The Effect of Cocoa Flavanols on β-Cell Mass and Function

Thomas John Rowley
Brigham Young University

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The Effect of Cocoa Flavanols on β-Cell Mass and Function

Thomas John Rowley

A thesis submitted to the faculty of
Brigham Young University
in partial fulfillment of the requirements for the degree of
Master of Science

Jeffery S. Tessem, Chair
Michael L. Dunn
Jason D. Kenealey

Department of Nutrition, Dietetics, and Food Science
Brigham Young University

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ABSTRACT

The Effect of Cocoa Flavanols on β-Cell Mass and Function

Thomas John Rowley
Department of Nutrition, Dietetics, and Food Science, BYU
Master of Science

A hallmark of type 2 diabetes (T2D) is β-cell dysfunction and the eventual loss of functional β-cell mass. Therefore, mechanisms that improve or preserve β-cell function could be used to improve the quality of life of individuals with T2D. Studies have shown that monomeric, oligomeric and polymeric cocoa flavanols have different effects on obesity, insulin resistance and glucose tolerance. We hypothesized that these cocoa flavanols may have beneficial effects on β-cell function. INS-1 832/13 derived β-cells and primary rat islets cultured with a monomeric catechin-rich cocoa flavanol fraction demonstrated enhanced glucose-stimulated insulin secretion, while cells cultured with total cocoa extract, oligomeric, or polymeric procyanidin-rich fractions demonstrated no improvement. The increased glucose-stimulated insulin secretion in the presence of the monomeric catechin-rich fraction corresponded with enhanced mitochondrial respiration, suggesting improvements in β-cell fuel utilization. Mitochondrial complex III, IV and V components were upregulated after culture with the monomer-rich fraction, corresponding with increased cellular ATP production. The monomer-rich fraction improved cellular redox state and increased glutathione concentration, which corresponds with Nrf2 nuclear localization and expression of Nrf2 target genes, including NRF-1 and GABPA, essential genes for increasing mitochondrial function. We propose a model by which monomeric cocoa catechins improve the cellular redox state, resulting in Nrf2 nuclear migration and upregulation of genes critical for mitochondrial respiration, and, ultimately, enhanced glucose-stimulated insulin secretion and β-cell function. These results suggest a mechanism by which monomeric cocoa catechins exert their effects as an effective complementary strategy to benefit T2D patients.

Keywords: cocoa; β-cell; catechin; insulin secretion; mitochondrial respiration; Nrf2
ACKNOWLEDGMENTS

I am indebted to so many people for their help with this program. First and foremost are my parents, whose example always encouraged me to pursue my educational dreams and whose love and support never ends. I would like to thank the NDFS faculty, who have always been available for informative and encouraging discussions every step of the way. I would particularly like to thank my graduate committee, Dr. Jeff Tessem, Dr. Mike Dunn, and Dr. Jason Kenealey, for their help and encouragement throughout my time in this program. My advisor, Dr. Tessem, has been a fantastic example and mentor in so many ways. The time and effort he has invested in me has helped me to grow as a student and as a man. I am grateful as well to all of my fellow lab members who have made research so enjoyable and who have become my true friends. I would be remiss if I did not thank my graduate secretary, Melanie Peine, whose dogged determination in helping her graduate students succeed and fantastic sense of humor have helped me get to this point. Brigham Young University has been a wonderful place to study and I feel extremely blessed to have had such an opportunity.
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MANUSCRIPT ONE: Mechanisms by which cocoa flavanols improve metabolic syndrome and related disorders $^f$

Karen M. Strat$^a$, Thomas J. Rowley IV$^b$, Andrew T. Smithon$^c$, Jeffery S. Tessem$^b$, Matthew W. Hulver$^{a,d}$, Dongmin Liu$^a$, Brenda M. Davy$^a$, Kevin P. Davy$^a$, Andrew P. Neilson$^c$

$^a$ Department of Human Nutrition, Foods and Exercise, Virginia Tech, Blacksburg, VA
$^b$ Department of Nutrition, Dietetics and Food Science, Brigham Young University, Provo, UT
$^c$ Department of Food Science and Technology, Virginia Tech, Blacksburg, VA
$^d$ Metabolic Phenotyping Core Facility, Virginia Tech, Blacksburg, VA

Corresponding author at: Virginia Tech; Department of Food Science and Technology; Integrated Life Science Building, Rm 1013, 1981 Kraft Dr., Blacksburg, VA, 24060; Tel.: +1-540-231-8391; fax: +1-540-231-9293; E-mail address: andrewn@vt.edu (A.P. Neilson).

$^f$ Grants, sponsors and funding sources: K.M. Strat, M.W. Hulver, B.M. Davy, K.P. Davy and A.P. Neilson currently have research support from The Hershey Co., Hershey, PA. No employee of The Hershey Co. assisted in the conceptualization, preparation or editing of this review.
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 prothrombotic 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.
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. 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 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) 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]. 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 assess 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.
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. 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].
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<th>Treatment/Delivery</th>
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<td>Matsui, 2005 [225]</td>
<td>Male Wistar rats</td>
<td>12.5% (w/w) cocoa powder, in food</td>
<td>7.040h</td>
<td>79,913c</td>
<td>Chronic, 3 weeks, high-fat diet</td>
<td>↓ final body weights, ↓ fatty acid synthesis</td>
</tr>
<tr>
<td>Ruzaidi, 2005 [66]</td>
<td>Male diabetic Wistar rats, (STZ-induced)</td>
<td>1, 2, 3% (w/w) cocoa extract, in food</td>
<td>Diabetic rats: 1% = 868 2% = 1,776 3% = 2,580 Normal Rats: 1% = 433 2% = 860 3% = 1,200</td>
<td>Diabetic rats: 1% = 9,853 2% = 20,160 3% = 29,286 Normal rats: 1% = 4,919 2% = 9,762 3% = 13,622</td>
<td>Chronic, 4 weeks, normal diet</td>
<td>↓ glycemia, ↓ hypercholesteremia</td>
</tr>
<tr>
<td>Tomaru, 2007 [67]</td>
<td>Female, db/db mice (obese, diabetic)</td>
<td>0.5%, 1.0% (w/w) cacao liquor proanthocyanidin, in food</td>
<td>0.5% = 1,107g 1.0% = 2,044</td>
<td>0.5% = 5,771 1.0% = 11,602</td>
<td>Chronic, 3 weeks, normal diet</td>
<td>↓ blood glucose in a dose dependent manner</td>
</tr>
<tr>
<td>Jalil, 2008 [158]</td>
<td>Male ob/db Sprague–Dawley rats (STZ induced)</td>
<td>Cocoa extract, by oral gavage</td>
<td>600</td>
<td>6,811</td>
<td>Chronic, 4 weeks, high-fat diet</td>
<td>↑ oxidative stress (8-isopostane)</td>
</tr>
<tr>
<td>Jalil, 2009 [68]</td>
<td>Male ob/db Sprague–Dawley rats (STZ induced)</td>
<td>Cocoa extract, by oral gavage</td>
<td>600</td>
<td>6,811</td>
<td>Chronic, 4 weeks, high-fat diet.</td>
<td>↑ glucose tolerance (OGTT- AUC), ↓ total cholesterol, ↓ triglycerides. No changes in insulin sensitivity</td>
</tr>
<tr>
<td>Perez-Berezo, 2011 [242]</td>
<td>Female Wistar rats</td>
<td>2%, 5%, or 10% (w/w) cocoa powder, in food</td>
<td>Unknown (food intake data not reported)</td>
<td>Unknown (food intake data not reported)</td>
<td>Chronic, 3 weeks, normal diet</td>
<td>↓ immune response (IgG1, IgG2, S-IgA) (5 and 10% treatments)</td>
</tr>
<tr>
<td>Si, 2011 [73]</td>
<td>Male db/db mice</td>
<td>0.25% epicatechin, in drinking water</td>
<td>150</td>
<td>851</td>
<td>Chronic, 15 weeks, normal diet.</td>
<td>↓ inflammatory markers (CRP, IL1B), oxidative stress (GSH, SOD), ↑ lifespan.</td>
</tr>
<tr>
<td>Study</td>
<td>Gender/Strain</td>
<td>Cocoa Source/Conc.</td>
<td>Treatment/Duration</td>
<td>Diet</td>
<td>Observations</td>
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<tr>
<td>Massot-Cladera, 2012</td>
<td>Female Wistar rats</td>
<td>10% (w/w) cocoa</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Chronic, 6 weeks, normal diet. Altered gut microbiome (↓ Bacteroides, Staphylococcus, Clostridium)</td>
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<td></td>
<td></td>
<td>powder, in food</td>
<td>(food intake data not reported).</td>
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<tr>
<td>Yamashita, 2012</td>
<td>Male C57BL/6 mice</td>
<td>0.5, 2.0% (w/w) cacao liquor procyanindins, in food</td>
<td>Normal diet: 0.5% = 588, 2.0% = 2,344; High-fat diet: 0.5% = 310, 2.0% = 1,532</td>
<td>Normal diet: 0.5% = 3,337, 2.0% = 13,304</td>
<td>Chronic, 13 weeks, control or high-fat diet ↓ fasting glucose (2.0% treatment); ↑ glucose tolerance (OGTT AUC). ↑ translocation of GLUT4, AMPK phosphorylation, UCP expression</td>
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<td></td>
<td>Unknown</td>
<td>Unknown</td>
<td>(food intake data not reported).</td>
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</tr>
<tr>
<td>Yamashita, 2012</td>
<td>Male C57BL/6 mice</td>
<td>0.5%, 1% (w/w) cocoa liquor procyanidins, in food</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Chronic, 1 week, normal diet ↑ glucose tolerance in a dose dependent manner (OGTT-AUC)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>(food intake data not reported).</td>
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<tr>
<td>Yamashita, 2012</td>
<td>Male ICR mice</td>
<td>Cocoa liquor</td>
<td>50 or 250</td>
<td>283 or 1,418</td>
<td>Acute ↑ glucose tolerance (OGTT-AUC) (250-mg/kg dose)</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>procyanidins, by oral gavage</td>
<td>0.01 or 0.06</td>
<td></td>
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</tr>
<tr>
<td>de Oliveira, 2013</td>
<td>Male Wistar STZ-induced diabetic rats</td>
<td>Cocoa liquor, by oral gavage</td>
<td>3,600 or 7,200</td>
<td>1,157 or 2,317</td>
<td>Chronic, 40 days, normal diet ↑ antioxidant capacity (ORAC, FRAP), no change in blood glucose levels</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>25 or 7200</td>
<td></td>
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<tr>
<td>Yamashita, 2013</td>
<td>Male ICR mice</td>
<td>Procyanidins, by oral gavage</td>
<td>0.01 or 0.06</td>
<td></td>
<td>Acute ↑ plasma insulin; ↑ GLP-1 levels</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>(food intake data not reported).</td>
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<tr>
<td>Dorenkott, 2014</td>
<td>Male C57L/6 mice</td>
<td>Monomeric, oligomeric and polymeric cocoa extract fractions, in food</td>
<td>25</td>
<td>142</td>
<td>Chronic, 12 weeks, high-fat diet Oligomeric fraction ↓ fasting blood glucose, ↑ glucose tolerance; ↑ insulin tolerance (OGTT); ↓ endotoxin</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>11,828 or 67,135</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gu, 2014</td>
<td>Male C57BL/6 J mice</td>
<td>8% (w/w) cocoa powder, in food</td>
<td>11,828</td>
<td>67,135</td>
<td>Chronic, 10 weeks, high-fat diet ↓ weight gain, ↑ fecal lipid content, ↑ insulin sensitivity (HOMA-IR), ↓ inflammatory markers (IL-6, MCP-1), no</td>
<td></td>
</tr>
<tr>
<td>Study, Year</td>
<td>Study Group</td>
<td>Intervention</td>
<td>Study Design</td>
<td>Effect Size</td>
<td>Study Duration</td>
<td>Findings</td>
</tr>
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<tr>
<td>Gu, 2014 [63]</td>
<td>Male C57BL/6 J mice</td>
<td>8% (w/w) cocoa powder, in food</td>
<td>Chronic, 18 weeks, high-fat diet</td>
<td>4,998n</td>
<td>28,367</td>
<td>↓ inflammation (adipose tissue NF-κB expression), ↑ insulin sensitivity (HOMA-IR), ↑ gut barrier function (plasma GLP-2), ↓ plasma endotoxin</td>
</tr>
<tr>
<td>Gutierrez-Salmean, 2014 [190]</td>
<td>High fat-fed, obese, male Wistar rats</td>
<td>(−)-epicatechin, by gavage</td>
<td>Chronic, 2 weeks, low fat or high-fat diet</td>
<td>1</td>
<td>11</td>
<td>↓ blood glucose, ↓ triglyceride levels, ↑ mitochondrial function (TFAM, mitofilin expression)</td>
</tr>
<tr>
<td>Gutierrez-Salmean, 2014 [72]</td>
<td>Male Wistar rats</td>
<td>(−)-epicatechin, by gavage</td>
<td>Chronic, 2 weeks, low fat or high-fat diet</td>
<td>1</td>
<td>11</td>
<td>↓ fasting glucose; ↑ glucose tolerance.</td>
</tr>
<tr>
<td>Matsumura, 2014 [65]</td>
<td>Male ICR mice</td>
<td>Flavanol fraction or (−)-epicatechin, by gavage</td>
<td>Acute</td>
<td>10</td>
<td>57</td>
<td>Flavanol fraction ↑ energy expenditure (REE), ↑ blood catecholamines</td>
</tr>
<tr>
<td>Osakabe, 2014 [3]</td>
<td>Male Wistar rats</td>
<td>0.2% (w/w) flavanols, in food</td>
<td>Chronic, 4 weeks, high-fat diet</td>
<td>78</td>
<td>890</td>
<td>↓ thermogenesis, ↓ lipolysis</td>
</tr>
<tr>
<td>Papadimitro u, 2014 [227]</td>
<td>Male SHRgrats, diabetic (STZ induced)</td>
<td>Cocoa powder, by gavage</td>
<td>Chronic, 16 weeks, normal diet</td>
<td>24</td>
<td>272</td>
<td>AMPK, ↓ NOX4 signaling</td>
</tr>
<tr>
<td>Watanabe, 2014 [70]</td>
<td>Male C57BL/J mice</td>
<td>Cocoa flavanolsg by gavage</td>
<td>Chronic, 2 weeks, normal diet</td>
<td>50</td>
<td>284</td>
<td>↓ plasma glucose, ↓ resting energy requirements, mitogenesis</td>
</tr>
<tr>
<td>Fernandez-Millan, 2015 [71]</td>
<td>Male Zucker diabetic fatty rat</td>
<td>10% (w/w) cocoa powder, in food</td>
<td>Chronic, 9 weeks, normal diet</td>
<td>8,311q</td>
<td>94,345</td>
<td>Prevented B cell mass loss, ↑ glucose tolerance (OGTT) insulin sensitivity (HOMA-IR), ↑ β cell function (HOMA-B), ↓ oxidative stress (carbonyl groups, TBARs)</td>
</tr>
</tbody>
</table>

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.
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.
<table>
<thead>
<tr>
<th>Author, year</th>
<th>Subjects</th>
<th>Health status</th>
<th>Treatment (daily dose)</th>
<th>Acute/Chronic (duration)</th>
<th>Outcomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nguyen, 1994</td>
<td>N = 10</td>
<td>Healthy</td>
<td>100-g chocolate bar,</td>
<td>Acute</td>
<td>Lesser but prolonged increase in glucose and insulin.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(45-g cocoa)</td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>Brand Miller, 2003</td>
<td>N = 10</td>
<td>Healthy</td>
<td>6 food pairs, one flavored with cocoa</td>
<td>Acute</td>
<td>↑ insulin response (insulin index) but not glycemic differences with chocolate flavored products.</td>
</tr>
<tr>
<td></td>
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<td></td>
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<tr>
<td>Basu, 2015</td>
<td>N = 14</td>
<td>Obese, Type 2 diabetic</td>
<td>Cocoa beverage (960-mg polyphenols, 480-mg flavanol)</td>
<td>Acute</td>
<td>↑ postprandial insulin secretion, no improvements in blood glucose or insulin resistance (except 4-h postmeal)</td>
</tr>
<tr>
<td>Grassi, 2005</td>
<td>N = 15</td>
<td>Healthy</td>
<td>100-g chocolate bar, (500-mg polyphenols)</td>
<td>Chronic (15 days), crossover design</td>
<td>↑ insulin sensitivity (HOMA-IR, QUICKI), ↑ glucose tolerance (OGTT).</td>
</tr>
<tr>
<td>Grassi, 2005</td>
<td>N = 20</td>
<td>Hypertensive</td>
<td>100-g chocolate bar, (88-mg flavanol)</td>
<td>Chronic (15 days), crossover design</td>
<td>↑ insulin sensitivity (HOMA-IR, QUICKI, ISI).</td>
</tr>
<tr>
<td>Muniyappa, 2008</td>
<td>N = 20</td>
<td>Hypertensive</td>
<td>150-ml beverage, 2 ×/day. (900-mg flavanol)</td>
<td>Chronic (2 weeks), crossover design</td>
<td>No effects on insulin sensitivity (QUICKI and clamp).</td>
</tr>
<tr>
<td>Grassi, 2008</td>
<td>N = 19</td>
<td>Hypertensive, impaired glucose tolerance</td>
<td>100-g chocolate bar, (1008-mg polyphenols)</td>
<td>Chronic (15 days), crossover design</td>
<td>↑ insulin sensitivity (HOMA-IR, QUICKI, SI), ↑ β cell function.</td>
</tr>
<tr>
<td>Davison, 2008</td>
<td>N = 49</td>
<td>Overweight and obese (BMI &gt; 25 kg/m2)</td>
<td>150-ml cocoa beverage (2 ×/day), high flavanol (902 mg) and low flavanol (36 mg)</td>
<td>Chronic (12 weeks), randomized arm</td>
<td>↑ insulin sensitivity (HOMA2-IR) at 6 and 12 weeks.</td>
</tr>
<tr>
<td>Mellor, 2010</td>
<td>N = 12</td>
<td>Type 2 diabetic</td>
<td>45-g chocolate (3 bars/day), (16.6-mg epicateching)</td>
<td>Chronic (8 weeks), crossover design</td>
<td>No change in glycemic control (HOMA-IR, HbA1c, fasting glucose). ↑ HDL cholesterol.</td>
</tr>
<tr>
<td>Almoosawi, 2012</td>
<td>N = 42</td>
<td>Healthy (BMI &lt; 25 kg/m2) compared to overweight (BMI &gt; 25 kg/m2)</td>
<td>20-g dark chocolate, (500-mg polyphenols)</td>
<td>Chronic (4 weeks), crossover design</td>
<td>Treatment prevented unfavorable changes in insulin sensitivity (QUICKI, HOMA-IR) seen in the placebo treatment.</td>
</tr>
<tr>
<td>Desideri, 2012</td>
<td>N = 90</td>
<td>Mild cognitive impairment</td>
<td>Cocoa beverage, (990-</td>
<td>Chronic (8 weeks),</td>
<td>High flavanol and intermediate flavanol</td>
</tr>
<tr>
<td>Study</td>
<td>N</td>
<td>Intervention</td>
<td>Design</td>
<td>Outcomes</td>
<td></td>
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<tr>
<td>Stote, 2012 [86]</td>
<td>19</td>
<td>Adults at risk for insulin resistance</td>
<td>Chron (5 days), crossover</td>
<td>No effects on glycemia (OGTT) or insulinemia (HOMA, QUICKI, ISI)</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Cocoa beverage (2 ×/day), (30-, 180-, 400- or 900-mg flavanols)</td>
<td>Arm</td>
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<td></td>
<td></td>
<td></td>
<td>Randomized arm</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Fast Glucose, HOMA-IR but not fasting insulin compared to low flavanol group.</td>
<td></td>
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<tr>
<td>Stellingwerff, 2013 [90]</td>
<td>16</td>
<td>Trained cyclists</td>
<td>Acute, crossover</td>
<td>↑ Blood glucose and ↑ insulin</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dark chocolate, (240-mg polyphenols)</td>
<td>Arm</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Randomized arm</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Haghighat, 2013 [81]</td>
<td>69</td>
<td>Hypertensive diabetic adults</td>
<td>Chronic (8 weeks), randomized</td>
<td>↓ Fasting glucose, ↓ HbA1c</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Arm</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ramirez-Sanchez, 2013 [87]</td>
<td>5</td>
<td>T2D/Stage II and Stage III heart failure patients</td>
<td>Chronic (3 months), parallel arm</td>
<td>No effects on glycemia/insulinemia. ↓ oxidative stress in mitochondria.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(compared with healthy controls)</td>
<td>Arm</td>
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</tr>
</tbody>
</table>

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.
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 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]. 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. 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
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
(IC50) of 6.9 μg/ml compared to GSE with an IC50 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 IC50 of 378 μg/ml. While total flavanol concentration plays a role in the observed IC50
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 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 IC50 values were 13 times lower compared to acarbose's inhibitory effect on α-amylase. Notably, both GSE (IC50 = 1.2 μg/ml) and white tea extract (IC50 = 2.5 μg/ml) were more potent α-glucosidase inhibitors than acarbose (IC50 = 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 (IC50 = 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.
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, noninsulinotropic 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. 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 \cite{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 \cite{129,130}. Further, links have been drawn between the composition of one's microbiome and their likelihood to present with obesity or metabolic disease \cite{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 \cite{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 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.

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], anticarcinogenic properties [155] and modulating immune function [24].

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 B2, 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 IC50 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 in 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 its 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.
As previously discussed in manuscript one, several studies have demonstrated that cocoa flavanols improve blood glucose levels. Pancreatic β-cells are central for the maintenance of healthy glucose levels in the body and cocoa flavanols have been shown to positively impact β-cells through enhanced survival, proliferation, and insulin secretion. However, the molecular mechanisms underlying these results were not identified in these studies. Therefore, we determined to construct a study that would define what molecular interactions may be responsible for improved β-cell function and published our results as part of the following manuscript.
MANUSCRIPT TWO: Monomeric cocoa catechins enhance β-cell function by increasing mitochondrial respiration


a Department of Nutrition, Dietetics and Food Science, Brigham Young University, Provo, UT
b Department of Physiology and Developmental Biology, Brigham Young University, Provo, UT
c Department of Food Science and Technology, Virginia Tech, Blacksburg, VA
d Current affiliation: University Hospitals, Cleveland, OH
e Current affiliation: DuPont Crop Protection, Newark, DE
f Current affiliation: SPF North America Inc., Greenville, SC
g Current affiliation: UC Irvine School of Medicine, Irvine, CA
h Corresponding author at: Brigham Young University; Department of Nutrition, Dietetics and Food Science; BYU ESC S-243, Provo, UT 84602; Tel.: +1-801-422-9082, Fax: +1-801-422-0258; E-mail address: jeffery.tessem@byu.edu (J.S. Tessem).
i Grants, sponsors and funding sources: J.S. Tessem has funding support from the Diabetes, Action, Research and Education Foundation and the American Diabetes Association. Funding for this work was provided, in part, to A.P. Neilson and S.F. O’Keefe by the Virginia Agricultural Experiment Station and the Hatch Program of the National Institute of Food and Agriculture, U.S. Department of Agriculture.
Abstract

A hallmark of type 2 diabetes (T2D) is β-cell dysfunction and the eventual loss of functional β-cell mass. Therefore, mechanisms that improve or preserve β-cell function could be used to improve the quality of life of individuals with T2D. Studies have shown that monomeric, oligomeric and polymeric cocoa flavanols have different effects on obesity, insulin resistance and glucose tolerance. We hypothesized that these cocoa flavanols may have beneficial effects on β-cell function. INS-1 832/13 derived β-cells and primary rat islets cultured with a monomeric catechin-rich cocoa flavanol fraction demonstrated enhanced glucose-stimulated insulin secretion, while cells cultured with total cocoa extract, oligomeric or polymeric procyanidin-rich fraction demonstrated no improvement. The increased glucose-stimulated insulin secretion in the presence of the monomeric catechin-rich fraction corresponded with enhanced mitochondrial respiration, suggesting improvements in β-cell fuel utilization. Mitochondrial complex III, IV and V components are upregulated after culture with the monomer-rich fraction, corresponding with increased cellular ATP production. The monomer-rich fraction improved cellular redox state and increased glutathione concentration, which corresponds with Nrf2 nuclear localization and expression of Nrf2 target genes including NRF-1 and GABPA, essential genes for increasing mitochondrial function. We propose a model by which monomeric cocoa catechins improve the cellular redox state, resulting in Nrf2 nuclear migration, and upregulation of genes critical for mitochondrial respiration, glucose-stimulated insulin secretion and ultimately improved β-cell function. These results suggest a mechanism by which monomeric cocoa catechins exert their effects as an effective complementary strategy to benefit T2D patients.

Keywords: cocoa; β-cell; catechin; insulin secretion; mitochondrial respiration; Nrf2
1. Introduction

The incidence of type 2 diabetes (T2D) is increasing at an alarming rate. There are an estimated 415 million people worldwide that are diabetic, with current projections of 642 million people being diabetic by 2040, with the vast majority suffering from T2D [1]. T2D is characterized by hyperglycemia and hyperlipidemia due to muscle, adipose and liver insulin resistance [2, 3]. In addition, studies have begun to demonstrate that decreased functional β-cell mass is an early and essential step in T2D disease progression [4].

The primary purpose of the β-cell is to maintain normoglycemia through glucose-stimulated insulin secretion. The β-cell dysfunction observed in T2D results in decreased and poorly controlled insulin secretion and, ultimately, β-cell death [4]. Mechanisms that increase functional β-cell mass could, therefore, be leveraged as a treatment for T2D.

Recent studies have shown that diets supplemented with cocoa or flavanols present in cocoa have potentially beneficial effects for the T2D patient [5]. Some of these studies have suggested that these effects may include increased cellular antioxidant abilities [6, 7]. Given the fact that β-cells are particularly sensitive to oxidative stress, flavanol compounds found in cocoa may have direct therapeutic applications for the control and prevention of T2D [8].

Cocoa contains a complex profile of monomeric flavanols (catechins such as epicatechin) and their oligomeric and polymeric forms (procyanidins) (Figure 1). Using fractionation methods, our group has begun to differentiate the effects of these distinct flavanol fractions. We have previously shown that these fractions have different whole-body effects in regard to diet-induced T2D. These findings demonstrated that oligomeric cocoa procyanidins have the greatest effect in preventing high fat feeding-induced weight gain while maintaining normal fasting glucose, insulin levels, glucose tolerance and insulin sensitivity [9]. However, these are whole-
body effects, and studies are needed to describe the effect of various cocoa flavanols fractions on various tissues affected by T2D. We recently demonstrated differential impacts of these fractions on insulin signaling and glucose utilization in skeletal muscle cells [10]. However, no data exist on how these fractions may affect pancreatic β-cell function, which is critical for preventing or ameliorating T2D.
Figure 1 - Structures of selected representative flavanols found in cocoa. (−)-epicatechin monomer and procyanidin oligomers/polymers comprised of (−)-epicatechin monomers linked (4β → 8).
Previous studies suggest that cocoa flavanols protect and/or improve β-cell function. These effects appear to include enhanced survival, insulin secretion, and proliferation. In vitro, cocoa flavanols protect against oxidative stress in INS-1 cells and increase insulin secretion from rat islets [11]. In Zucker diabetic fatty rats, dietary cocoa prevents β-cell apoptosis by reducing oxidative stress, and also increases small islet size and maintenance of total islet mass, suggesting induction of β-cell proliferation [7]. In ob/ob and normal rats, cocoa flavanol treatment enhances insulin secretion [6]. In a non-obese Type 1 Diabetic model, application of cocoa flavanols increases β-cell mass [12]. Additionally, cocoa flavanol treatment enhances DNA replication and regeneration of β-cells in rats [13-15]. Finally, in humans, cocoa flavanols enhance postprandial insulin secretion [16]. Despite these promising results, the molecular mechanisms by which these improvements occur are yet to be elucidated.

The transcription factor Nrf2 (Nuclear Factor, Erythroid 2 Like 2, also known as NFE2L2) controls the cellular response to oxidative stress [17]. Under normal conditions, Nrf2 is bound in the cytosol by the Kelch-like ECH-associated protein 1 (Keap1) and targeted for proteolytic degradation, thus suppressing Nrf2-mediated transcriptional activity [18]. Under oxidative stress conditions, however, Keap1 cysteine residues are oxidized, resulting in Nrf2 release and nuclear translocation [19]. Upon nuclear translocation, Nrf2 induces expression of genes whose promoters contain antioxidant response elements (ARE) [20]. Many of these Nrf2 regulated genes are involved in processes to quench free radicals and ultimately upregulate the cell’s ability to decrease oxidative stress. While direct Nrf2 targets involved in antioxidant response have been defined for some time, recent studies have demonstrated that Nrf2 also enhances expression of genes that regulate mitochondrial function [21].
The transcription factors NRF-1 (Nuclear Respiratory Factor 1) and GABPA (GA Binding Protein Transcription Factor Alpha Subunit, also known as Nuclear Respiratory Factor 2) are essential for mitochondrial function and biogenesis [22]. These transcription factors directly control expression of genes involved in the electron transport chain, as well as enhance expression of genes such as TFAM that drive mitochondrial production [22, 23]. Cellular response to events that require increased mitochondrial function are dependent on the function of these transcription factors. Given that glucose-stimulated insulin secretion is intimately connected to mitochondrial respiration, these transcription factors are especially important for β-cell function [24]. Therefore the goal of this study was to determine the effect of flavanol fractions on glucose stimulated insulin secretion, and to define the mechanism by which these compounds exert their effects.

2. Materials and Methods

2.1 Animal Husbandry and Islet Isolation

Wistar rat breeding pairs were purchased from Harlan and maintained on standard chow diet (Teklad 7001; Harlan). Pups were weaned at 21 days. Male rats were allowed to feed ad libitum and were maintained on a 12-hour light-dark cycle. Rats were age- and weight-matched for all islet experiments. Pancreatic islets were isolated from 5-week-old male rats as previously described [25-28]. Primary rat islets were cultured in RPMI 1640 and supplemented with 10% FBS, 1% Fungizone antimycotic (Life Technologies), and 1% HEPES. Islet medium was changed every 24 hours. All animal studies were approved and performed in accordance with Brigham Young University’s IACUC guidelines (Protocol #16-0902).
2.2 Cocoa Extraction and Fractionation

We selected fractionation in order to examine the activities of groups of compounds together, as opposed to individually, due to the scarcity of commercial standards for procyanidins larger than tetramers. A flavanol-rich cocoa extract was produced from commercially available cocoa powder, and fractionated into monomeric catechin-rich, oligomeric procyanidin-rich and polymeric procyanidin-rich fractions. The production and composition of these fractions has been described previously [9]. Detailed methodologies are presented in Supplementary Information. Characterization and enrichment levels of monomeric catechins and procyanidins in the extract and individual fractions are presented in Supplementary Material (Table S1, Figures S1-S2). Briefly, the whole cocoa extract is rich in monomeric catechins and oligomeric and polymeric procyanidins extracted from cocoa. The catechin monomer-rich fraction is highly enriched for catechin monomer species [particularly (-)-epicatechin and (±) catechin] and depleted of procyanidins compared to the whole cocoa extract. The oligomeric procyanidin-rich fraction is highly depleted of monomers compared to CE, but was greatly enriched for dimeric through hexameric procyanidins compared to the whole cocoa extract. Finally, the polymeric fraction was highly depleted for catechin monomers and dimeric through hexameric procyanidins, and highly enriched in polymeric procyanidins (heptameric and above).

2.3 Cell Culture

The INS-1 derived 832/13 rat insulinoma cell line was maintained in complete RPMI 1640 medium with L-glutamine and 11.2 mM glucose supplemented with 50 U/ml penicillin, 50 μg/ml streptomycin, 10 mM HEPES, 10% fetal bovine serum, and INS-1 supplement, as previously described [29]. For all glucose-stimulated insulin secretion, RT-PCR, western blotting, respiration and biochemical assays, cells were plated at 0 hours, cocoa extract/fractions or
DMSO treatment were given at 24 hours, and cells were harvested following 24 hours of treatment. Stock solutions of whole cocoa extract, monomeric, polymeric and oligomeric catechin fractions were diluted in DMSO at a concentration of 25 mg/ml. Final treatment concentrations for cell culture were 0-25 µg/ml whole cocoa extract/fractions or DMSO alone for control, using 1µl DMSO/ml media or less in all conditions. The final concentration of DMSO in media was 0.1% v/v [30]. The concentrations of individual compounds in the highest concentration are shown in Supplementary Information (Table S2). Concentrations of individual compounds ranged from not detectable to 2600 nM (in the highest dose employed), and were generally on the order of 10-500 nM. The only compound to exceed 1000 nM (1 µM) was epicatechin in the cocoa monomer fraction. The concentrations of catechin/epicatechin are feasibly obtainable in circulation following consumption of cocoa/chocolate (up to 2-3 µM epicatechin as the major bioavailable species) [31]; concentrations of larger species (procyanidins greater than trimers) are likely on the high end of what that is obtainable in circulation. However, all concentrations are generally lower than concentrations used in flavanol cell culture studies (typically 1-50 µM).

2.4 Glucose-Stimulated Insulin Secretion.

Glucose-stimulated insulin secretion (GSIS) was performed as previously described [29]. Briefly, cells were grown to confluency, washed with PBS and preincubated in secretion assay buffer (SAB) for 1.5 hours (114 mM NaCl, 4.7 mM KCl, 1.2 mM KH2PO4 1.16 mM MgSO4, 20 mM HEPES, 2.5 mM CaCl2, 0.2% BSA, pH 7.2) containing 2.5 mM glucose. glucose-stimulated insulin secretion (GSIS) was performed by incubating quadruplicate replicate wells of cells cultured with varying levels of cocoa monomer fraction, oligomer fraction, polymer fraction, whole cocoa extract or DMSO vehicle control (1 µl DMSO/ml media for cocoa extract and
fractions, as well as DMSO control) in SAB containing 2.5 mM glucose for 1 hour, followed by
1 hour in SAB with 16.7 mM glucose, followed by collection of the respective buffers, as
previously described. For total insulin content, cells were lysed in RIPA buffer with protease
inhibitors (Life Technologies). Secreted insulin and total insulin was measured in SAB using a
rat insulin RIA kit (MP Biomedicals) as previously described [29, 32]. Insulin secretion from
isolated rat islets was completed as previously described [29, 32, 33].

2.5 Oroboros Respiration

Experiments were done with permeabilized INS-1 832/13 cells cultured with cocoa monomer
using a Clarke Oxygen electrode high-resolution respirometer (Oxygraph-2K; Oroboros
Instruments, Innsbruck, Austria). For permeabilization, 832/13-derived cells were harvested in
MiR05 respiration media (0.5 mM EGTA, 3 mM MgCl2, 60 mM K-lactobionate, 20 mM
taurine, 10 mM KH2PO4, 20 mM HEPES, 110 mM sucrose, and 1 g/l BSA, pH 7.1) [34, 35]. All
experiments were run in pairs with a cocoa monomer treated sample (25μg/ml at 1μl DMSO/ml
media) and a corresponding DMSO control (1 μl DMSO/ ml media). Respiration experiments
were done with an oxygen concentration ranging between 250 and 300 nmol/ml at 37°C [36]. A
basal, nonphosphorylating respiratory state (GM-Glutamate and Malate) was determined in the
presence of Glutamate (10 mM) and Malate (2 mM). The respiratory capacity of mitochondria in
the ADP-stimulated state of oxidative phosphorylation was assessed upon the addition of ADP
(2.5 mM; GMD-Glutamate and Malate and ADP) and succinate (10 mM; GMDS-Glutamate and
Malate and ADP and Succinate). Mitochondrial integrity was tested by adding cytochrome c (10
μM). Uncoupled respiratory capacity of the electron transfer system was measured after a
stepwise addition of 0.5 μM FCCP (FCCP). Residual oxygen consumption was determined
following inhibition of complex III with the addition of rotenone and antimycin A. This state of
residual oxygen consumption served as a baseline correction for all of the other states [36].
Respiratory control ratio (RCR) was determined by dividing the GMD value by the GM value.
Analysis of complex-supported mitochondrial respiration specifically involving succinate (10 mM) was completed with the addition of rotenone (0.5 μM) for complex II-mediated leak respiration and then ADP (2.5 mM) addition for complex II-mediated oxidative phosphorylation. Finally, maximal electron transport system-supported respiration via complex II was determined by the addition of FCCP (0.05 μM). All data were normalized to protein content of each chamber, determined by BCA assay.

2.6 MTT Assay
INS-1 832/13 cells were plated in 96 well plates at a concentration of 2 x 10^5 cells/ml. Cells were treated with or without cocoa monomer. After 24 hours of exposure cells underwent MTT assay following protocols as outlined elsewhere [37, 38]. Relative absorbance was measured with a BioTek Synergy 2 plate reader and normalized to total protein content by BCA.

2.7 Alamar Blue Assay
INS-1 832/13 cells were plated in 96 well plates at a concentration of 2 x 10^5 cells/ml. Cells were treated with or without cocoa monomer. After 24 hours of exposure cells underwent Alamar Blue assay following protocols in use by our research group [37, 38]. Relative absorbance was measured with a BioTek Synergy 2 plate reader and normalized to total protein content by BCA.

2.8 MitoTracker Assay
INS-1 832/13 cells were plated in 96 well plates at a concentration of 2 x 10^5 cells/ml. Cells were treated with or without cocoa monomer. After 24 hours of exposure were loaded with 25 nM MitoTracker Red CMXRos-FM for 15 min, following the manufacturer's protocol (Life
Technologies). Relative mitochondrial content was measured by fluorescence in a BioTek Synergy 2 plate reader and normalized to total protein content by BCA.

2.9 ATP Assay

INS-1 832/13 cells were treated with or without cocoa monomer for 24 hours. Following exposure, cells were cultured in SAB buffer with 2.5mM glucose for 2 hours, washed and then transferred to SAB buffer with either 2.5mM glucose or 16.7mM glucose for 1 hour. Cells were harvested and ATP content was measured by using the ATP determination kit, following the manufacturer's specifications (Molecular Probes) [38].

2.10 HPLC GSH Assay

To assay the glutathione (GSH), glutathione disulfide (GSSG) and cellular GSH redox states (E_h), we measured GSH and GSSG concentrations by HPLC as S-carboxymethyl, N-dansyl derivatives using γ-glutamyl-glutamate as an internal standard as previously described [39, 40]. Cells were washed with cold PBS and the collected in cold 5% perchloric acid buffer containing 10 µM γ-glutamyl-glutamate, used as an internal standard. Protein pellets were measured via BCA protein assay methods for sample to sample normalization as described elsewhere [41]. Samples were subsequently derivatized, analyzed by HPLC, and intracellular GSH and GSSG levels were determined by comparison with the internal standard. Concentrations were then used in the Nernst equation to determine the GSH/GSSG E_h.

2.11 Apoptosis Assay

INS-1 832/13 cells were cultured in the presence or absence of cocoa monomer for 72 hours. During the last 18 hours of culture, cells were exposed to 1mM palmitate or control media. Percent cell viability was determined by dividing the number of cells counted for the cocoa monomer untreated and treated groups after palmitate exposure by the number of cells...
counted with vehicle treatment after the respective treatment with or without cocoa monomer (i.e. \([\text{Cell count}_{\text{CM+Palmitate}}]/[\text{Cell count}_{\text{CM-Palmitate}}]\)) as previously described [32, 37].

2.12 Nrf2 Immunocytochemistry

INS-1 832/13 cells were seeded on poly-D-lysine coated coverslips (BD). Cells were cultured in the presence or absence of cocoa monomer fraction for 24 hours. Cells were fixed in 4% PFA, permeabilized with 0.5% Triton-X 100 PBS. Cells were stained for insulin (1:1,000, DAKO), Nrf2 (1:500, GeneTex) and DAPI. Cells were imaged using 40x magnification using an Olympus BX43 microscope with an Olympus U-HGCLGPS light source. Images were combined using Photoshop and quantified using ImageJ (NIH).

2.13 Immunoblot Analyses

INS-1 832/13 cells were cultured in the presence or absence of cocoa monomer fraction for 24 hours. Following culture, cells were harvested and lysed in 100 μL RIPA buffer with protease inhibitors (Life Technologies). Clarified cell lysates were run on 10% PAGE gels and transferred to poly (vinylidene fluoride) membranes. Membranes were probed using the total OXPHOS Complex Monoclonal Antibody cocktail (1:1000, ThermoFisher). Sheep anti-mouse (1:10,000) and goat anti-rabbit (1:10,000) antibodies (GE Healthcare) coupled to peroxidase were used to detect the primary antibodies. For nuclear and cytoplasmic extracts, cells were processed using the NE-PER Nuclear Lysate Kit (Pierce) per the manufacturer's instructions. Clarified lysates were run on 10% PAGE gels and transferred to poly (vinylidene fluoride) membranes. Membranes were probed with diluted antibodies raised against Nrf2 (1:500, GeneTex), Histone H3 (1:1,000, Cell Signaling) and γ-tubulin (1:1000, DSHB). Sheep anti-mouse (1:10,000) and goat anti-rabbit (1:10,000) antibodies (GE Healthcare) coupled to peroxidase were used to detect
the primary antibodies. Blots were developed with ECL advance reagent (GE Healthcare). Quantitation of immunoblots was performed using ImageJ (NIH).

2.14 Quantitative RT-PCR

RNA was harvested using TriReagent (Life Technologies) and cDNA was synthesized using the High-Capacity cDNA Reverse Transcription kit (Life Technologies), per the manufacturer's instructions [29, 32, 38]. Real-time PCR was performed using the Life Technologies One Step Plus Sequence Detection System and Software (Life Technologies). SYBR green based PrimeTime assays (IDT) were used to detect rat Hmox1, Gclc, Nqo1, Nrf1, GABPA, Nrf2, Nr4a1, Nr4a2, Nr4a3 and peptidylprolyl isomerase A (PPIA, internal control). All primer sequences are available upon request.

2.15 Statistical Analysis

For analysis between two groups, Student's \( t \)-test was used, and differences were considered significant when \( P < 0.05 \). One-way analysis of variance (ANOVA) was used in experiments that had three or more groups. Differences within ANOVA were determined using Tukey's post hoc tests, and differences were considered significant when \( P < 0.05 \). All data are reported as means ± SD.

3. Results

3.1 Cocoa monomeric fraction enhances glucose-stimulated insulin secretion in INS-1 832/13 β-cells and primary rat islets.

Various studies have suggested that cocoa-derived compounds help maintain normoglycemia. Catechins are particularly abundant in cocoa and catechin-rich supplements have been the focus of these studies [12]. Recent studies, however, have demonstrated that cocoa flavanols of different polymerization levels (i.e. catechins and procyanidins) have different
effects in terms of their impact on the phenotypes characteristic of T2D [9]. Therefore, based on these recent results, we sought to determine the effect of cocoa flavanols, fractionated based on degree of polymerization, on glucose-stimulated insulin secretion. We cultured INS-1 832/13-derived β-cells in the presence of unfractionated cocoa extract, or cocoa extract fractionated to enrich for monomeric catechins, oligomeric procyanidins or polymeric procyanidins. The 832/13 β-cells were cultured in the presence of serial dilutions of total cocoa extract, or the three fractions. An increase in glucose-stimulated insulin secretion versus the control was observed with 25ug/ml for the monomeric fraction (Figure 2A). Interestingly, treatment with the cocoa extract, oligomeric and polymeric fractions all resulted in a decrease in insulin secretion (Figure 2B-D). No change in total insulin content was observed for the monomer or polymer treated cells, with small decreases in content for the oligomer and cocoa extract treated cells (Figure 2E-H). Finally, as the highest dose of the monomer fraction was the only effective treatment for enhancing insulin secretion, we cultured primary rat islets in 25ug/ml cocoa monomer fraction for 24 hours, and measured the insulin secretion rate. A significant increase in insulin secretion was observed (Figure 2I) without changes in insulin content (Figure 2J). These data demonstrate that INS-1 832/13 β-cells or primary rat islets cultured with cocoa monomer fraction have enhanced glucose-stimulated insulin secretion. Therefore, we focused on this fraction for the remaining experiments.
Figure 2 - Culture of INS-1 832/13 β-cells or primary rat islets with monomeric catechin-rich cocoa fraction enhances glucose-stimulated insulin secretion. Glucose-stimulated insulin secretion was measured after culturing INS-1 832/13 β-cells for 24 hours in (A) monomeric catechin-rich cocoa fraction, (B) oligomeric procyanidin-rich cocoa fraction, (C) polymeric procyanidin-rich cocoa fraction and (D) whole cocoa extract with concentrations from 0.75-25 ug/ml. Total cellular insulin content was measured after culturing INS-1 832/13 β-cells for 24 hours in (E) monomeric catechin-rich cocoa fraction, (F) oligomeric procyanidin-rich cocoa fraction, (G) polymeric procyanidin-rich cocoa fraction and (H) whole cocoa extract with concentrations from 0.75-25 ug/ml. Glucose-stimulated insulin secretion (I) and total insulin content (J) was measured from primary rat islets cultured in the presence or absence of 25 ug/ml cocoa monomer fraction. n=4 individual replicates per conditions, run in quadruplicate for each replicate. * p<0.05 vs. control. For ANOVA (Figure 2A-H), means sharing the same letter are not significantly different from the control (p>0.05) or means that have no letter in common are significantly different from the control (p<0.05)
3.2 Cocoa monomer fraction treatment enhances β-cell mitochondrial respiration and ATP production.

Insulin secretion is directly connected to mitochondrial respiration. Therefore, we hypothesized that the increase in glucose-stimulated insulin secretion could be due to enhanced mitochondrial respiration. We sought to determine if culturing INS-1 832/13 β-cells with the cocoa monomer fraction would affect β-cell mitochondrial respiration. Treatment of INS-1 832/13 β-cells with the cocoa monomer fraction resulted in significantly increased respiratory function in GM, GMD, GMDS and FCCP respiratory measurements (Figure 3A). This increase was not due to increased complex II (CII)-mediated respiration (Figure 3B), nor were significant differences in mitochondrial “health” observed (Figure 3C), as determined via respiratory control ratio (RCR). Finally, these results were substantiated by increased levels of ATP observed after culturing INS-1 832/13 β-cells with the cocoa monomer fraction under stimulatory conditions (16.7mM glucose) with no change under non-stimulatory conditions (2.5mM) (Figure 3D). Taken together, these data demonstrate an overall favorable alteration in mitochondrial physiology; namely, increased respiration associated with enhanced ATP formation.
Figure 3 - Culture of β-cells with monomeric catechin-rich cocoa fraction enhances mitochondrial respiration and function. Mitochondrial respiration (A) was measured in INS-1 832/13 β-cells following 24 hours of culture with 25 μg/ml a monomeric catechin-rich cocoa fraction. GM-glutamate and malate, GMD-glutamate, malate and ADP, GMDS-glutamate, malate ADP and succinate, and FCCP-Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (uncoupling reagent). No significant changes were observed in the complex II respiration (CII Factor, B) or in the overall mitochondrial health as measured by RCR (C). (D) Total cellular ATP was measured in INS-1 832/13 β-cells following 24 hours of culture with 25 μg/ml monomeric catechin-rich cocoa fraction and exposure to either unstimulatory (2.5mM) or stimulatory (16.7mM) levels of glucose. n=4 individual replicates per conditions. * p<0.05, **p<0.01 vs. control.
3.3 Cocoa monomer-induced enhancement of β-cell mitochondrial respiration is not due to increased mitochondrial viability or mitochondrial content.

The observed increase in mitochondrial respiration could be due to increased mitochondrial viability or increased mitochondrial content. To determine if mitochondrial viability was enhanced, we used the Alamar Blue and MTT assays. Neither assay, however, demonstrated any increase in viability due to culture with the cocoa monomer fraction (Figure 4A-B). To determine if total mitochondrial content was increased, we stained the cells with the fluorescent mitochondrial-specific stain MitoTracker Red. Total fluorescence of the samples demonstrated no significant differences between the two groups on a per cell basis (Figure 4C). These data indicate that culture of INS-1 832/13 β-cells with cocoa monomer fraction does not increase mitochondrial viability or mitochondrial number.
Figure 4 -Culture of β-cells with monomeric catechin-rich cocoa fraction does not improve mitochondrial respiration or function through increased mitochondrial viability or mitochondrial number. No differences in mitochondrial viability were observed after culturing INS-1 832/13 β-cells with a monomeric catechin-rich cocoa fraction as measured by (A) Alamar Blue or (B) MTT staining. No differences in total mitochondrial content were observed by (C) MitoTracker staining of INS-1 832/13 β-cells cultured with monomeric catechin-rich cocoa fraction. n=4 individual replicates per conditions.
3.4 Culture of β-cells with cocoa monomers increases levels of mitochondrial complex proteins.

The enhanced mitochondrial respiration observed in INS-1 832/13 β-cells cultured in the presence of cocoa catechin monomers could be due to enhance mitochondrial function. Therefore, we measured the expression of mitochondrial complexes in INS-1 832/13 β-cells after culturing in the presence or absence of cocoa monomer fraction for 24 hours. A significant increase was observed in the protein levels of mitochondrial respiratory complex III, IV and V, while no significant changes were observed for complex I or II (Figure 5A-B). The significant increase in complex V and the lack of change in complex II levels substantiate the findings regarding ATP levels and CPII factor. These results strongly suggest that culture of INS-1 832/13 β-cells with cocoa catechin monomers results in increased insulin secretion due to increased ATP production and mitochondrial respiration through the increased expression of components of the electron transport chain.
Figure 5 - Culture of β-cells with monomeric catechin-rich cocoa fraction increases electron transport chain complex V, III and IV levels. Mitochondrial electron transport chain complex levels (A) were measured by western blotting and quantified (B) demonstrating increased levels of complex V, IV and III. n=3 individual replicates. * p<0.05, **p<0.001, ***p<0.0001 vs. control.
3.5 Culture of β-cells with cocoa monomer fraction initiates an antioxidant response.

Previous studies have suggested that cocoa flavanols act as mild oxidants to initiate an antioxidant response [42]. To verify these findings, we measured potential changes in $E_h$, GSH and GSSG levels, as well as any potential apoptotic protection the monomeric fraction might confer in the presence of palmitate. INS-1 832/13 cells cultured with cocoa monomer fraction improved the cellular redox state (Figure 6A). Furthermore, these cells had elevated levels of GSH (Figure 6B) while maintaining the cellular GSSG levels (Figure 6C). This, however, does not impede cellular proliferation rates (Figure 6D), indicating that treatment of 832/13 cells with cocoa monomer fraction does not decrease cellular viability. Finally, it has been shown that culture of β-cells in the presence of 1.0 mM palmitate induces apoptosis through reactive oxygen species production. Therefore, we determined the susceptibility of 832/13 cells to palmitate-induced apoptosis after culturing in the presence of cocoa monomer for 72 hours. We observed that the monomeric fraction attenuated palmitate-induced apoptosis (Figure 6E). These data suggest that culture with the cocoa monomer fraction promotes an antioxidant response capable of protecting β-cells from apoptotic stimuli.
Figure 6 - Culture of β-cells with monomeric catechin-rich cocoa fraction improves cellular redox potential through increased GSH levels, and results in increased protection against ROS induced apoptosis. INS-1 832/13 β-cells cultured for 24 hours with 25 μg/ml monomeric catechin-rich cocoa fraction have improved cellular redox potential (A). Increased GSH levels (B) were observed while GSSG levels (C) remained constant. Culture with monomeric catechin-rich cocoa fraction (D) maintains 832/13 β-cell proliferation, and (E) protects against palmitate induced apoptosis. n=6 individual replicates. ****p<0.0001 vs. control.
3.6 Culture of β-cells with cocoa monomer fraction leads to nuclear translocation and activation of Nrf2.

Increased glutathione levels and improved cellular redox states are indicative of the activation of the transcription factor Nrf2. Under normal conditions, Nrf2 is bound by KEAP1 in the cytosol where it is constantly degraded through the proteasome. However, under conditions of oxidative stress, Nrf2 is released from KEAP1, is allowed to translocate to the nucleus, and induces expression of target genes involved in glutathione production, reactive oxygen species (ROS) quenching and NAD(P)H cycling. The increased glutathione levels and an improved cellular redox state are suggestive of Nrf2 nuclear translocation and transcriptional transactivation. To determine if Nrf2 is migrating to the nucleus we performed immunofluorescence assays (Figure 7A). These studies demonstrate that Nrf2 staining after 24 hours of culture with cocoa monomer fraction results in increased nuclear localization. Furthermore, nuclear and cytosolic fractionation demonstrate that a significantly increased level of Nrf2 is observed in the nucleus of cocoa monomer fraction treated 832/13 β-cells (Figure 7B-E). Finally, expression of three hallmark Nrf2 target genes, Hmox1, Gclc, and Nqo1, were all upregulated (Figure 7F-H). These data demonstrate that culture of INS-1 832/13 β-cells with cocoa monomer fraction results in nuclear migration and activation of Nrf2.
Figure 7 - Culture of β-cells with monomeric catechin-rich cocoa fraction induces nuclear localization and transcriptional activation of Nrf2. INS-1 832/13 β-cells cultured for 24 hours with 25 ug/ml monomeric catechin-rich cocoa fraction have increased Nrf2 nuclear localization as measured by (A) immunoflorescence staining of treated and untreated INS-1 832/13 β-cells, (B, D) western blotting of cytoplasmic and (C, E) nuclear fractions, and increased expression of Nrf2 target genes (F) Hmox1 (G) Gclc and (H) Nqo1. For immunoflorescence assay n=5 per condition, for western blotting n=4 per condition, for RT-PCR assays n=6 per condition. * p<0.05, **p<0.01 vs. control.
3.7 Culture of β-cells with cocoa monomer fraction leads to increased expression of genes responsible for mitochondrial complex biogenesis.

Recent studies have begun to define the Nrf2 target genes. Among the genes that have been defined as targets of Nrf2 is the transcription factor Nrf1. Nrf1 and GABPA are known as the nuclear respiratory factors and are critical for upregulation of metabolic enzymes necessary for mitochondrial respiration. Therefore, given the connection between Nrf2 and Nrf1, and the increase in mitochondrial electron transport chain components, we measured expression of Nrf1, GABPA and Nrf2. Our results demonstrate that culture with cocoa monomer fraction results in increased expression of both Nrf1 and GABPA (Figure 8A-B). Interestingly, our results also demonstrated increased expression of Nrf2 (Figure 8C), suggesting that this cycle may continue as long as the 832/13 β-cells remain exposed to cocoa monomer fraction. Finally, we have previously shown that the orphan nuclear receptors Nr4a1, Nr4a2 and Nr4a3 are critical for β-cell glucose metabolism and insulin secretion [38]. Treatment of INS-1 832/13 cells with cocoa monomer does not induce expression of any Nr4a family members (Figure 8D-F), demonstrating that the change in insulin secretion is independent of this previously defined pathway. These data suggest that the increased expression of mitochondrial respiratory complexes, which ultimately results in increased β-cell insulin secretion, is due to the nuclear migration of Nrf2 and upregulation of NRF-1 and GABPA expression.
Figure 8 - Culture of β-cells with monomeric catechin-rich cocoa fraction induces upregulation of the transcription factors Nrf1 and GABPA that are essential for expression of mitochondrial electron chain components. INS-1 832/13 β-cells cultured for 24 hours with 25 μg/ml monomeric catechin-rich cocoa fraction have increased expression of the nuclear respiratory factors (A) Nrf1 and (B) GABPA. Furthermore, expression of (C) Nrf2 is also increased after culture conditions. No change was observed for the orphan nuclear receptors (D) Nr4a1, (E) Nr4a2, and (F) Nr4a3, which have been shown to play a role in glucose metabolism and β-cell insulin secretion. n=8 per condition, each run in triplicate. **p<0.01, ***p<0.001 vs. control.
4. Discussion

Over 9% of the American population has T2D and with over 1.7 million new cases each year, it is proving to be a significant healthcare concern [43]. While T2D is initially characterized by decreased insulin sensitivity in peripheral tissue, progression of the disease ultimately leads to destruction of pancreatic β-cells [44, 45]. Strategies that improve the insulin secretion rate or promote β-cell viability have direct application in first preventing T2D progression by inhibiting β-cell destruction, as well as treating patients suffering with prolonged T2D. Mitochondrial function is vital to the functionality and viability of pancreatic β-cells, with glucose-stimulated insulin secretion relying on mitochondrial respiration through the components of the electron transport chain [46].

Recent studies have found that cocoa flavanols may improve glucose homeostasis and protect pancreatic β-cells from apoptotic stimuli. Interestingly, purified monomeric, oligomeric, and polymeric cocoa flavanol fractions may exert different levels of benefit with regard to β-cell function and viability. Given these findings, cocoa flavanols may represent a therapeutic option for increasing functional β-cell mass.

The purpose of this study was to 1) determine the effect of different cocoa flavanol fractions on β-cell function and 2) identify potential mechanisms underlying these effects. We have shown that a cocoa monomer-rich fraction enhances glucose-stimulated insulin secretion and have identified a potential mechanism of action explaining this effect. We show that 24-hour cocoa monomer treatment of 832/13 β-cells is sufficient to initiate Nrf2 translocation to the nucleus, leading to upregulation of antioxidant genes as well as those vital to mitochondrial biogenesis (Figure 9). This is consistent with previous studies showing that flavanol consumption may increase mitochondrial copy number in skeletal muscle [47]. These data were
substantiated as we saw increased glutathione levels as well as increased levels of mitochondrial complexes III-V after administration of the monomer fraction. Cocoa monomer treatment led to higher levels of ATP, which would be expected with improved mitochondrial function and enhancement of insulin secretion. These data provide the first definitive mechanism by which cocoa monomeric catechins enhance insulin secretion in the β-cell.
Figure 9 - Monomeric cocoa catechins enhance β-cell glucose-stimulated insulin secretion. Exposure of β-cells to a monomeric catechin-rich cocoa fraction results in 1) oxidation of KEAP1 which releases Nrf2, 2) nuclear translocation of Nrf2, 3) transcriptional activation of Nrf2 targets such as Nqo1, Gclc, Hmox1, Nrf1, GABPA and Nrf2. This results in 4) a more reduced redox state, increased glutathione concentrations, increased ROS detoxification and improved NAD(P)/NAD(P)H cycling. Upregulation of Nrf1 and GABPA results in 5) induction of the electron chain components complex V, III and IV. Increased expression of these integral mitochondrial components 6) enhances mitochondrial respiration resulting in 7) increased cellular ATP levels and 8) ultimately increased levels of glucose-stimulated insulin secretion.
Our data in this study demonstrate that monomeric catechin-rich cocoa fraction enhance insulin secretion. Previously published research from our group demonstrated that only oligomeric flavanol cocoa fractions normalize blood glucose levels in C57Bl/6J mice fed a 12-week high fat diet. This apparent discrepancy in findings may be a function of the method used to induce T2D in the animal model. In T2D, hyperglycemia has been shown to initially be a result of peripheral tissue insulin resistance, with β-cell loss coming after long-term hyperglycemia and hyperlipidemia. It is likely that 12 weeks of high fat feeding was sufficient to induce peripheral insulin resistance, however the duration was not enough to cause β-cell derangement. High fat diet feeding of C57Bl/6J mice has been used to induce the phenotypes of T2D, however the length of time necessary for different phenotypes associated with T2D varies. Feeding times of 16-20 weeks are recommended to observe obesity, insulin resistance, and β-cell defects. This is supported by two recent studies, where 12-14 weeks of high fat feeding of C57Bl/6J mice resulted in the presence or absence of insulin secretion defects. Since our previous study did not directly measure β-cell insulin secretion or mass, we are unable to determine if β-cell defects were present after the feeding regimen. Future studies to translate our findings into a T2D model with β-cell dysfunction are planned.

In addition, our data demonstrate that culture of the INS-1 832/13 cells with the oligomeric, polymeric and cocoa extract resulted in decreased glucose stimulated insulin secretion. As the purpose of this study was to determine if insulin secretion could be enhanced, we have not yet pursued a mechanism by which culture with the three other fractions inhibited insulin secretion. These studies are currently ongoing.

Our data demonstrate Nrf2 nuclear localization after culture with cocoa monomer. Nrf2 is typically found in the cytosol bound to Keap1. When confronted with reactive oxygen species,
Nrf2 can be liberated to migrate to the nucleus and upregulate transcription of antioxidant genes. Flavanols have been shown to present a mild generation of reactive oxygen species [48]. This relatively small oxidative burden can lead to disruptive interactions with Keap1 cysteine thiol bonds, allowing Nrf2 translocation and initiation of an antioxidant response. It is important to note, however, that Nrf2 translocation can be induced through non-oxidative stress mediated pathways. MAPKs and PI3K have both been shown to phosphorylate Nrf2 and mediate its nuclear translocation [49]. Furthermore, INS-1E cells cultured with epicatechin have been shown to induce no increase in reactive oxygen species [50]. While our data clearly show that Nrf2 translocates to the nucleus, our data do not define the mechanism by which this occurs. Therefore it is possible that monomeric catechins activate MAPKs or PI3K which results in Nrf2 phosphorylation, stabilization and nuclear localization.

In addition to ARE-promoter binding and subsequent upregulation of antioxidant genes, Nrf2 has been shown to upregulate NRF-1 and GABPA, integral transcription factors in mitochondrial biogenesis [51-54]. Activation of NRF-1, in particular, has shown to be sufficient to induce mitochondrial biogenesis through significant upregulation of Tfam [22, 23, 55, 56].

An exciting finding of this paper is that the monomeric fraction was found to have the greatest bioactivity in β-cells, compared to the larger procyanidins. This is particularly important, given the fact that monomeric flavanols have much greater oral bioavailability than oligomeric and polymeric forms [57-59]. Therefore, the finding that the most bioavailable cocoa flavanols are also the most bioactive in this context suggests great potential for translation from *in vitro* cell culture to *in vivo* efficacy in animals and humans. While our data clearly show an effect of monomeric flavanols, they do not negate the possibility that the larger unabsorbed dietary flavanols may still have systemic effects due to their transformation to bioavailable microbial
metabolites by the commensal gut microbiota[60-62]. Therefore, while monomeric cocoa flavanols appear to have the greatest impact on β-cell function of the native flavanols, the impact of these microbial metabolites remains unknown. These metabolites have been identified as a potential “missing link” between dietary polyphenols with potent activities despite poor systemic bioavailability[63]. Therefore, investigations are warranted to elucidate whether the systemic impacts of these cocoa flavanols in vivo, particularly the larger compounds, are mediated by microbial biotransformation in the gut. These studies are currently ongoing.

As has been noted, prolonged T2D is characterized by a reduction in functional β-cell mass. While insulin therapy is the predominant treatment, islet transplantation has shown promise along with pharmacological intervention aimed at preserving and enhancing residual β-cell mass. We have demonstrated that this cocoa monomer fraction promotes β-cell stability and enhances function through upregulation of key genes encoding antioxidant and mitochondrial respiratory complex proteins. These pathways could be leveraged to provide ex-vivo islet expansion and transplantation or cocoa-derived medications that enhance insulin secretion. In this way, cocoa flavanols may provide a novel approach to ameliorating the effects of T2D.

Disclosure

The authors have declared no conflict of interest.

Acknowledgments

The authors would like to thank Drs. Joshua L. Andersen and Jason D. Kenealey, Brigham Young University, Provo, Utah for their guidance and discussion regarding the presented research. We thank all members of the Tessem laboratory for constructive discussion and analysis of data. We thank the American Diabetes Association (1-17-IBS-101 to JST), the
Diabetes Action Research and Education Foundation (grant to JST), BYU MEG program (grant to JST) and the BYU ORCA program (grant to BFB) for funding.
FUTURE DIRECTION

Having found that monomeric cocoa catechins enhance glucose-stimulated insulin secretion at a concentration of 25 ug/mL, our recent study focused on that concentration with regard to other aspects of beta cell function, such as proliferation and protection against apoptosis-inducing factors. Currently, our research group is investigating the effects of a wide range of cocoa monomer, oligomer, polymer fraction and whole cocoa extract concentrations on beta cell proliferation and protection against palmitate. This will provide a broader understanding of the impact of different cocoa fractions on functional beta cell mass. Such knowledge could then be leveraged in applications such as ex-vivo islet expansion, where researchers could treat transplant-ready islets with an ideal concentration of a beneficial cocoa fraction in order to increase the amount of available islets for T2D patients.

Oligomeric and polymeric procyanidins did not enhance glucose-stimulated insulin secretion in this study, but our research group has shown in previous in vivo studies that these larger fractions are associated with greater protection against diabetes onset when compared to monomeric forms. Although monomeric cocoa catechins have a much higher bioavailability than larger cocoa fractions, their effect in vivo could be explained by their transformation to bioavailable metabolites by microbiota in the gut. To pursue this theory, our research group is currently investigating the impact of different cocoa breakdown metabolites on beta cell viability and function.

Transition to animal studies represents the next step in assessing the impact of various cocoa fractions on diabetic symptoms in vivo.
REFERENCES

Manuscript one references


Manuscript two references


APPENDIX

Supplementary information

For

Monomeric cocoa catechins enhance β-cell function by increasing mitochondrial respiration

Running Title: Monomeric cocoa catechins enhance β-cell function


a Department of Nutrition, Dietetics and Food Science, Brigham Young University, Provo, UT
b Department of Physiology and Developmental Biology, Brigham Young University, Provo, UT
c Department of Food Science and Technology, Virginia Tech, Blacksburg, VA
d Current affiliation: University Hospitals, Cleveland, OH
e Current affiliation: DuPont Crop Protection, Newark, DE
f Current affiliation: SPF North America Inc., Greenville, SC
g Current affiliation: UC Irvine School of Medicine, Irvine, CA
h Corresponding author at: Brigham Young University; Department of Nutrition, Dietetics and Food Science; BYU ESC S-243, Provo, UT 84602; Tel.: +1-801-422-9082, Fax: +1-801-422-0258; E-mail address: jeffery_tessem@byu.edu (J.S. Tessem).
i Grants, sponsors and funding sources: J.S. Tessem has funding support from the Diabetes, Action, Research and Education Foundation and the American Diabetes Association. Funding for this work was provided, in part, to A.P. Neilson and S.F. O’Keefe by the Virginia Agricultural Experiment Station and the Hatch Program of the National Institute of Food and Agriculture, U.S. Department of Agriculture.
MATERIALS AND METHODS

All methods were originally described in Dorenkott et al. [1].

Cocoa Flavanol Extraction. A flavanol-rich cocoa extract (CE) was produced following the methods of Adamson et al. [2] and Robbins et al. [3] with modifications. Commercially-available non-alkalized natural cocoa powder (The Hershey Co., Hersey, PA) was defatted by dispersing 100 g cocoa powder in 400 mL hexane, sonication (10 min), stirring (5 min) and centrifugation (5 min, 20 °C, 5,000 x g). The supernatant was discarded and the extraction repeated. Residual hexane was evaporated from defatted cocoa at room temperature. Flavanols were extracted by dispersing defatted cocoa in 400 mL acetone: water: glacial acetic acid (70:28:2 v/v/v) solution, sonication (10 min), stirring (5 min) and centrifugation (5 min, 20 °C, 5,000 x g). The supernatant was collected and the extraction was repeated. The supernatants were pooled, acetone was evaporated using a rotary evaporator (40-45 °C), and the remaining extract (predominantly water) was frozen at −80 °C and freeze-dried for ≥2 d. After freeze-drying, CE was crushed into a powder, weighed, and stored at −80 °C. The final extraction yield from 100 g cocoa powder was ~12.8 g CE.

Cocoa Extract Fractionation. CE was fractionated by solid phase extraction to produce three fractions with different mDP values (monomers, oligomers, and polymers) according to the method used by Sun et al. [4] with modifications. A tC18 Sep-Pak SPE column (20 cc, 5 g sorbent) (Waters, Milford, MA) was attached on top of a C18 Sep-Pak SPE column (20 cc, 5 g sorbent). The columns were preconditioned with 10 mL methanol (MeOH) followed by 10 mL distilled, deionized water (ddH2O, pH 7.0) on a vacuum manifold. CE was dissolved in acetone: water: glacial acetic acid (70:28:2 v/v/v) to 0.1 g/mL and 1.5 mL of the CE solution was loaded onto the column. Highly polar compounds (primarily phenolic acids) were eluted with 10 mL ddH2O and discarded. Monomers and oligomers were eluted together with 35 mL ethyl acetate, concentrated
by rotary evaporation, and set aside. The polymer fraction was then eluted with 40 mL MeOH, concentrated by rotary evaporation, dispersed in ddH₂O and freeze-dried as described above until a dark brown powder remained. A new set of tC₁₈ Sep-Pak + C₁₈ Sep-Pak columns was then preconditioned, and the combined monomer/oligomer fraction (dissolved in 1.5 mL of the acetone: water: glacial acetic acid solution) was loaded onto the column. Monomers were eluted with 35 mL diethyl ether, concentrated by rotary evaporation, dispersed in ddH₂O and freeze-dried until a light brown powder remained. Oligomers were eluted with 40 mL MeOH, concentrated by rotary evaporation, dispersed in ddH₂O and freeze-dried until a cream-colored powder remained. All three fractions were stored at −20 °C for 2 d, weighed, and stored −80 °C. An average of ~155 mg monomer, ~282 mg oligomer, and ~502 mg polymer were produced from each batch of 10 replicate fractionations (a total of 15 mL containing 1.5 g CE, or 1.5 mL containing 0.15 g CE per replicate). Fractions produced from separate batches were combined to produce a single uniform lot of each fraction.

Normal-Phase HPLC of Cocoa Procyanidins. Cocoa fractionation was further evaluated by normal-phase HPLC profiling [1]. Analyses were performed on an Agilent Technologies (Santa Clara, CA) 1260 Infinity HPLC equipped with a solvent degasser, quaternary pump, an autosampler with temperature control, a thermostat column compartment, and a fluorescence detector (FLD). Separations were carried out using a Develosil Diol column (100 Å, 250 × 4.6 mm, 5 μm particle size) equipped with a Luna HILIC guard column (4 × 3.0 mm ID SecurityGuard cartridge and cartridge holder) (both from Phenomenex, Torrance, CA). The column temperature was 35°C. Binary gradient elution employing 2% acetic acid (v/v) in ACN (phase A) and 2% acetic acid (v/v) and 3% ddH₂O (v/v) in MeOH (phase B) was performed at a flow rate of 1 mL/min. The gradient was as follows: 93% A at 0 min, 93% A at 3 min, 62.4% A at 60 min, 0.0% A at 63 min,
0.0% A at 70 min, 93.0% A at 76 min, 7.0% B at 0 min, 7.0% B at 3 min, 37.6% B at 60 min, 100.0% B at 63 min, 100.0% B at 70 min, and 7.0% B at 76 min. FLD excitation and emission wavelengths were 230 nm and 321 nm, respectively. CE and cocoa fractions were prepared at 10 mg/mL in acetone: water: acetic acid (70:28:2, v/v/v) immediately prior to analysis. All the samples and standards were held at 5 °C in the autosampler before injection. Injection volume was 5 μL. Mixtures of authentic standards consisting of monomers (DP 1: C, EC, ECG), PC oligomers (dimers-hexamers), and PC polymers (heptamers-decamers) were prepared and used as a reference for comparison of elution profiles.

UPLC-MS/MS Analysis of Cocoa Procyanidins. CE and cocoa fractions were analyzed by UPLC-MS/MS (n=3) to quantify individual monomer and procyanidin species up to decamers [1]. CE and cocoa fractions were prepared by diluting to 0.05 mg/mL in 0.1% formic acid in water/0.1% formic acid in ACN (95:5). UPLC separations and MS/MS analyses were performed on the Acquity UPLC-TQD and UPLC HSS T3 column described above (2.1 mm × 100 mm, 1.8 µm particle size). Column temperature, sample temperature, mobile phases, flow rate, gradient, ESI parameters, MS/MS CID settings, data collection parameters, and Intellistart tuning of authentic standards to generate MRM functions were the same as described above. MRM parameters for monomers and PCs are listed in Table S1.
Table S1. MS/MS Settings for MRM Detection of Monomers and Procyanidins.

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<th>Compound</th>
<th>( t_R ) (min)</th>
<th>MW (g mol(^{-1}))</th>
<th><a href="m/z">M – H(^{-})</a></th>
<th>Daughter Ion (m/z)</th>
<th>Cone Voltage (V)</th>
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\(^a\) retention time

\(^b\) All MRMs used singly-charged parent ions except for cinnamtannin tetramer A\(_2\), pentamers, hexamers, heptamers, octamers, which were doubly-charged ([M – 2H]\(^{2+}\)), and nonamers and decamers, which were triply-charged ([M – 3H]\(^{3+}\))

\(^c\) likely procyanidin dimers B\(_3\), B\(_4\), and either B\(_6\), B\(_7\) or B\(_8\)

The levels of PCs with DP 1-6 in CE and cocoa monomer, oligomer, and polymer fractions are shown in Fig. S1. CE contains moderate levels of monomers, dimers, and DP 3-6 compared to each enriched fraction. The monomeric PC fraction is enriched for monomers and dimers, but depleted of DP 3-6, compared to CE. The oligomeric PC fraction is depleted of monomers, but enriched for dimers and DP 3-6, compared to CE. The polymeric PC fraction was depleted for monomers, dimers and DP 3-6 and slightly enriched for PCs DP 7-10 compared to CE. As the
reverse-phase UPLC analysis only quantified PCs up to DP 10, the composition of larger PCs that make up a large portion of CE and the majority of the polymer fraction (see Fig. S2) are not reflected in the total composition values in Fig. S1.

Normal-phase HPLC elution profiles of CE and PC fractions are shown in Fig. S2. In this method, retention time roughly correlate to DP (larger DP elute later, see elution profiles of standards, top of Fig. S2), confirming that monomeric, oligomeric, and polymeric fractions are enriched for smaller, intermediate, and larger PCs, respectively. This also confirms the presence of larger PCs in the polymeric fraction that were not detected in Fig. S1.

Figure S1. Levels of procyanidins in cocoa extract (CE) and individual cocoa fractions. Data reported as mean ± SEM from \( n=3 \) replicates. Treatments with different letter superscripts are significantly different (\( P<0.05 \), one-way ANOVA with Fisher’s LSD post-hoc test). Note that the polymer fraction contains the lowest levels of all groups of compounds, since polymers were not quantified; the presence of these large, non-quantified compounds was confirmed by normal-phase HPLC (see Fig. S2). Data adapted with permission from Dorenkott, Melanie R., et al. "Oligomeric cocoa procyanidins possess enhanced bioactivity compared to monomeric and polymeric cocoa procyanidins for preventing the development of obesity, insulin resistance, and impaired glucose tolerance during high-fat feeding." *Journal of Agricultural and Food Chemistry* 62.10 (2014): 2216-2227; Copyright 2014, American Chemical Society.
Figure S2. Fluorescence profiles ($\lambda_{ex} = 230$ nm, $\lambda_{ex} = 321$ nm) of normal-phase HPLC chromatograms of authentic standards [monomers, oligomers (DP 2-6), and polymers (DP 7-10)] and the profiles of the cocoa extract, monomer, oligomer, and polymer fractions used in the mouse diets. Note that the scale is different for each chromatogram to emphasize relative peak heights within each trace. The scale for each chromatogram in fluorescence units (LU) is as follows: monomer standards, 740 LU; oligomer standards, 72 LU; polymer standards, 46 LU; CE, 230 LU; monomer-rich fraction, 160 LU; oligomer-rich fraction, 150 LU; polymer-rich fraction, 40 LU. Figure reproduced with permission from Dorenkott, Melanie R., et al. "Oligomeric cocoa procyanidins possess enhanced bioactivity compared to monomeric and polymeric cocoa procyanidins for preventing the development of obesity, insulin resistance, and impaired glucose tolerance during high-fat feeding." *Journal of Agricultural and Food Chemistry* 62.10 (2014): 2216-2227; Copyright 2014, American Chemical Society.
Table S2. Media concentrations of measured cocoa flavanols in the highest concentration employed in cell culture studies (25 μg extract or fraction/mL media)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Media concentrationa (nM)</th>
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<th>Monomer</th>
<th>Oligomer</th>
<th>Polymer</th>
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a25 μg extract or fraction/mL; media concentrations calculated from extract/fraction concentrations reported in Dorenkott, Melanie R., et al. "Oligomeric cocoa procyanidins possess enhanced bioactivity compared to monomeric and polymeric cocoa procyanidins for preventing the development of obesity, insulin resistance, and impaired glucose tolerance during high-fat feeding." Journal of Agricultural and Food Chemistry 62.10 (2014): 2216-2227; Copyright 2014, American Chemical Society.
REFERENCES


