, an understanding of whether native flavanols, or their microbial metabolites, are primarily responsible for the observed benefits would be useful to design strategies to increase native flavanol bioavailability or increase microbial metabolism of flavanols.

Lack of definitive mechanistic data limits current flavanol intervention strategies to "shot in the dark" approaches within a specific target dose, which may result in suboptimal efficacy and attempted

use in populations that may not benefit from cocoa interventions. Therefore, clarification of the mechanisms involved is essential to improve clinical utility of cocoa flavanols.

5.2. Implications of bioavailability on mechanism

Some of the proposed mechanisms suggest that cocoa flavanols may improve glucose control at least in part by acting locally in the gut lumen. This is critical due to the fact that flavanols, particularly the PCs (*i.e.*, the larger flavanols), have poor systemic bioavailability [29–31,202–205]. Reported oral bioavailability of flavanols is generally <10% for monomeric catechins [206,207] (when Phase-II metabolites are accounted for, bioavailability of monomers from catechins has been reported as high as 55%), much lower for small PCs (dimers-, trimers) and essentially 0% for larger PCs [29,39,207,208].

Poor bioavailability likely limits flavanol activities in peripheral tissues compared to the gut. Following cocoa consumption, concentrations of major flavanols (epicatechin, procyanidin B_2, *etc.*) in circulation are typically 0.010–6.0 μM [37,43–45]. By comparison, consuming a 5-g serving of cocoa powder (~6-mg catechin, 25-mg epicatechin and 235-mg PCs) would result in gut concentrations of ~10-μM catechin, 43-μM epicatechin and 67-μM PCs (assuming intermediate size PCs and gastrointestinal fluid volume of 2 L) [209]. Therefore, cocoa flavanols are typically much more concentrated in the gut compared to peripheral tissues. The hypothesis of gut activity is strengthened by an intriguing study demonstrating that orally administered flavanols improved glucose tolerance in animals when glucose was administered orally, but not when glucose was administered by intraperitoneal injection [120]. Despite this evidence, the gut-located activities (inhibition of digestive enzymes, improved barrier to endotoxin, stimulation of GLP-1 secretion, *etc.*) of cocoa flavanols have not yet been rigorously tested nor targeted *in vivo* for inhibition or improvement of metabolic syndrome. Mechanistic animal and human clinical experiments are needed in order to demonstrate the ability of cocoa flavanols to act specifically by gut-mediated mechanisms. Demonstration that cocoa flavanols act through gut mechanisms is needed so that delivery and dosing strategies may be designed to specifically target these mechanism(s) and optimize intervention efficacy, as well as identify behaviors and nutrition profiles that optimize the efficacy of these digestive effects.

5.3. Implications of mechanism on dose distribution

Acute human studies demonstrate that consuming flavanols with a meal can lower postprandial hyperglycemia [210–214]. Thus, co-consuming flavanols with meals may be a viable strategy for improving both acute and long-term blood glucose control, as well as reducing dyslipidemia. However, several of the proposed activities of cocoa flavanols (inhibiting carbohydrate/lipid digestion and improving the "incretin effect") require the presence of flavanols in the lumen of the gut concurrent with macronutrients during digestion, similar to acarbose or orlistat. If co-consumption of flavanols with meals significantly improves acute glucose control and blood lipid profiles, it follows that chronic flavanol co-consumption with meals should maximize their activities compared to consumption at other times. Conversely, if acute effects require co-consumption with meals, consuming flavanols between meals may reduce their potential benefits; cocoa flavanols cannot inhibit macronutrient digestion if the two are not present at the required concentrations in the gut lumen simultaneously. However, it remains largely unknown whether consuming flavanols with meals (*vs.* other patterns) maximizes their efficacy or if dose distribution does not affect efficacy.

Most animal studies [68,69,158,215], including those in our lab [17,91], administer flavanols incorporated into the diet (thus, flavanols and macronutrients are always co-consumed). Human interventions are not necessarily designed to recapitulate animal dosing patterns; rather, emphasis is simply placed on translating the effective dose from animals to humans. This may account for partial loss of efficacy during translational research. In at least four out of the reported effective chronic flavanol clinical interventions, dosing was synchronized with meals or distributed widely throughout the day [78–82,216,217]. Conversely, only one of the reported ineffective interventions was synchronized with a meal [84,86,218,219]. The preliminary evidence therefore suggests that dosing strategies may matter in terms of flavanol efficacy. Consuming flavanols with meals, or evenly throughout the day, appears to maximize efficacy. Variations in design make it impossible to definitively assess the impact of dosing strategy from published studies [78–82,84,86,216–219]. However, to our knowledge, the impact of different flavanol dosing strategies on biomarkers of metabolic syndrome has not been rigorously tested. Studies are needed which examine the impact of dose distribution on efficacy.

5.4. Relationship between mechanism and effective dose

Animal and clinical studies alike have used drastically different doses of cocoa treatments, including doses that are likely not translatable to humans [17,71,85,86,190,220]. Different mechanisms likely have distinct effective doses; since the mechanisms behind the beneficial health outcomes associated with cocoa have yet to be determined, it may be difficult to pinpoint an ideal dose before the mechanisms are defined. However, the "more is better" concept often used for phytochemical is inherently flawed, as many phytochemicals exhibit U-shaped dose response curves where lower doses are more effective, likely due to lower levels of detoxification pathway expression and different binding efficiencies for receptors and enzyme active sites and others [221,222] (this is known as "hormesis") [223,224]. Higher doses can result in reduced efficacy compared to lower doses, no effect or even toxicity. The use of high doses can therefore mask potential efficacy of mechanisms that may be relevant to humans at translatable doses. Furthermore, nontranslatable doses may modulate mechanisms that are not impacted at lower doses, thus suggesting potential mechanisms of action that are unlikely to be modulated once translated to human dosing. Therefore, future studies should ideally be designed to examine the effects of lower, translatable doses of cocoa flavanols.

5.5. Relationship between flavanol structure and mechanism

Cocoa flavanols exist in a broad range of polymerization states. Different flavanols likely act through distinct mechanisms due to differences in structure as well as bioavailability. Animal studies have generally focused on whole cocoa or chocolate [71,74–76,215,225–227], extracts [62,66–69,228,229] and flavanol monomers (catechins) [65,190,214,230]. Little data exist on the bioactivities of larger flavanols (PCs), partly due to difficulty of isolation, complexity of analytical characterization and lack of commercially available standards. However, recent data have suggested that the PCs may possess distinct (and in some cases, enhanced) activities for improvement of glucose homeostasis compared to flavanol monomers. Gu *et al.* [102] demonstrated that flavanol DP was inversely correlated to the IC_{50} of digestive enzyme inhibition (larger cocoa flavanols were more effective inhibitors). Yamashita *et al.* [231] showed that a fraction composed of smaller cocoa flavanols (DP≤3) more effectively stimulated glucose uptake, GLUT4 translocation and AMPK phosphorylation in skeletal muscle cells than a fraction composed of larger PCs (DP≥4). However, in the same study, the larger flavanols were more effective α-glucosidase inhibitors than the smaller flavanols. Yamashita *et al.* [64] further demonstrated that cinnamtannin A2 (a DP 4 cocoa flavanol) increased circulating GLP-1, insulin levels and

activation of the insulin-signaling pathway ins skeletal muscle in mice, whereas cocoa flavanols with DP 1-3 had little to no effect. Subsequently, we demonstrated that oligomeric cocoa flavanols more effectively inhibited the onset of diet-induced obesity and glucose intolerance compared to a crude cocoa polyphenol extract, monomeric cocoa flavanols and polymeric cocoa flavanols [17]. This finding was intriguing, as the bioavailability of oligomeric flavanols is lower than that of monomeric flavanols (see above). Thus, these enhanced activities may be due to mechanisms that do not require bioavailability.

These data suggest that cocoa flavanols of different DP may possess distinct activities. As cocoa contains a wide distribution of flavanol DPs, this emphasizes that the observed bioactivities are likely due to a variety of compounds acting through various mechanisms synergistically. Understanding the relationship between flavanol DP and bioactivities will facilitate an understanding of how cocoa composition impacts potential health benefits. Despite the cost and complexity associated with preparing or obtaining these larger flavanols, the influence of DP on flavanol bioactivity warrants further investigation. This is another emerging area with the potential to yield highly valuable, novel data to clarify the role of cocoa flavanols in metabolic syndrome. Efforts to isolate, purify, characterize and make these compounds available to other diabetes researchers will be central to this effort.

6. Conclusions

In conclusion, cocoa flavanols appear to alleviate metabolic syndrome, and specifically, derangements in glucose homeostasis, by several intermediate mechanisms. First, cocoa may reduce glucose excursion after a meal by inhibiting digestive enzymes, inhibiting glucose transporters and promoting an incretin response. These outcomes are most likely to be observed after an acute dose of cocoa, and since these mechanisms predominantly occur in the gut, the poor bioavailability of flavanols is not a limiting factor for these activities.

Second, chronic cocoa consumption may lead to beneficial changes in the gut microbiota, resulting in improved gut barrier function, reduced circulating endotoxin and uninhibited insulin signaling mechanisms. PCs are stable through gastric and intestinal transit so they will reach the colon intact. Again, bioavailability is not a limiting factor.

Third, cocoa flavanols can act in peripheral tissues (improved β-cell function and insulin sensitivity in skeletal muscle, *etc.*). These effects are limited by the poor bioavailability of many cocoa flavanols. Demonstration of the activities of flavanol microbial metabolites may be the missing link between oral flavanol consumption and activity in peripheral tissues.

It is likely that the potential benefits of cocoa consumption are mediated by all of these mechanisms to some extent. However, it remains unknown which, if any, of these mechanisms are primarily responsible for observed effects *in vivo*. Furthermore, the primary molecular mechanisms by which these intermediate mechanisms occur are generally unknown. Therefore, additional *in vivo* mechanistic studies are needed in order to isolate and assess individual primary and intermediate mechanisms of action.

There are many elements of this puzzle that are still unknown. First, it is unknown what acute effects cocoa may have on carbohydrate digestion in a population with existing prediabetes or T2DM. So far, to our knowledge, the only acute studies (in both animal and clinical models) have examined healthy subjects or animals. Individuals with metabolic disorders will benefit greatly from a supplement to control glucose excursions, but it is unclear to what extent cocoa can be helpful in this population. Second, little is known regarding the impact of cocoa on human subjects with differing substates along the continuum of diabetes. In addition, studies examining the impacts of cocoa and its mechanisms of action when administered in conjunction with common diabetes medications in subjects with T2DM (which is likely to occur in real-world clinical settings) are needed. Third, cocoa is metabolized in the colon by the microbiota into many metabolites and it is unknown what functions, if any, that these metabolites have on human health. Third, it is hypothesized that *Akkermansia* has beneficial effects on gut barrier function, but it is still unknown if cocoa can modulate this species, but this may be a worthwhile study to pursue. Lastly, it is unknown what doses of cocoa (for either acute or chronic outcomes) elicit the most beneficial outcomes related to metabolic syndrome.

Therefore, highly mechanistic clinical and animal studies are needed, in addition to the largely descriptive studies done thus far. Based on the proposed mechanisms, acute and chronic cocoa studies should be designed to assess mechanism. Acute studies should focus on the impact of cocoa consumption on starch, disaccharide and triglyceride digestion (to assess the impact of cocoa on α-amylase, α-glucosidase and lipase activity, respectively) following mixed meals as well as individual macronutrient doses and postprandial hormone secretion (GLP-1, GIP, insulin, *etc.*) following mixed meals as well as simple sugar and complex carbohydrate doses.

Chronic studies should focus on gut permeability, fasting and postprandial circulating endotoxin levels, fasting and postprandial circulating hormone levels (GLP-1, GIP, insulin, *etc.*), skeletal muscle metabolism, effects of gut microbiota/metabolites and dose synchronization with meals. Such studies will greatly improve the depth of our understanding of the impacts of cocoa consumption on human physiology. In order to probe the impact of cocoa flavanols on incretin pathways (secretion, action and degradation), various techniques can be employed, including incretin or incretin receptor knockout models, DPP-4 knockout or overexpression models, incretin receptor antagonists and others. To explore the impact of dose synchronization with meals, various patterns of dosing can be employed in both animals and human subjects (single daily flavanol dose with a meal, dose single daily dose in the fasted state, multiple daily doses with meals, multiple daily doses in between meals, *etc.*). Finally, to determine the impact of gut microbiota (and flavanol metabolites produced by gut microbiota) in mediating the effects of flavanol consumption, studies can be performed in germ-free, gnotobiotic or antibiotic-treated animals and compared with results of normal, fully-colonized animals. This will facilitate identification of effects dependent upon the presence of gut microbiota.

In addition, use of advanced physiology assays in chronic human studies is needed to delineate the precise metabolic effects of chronic cocoa exposure in study subjects. Specifically, the insulin-augmented intravenous glucose tolerance test (IVGTT) could be performed to simultaneously assess glucose effectiveness (ability of the body to stimulate glucose uptake and suppress endogenous glucose production due to the presence of glucose), insulin response to glucose and insulin sensitivity. Alternatively, the hyperglycemic glucose clamp or hyperinsulinemic–euglycemic clamp techniques could be employed. Such studies are needed in order to go beyond fasting glucose/insulin levels, postprandial oral glucose tolerance and the homeostatic model assessment (HOMA) protocols commonly used [232]. In addition, skeletal muscle biopsies followed by metabolism and energetics assays could reveal much information regarding the impact of cocoa on substrate metabolism, metabolic flexibility and muscle function (and improvement on deranged metabolic states observed during metabolic syndrome) [233–240]. While these assays are more complex, burdensome to subjects and expensive, they are needed to advance our knowledge of the mechanisms by which cocoa exerts it effects. Perhaps most importantly, additional long-term (1 month or longer) intervention trials are needed in individuals with prediabetes or diabetes in order to determine the clinical utility of cocoa flavanols for successful prevention or amelioration of these diseases.

In future studies, it is critical that all trials publish a full characterization of the cocoa utilized in the study, due to the impact of flavanols structure on potential mechanisms of action. Clinical studies should report the food matrix used in the treatment, and animal studies should report food intake. Finally, utilizing acute and chronic study designs will be important to characterize the mechanisms of action of cocoa flavanols.

Insights into the mechanisms by which cocoa flavanols act and the substates of diabetes modulated by cocoa flavanols will refine the ability of clinicians to effectively use cocoa, in combination with diet, exercise and medications, to effectively combat prediabetes and T2DM.

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