UNIVERSITY OF CALIFORNIA, MERCED

Cannabidiol Compounds Correct Multiple Cluster Factor Conditions of Metabolic Syndrome

A dissertation submitted in partial satisfaction of the requirements for the degree
Doctor in Philosophy

In

Quantitative and Systems Biology

By

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The dissertation of Jessica N. Wilson is approved and is acceptable in quality and form for publication on microfilm and electronically.

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List of Abbreviations

3MH	3-methylhistdine
4HNE	4-hydroxynonenal
Acox1	acyl-CoA oxidase 1
Akt	protein kinase B
Aldo	aldosterone
AMPK	AMP-activated protein kinase
Ang1-7	angiotensin 1-7
AngII	angiotensin II
ApoB	apolipoprotein B
AT_1	angiotensin receptor type I
AUC	area under the curve
BAIBA	3(β)-aminoisobutyric acid
BM	body mass
CBD	cannabidiol
CD36	cluster of differentiation 36
CPT1A	carnitine palmitoyltransferase 1A,
Cr	creatinine
CVD	cardiovascular disease
DGAT1	diacylglycerol O-acyltransferase 1
DSI	Data Sciences International
Epi	epididymal fat
FATP2	fatty acid transport protein 2
FATP5	fatty acid transport protein 5
FBG	fasting blood glucose
FI	food intake
FM	fat mass
GLUT4	glucose transporter type IV
GPAM	glycerol-3-phosphate acyltransferase 1
GSK3	glycogen synthase kinase 3
H2CBD	8,9-dihydrocannabidiol
H4CBD	1,2,8,9-tetrahydrocannabidiol
HO-1	hemeoxygenase 1
HPLC	high-performance liquid chromatography
IkBα	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, *alpha*
IL1β	interleukin 1 beta
IL6	interleukin 6
IR	insulin receptor
IRI	insulin resistance index
Keap1	kelch-like ECH associated protein 1
LBM	lean body mass

Lep/Adi	leptin to adiponectin ratio
LETO	Long Evans Tokushima Otsuka rat
LM	lean muscle
LV	left ventricle/ventricular
Mas1	mitochondrial assembly 1
MetS	metabolic syndrome
MS	mass spectrophotometry
NAFLD	non-alcoholic fatty liver disease
NEFA	non-esterified fatty acid
NF-kB	nuclear factor kappa B
Nrf2	nuclear factor erythroid 2-related factor 2
oGTT	oral glucose tolerance test
OLETF	Otsuka Long Evans Tokushima Fatty rat
pAkt	phosphorylated protein kinase B
pAMPK	phosphorylated AMP-activated protein kinase
pIR	phosphorylated insulin receptor
PRDX6	peroxiredoxin 6
Retro	retroperitoneal fat
SBP	systolic blood pressure
T2DM	type II diabetes
TBW	total body water
TG	triglycerides
TNFα	tumor necrosis factor alpha
TP	total protein

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Curriculum Vitae

EDUCATION

Doctor of Philosophy University of California, Merced	December 2022
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Associate of Arts in Natural Sciences (Walnut, CA)	May 2012
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RESEARCH EXPERIENCE

Graduate Student Researcher University of California, Merced May 2019-Fall 2022

- Laboratory of Rudy M. Ortiz, PhD, FAPS, FAHA
- Determine the ability of cannabidiol (CBD) to affect hypertension in a metabolic syndrome rat model.

Graduate Student Researcher University of California, Merced August 2017-May 2019

- Laboratory of Kirk Jensen, PhD
- Dissected the extracellular parasite moiety required for immunity to secondary exposure in a resistant and a susceptible murine host model.

Graduate Student Researcher of Parasite Burden CSU, Fresno August 2015-May 2017

- Laboratory of Paul Crosbie, PhD
- Investigated the endoparasitic burden of the endangered San Joaquin Valley Kit Fox.

Undergraduate Researcher of Parasite Burden CSU, Fresno April 2014-August 2015

- Laboratory of Paul Crosbie, PhD
- Investigated the endoparasitic burden of the endangered San Joaquin Valley Kit Fox.

Undergraduate Researcher of Cancer Metabolism CSU, Fresno October 2012-April 2014

- Laboratory of Laurent Dejean, PhD
- Investigated the consequences of the over-expression of specific Bcl-2 Family proteins on the glycolytic pathway in an *in vitro* model of Non-Hodgkin's Lymphoma.

PUBLICATIONS

1. **Wilson JN**, Shevchenko N, Mascal M, Lund K, Fitzgerald R, Fiehn O, Newman J, DiPatrizio NV and Ortiz RM. *Working title: Effects of cannabidiol compounds (CBD and H2CBD) on primary metabolites during metabolic syndrome.* In progress.
2. **Wilson JN**, Shevchenko N, Mascal M, Lund K, Fitzgerald R, DiPatrizio NV and Ortiz RM. *Working title: Cannabidiol (CBD) reduces arterial pressure independent of RAS*. In progress.

3. **Wilson JN**, Shevchenko N, Mascal M, Lund K, Fitzgerald R, DiPatrizio NV and Ortiz RM. *Working title: Hydrogenated cannabidiol (H4CBD) effects on renal function.* In progress.
4. **Wilson JN**, Shevchenko N, Mascal M, Mendez DA, Lund K, Fitzgerald R, DiPatrizio NV and Ortiz RM. Cannabidiol compound (H4CBD) reduced body mass and visceral adiposity and improved lipid metabolism in advanced metabolic syndrome (OLETF) rats. Metabolism. *In revision*.
5. **Wilson JN**, Shevchenko N, Mascal M, Lund K, Fitzgerald R, DiPatrizio NV and Ortiz RM. Cannabidiol compound (H4CBD) improves glucose tolerance and insulin resistance during advanced metabolic syndrome in OLETF rats independent of sustained increase in insulin signaling. American Journal of Physiology: Regulatory, Integrative and Comparative Physiology. *In review.*
6. Souza SP, Splitt SD, Sanchez-Arcila JC, Alvarez JA, **Wilson JN**, Wizzard S, Luo Z, Baumgarth N, Jensen KDC. Genetic mapping reveals Nfkbid as a central regulator of humoral immunity to Toxoplasma gondii. PLOS Pathogens. In press.
7. **Wilson JN**. More Is Better: Resistant and Susceptible Mouse Model Reveals *Toxoplasma gondii* Glycophosphatidylinositol Anchor to be a Common Natural Antibody Epitope. eScholarship. In press.

ORAL PRESENTATIONS

University of California, Doctoral Dissertation Defense (Passed) *Fall 2022*
Cannabidiol Compounds Correct Multiple Cluster Factor Conditions of Metabolic Syndrome.

California Society for Physiologist (APS Chapter) *Fall 2022*
CBD compounds preserve brown adipose tissue via increase in BAIBA. *Awarded 2nd place for best pre-doctoral presentation.*

Closed Seminar for ChemoMetec *Fall 2022*
CBD compounds correct multiple cluster factor conditions of metabolic syndrome.

Metabolomic Workshop *Fall 2022*
Metabolite Mapping Basics: From data receipt to network map. Explanation of MetaMapp and Cytoscape.

Guest Lecturer University of California, Merced *Fall 2022*
Human Physiology (BIO161): History of Physiology and Renal Function.

Medical College of Wisconsin Hypertension Group Seminar *Fall 2022*
Invited speaker seminar/Post-doctoral interview. CBD compounds correct multiple cluster factor conditions of metabolic syndrome.

Quantitative and Systems Biology Program Retreat *Spring 2022*

Invited speaker; "CBD: Wonder Drug or Dud?"

University of California Grad Slam ("CBD: Wonder Drug or Dud?") *Spring 2022*
UCOP Competition; represented Merced campus

Molecular and Cell Biology Research in Progress Seminar *Spring 2022*
Cannabidiol (CBD) Compounds Correct Multiple Cluster Factor Conditions of Metabolic Syndrome.

University of California Merced Grad Slam *Spring 2022*
Campus Champion; "CBD: Wonder Drug or Dud?"

Center for Medical Cannabis Research Symposium *Spring 2022*
Cannabidiol (CBD) reduced arterial blood pressure and body mass and improved insulin resistance during metabolic syndrome in OLETF rats.

Guest Lecturer University of California, Merced Fall 2021
Human Physiology (BIO161): Energy Metabolism.

California Society of Physiologists Inaugural Meeting (USC) *Fall 2019*
More Is Better: Resistant and Susceptible Mouse Model Reveals *Toxoplasma gondii* Glycophosphatidylinositol Anchor to be a Common Natural Antibody Epitope

University of California, Merced Thesis Defense (Passed) *Spring 2019*
More Is Better: Resistant and Susceptible Mouse Model Reveals *Toxoplasma gondii* Glycophosphatidylinositol Anchor to be a Common Natural Antibody Epitope

Central California Research Symposium *Spring 2017*
Endoparasite Burden of the Endangered San Joaquin Valley Kit Fox
Awarded Best Graduate Oral Presentation ($250 Award)

POSTER PRESENTATIONS

Gordon Research Conference on Angiotensin (Ventura, CA) *Fall 2022*
Cannabidiol (CBD) Attenuates Metabolic Syndrome-Associated Hypertension.

Gordon Research Seminar on Angiotensin (Ventura, CA) *Fall 2022*
Cannabidiol (CBD) Attenuates Metabolic Syndrome-Associated Hypertension.

California Society for Physiologist (APS Chapter) *Fall 2022*
CBD compounds preserve brown adipose tissue via increase in BAIBA.

International Society of Hypertension (Kyoto, Japan) *Fall 2022*
Oral cannabidiol reduces arterial blood pressure during early phase metabolic syndrome in OLETF rats. **Wilson JN**, DiPatrizio N., Ortiz RM.

Center for Medical Cannabis Research Symposium (Virtual) *Spring 2022*

Cannabidiol (CBD) reduced arterial blood pressure and body mass and improved insulin resistance during metabolic syndrome in OLETF rats. **Wilson JN**, Lund K, Fitzgerald R, Ortiz RM.

Experimental Biology (Philadelphia, PA) *Spring 2022*

Hydrogenated Cannabidiol Reduces Body Mass and Visceral Adiposity But Not Blood Pressure in Rats With Advanced Metabolic Syndrome. **Wilson JN**, Mendez DA, Mascal M, Fitzgerald R, Ortiz RM.

Central California Research Symposium *Spring 2014*

Bcl-2 Family protein over-expression differentially effects glycolysis, oxidative phosphorylation and lactate dehydrogenase production.

California State University Program for Education and Research in Biotechnology *Spring 2013*

Bcl-2 Family protein over-expression differentially effects glycolysis, oxidative phosphorylation and lactate dehydrogenase production.

Central California Research Symposium *Spring 2013*

Bcl-2 Family protein over-expression differentially effects glycolysis, oxidative phosphorylation and lactate dehydrogenase production.

MENTORED POSTERS

SACNAS (Virtual) Conference *Summer 2021*

5-weeks Oral Cannabidiol Reduces Systolic Blood Pressure in Metabolic Syndrome (OLETF) Rats Authors: Jennifer X. Mendez, **Jessica N. Wilson M.S**. and Rudy M. Ortiz Ph.D

Undergraduate Research Summer (Virtual) Symposium (UCSD) *Summer 2021*

5-weeks Oral Cannabidiol Reduces Systolic Blood Pressure in Metabolic Syndrome (OLETF) Rats Authors: Jennifer X. Mendez, **Jessica N. Wilson M.S**. and Rudy M. Ortiz Ph.D

Undergraduate Research Opportunity Center Summer (Virtual) Symposium (UCM) *Summer 2021*

5-weeks Oral Cannabidiol Reduces Systolic Blood Pressure in Metabolic Syndrome (OLETF) Rats Authors: Jennifer X. Mendez, **Jessica N. Wilson M.S**. and Rudy M. Ortiz Ph.D

Undergraduate Research Summer (Virtual) Symposium (UCSD) *Summer 2021*

5-weeks Oral Cannabidiol Improved Blood Glucose Tolerance in OLETF Rats

Authors: Erick Macario, **Jessica N. Wilson M.S**. and Rudy M. Ortiz Ph.D

Undergraduate Research Opportunity Center Summer (Virtual) Symposium *Summer 2021*
5-weeks Oral Cannabidiol Improved Blood Glucose Tolerance in OLETF Rats
Authors: Erick Macario, **Jessica N. Wilson M.S**. and Rudy M. Ortiz Ph.D

Undergraduate Research Opportunity Center Summer (Virtual) Symposium *Summer 2021*
Brown Adipose Tissue Abundance Increased in Cannabidiol-Treated OLETF Rats
Authors: Suzzy Emily Campero, **Jessica N. Wilson M.S**. and Rudy M. Ortiz Ph.D

AWARDS & SCHOLARSHIPS

Best Pre-Doctoral Presentation 2nd Place (CASP) *Fall 2022*

Travel Grant for California Society for Physiologists (CASP) *Fall 2022*
Conference Attendance ($250)

QSB Travel Fellowship ($1,000) *Fall 2022*
Funds to facilitate travel to conference (ISH2022)

Fellowship Incentive Award ($200) *Fall 2022*
Award for application to external funding source above $12k.

ISH2022Kyoto Travel Grant ($600) *Fall 2022*
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University of California Merced Grad Slam Champion ($5000) *Spring 2022*
TED talk style 3-minute presentation on dissertation research

QSB Graduate Student Research Improvement Award ($300) *Spring 2022*
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University of California Merced Grad Slam Finalist ($250) *Spring 2022*
TED talk style 3-minute presentation on dissertation research

QSB Travel Fellowship ($1,000) *Spring 2022*
Funds to facilitate travel to conference (EB 2022)

Quantitative and Systems Biology Remote Teaching and Research Fellowship *Summer 2021*
The Remote Teaching and Research Fellowship is designed as a recognition for the extraordinary effort QSB graduate students have demonstrated in the 20/21 academic year. $250

Center for Medical Cannabis Research, Pilot Research Award to RMO *Fall 2020*
Title: Effects of CBD on blood pressure and substrate metabolism during metabolic syndrome
Program: Center for Medicinal Cannabis Research, Pilot Application

Primary Investigator: Rudy M. Ortiz, PhD, FAPS, FAHA
Assisted in authorship.

Quantitative and Systems Biology Summer Fellowship *Summer 2020*
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Declined to pursue opportunity with UROC.

Graduate Group Recruitment Fellowship *Fall 2017*
$5,000 stipend awarded to recruit and support the best PhD students with distinguished academic records to University of California, Merced's Quantitative and Systems Biology Graduate Program.

Dean's Relocation Grant *Fall 2017*
$500 to aid in relocation to Merced, CA.

Phi Kappa Phi Honors Society *Spring 2017*
Awarded for academic excellence at California State University, Fresno.
4.0 grade point average for four consecutive semesters.

Best Graduate Oral Presentation *Spring 2017*
Central California Research Symposium
$250 Cash Prize

President's Award for Volunteer Service *Fall 2012*
$2,500 grant awarded for 2,000 hours of outstanding service to the community.
Community service project served military veterans and children with special needs through equine therapy.

AFFILIATIONS

American Physiological Society: Graduate Student Member 00323365 (2021-2022)

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Nicotine and Cannabis Policy Center Member (2019-2022)

California Society for Physiologists Member (APS Chapter) (2019-2022)

Phi Kappa Phi Honors Society Member (2017-*lifetime*)

Cannabidiol Compounds Correct Multiple Cluster Factor Conditions of Metabolic Syndrome

Jessica N. Wilson
Doctor of Philosophy in Quantitative and Systems Biology
University of California, Merced, 2022
Chair: Dr. Rudy M. Ortiz

Abstract

Cardiovascular disease (CVD) is the leading cause of death in the United States and is a primary consequence of metabolic syndrome (MetS)[1–5]. MetS is progressive and diagnosed by the presentation of at least 3 of 6 cluster conditions, including obesity, dyslipidemia and elevated arterial pressure, which can contribute to the development of CVD[2,3]. Therefore, identification of treatments that beneficially target multiple cluster factor conditions of MetS are of great interest. Chronic *Cannabis* consumption has been associated with reduced MetS risk factors[6–12]. The most abundant constituent, cannabidiol (CBD), is of particular interest for pharmacological investigation due to recognized antioxidative properties and non-intoxicating, wide dose range. Clinical and pre-clinical studies using CBD are sparse but CBD has been shown to attenuate isolated conditions associated with MetS (e.g. hypertension, obesity). However, it is unclear if these effects would be observed in a translational model of cluster factor conditions (e.g., diabetes with insulin resistance, dyslipidemia, etc.). The Otsuka Long-Evans Tokushima Fatty (OLETF) rat is an obese, hypertensive, translational model of MetS that closely mimics the human condition. With this model, we probed the effects of CBD on clustered conditions of MetS. In the second chapter, we demonstrate that a chronic, high dose of oral CBD reduces hypertension in the presence of MetS comorbidities but does not appear to do so via modulation of the renin-angiotensin-aldosterone system (RAAS). In the third chapter, we demonstrate that CBD reduces adiposity and dyslipidemia. Metabolomic analyses suggested a shift in the metabolome and revealed the most significantly increased metabolite to be β-aminoisobutyric acid (BAIBA), which is a novel myokine implicated in the browning of white adipose tissue (WAT) and enhancement of lipolysis. Importantly, we note that CBD compounds do not correct the diabetic phenotype, despite reductions in adiposity, even in more severely impaired metabolism, as demonstrated in Chapter 4 and 5. Our findings contribute to the expanding pharmacology of CBD compounds and reveal novel implications for the use of CBD compounds in conditions of metabolic dysfunction.

Chapter 1: Introduction

Cardiovascular disease is a significant health burden

Cardiovascular disease (CVD) is the leading cause of death in the U.S. (1 in 4) and costs approximately $219 billion annually[1]. CVD constitutes an umbrella term for a variety of diseases including left ventricular hypertrophy and dysfunction, myocardial infarction and congestive heart failure[13]. Diagnosis of cardiovascular complications requires costly and burdensome imaging, emphasizing the importance of early diagnosis and intervention. The development of CVD is complex but risk factors have been identified to be high blood pressure (hypertension), high blood cholesterol, and smoking and, of these, 47% of Americans have at least one risk factor[5]. The presentation of hypertension with comorbidities, like obesity, significantly contributes to the cardiovascular risk of patients[2–4]. CVD is a primary outcome of metabolic syndrome (MetS)[2–4], which is conservatively estimated to effect 24% of the U.S. population[4,14]. MetS is characterized by the simultaneous presentation of 3 of 6 defining conditions, which include high blood pressure and poor glucose tolerance[2,3]. As a cluster factor condition, determination of the primary etiological event of MetS or CVD is difficult, but because MetS is associated as a precursory condition to multiple life-threatening diseases (e.g., CVD type II diabetes, etc.), MetS is a viable target for pharmaceutical intervention and therefore a key component of pre-clinical investigations of therapeutic approaches for the prevention of hypertension and later onset CVD.

CVD is a primary outcome of metabolic syndrome.

CVD is a primary outcome of metabolic syndrome (MetS)[1–4], which is conservatively estimated to effect 24% of the U.S. population[4,14]. MetS is characterized by the simultaneous presentation of 3 of the 6 defining conditions: **1)** abdominal obesity, **2)** atherogenic dyslipidemia, **3)** elevated arterial pressure, **4)** glucose intolerance ± insulin resistance, **5)** pro-inflammatory state, and **6)** prothrombotic state[2,3]. MetS is also associated with T2DM as a precursory condition, which is estimated to afflict 30 million Americans (1 in 10) as of 2019[15], but is distinguished by loss of insulin sensitivity and secretion[16]. Because MetS is associated as a precursory condition to multiple life-threatening diseases, MetS is a viable target for pharmaceutical intervention. That said, the specific cause for spontaneous metabolic dysfunction is unknown and the complex, multi-system contributions toward disease progression makes intervention difficult. Therefore, application of an intervention which beneficially affects multiple systems to slow or stop the progression of MetS would be of great value.

Inappropriately elevated angiotensin II promotes hypertension

Currently, first-line management of hypertension targets the renin-angiotensin-aldosterone system (RAAS)—a homeostatic regulator of blood pressure[13,17]. RAAS is inappropriately elevated during MetS-associated hypertension[18–25]. Of the limited pharmaceutical treatments available, RAAS disruptors only manage high blood pressure and often require concurrent pharmaceutical intervention[17]. Moreover, patients often develop tolerance to individual RAAS disruptors and must be cycled through other drugs in the same class. Unfortunately, none of the drug therapies available reverse hypertension once diagnosed and established. Importantly, there has not been a significant advancement in the pharmaceutical management of CVD in 30 years, since the introduction of statins (low-density lipid synthesis inhibitor), which underscores the urgency for investigation of alternative approaches to treat or prevent CVD[26] associated with hypertension.

Cannabis consumption is associated with lower BMI but rimonabant was a failure.

Chronic *Cannabis* ingestion is commonly associated with lower body mass index (BMI) suggesting that cannabinoids have profound metabolic benefits. An epidemiological study using NHANES data correlated *Cannabis* consumption and lowered risk factors for MetS including lower waist circumference in men, decreased fasting glucose, and reduction of other MetS indicators[12]. However, a similar study from Sweden using male patient data reached an opposing conclusion[27]. The diversity of the phytocannabinoid composition of *Cannabis* makes attribution of any number of pharmacological activities difficult[28]. *Cannabis* contains more than 400 distinct molecular constituents of which, cannabidiol (CBD) is the most abundant at 40% followed by Δ^9-tetrahydrocannabinol (THC) at approximately 20%[29]. The often-unwanted psychoactive properties mediated by THC typically confounds the preference for whole *Cannabis* ingestion as a means of treatment for various conditions. Efforts to find the receptor that mediates THC activity lead to the discovery of the endogenous cannabinoid system (ECS) receptors CB1 and CB2 in the 1980's[30–32]. Since then, multiple endeavors to manipulate the ECS to pinpoint the mechanism by which *Cannabis* reduces BMI have been realized[33–42]. The most notable was rimonabant (SR141716A), which blocked CB1 to significantly reduce overall body mass, waist circumference and improved lipid and glucose metabolism, thereby reducing multiple risk factors for MetS, T2DM, and CVD[43–45]. Unfortunately, the beneficial actions of rimonabant were substantially overshadowed by intense, negative psychological side-effects, which resulted in the abandonment of the entire class of compounds[46,47]. Despite the failure of rimonabant, there remains an active interest in the ECS and *Cannabis* constituents for the purpose of favorably modulating metabolism.

CBD does not directly engage the ECS

The investigation of cannabis constituents, particularly THC, have led to the discovery of the endocannabinoid system (ECS)[30,31]. These highly conserved cannabinoid receptors type 1 and type 2, CB1 and CB2, respectively, are receptor proteins categorized under the extensive G-coupled protein receptor family and located on the cell surface of all tissues tested but especially concentrated in nervous tissue[29], as well as the small intestine in conditions of increased adiposity[48]. Importantly, CBD acts as an inverse agonist due to its weak affinity for the cannabinoid receptors[32,49] and engagement of these receptors occurs primarily via endogenous cannabinoid ligands anandamide (AEA) and 2-archidonylglycerol (2-AG). CBD acts to elevate systemic AEA by inhibiting the enzyme responsible for endocannabinoid ligand breakdown, fatty acid amine hydrolase (FAAH)[50]. That said, direct receptor targets of CBD constitute an active area of research. Candidates for a third endocannabinoid receptor include GPR55, GPR12, GPR6 and GPR3[51–55]. CBD is also known to differentially engage transient receptor potential villanoid type 1 (TRPV1) and the serotonin receptor, 5-HT3, which have been found to have a hypotensive effect[56,57]. These receptors have been implicated in a rapid, transient release of calcium suggesting that they may contribute to cardiac contraction. Indeed, acutely administered CBD reduced arrhythmia and infarct size in models of ischaemia[58,59]. Additionally, CBD demonstrated powerful antioxidant and anti-inflammatory effects, which were implicated in the restoration of left ventricular function in a murine model of type I diabetes[60], vascular endothelial barrier function[61] and cisplatin-induced nephropathy[62]. In addition to direct effects on cardiomyocytes and vasculature, CBD reduced body mass in obesity-prone Wistar rats over ten days of administration[63] and ameliorated the dyslipidemia in murine models[64–66]. However, these effects have not been investigated in the complex condition of MetS. Importantly, these obese murine and rat models do not demonstrate the previously articulated conditions of MetS (section

A2) that are critical for the examination of CBD on MetS-associated hypertension and substrate metabolism. To date, there are no studies that sufficiently model MetS to probe the effects of CBD.

CBD may beneficially modulate metabolism

Observations of cardiovascular and/or metabolic improvements conferred by CBD or CBD-dominant cannabis have prompted inquiry into other applications for CBD[6–10,12]. Clinical trials determined 100mg CBD, twice daily, to be well-tolerated in patients with T2DM and made improvements in circulating resistin and gastric inhibitory peptide but did not reverse the T2DM phenotype[67]. A much higher dose (600mg) of CBD acutely reduced arterial blood pressure in healthy males[68], however, this effect was lost after a 7-day dose regimen[69].

There are sparse pre-clinical data on the effects of CBD in a model of metabolic dysfunction. Natural (extracted) CBD reduced the incidence of diabetes in diet-induced obese (DIO) and non-obese diabetic mice[66,70], which provides some reasonable evidence that CBD has the potential to similarly ameliorate dysregulated metabolism. CBD administered over 4 weeks attenuated dysfunctional hemodynamics and cardiac fibrosis in mice with induced type I diabetes[71]. Yet 2 weeks of 10mg/kg CBD did not reduce arterial blood pressure in spontaneous hypertensive or deoxycorticosterone salt induced hypertensive rats though significant improvements to oxidative stress and lipid metabolism were observed[72]. Taken together, these data suggest that longer duration treatment may be necessary to achieve improvements in hypertension.

Pseudocannabinoids offer Cannabis-free alternatives

The legislative ambiguity and increasing ease-of-access to unregulated cannabis constituents have prompted endeavors to synthetize the cannabis constituent molecule of interest without use of whole plant substrate. Thus, circumventing violation of laws surrounding production of, or with, a Schedule I compound while simultaneously providing a chemically pure compound free from the growing concerns of unregulated pesticide use on whole plants. Synthetically derived pseudocannabinoids, such as dihydrocannabidiol (H2CBD), offer similar effects of natural CBD molecules and have been shown to equally reduce seizure frequency and severity in PTZ-induced rats[73]. Tetrahydrocannabidiol (H4CBD) is a another synthetic pseudocannabinoid that differs by the saturation of the exocyclic carbon-carbon double bond and sole endocyclic carbon-carbon double bond of the attached aromatic ring. Like natural CBD, both H2CBD and H4CBD have little affinity for the endocannabinoid receptors responsible for cannabis intoxication[74].

Considerations of cannabinoid therapies by older adults

The popularity of cannabis use in older adults (>55 years of age) in the United States has more than doubled over the past two decades[75–78]. Conditions for which older adults typically seek medicinal cannabis are often pain-associated[77–79]. A common consequence of aging is impaired substrate metabolism, which could result in the onset of metabolic conditions ranging in severity from risk factors for metabolic syndrome (MetS)[3], like obesity, to frank type II diabetes mellitus (T2DM)[5]. The prevalence of MetS is 55% among people ≥60 years of age[5] and the average age at diagnosis of T2DM is approximately 46 years of age with equal abundance across racial and ethnic groups[80]. The therapeutic risks and benefits of cannabis and cannabinoids in metabolic dysfunction have yet to be defined.

Premise for study

This work is inherently innovative due to the overwhelming gap in the knowledge of the pharmacology of CBD and the paucity of relevant, translational studies to the application of CBD for use in MetS condition. Specifically, to our knowledge, investigation on the effects of chronic, oral administration of CBD on the development and progression of hypertension associated with MetS has not been realized. Moreover, there have been no other studies using CBD, which have adequately modeled the complex metabolic conditions that manifest CVD in humans. Our rat model of MetS has been used extensively by our lab to investigate the mechanisms that perpetuate the MetS condition[23,25,81–88]. This study, therefore, will bring much needed context and potentially yield paradigm shifting results around the therapeutic potential of CBD for the treatment of MetS and MetS-associated CVD. To our knowledge, there are no studies which address the effects of CBD on hypertension and simultaneous cluster factor conditions of MetS.

Chapter 2. Cannabidiol reduces metabolic syndrome-associated hypertension independent of RAAS modulation in OLETF rats

Abstract

Cardiovascular disease (CVD) is the leading cause of death in the U.S. and a primary outcome of metabolic syndrome (MetS). MetS is diagnosed by the presentation of at least 3 of the 6 conditions: 1) abdominal obesity, 2) atherogenic dyslipidemia, 3) elevated arterial pressure, 4) glucose intolerance ± insulin resistance, 5) pro-inflammatory state and 6) prothrombotic state. Pinpointing the root cause of these conditions individually is difficult but identification of treatments that potentially affect multiple conditions of MetS has greater potential to ameliorate later-onset CVD. The therapeutic potential of cannabidiol (CBD) is widely recognized for powerful anti-inflammatory and antioxidative properties, which have been shown to be cardioprotective. However, these effects have not been probed in the context of MetS. Otsuka Long-Evans Tokushima Fatty (OLETF) rats demonstrate a timed progression of MetS that closely mimics the human condition. OLETF rats were implanted with blood pressure monitors [DSI; HD-S10] and administered oral CBD extract (175mg/kg/day) for 5 weeks. Arterial pressure reduced within the first week of dosing (6±1.4mmHg; 4.1%; $p<0.05$ vs. OLETF control) and, after 5 weeks, by 9.7±1.7mmHg (6.5%; $p<0.05$ vs. OLETF control) from baseline. The renin-angiotensin-aldosterone system (RAAS) is a known driver of hypertension in humans with MetS and in the OLETF rat model. Angiotensin II (AngII) levels were 60% lower ($p<0.05$) in OLETF compared to LETO and both CBD and H2CBD increased AngII more than 200% ($p<0.05$) after 5 weeks. Angiotensin 1-7 (Ang 1-7) and aldosterone were not affected by cannabinoid treatment. These data suggest that CBD compounds are likely not capable of modulating classical or non-classical RAAS tone or signal transduction but may promote vasodilatory effects on the vasculature via antioxidant properties or effect on endothelial nitric oxide synthase abundance/activity. Taken together, chronic CBD regimen could provide antihypertensive actions despite MetS cluster factor conditions.

Introduction

Hypertension is a cluster factor condition of metabolic syndrome (MetS)[1–4] and is defined by elevated blood pressure. As of 2021, the World Health Organization (WHO) estimates hypertension to conservatively affect 1.28 billion adults worldwide[89,90]. An estimated 46% of adults with hypertension are unaware of onset and less than half of adults with hypertension are diagnosed and treated[89,90]. Unfortunately, only 1 in 5 adults (21%) with hypertension have it under control[89,90]. Currently, first-line management of hypertension targets the renin-angiotensin-aldosterone system (RAAS)—a homeostatic regulator of blood pressure[13,17]. RAAS is inappropriately elevated during MetS-associated hypertension[18–25]. Of the limited pharmaceutical treatments available, RAAS disruptors only manage high blood pressure and often require concurrent pharmaceutical intervention[17]. Moreover, patients often develop tolerance to individual RAAS disruptors and must be cycled through other drugs in the same class. Unfortunately, none of the drug therapies available reverse hypertension once diagnosed and established. Importantly, there has not been a significant advancement in the pharmaceutical management of CVD in 30 years, since the introduction of statins (low-density lipid synthesis inhibitor), which underscores the urgency for investigation of alternative approaches to treat or prevent CVD[26] associated with hypertension.

Cannabidiol (CBD) is an abundant non-psychoactive constituent of *Cannabis sativa*, which is of particular interest for pharmacological investigation. The legislative ambiguity and increasing ease-of-access to unregulated cannabis constituents have prompted endeavors to synthetize the cannabis constituent molecule of interest without use of whole plant substrate. Thus, circumventing violation of laws surrounding production of, or with, a Schedule I compound while simultaneously providing a chemically pure compound free from the growing concerns of unregulated pesticide use on whole plants. Dihydrocannabidiol (H2CBD) is a synthetic pseudocannabinoid CBD molecule that differs by the saturation of the exocyclic carbon-carbon double bond and sole endocyclic carbon-carbon double bond of the attached aromatic ring. Pseudocannabinoid CBD molecules offer similar effects of natural CBD molecules and have been shown to equally reduce seizure frequency and severity in PTZ-induced rats[73]. Like natural CBD, H2CBD has little affinity for the endocannabinoid receptors responsible for cannabis intoxication[74]. However, use *in vivo* has not been previously realized with this synthetic CBD product but is expected to exert similar effects as natural CBD.

Convincing clinical evidence has been provided for the efficacy of CBD to reduce seizure frequency and severity in children and adults. Epidiolex® is currently the only FDA-approved, cannabis-derived medication for the treatment of Lennox-Gastaut and Dravet syndrome[91]. Tangential observations of cardiovascular and metabolic improvements have prompted inquiry into other applications for CBD[67], for which the greatest interest is held for the treatment of CVD, T2DM and MetS. A relatively high dose (600mg) of CBD acutely reduced arterial blood pressure in healthy males[68], however, this effect was lost after a 7 day dose regimen[69]. CBD is also known to differentially engage transient receptor potential villanoid type 1 (TRPV1) and the serotonin receptor, 5-HT3, which have been found to have a hypotensive effect[56,57]. These receptors have been implicated in a rapid, transient release of calcium suggesting that they may contribute to cardiac contraction. Indeed, acutely administered CBD reduced arrhythmia in models of ischaemia[58,59] and CBD demonstrated powerful antioxidant and anti-inflammatory effects,

which were implicated in the improvement of cardiovascular hemodynamics in a murine model of type I diabetes[60], vascular endothelial barrier function[61] and cisplatin-induced nephropathy[62]. Notwithstanding, 2 weeks of 10mg/kg CBD did not reduce arterial blood pressure in spontaneous hypertensive or deoxycorticosterone salt induced hypertensive rats, though significant improvements to oxidative stress and lipid metabolism were observed[72], which suggests that a longer treatment duration may be necessary to achieve improvements in hypertension.

Therefore, the goals of this study were to assess the effect of CBD compounds on hypertension in a translational model of impaired substrate metabolism akin to MetS. Because of the increasing interest in cannabinoids and the relatively high incidence of MetS, a critical intersection of cannabis constituent use and metabolic dysfunction exists. The Otsuka Long-Evans Tokushima Fatty (OLETF) rat is a monogenic model of diet-induced obesity accelerated by a mutation in the CCK receptor[92]. These rats have a predictable, timed progression toward non-insulin dependent diabetes mellitus (T2DM) (>20 weeks), marked by a linear phase progression of obesity and poor glucose tolerance (8-20 weeks), which closely resembles symptoms displayed by human MetS symptoms. At 15 weeks of age, OLETF rats increasingly obese suffer from severe hypertension and metabolic dysfunction, including visceral adiposity, dyslipidemia, and insulin resistance[92,93]. We hypothesized that a high, chronic dose of CBD would be sufficient to reduce elevated arterial pressure in the presence of MetS cluster factor conditions and that this reduction would likely be mediated by RAAS modulation.

Methods

All animal procedures were reviewed and approved by the institutional animal care and use committees of the University of California, Merced.

Animals

Male lean Long Evan's Tokushima Otsuka (LETO) rats and obese Otsuka Long Evans Tokushima Fatty (OLETF) rats (Japan SLC Inc., Hamamatsu, Japan), both 14 weeks of age were assigned to the following groups (n=7-8/group): **(1)** vehicle-dosed LETO (LETO), **(2)** vehicle-dosed OLETF (OLETF), **(3)** CBD-treated (175mg/kg/day x 5 weeks) OLETF (CBD) and **(4)** H2CBD-treated (175mg/kg/day x 5 weeks) OLETF (H2CBD). Rats were maintained in a specific pathogen-free, climate-controlled facility at the University of California, Merced on a 12-hour light:dark cycle (07:00-19:00 and 19:00-07:00, respectively). All animals had free access to water and were fed rat chow (Teklad Global; fat 9.0%; carbohydrate 44.9%; protein 19.0%) *ad libitum*. Treatment intervention was initiated at 15 weeks of age.

Telemeter implantation

At 13 weeks, LETO (n=3) and OLETF rats (n=6) were implanted with DSI telemeters (HD-S10) into the abdominal cavity to measure systolic blood pressure as previously described[88]. Activity was measured every hour for 15 minutes for the duration of the study. Data were analyzed using A.R.T. software (DSI, St. Paul, MN).

Drug preparation & administration

Cannabidiol extract was obtained from MissU. Both natural CBD extract (~55%) and purified H2CBD (>99%) were separately suspended in sesame oil and administered by oral gavage at a dose of 175mg/kg/day. A similar dose (200mg/kg) has been shown to be similarly effective on seizure frequency and severity in rats compared to natural CBD extract[73], which gave reasonable cause for efficacy of H2CBD at the same dose and is well below documented toxicity of CBD (>600mg/kg) in rodents[94].

Drug bioavailability detection

CBD and H2CBD bioavailability in heparinized plasma was determined via HPLC and MS chromatography as described previously[95].

Tissue collection

After the 5-week study period, animals were fasted overnight, and tissues were collected at sacrifice the following morning. BM was recorded before animals were decapitated and trunk blood was collected, as previously described[23,87,96]. Briefly, animals were fasted overnight and sacrificed by decapitation without sedation. Whole trunk blood was collected into glass collection tubes preloaded with either 1% heparin and 1% protease inhibitor cocktail [Sigma Aldrich] or 1% EDTA with 1% ACEi [Lisinopril] and 1% protease inhibitor cocktail. Plasma was separated via centrifugation (3000RPM x 30 minutes at 4℃) and the supernatant was stored at -80℃ until use.

Plasma analysis

Plasma concentrations of aldosterone (R&D Systems; KGE016), angiotensin 1-7 (Phoenix Pharmaceuticals; EKE-002-01) and angiotensin II (Phoenix Pharmaceuticals; EK-002-12CE), were measured in fasted, end of study plasma samples. All samples were analyzed in duplicate and run in a single assay with intra-assay and percent CV of <10% for all assays.

Statistics

All values are represented as mean ± standard error mean (SEM) unless otherwise indicated. Means were compared by one-way or two-way (if change over time) ANOVA followed by Tukey's honest significant difference or unpaired, one-tailed t-test was to assess significant differences among groups. Means and regressions were considered significant at $p<0.05$. Outliers were detected by ROUT (Q=1.0%) and removed. All statistical procedures were performed using GraphPad Prism 7 (GraphPad Software, Inc., San Diego, CA, USA).

Results

CBD compounds are bioavailable and uniquely detectable

Bioavailability of H4CBD compound was validated in end of study plasma and confirmed that only treated animals received drug and that the dose was sufficient to increase circulating levels (**Figure 1B-C**).

CBD reduces MetS-associated hypertension

Systolic blood pressure (SBP) was monitored throughout the study via radiotelemetry. Arterial pressure reduced within the first week of dosing (6±1.4mmHg; 4.1%; $p<0.05$ vs. OLETF control)

and, after 5 weeks, by 9.7±1.7mmHg (6.5%; $p<0.05$ vs. OLETF control) from baseline (**Figure 2A**).

CBD does not modulate RAAS

End of study plasma was measured for RAAS-modulating peptides. AngII was 60% lower in OLETF compared to LETO ($p<0.05$) (**Figure 2B**). CBD increased plasma AngII over 200% compared to OLETF ($p<0.05$) and H2CBD increased AngII over 290% ($p<0.05$) (**Figure 2B**). Although plasma Ang 1-7 was slightly reduced (non-significant) in OLETF compared to LETO, neither CBD nor H2CBD increased levels (**Figure 2C**). Plasma aldosterone was not changed amongst groups, however the CBD and H2CBD groups demonstrate similar bimodal distribution, which may suggest differential response (**Figure 2D**). Taken together, there is no indication that CBD compounds modulate non-classical RAAS.

Discussion

High blood pressure is a cluster factor condition of metabolic syndrome (MetS)[1–4] and often poorly controlled in adults formally diagnosed with hypertension[89,90]. Importantly, there has not been a significant advancement in the pharmaceutical management of CVD in 30 years, since the introduction of statins (low-density lipid synthesis inhibitor), which underscores the urgency for investigation of alternative approaches to treat or prevent CVD[26] associated with hypertension. Since MetS is associated with development of lethal conditions, such as CVD and T2DM, intervention strategies that affect multiple cluster conditions of MetS are of critical interest. The effects of CBD compounds on metabolic syndrome pathophysiology have not been described though a few studies have shown beneficial[60,63] or null effects[72] of CBD on isolated MetS risk factors. However, these effects have not been explored in context as cluster factors. Therefore, the aim of this study was to preclinically assess the therapeutic effect of a cannabinoid compound on elevated arterial pressure in the condition of MetS. Additionally, we chose for this purpose to evaluate the effects of the non-narcotic pseudocannabinoid 8,9-tetrahydrocannabidiol (H2CBD), due to its synthetic accessibility in pure form and potential for greater adoption than CBD, which is subject to multiple regulatory restrictions worldwide.

CBD may modulate RAAS-alternative mediators of hypertension

CBD reduced MetS-associated elevated SBP and end of study plasma revealed an increase in AngII. Although the exact mechanisms promoting the strain-associated hypertension of OLETF are not well defined, elevated RAAS is thought to be a contributing factor[23,25,88]. The increase in plasma AngII may indicate a partial inhibition of AngII binding at the level of the AT_1, which is evidenced in ARB-treated OLETF[84]. However, the reduction of AngII in the OLETF control is fundamentally incongruent with the model literature and negates this line of reasoning. The absence of CBD-induced change to Ang 1-7 and aldosterone levels suggest AngII is not being actively funneled to the non-classical RAAS pathway, which is implicated in anti-hypertensive counterbalance of classical RAAS signaling. Therefore, alternative mechanisms for the reduction of hypertension may include modulation of nitric oxide synthase (NOS) activity in the vasculature. Elevated NOS expression is associated with elevated RAAS tone [97–100] and elevated in the

ARB-sensitive OLETF model[83]. CBD has been previously shown to reduce NOX1 and NOX4 in the kidneys of cisplatin-treated mice[62], elevate glutathione (GSH) in cardiac tissue of T1D mice[60] and increase inducible NOS expression in human coronary artery endothelial cells exposed to high glucose conditions *in vitro*[61]. It remains unclear if these effects can be appreciated in the clustered conditions of MetS. To this end, the measurement of NOS expression in the endothelium of these animals would provide great insight into the ability of CBD to drive expression as a key anti-hypertensive mechanism during MetS.

Limitations and considerations

Unfortunately, due to multiple COVID-related delays, several telemeters that were repurposed for BP measurements lost power well before completion of the study. The treatment group particularly affected was the synthetic CBD (H2CBD) group. Of the 3 animals implanted with BP telemeters and dosed with H2CBD, only 1 telemeter retained power for the entirety of the study, which precludes statistical comparison. Therefore, it is unclear if H2CBD reduces hypertension equally to CBD extract.

Conclusion

The present study demonstrates that a long-term, high dose treatment of H4CBD reduces hypertension in the presence of cluster factor conditions that define MetS. However, it is unclear if cannabinoids interact with ARB and this point should be explored to avoid inappropriate self-prescription of CBD in individuals prescribed ARB. Future pre-clinical research should determine not only the ideal timepoint to initiate a therapeutic intervention but minimum dose to effect, ideal dose duration, as well as the efficacy of intermittent dosing. The benefits observed here are encouraging and contribute to establishing a foundation from which to inform future studies on the effects of CBD compounds on metabolic disorders and associated cardiovascular dysfunction.

Chapter 3. Cannabidiol improves overall metabolism and preserves brown adipose tissue via increased BAIBA during progressive metabolic syndrome

Abstract

Objective: Metabolic syndrome (MetS) is estimated to affect over 1 billion people globally and is a precursory condition of lethal conditions, including cardiovascular disease. *Cannabis* been implicated to improve risk factors of MetS, including obesity and dyslipidemia, but the intoxicating properties of whole plant consumption limit such application. Cannabidiol (CBD) is an abundant, non-intoxicating constituent of *Cannabis sativa*, which ameliorates isolated MetS risk factors but has not been investigated in context as risk factors typically present as a cluster of conditions. Of those risk factors, the loss of brown adipose tissue (BAT), which is an indicator for impaired overall metabolism, has not been investigated.

Methods and materials: To assess the effects of a CBD compounds on progressive MetS, a cohort of 15-week-old Otsuka Long-Evans Tokushima Fatty (OLETF) rats were administered ~200mg CBD or H2CBD/kg/day by oral gavage for 5 weeks. Animals were fed *ad libitum* and monitored in metabolic cages alongside vehicle-treated OLETF and lean strain control Long-Evans Tokushima Otsuka (LETO) rats.

Results: CBD compounds prevented body mass gain in MetS-afflicted rats, which resulted in the prevention of adipose accumulation in the abdomen but not a correction of the diabetic phenotype in OLETF. Indeed, CBD reduced relative abdominal WAT mass (39%; $p<0.05$), which was associated with a body mass (BM) disparity of 79g (15%, $p<0.05$) compared to OLETF. While CBD compounds did not reverse the diabetic phenotype overall, it did reduce plasma triglycerides (TG) by 53% (CBD; $p<0.05$) and 40% (H2CBD; $p<0.05$) and CBD alone increased plasma non-esterified fatty acids (NEFA) ratio to TG by 61% ($p<0.05$), which suggests there is an increase in lipase activity or at least a shift away from lipogenesis and lipid storage. This is corroborated by a reduction in adipocyte morphology whereby CBD reduced large adipocyte (>100µm) abundance by 61% ($p<0.05$) compared to OLETF. Metabolomics revealed distinct profiles among treatment and control groups with the most significant metabolite shift in CBD-treated animals identified to be a 2.1-fold-change increase in BAIBA ($p<0.01$) (H2CBD 1.6 fold increase; $p<0.01$) compared to OLETF, which is associated with browning of WAT. A crude 2D quantification of BAT at dissection revealed CBD preserved 55% ($p<0.05$) of BAT masses lost in OLETF control, which suggests a beneficial shift in whole-body substrate metabolism.

Conclusions: Chronic CBD regimen may drive BAIBA increase to improve overall substrate metabolism via browning of WAT or preservation of BAT. Although H2CBD treatment realized similar benefits with respect to adipose accumulation and BAT preservation but was overall not as potent as treatment with CBD extract. Taken together, these data suggest a promising use for the prevention of impaired substrate metabolism progression after MetS onset.

Introduction

Metabolic syndrome (MetS)[1–4] is conservatively estimated to effect 24% of the U.S. population[4,14]. MetS is characterized by the simultaneous presentation of 3 of the 6 defining conditions: **1)** abdominal obesity, **2)** atherogenic dyslipidemia, **3)** elevated arterial pressure, **4)** glucose intolerance ± insulin resistance, **5)** pro-inflammatory state, and **6)** prothrombotic state[2,3]. MetS is also associated with T2DM as a precursory condition, which is estimated to afflict 30 million Americans (1 in 10) as of 2019[15], but is distinguished by loss of insulin sensitivity and secretion[16]. Because MetS is associated as a precursory condition to multiple life-threatening diseases, MetS is a viable target for pharmaceutical intervention. That said, the specific cause for spontaneous metabolic dysfunction is unknown and the complex, multi-system contributions toward disease progression makes intervention difficult.

Obesity has long been a recognized risk factor for MetS and T2DM[101]. The development of obesity is fundamentally an excess in caloric intake juxtaposed with insufficient energy expenditure. Once established, adipokine imbalance contributes to propagation of more (and larger) white adipocytes, inflammation as well as insulin resistance[102,103]. A consequence of increased white adipose tissue (WAT) is the loss of brown adipose tissue (BAT), the abundance of which is considered an indicator of metabolic health[104]. BAT is dense in mitochondria and thought to play a key role in thermogenesis[104]. Although BAT stores are abundant during developmental periods of growth and diminish over time, consistent exercise has been shown to preserve and even promote BAT abundance[104]. Recently, beta-aminoisobutyric acid (BAIBA), a myokine secreted by the skeletal muscle during exercise, has been shown to promote BAT abundance[105], reduce cluster factor conditions of MetS[106–108] and is inversely correlated with cardiometabolic risk factors[105]. Exogenous administration of BAIBA reduces plasma and liver triglycerides via increased β-oxidation[105] and prevents diet induced obesity[108], which implicates BAIBA as a regulator of lipid metabolism[109].

Cannabidiol (CBD) is an abundant non-psychoactive constituent of *Cannabis sativa*, which is of particular interest for pharmacological investigation. There are sparse pre-clinical data on the effects of CBD in various models of metabolic dysfunction. CBD reduced body mass gain in obesity-prone Wistar rats over ten days of administration[63] and ameliorated dyslipidemia in murine models[64–66]. CBD was also found to reduce the incidence of diabetes in diet-induced obese (DIO) and non-obese diabetic mice[66,70]. However, these effects have not been investigated in the complex, clustered conditions of MetS and there exists an overwhelming gap in the understanding of the pharmacology of CBD compounds.

Therefore, the goals of this study were to assess the effect of CBD compounds on impaired substrate metabolism during MetS. Because of the increasing interest in cannabinoids and the relatively high incidence of MetS, a critical intersection of cannabis constituent use and metabolic dysfunction exists. The Otsuka Long-Evans Tokushima Fatty (OLETF) rat is a monogenic model of diet-induced obesity accelerated by a mutation in the CCK receptor[92]. These rats have a predictable, timed progression toward non-insulin dependent diabetes mellitus (T2DM) (>20 weeks), marked by a linear phase progression of obesity and poor glucose tolerance (8-20 weeks), which closely resembles symptoms displayed by human MetS symptoms. At 15 weeks of age, OLETF rats are increasingly obese and suffer from severe metabolic dysfunction, including abdominal adiposity,

dyslipidemia, and insulin resistance[92,93]. We hypothesized that CBD selectively shifts substrate metabolism in conditions of poor glucose tolerance to improve overall metabolism.

Methods

All animal procedures were reviewed and approved by the institutional animal care and use committees of the University of California, Merced.

Animals

Male lean Long Evans Tokushima Otsuka (LETO) rats and obese Otsuka Long Evans Tokushima Fatty (OLETF) rats (Japan SLC Inc., Hamamatsu, Japan) at 14 weeks of age were assigned to the following groups (n=8/group): **(1)** vehicle-dosed LETO (LETO), **(2)** vehicle-dosed OLETF (OLETF), and **(3)** CBD-treated (200mg/kg/day x 5 weeks) OLETF (CBD). Rats were maintained in a specific pathogen-free, climate-controlled facility at the University of California, Merced on a 12-hour light:dark cycle (07:00-19:00 and 19:00-07:00, respectively). All animals had free access to water and were fed rat chow (Teklad Global; fat 9.0%; carbohydrate 44.9%; protein 19.0%) *ad libitum*. Treatment intervention was initiated at 15 weeks of age.

Body mass and food intake

Body mass (BM) and food intake were measured daily to calculate the appropriate drug and vehicle dose.

Drug preparation & administration

Cannabidiol (CBD) extract was obtained from MissU. 8,9-dihydrocannabidiol (H2CBD) was synthesized as previously described [73]. Both natural CBD extract (~55%) and purified H2CBD (>99%) were separately suspended in sesame oil and administered by oral gavage at a dose of 175mg/kg/day. A similar dose (200mg/kg) has been shown to be similarly effective on seizure frequency and severity in rats compared to natural CBD extract[73], which gave reasonable cause for efficacy of H4CBD at the same dose and is well below documented toxicity of CBD (>600mg/kg) in rodents[94].

Bioavailability detection

CBD bioavailability in heparinized plasma was determined via HPLC and MS chromatography as described previously[95].

Oral glucose tolerance test (oGTT)

At approximately 20 weeks of age and following an overnight fast, oGTTs were performed as previously described[96]. Briefly, animals were fasted overnight and blood glucose was measured via commercially available glucometer from blood obtained via tail nick. A glucose bolus (2g D-glucose/kg body mass) was then administered orally and blood was taken at 10, 30, 60 and 120 minutes after for blood glucose measurement and approximately 100-200uL of whole blood was collected at each time point for assessment of insulin response. The positive incremental areas under the curve for glucose ($AUC_{glucose}$) and insulin ($AUC_{insulin}$) were calculated by the trapezoidal method[110].

Tissue collection

After the 5-week study and 3 days following the oGTT, animals were fasted overnight and tissues were collected the following morning as previously described[23,87,96]. Briefly, animals were fasted overnight and sacrificed by decapitation without sedation. Whole trunk blood was collected into glass collection tubes preloaded with either 1% heparin and 1% protease inhibitor cocktail [Sigma Aldrich] or 1% EDTA with 1% ACEi [Lisinopril] and 1% protease inhibitor cocktail. Plasma was separated via centrifugation (3000RPM x 30 minutes at 4℃) and the supernatant was stored at -80℃ until use.

Plasma analysis

Plasma concentrations of non-esterified fatty acid (NEFA; Wako Chemicals) and triglycerides (TG; Cayman Chemical; 10010303) were measured in fasted, end of study plasma samples. All samples were analyzed in duplicate and run in a single assay with intra-assay and percent CV of <10% for all assays.

Metabolomics and network mapping

Fold changes were calculated as mean disease or treated condition/mean control condition after mTIC normalization. Heatmaps of selected metabolites by pathway represent log2 fold change to show directionality and p-values <0.05 following Student's t-test are indicated separately. Metabolic maps were plotted by comparison for metabolites with $p<0.09$ (Student's t-test) and metabolites across comparisons (e.g. what is significantly changed in on comparison is not necessarily significantly changed in another) using MetaMapp[111]. Metabolic maps were visualized using Cytoscape 3.9.1 with organic layout. Nodes with significant difference after FDR correction ($q<0.2$) were highlighted for fold-change and direction of change. A threshold of 0.2 was established for the FDR correction to provide enough statistical power to detect significant differences with no greater than 1% (<2 metabolites) false positives per comparison. Metabolites were classified into respective pathways based on their entry on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database[112].

Brown adipose tissue quantification

At dissection, we visually quantified visible periaortic brown adipose tissue (BAT) proximal to the left kidney via pictures taken with an iPhone 11. Those 2D images were arbitrarily scaled to the left kidney across images and BAT was traced and quantified via pixel area in ImageJ in a single-blinded fashion.

Calculations & statistics

Insulin resistance index (IRI) was calculated by $AUC_{glucose}$ x $AUC_{insulin}/100$, as previously described[96,113,114]. All values are represented as mean ± standard error mean (SEM) unless otherwise indicated. Means were compared by one-way ANOVA followed by Tukey's honest significant difference or unpaired, one-tailed t-test to assess significant differences among groups. Means and regressions were considered significant at $p<0.05$. Means and regressions were considered a trend between p=0.09-0.051 Outliers were detected by ROUT (Q=1.0%) and removed. Statistical procedures independent of omics analyses were performed using GraphPad Prism 7 (GraphPad Software, Inc., San Diego, CA, USA).

Results

CBD compounds prevent body mass gain but are initially anorexigenic

Body mass (BM) and food intake were measured to determine the effect of CBD compounds on phenotypic indicators of metabolic dysfunction. OLETF BM was 28% ($p<0.05$) higher than LETO at 20 weeks of age (**Figure 3A**). By the end of the study, the CBD-treated group was 14% ($p<0.05$) lower than OLETF control and the H2CBD-treated group was 10% ($p<0.05$) lower than OLETF (**Figure 3A**). Both CBD compounds prevented gains in BM compared to OLETF and LETO ($p<0.05$) (**Figure 3B**). CBD reduced relative food intake for the first week by 31% ($p<0.05$) compared to LETO and 22% ($p<0.05$) compared to OLETF (**Figure 3C-D**). H2CBD reduced relative food intake for the first week by 21% ($p<0.05$) compared to LETO and 10% ($p<0.05$) compared to OLETF (**Figure 3C-D**). However, relative food intake stabilized to comparable lean strain control levels for the remainder of the study (**Figure 3C & E**).

CBD compounds ameliorated dynamic insulin response to glucose

Glucose tolerance and insulin response are key indicators for metabolic dysfunction and measured via the gold standard oral glucose tolerance test (oGTT). While CBD compounds did not obviously improve glucose tolerance (**Figure 4A-B**), animals given CBD extract were observed to demonstrate a modest delay in blood glucose spike at 10 min after glucose bolus (**Figure 4A**), which reduced AUC for the first 10 minutes by 22% ($p<0.05$). Although the height of the peak of the GTT curve was not reduced by CBD compounds, blood glucose clearance in the CBD-treated group after 60min was enhanced 2.6 times the rate of clearance of control OLETF and 28% greater than H2CBD-dosed animals, though this did not reach statistical significance (**Figure 4A**). $AUC_{glucose}$ of all timepoints were not reduced among treated groups compared to OLETF (**Figure 4B**). Insulin response to glucose challenge was exaggerated in OLETF compared to LETO (**Figure 4C**) and $AUC_{insulin}$ was over 800% higher ($p<0.05$) (**Figure 4D**). CBD compounds reduced the insulin response curve to comparable LETO response levels (**Figure 4C**) and reduced $AUC_{insulin}$ by 79% (CBD; $p<0.05$) and 77% (H2CBD; $p<0.05$) compared to OLETF (**Figure 4D**). The inclusion of $AUC_{glucose}$ and $AUC_{insulin}$ for insulin resistance index (IRI) analysis, therefore, reflects an increase of 1472% ($p<0.05$) IRI in OLETF compared to LETO, which was reduced 79% ($p<0.05$) in CBD-treated animals and reduced 77% ($p<0.05$) in H2CBD-treated animals compared to OLETF (**Figure 4E**).

CBD compounds reduce abdominal adiposity, adipocyte morphology and plasma triglycerides

Abdominal adipose accumulation, adipocyte morphology and plasma triglycerides (TG) were measured to assess shifts in lipid catabolism. At 20 weeks, OLETF possesses 155% more relative retroperitoneal fat than LETO (**Figure 5A**). CBD and H2CBD reduced relative retroperitoneal fat mass, but not relative epididymal fat mass by 48% ($p<0.05$) and 32% ($p<0.05$), respectively, compared to OLETF (**Figure 5A & B**). Relative abdominal fat mass (retroperitoneal + epididymal) overall was reduced by 39% in CBD-treated animals and 25% in H2CBD-treated animals compared to OLETF (**Figure 5C**). Adipocyte morphology was comparable below 100μm among groups (**Figure 5D-H**). However, CBD treatment alone reduced the frequency of large adipocytes (100-150μm) by 61% ($p<0.05$) compared to OLETF (**Figure 5H**). Fasted plasma TG, a substrate of lipolysis, was 92% higher in OLETF compared to LETO ($p<0.05$) (**Figure 6A**), which is consistent

with the obese OLETF phenotype. Both CBD compounds reduced fasted plasma TG to LETO control levels (53% CBD and 40% H2CBD reduced from OLETF; $p<0.05$) (**Figure 6A**). Non-esterified fatty acids (NEFAs) are freed in the breakdown of TGs and are considered an indirect measurement of lipolysis or lipase activity. Plasma NEFA was 37% higher in OLETF compared to LETO and only H2CBD reduced plasma NEFA by 28% ($p<0.05$) compared to OLETF (**Figure 6B**). The ratio of NEFA to TG in plasma may indicate a shift toward lipolysis. NEFA/TG ratio is comparable between LETO and OLETF (*though OLETF is non-significantly reduced by 21% compared to LETO*) but CBD increases this ratio by 61% ($p<0.01$) compared to OLETF (**Figure 6C**).

CBD compounds shift metabolites associated with browning of white adipose tissue

PLSDA analysis of metabolites revealed a clear distinction in the metabolomic profile amongst the groups (**Figure 7**). Statistical comparison of discrete metabolites revealed the most significantly perturbed metabolites in OLETF compared to LETO included a 1.9-fold increase in linolenic acid ($p<0.01$; $q<0.02$), 1.1-fold increase in isoleucine ($p<0.05$; $q<0.02$), 0.9-fold decrease in glucose ($p<0.05$; $q<0.02$), 1.1-fold increase in leucine ($p<0.05$; $q<0.02$) and 1.2-fold increase in succinic acid ($p<0.05$; $q<0.02$) (**Figure 8; Table 1**). Respectively, these metabolites are involved in lipid metabolism, amino acid (AA) metabolism, carbohydrate metabolism, AA metabolism and energy metabolism (**Table 1**). CBD induced the following perturbations of metabolites compared to OLETF: 2.1-fold increase in 3(β)-aminoisobutyric acid (BAIBA) ($p=0.001$; $q<0.02$), 1.4-fold increase in hypotaurine ($p<0.01$; $q<0.02$), 1.4-fold increase in glutamine ($p<0.01$; $q<0.02$), 1.7-fold increase in 5-aminovaleric acid ($p<0.05$; $q<0.02$) and 0.3-fold decrease in taurine ($p<0.05$; $q<0.02$) (**Figure 8; Table 1**). Respectively, these metabolites are involved in browning of white adipose tissue (WAT), energy metabolism, AA metabolism, AA metabolism and energy metabolism (**Table 1**). H2CBD induced the following perturbations of metabolites compared to OLETF: 0.5-fold decrease in linolenic ($p<0.01$; $q<0.02$), 0.7-fold decrease in isocitric acid ($p<0.01$; $q<0.02$), 0.5-fold decrease in campesterol ($p<0.01$; $q<0.02$), 0.1-fold decrease in taurine ($p<0.01$; $q<0.02$) and 1.6-fold increase in BAIBA ($p<0.01$; $q<0.02$) (**Figure 8; Table 1**). Respectively, these metabolites are involved in lipid metabolism, carbohydrate metabolism, biosynthesis of terpenoids, energy metabolism and browning of WAT (**Table 1**). The increase in BAIBA was amongst the top 5 significantly increased metabolites in both CBD and H2CBD-treated animals compared to OLETF (**Figure 8; Table 1**).

CBD compounds may increase BAIBA to preserve brown adipose tissue

BAIBA mean peak area was comparable between LETO and OLETF but was increased 110% ($p<0.01$) in CBD-treated animals and increased 60% ($p<0.01$) in H2CBD-treated animals compared to OLETF (**Figure 9A-B**). Periaortic brown adipose tissue (BAT), proximal to the left kidney, was visually quantified by pixel area on a 2D plane (*from a scaled photo*) at dissection (**Figure 9C-E**) and revealed BAT abundance in OLETF was 52% lower compared to LETO ($p<0.001$) (**Figure9F & G**). CBD and H2CBD appeared to preserve or rescue BAT abundance by 55% ($p<0.05$) and 52% ($p<0.05$) compared to OLETF, respectively (**Figure9F & G**).

Discussion

Obesity is a recognized risk factor in the development of MetS and CBD has been shown to exert therapeutic effects in obesogenic conditions. However, the mechanism of action of CBD is not well understood at any level of metabolic function. The present study models a progressive condition of MetS with the OLETF rat and demonstrates that a high, chronic dose of CBD compound confers benefits in the reduction of adiposity in spite of concurrent comorbidities that define MetS. However, H2CBD-induced effects, though similar, were not as profound as those induced by CBD extract. This could be because the extracted compound is not completely free of other cannabinoids, which may facilitate some of the effects observed here. Regardless, the magnitude of benefit here is comparable between CBD extract and pseudocannabinoid H2CBD observed elsewhere[73].

A striking and novel discovery of the present study is the CBD-induced increase in BAIBA after 5 weeks. The therapeutic benefit to adiposity, dyslipidemia and potentially insulin resistance have been similarly demonstrated in streptozotocin (STZ)-treated mice (a model of type 1 diabetes) administered BAIBA for 4 weeks[107]. It is not clear, however, that CBD compounds directly increase BAIBA or, alternatively, promote energy expenditure in the form of physical activity. It is known that plasma BAIBA levels increase during exercise [105,115] and, though not shown here, we note that CBD compound tends to increase activity by 2-fold in our study discussed later on in chapter 4 of this dissertation. Therefore, continuation of this work would aim to distinguish the effects of CBD on skeletal muscle BAIBA secretion in vitro using myocytes[105]. We note that though glucose tolerance in this study was not improved overall, the reduction of insulin secretion to achieve the same, albeit delayed, blood glucose clearance may suggest an increase in insulin receptor sensitivity. Others have shown BAIBA to promote glucose uptake in a dose dependent fashion during conditions of insulin resistance through an AMPK-mediated pathway[108]. On the other hand, both groups treated with CBD compound were found to have significantly decreased (>80% compared to OLETF; $p<0.0001$) circulating plasma leptin levels (*data not shown*), which could account for potential insulin receptor sensitization in response to significant adipose reduction[116,117]; without plasma adiponectin to contrast, however, it is difficult to conclusively attribute reduced insulin resistance to shifts in adipokine levels. To the extent of our knowledge, this is the first study to demonstrate CBD compounds increase plasma levels of the novel myokine, BAIBA.

Although glucose tolerance was not improved overall, CBD compounds may modulate glucose transport across the intestinal lumen. We note that in CBD treated animals, the blood glucose peak of the GTT curve was delayed and clearance after 60 minutes post-challenge had increased. It could be that the peak was simply shifted to the right and the absence of additional measurements between 10- and 60-minutes post-challenge may sacrifice some dynamic resolution. Of course, these subtle improvements were not captured with GTT AUC analysis of all timepoints, which suggested the modest improvements observed from the curve itself were not sufficient to significantly distinguish glucose tolerance between OLETF control and CBD-treated OLETF in early MetS condition. That said, the delay in measured blood glucose may indicate a delay in glucose transport across lumen. Although the specific targets of CBD are a contentious topic, CBD administration has been shown to enhance endogenous cannabinoid (endocannabinoid) abundance, specifically anandamide (AEA), in the plasma[56,118]. Endocannabinoid signaling in the gut and peripherally has been shown to modulate nutrient absorption and uptake[119,120]. Taken together, this may suggest CBD exerts beneficial delays in glucose transport into the blood stream, which would imply CBD directly

or indirectly acts on glucose transporters in the gut. Future work to address this hypothesis would involve a comparison of glucose response to oGTT and intraperitoneal (i.p.) GTT. The latter should not demonstrate a delay in blood glucose increase in response to challenge.

Limitations and considerations

Our interests in BAT may require a more meaningful method of quantification. Our first pass quantification utilized an image which was scaled arbitrarily to the left kidney. We then traced visible BAT, distinguished from WAT or other anatomical structures by visual assessment of tissue color, and used ImageJ to assign pixel area to our free-hand trace. Although this method maybe commonplace for quantification of differential staining, our BAT pictures limit the accuracy of the software because BAT is not on a 2D plane. Unlike histology images, photography at dissection introduces sheen, light variance and distance between lens and target fluctuation between subjects. We would aim for a more standardized quantification strategy, potentially proteomics, to not only corroborate our findings but to potentially contribute to the scientific community.

Chapter 4: Pseudocannabinoid (H4CBD) reduced abdominal adiposity but not hypertension in advanced metabolic syndrome

Abstract

Objective: The prevalence of metabolic syndrome (MetS) is 55% among people ≥60 years of age and is a precursor for cardiovascular disease and type II diabetes. Cannabidiol (CBD) use has grown more popular in the last two decades, particularly amongst adults >55 years of age and synthetic analogues of CBD have generated great interest because they can offer a chemically pure product, free of undesired compounds. However, the effects of chronic CBD use during age-associated cardiometabolic dysfunction have not been examined.

Methods and materials: To assess the effects of a synthetic analogue of CBD (H4CBD) on advanced MetS, a cohort of 41-week-old Otsuka Long-Evans Tokushima Fatty (OLETF) rats were administered 200mg H4CBD/kg by oral gavage daily for 4 weeks. Animals were fed *ad libitum* and monitored alongside vehicle-treated OLETF and lean strain control Long-Evans Tokushima Otsuka (LETO) rats.

Results: H4CBD reduced body mass (BM) (22%; $p<0.05$) to lean strain control levels within the first week but did not reverse the diabetic nor hypertensive phenotype of the aged OLETF. H4CBD reduced abdominal fat mass (41%; $p<0.05$) and nearly ablated large adipocyte (>100μm) abundance ($p<0.05$). However, urinary 3-methylhistidine to creatinine ratio tended to be higher (77%; $p=0.07$) in H4CBD, which suggested muscle was lost alongside fat mass. Estimation of muscle mass from total body water (TBW) revealed H4CBD reduced muscle mass by 34% ($p<0.05$) and lean BM overall by 20% ($p<0.05$). H4CBD reduced plasma triglycerides (TG) by 45% ($p<0.05$) and reduced liver TG content (28%; $p<0.05$). Although H4CBD increased expression of hepatic fatty acid (FA) transporters, CD36 (48%; $p<0.05$) and FATP2 (60%; $p<0.05$), increases in hepatic targets associated with hepatic FA beta-oxidation were not observed.

Conclusions: Chronic pseudocannabinoid (H4CBD) regimen may drive fat loss via increased energy expenditure in conditions of severely impaired glucose metabolism. That said, observed lean tissue loss alongside fat mass reduction may signal contraindication for use in conditions of sarcopenic obesity.

Introduction

The popularity of cannabis use in older adults (>55 years of age) in the United States has more than doubled over the past two decades[75–78]. Conditions for which older adults typically seek medicinal cannabis are often pain-associated[77–79]. A common consequence of aging is impaired substrate metabolism, which could result in the onset of metabolic conditions ranging in severity from risk factors for metabolic syndrome (MetS)[3], like obesity, to frank type II diabetes mellitus (T2DM)[5]. The prevalence of MetS is 55% among people ≥60 years of age[5] and the average age at diagnosis of T2DM is approximately 46 years of age with equal abundance across racial and ethnic groups[80]. The therapeutic risks and benefits of cannabis and cannabinoids in metabolic dysfunction have yet to be defined.

Cannabidiol (CBD) is an abundant non-psychoactive constituent of *Cannabis sativa*, which is of particular interest for pharmacological investigation. The legislative ambiguity and increasing ease-of-access to unregulated cannabis constituents have prompted endeavors to synthetize the cannabis constituent molecule of interest without use of whole plant substrate. Thus, circumventing violation of laws surrounding production of, or with, a Schedule I compound while simultaneously providing a chemically pure compound free from the growing concerns of unregulated pesticide use on whole plants may be advantageous. Synthetically derived CBD analogues, like 8,9-tetrahydrocannabidiol (H2CBD), offer similar effects to natural CBD molecules. For example, H2CBD reduced seizure frequency and severity in PTZ-induced rats similarly to CBD [73]. 1,2,8,9-tetrahydrocannabidiol (H4CBD) is a synthetic analogue of CBD that differs by the saturation of the exocyclic carbon-carbon double bond and sole endocyclic carbon-carbon double bond of the attached aromatic ring. Like natural CBD, H4CBD has little affinity for the endocannabinoid receptors responsible for cannabis intoxication[74]. However, use *in vivo* has not been previously described, although in vitro use has yielded promising effects consistent with natural CBD [74,121,122]. Therefore, H4CBD is expected, like H2CBD, to exert similar effects to those of natural CBD.

Primarily, convincing clinical evidence has been provided for the efficacy of CBD to reduce seizure frequency and severity in children and adults. Epidiolex® is currently the only FDA-approved, cannabis-derived medication for the treatment of Lennox-Gastaut and Dravet syndrome[91]. Observations of cardiovascular and/or metabolic improvements conferred by CBD or CBD-dominant cannabis have prompted inquiry into other applications for CBD[6–10,12]. Clinical trials determined 100mg CBD, twice daily, to be well-tolerated in patients with MetS or T2DM and made improvements in circulating resistin and gastric inhibitory peptide but did not reverse the T2DM phenotype[67]. A much higher dose (600mg) of CBD acutely reduced arterial blood pressure in healthy males[68], however, this effect was lost after a 7-day dose regimen[69].

There are sparse pre-clinical data on the effects of CBD in a model of metabolic dysfunction. Natural (extracted) CBD reduced the incidence of diabetes in diet-induced obese (DIO) and non-obese diabetic mice[66,70], which provides some reasonable evidence that synthetic CBD has the potential to similarly ameliorate dysregulated metabolism. CBD administered over 4 weeks attenuated cardiac dysfunction and cardiac fibrosis in mice with induced type I diabetes[71]. Yet 2 weeks of 10mg/kg CBD did not reduce arterial blood pressure in spontaneous hypertensive or deoxycorticosterone salt induced hypertensive rats, significant improvements to oxidative stress and lipid metabolism were observed[72], suggesting that longer duration treatment may be necessary to achieve improvements in arterial pressure.

Therefore, the goals of this study were to assess the effect of H4CBD on MetS cluster conditions in the advanced stages of MetS disease progression. Because of the increasing interest in cannabis, cannabis constituents and pseudocannabinoids in older adults, and the relatively high incidence of MetS and CVD in this population, a critical intersection of cannabinoid use, and advanced metabolic dysfunction, exists. The Otsuka Long-Evans Tokushima Fatty (OLETF) rat is a monogenic model of diet-induced obesity accelerated by a mutation in the CCK receptor[92]. These rats have a predictable, timed progression toward non-insulin dependent diabetes mellitus (T2DM) (>20 weeks), marked by a linear phase progression of hypertension (8-20 weeks), which closely resembles symptoms displayed by human T2DM symptoms including visceral adiposity, dyslipidemia, and insulin resistance[92,93]. At >40 weeks of age, OLETF rats suffer from severe metabolic dysfunction and therefore serve as a model of aged, severe MetS. We hypothesized that H4CBD is not a viable intervention therapy for severe hypertension but would improve parameters of lipid metabolism to reduce cluster factors of MetS.

Methods

All animal procedures were reviewed and approved by the institutional animal care and use committees of the University of California, Merced.

Animals

Male lean Long Evans Tokushima Otsuka (LETO) rats and obese Otsuka Long Evans Tokushima Fatty (OLETF) rats (Japan SLC Inc., Hamamatsu, Japan) at 14 weeks of age were assigned to the following groups (n=8/group): **(1)** vehicle-dosed LETO (LETO), **(2)** vehicle-dosed OLETF (OLETF), and **(3)** H4CBD-treated (200mg/kg/day x 4 weeks) OLETF (H4CBD). Rats were maintained in a specific pathogen-free, climate-controlled facility at the University of California, Merced on a 12-hour light:dark cycle (07:00-19:00 and 19:00-07:00, respectively). All animals had free access to water and were fed rat chow (Teklad Global; fat 9.0%; carbohydrate 44.9%; protein 19.0%) *ad libitum*. Treatment intervention was initiated at 41 weeks of age.

Body mass and water and food intake

BM was measured daily to calculate the appropriate drug and vehicle dose. Water intake, urine excretion, and food consumption were also measured daily throughout the study.

Telemeter implantation

At 16 weeks, OLETF rats (n=5) were implanted with DSI telemeters (HD-S10) into the abdominal cavity to measure systolic blood pressure as previously described[88]. LETO rats were not implanted; however, we have extensive telemetry data in these controls to demonstrate the maintenance of normotensive conditions including at a similar advanced age[123]. Activity was measured every hour for 15 minutes for the duration of the study. Data were analyzed using A.R.T. software (DSI, St. Paul, MN).

Drug preparation & administration

H4CBD (1,2,8,9-tetrahydrocannabidiol, systematic name 2-(2-isopropyl-5-methylcyclohexyl)-5-pentylbenzene-1,3-diol), was prepared by reduction of synthetic H2CBD (8,9-tetrahydrocannabidiol) with hydrogen and a Pd/C catalyst in acetic acid solvent and was purified by distillation *in vacuo*. H2CBD was synthesized from olivetol and food-grade α-phellandrene

according to the published procedure[73]. All chemicals were purchased from Millipore Sigma and used as received.

Purified H4CBD (>99%) was suspended in food grade sesame oil and administered by oral gavage at a dose of 200mg/kg/day. A volumetrically equivalent dose of vehicle was administered to LETO and OLETF control groups by oral gavage. This dose, administered via intraperitoneal injection, has been shown to be similarly effective for the mitigation of seizure frequency and severity in rats compared to natural CBD[73], which gave reasonable cause for efficacy of H4CBD at the same dose, which is well below documented toxicity of CBD (>600mg/kg) in rodents[94]. While this is a relatively high dose, the current study provides a basis to justify this dose as the highest needed.

Drug bioavailability detection

H4CBD bioavailability in heparinized plasma was determined via HPLC and MS chromatography as described previously[95].

Oral glucose tolerance test (oGTT)

At approximately 45 weeks of age and following an overnight fast, oGTTs were performed as previously described[96]. The positive incremental areas under the curve for glucose ($AUC_{glucose}$) and insulin ($AUC_{insulin}$) were calculated by the trapezoidal method[110].

Tissue collection

After the 4-week study and 3 days following the oGTT, animals were fasted overnight and tissues were collected the following morning as previously described[23,87,96].

Plasma analysis

Plasma concentrations of adiponectin (Millipore Sigma; EZRADP-62K), aldosterone (R&D Systems; KGE016), angiotensin 1-7 (Ang 1-7; Phoenix Pharmaceuticals; EKE-002-01), angiotensin II (Ang II; Phoenix Pharmaceuticals; EK-002-12CE), corticosterone (B; R&D Systems; KGE009), ghrelin (Sigma Aldrich; RAB0207), glucagon (Crystal Chemicals; 81519), leptin (Millipore Sigma; EZRL-83K), non-esterified fatty acid (NEFA; Wako Chemicals) and triglycerides (TG; Cayman Chemical; 10010303) were measured in fasted, end of study plasma samples. All samples were analyzed in duplicate and run in a single assay with intra-assay and percent CV of <10% for all assays.

Urinalysis

Urine was collected as previously described[88]. Urine was thawed on ice to measure 3-methylhistidine (3MH; Abbexa; 257295) and creatinine (Invitrogen; EIACUN). Excretion was calculated for both by multiplying the 24-hr urine volume by the measured concentration ($U_xV = V * [x]$), where x = creatinine or 3MH.

Lipase activity assay

White adipose tissue (WAT), liver, and end of study plasma was assayed for lipase activity, as previously performed[124].

Adipocyte morphological quantification

WAT was mounted and stained using hematoxylin and eosin (H&E) stain. Each slide was imaged at 10X using a Keyence BZ-X Series microscope at three distinct locations and scaled[23]. The images were coded and analyzed for single-blind adipocyte count and area using ImageJ software.

Liver damage assessment

An aliquot of liver was homogenized and assayed for TG content (Cayman Chemical; 10010303), and 4-hydroxynonenal (4HNE) accumulation (myBioSource; MBS736336) and collagen type IV (myBioSource; MBS732756) deposition were measured by ELISA via manufacturer's recommendation.

Western blot

An aliquot of liver was used to measure proteins involved in fatty acid uptake, metabolism, and storage as previously described[125]. Densitometry values were quantified using ImageJ software (NIH) and normalized by correcting for densitometry values of representative protein bands below 37kDa stained with Ponceau S. Results are reported as expression (%) compared to LETO.

Real-time qPCR

Gene expression was quantified by qPCR on cardiac tissue a previously performed[87]. Primers used were acquired from Integrated DNA Technologies and listed here 5'-3': MAS1 receptor (Mas1) Fw: AACACATGGGCCTCCCATTC, Rv: AACAGGTAGAGGACCCGCAT; Ang II receptor (AT_{1a}) Fw: CTTGTTCCCTTTCCTTATCA, Rv: CGTTTCTTGGTTTGTTCTTT.

Calculations & statistics

Insulin resistance index (IRI) was calculated by $AUC_{glucose}$x $AUC_{insulin}$/100, as previously described[96,113,114]. [creatinine clearance] All values are represented as mean ± standard error mean (SEM) unless otherwise indicated. Means were compared by one-way ANOVA followed by Tukey's honest significant difference or unpaired, one-tailed t-test to assess significant differences among groups. Means and regressions were considered significant at $p<0.05$. Means and regressions were considered a trend between p=0.09-0.051 Outliers were detected by ROUT (Q=1.0%) and removed; however, it should be noted that it was necessary for only 6 occurrences. All statistical procedures were performed using GraphPad Prism 7 (GraphPad Software, Inc., San Diego, CA, USA).

Results

H4CBD was only detected in plasma from treated rats

Bioavailability of H4CBD compound was validated in end of study plasma and confirmed that **1)** only treated animals received drug and **2)** that the dose was sufficient to increase circulating levels (**Table 1**).

H4CBD ameliorated dynamic glucose response and IRI

oGTTs were performed to determine the effects of H4CBD on MetS-associated glucose intolerance and degree of insulin response status during advanced MetS. Fasting blood glucose (FBG) was comparable between LETO and OLETF while FBG in H4CBD-treated animals was 56% higher than OLETF ($p<0.01$) (**Table 1**). $AUC_{glucose}$ was 49% higher in OLETF compared to LETO

(p<0.001) and H4CBD reduced $AUC_{glucose}$ 29% from OLETF (p<0.001) (**Table 1**). Plasma insulin response was abolished in OLETF (p<0.05) and not rescued by H4CBD treatment (**Table 1**). The insulin resistance index (IRI) status was similar between aged LETO and OLETF rats but was reduced by 23% in H4CBD (p<0.05) (**Table 1**). Fasting plasma insulin was 64% higher in OLETF compared to LETO (p<0.01), which was not affected by H4CBD (**Table 1**). Plasma glucagon was 63% lower in OLETF compared to LETO (p<0.05) but not different from H4CBD (**Table 1**). H4CBD increased fasting glucose to insulin ratio by 47% (p<0.05), and fasting insulin to glucagon ratio by 64% (p<0.05), compared to OLETF (**Table 1**).

H4CBD reduced BM despite increased food consumption

BM, relative food consumption, water consumption and activity were measured daily to determine the effect of CBD on phenotypic indicators of metabolic dysfunction. H4CBD reduced BM in OLETF to LETO levels within the first week and were maintained for the duration of the study (**Figure 1A & B**). BM of untreated OLETF was 16% higher than LETO and 15% higher than H4CBD-treated OLETF after the first week of the study (day 10-30). Despite an initial decrease in relative food intake, H4CBD surpassed OLETF relative consumption 24% by day 11 and maintained for the remainder of the study (**Figure 1C**) without BM regain. Water intake was >200% higher in OLETF compared to LETO for the duration of the study (**Figure 1D**). By day 13, water intake in H4CBD-treated OLETF was 90% higher than OLETF control and >600% higher than LETO through the end of the study (**Figure 1D**). Treated OLETF (H4CBD; n=3) displayed a trend (p=0.0985) toward increased activity that was nearly double that of OLETF control (**Figure 1E**).

H4CBD elevated urinary indicators of lean tissue catabolism

Urine was collected over a 24-hour period on the final day of treatment and analyzed for 3-methylhistidine (3MH), creatinine (Cr), and total protein excretion to determine if H4CBD promoted muscle wasting. H4CBD urine output and proteinuria was comparable to control groups (**Figure 2A-B**). H4CBD treatment reduced creatinine excretion by 42% compared to OLETF (**Figure 2C**). 3-MH excretion is a product of amino acid breakdown and urine 3-MH to Cr ratio (3-MH/Cr) is used clinically to assess muscle wasting[126]. 3-MH excretion of the H4CBD group was comparable to OLETF (**Figure 2D**). OLETF 3-MH/Cr tended to be 128% higher (p=0.06) than LETO and 77% higher still in H4CBD (p=0.07) (**Figure 2E**).

Fat loss promoted by H4CBD comes at the expense of some lean tissue loss

End of study BM, FM and lean organ mass were measured to determine the amount of abdominal fat lost and estimate the degree of lean tissue lost in treated animals. At 45 weeks of age, H4CBD reduced OLETF BM by 22% (p<0.05) (**Figure 3A**). Abdominal fat masses (retroperitoneal and epididymal) were dissected and quantified to determine the effect of H4CBD on abdominal adiposity. Relative retroperitoneal fat, but not relative epididymal fat, was 267% more abundant in OLETF than LETO (p<0.0001) (**Table 2; Figure 4A-C**). H4CBD reduced relative retroperitoneal fat by 24% (p<0.05) and relative epididymal fat by 35% (p<0.05) compared to OLETF control (**Table 2; Figure 4A-C**). Relative combined adipose was 149% higher in OLETF compared to LETO (p<0.05), which was reduced 26% (p<0.05) by H4CBD (**Figure 3B; Table 2; Figure 4A-C**). To estimate total body water (TBW) and lean tissue loss, equations extrapolated from the literature were used[127]. Conservatively, TBW was reduced by 15% (p<0.05) (**Figure 3C; Table**

2), muscle mass was reduced by 34% ($p<0.05$) (**Figure 3D; Table 2**) and lean tissue overall was reduced by 20% ($p<0.05$) (**Table 2**).

H4CBD reduces large adipocyte abundance

Adipocyte morphology was comparable below 100μm among groups (**Figure 4D-E**). However, H4CBD treatment reduced the frequency of large adipocytes (100-150μm) by 95% ($p<0.05$) compared to OLETF (**Figure 4E**).

H4CBD ablates plasma triglycerides

Liver and adipose lipase activity were measured to determine the effect of H4CBD. The reduction in visceral adiposity and adipocyte size suggests that H4CBD activated and enhanced lipid catabolism as an alternative substrate for metabolism in a diabetic condition. Adipose lipase activity was 31% ($p<0.05$) lower in OLETF compared to LETO and H4CBD tended to rescue adipose lipase activity at an average increase of 22% ($p=0.094$) (**Figure 5A**). Liver endothelial membrane-bound lipases are known to contribute to plasma TG breakdown [128–130]. However, hepatic membrane-bound and cytosolic lipase activity was not different among groups (**Figure 5B-C**). Fasted plasma TG, the substrate of lipolysis, was 147% higher in OLETF compared to LETO ($p<0.0001$) and H4CBD treatment reduced fasted plasma TG to LETO control levels ($p<0.0001$ OLETF vs. H4CBD) (**Figure 5D**). Fasted plasma NEFA and plasma lipase activity were not different among groups (**Figure 5E &F**).

H4CBD promoted FA transporter expression but did not increase TG accumulation

Hepatic lipid handling proteins were measured to assess the impact of H4CBD on TG synthesis and accumulation versus lipid commitment to beta-oxidation. FATP5 expression was increased 109% ($p<0.01$) in OLETF compared to LETO and was not affected by H4CBD treatment (**Figure 6A**). FATP2 expression was comparable between LETO and OLETF but increased by 60% ($p<0.05$) in the H4CBD treated group (**Figure 6B**). CD36 expression was 41% ($p<0.05$) lower in OLETF compared to LETO and H4CBD treatment rescued CD36 expression by 48% ($p<0.05$) (**Figure 6C**). H4CBD tended to reduce hepatic ApoB by 20% ($p=0.08$), which could partially explain the reduction in plasma TG (**Figure 6D**). GPAM and DGAT1, targets involved in TG synthesis, were not affected (*data not shown*) and liver TG content overall was reduced in H4CBD-treated animals 28% ($p<0.05$) (**Figure 6E**). Taken together, these data suggest utilization of TG for breakdown and subsequent FA oxidation. That said, hepatic CPT1A, ACOX1 and PRDX6, targets involved in the shuttling of FA to the mitochondria, initiation of beta-oxidation and downstream anti-oxidant, respectively, were unchanged (*data not shown*).

H4CBD did not contribute to liver damage

Indicators of damage were measured in liver tissue to assess the effects of H4CBD on hepatotoxicity. End of study liver 4HNE was not different among groups (**Figure 7A**). Type IV collagen deposition is an indicator of liver fibrosis, which is useful in the diagnosis of NAFLD in elderly individuals[131] and models of NAFLD like OLETF[132]. Liver collagen deposition was 33% ($p<0.05$) higher in OLETF compared to LETO and H4CBD treatment had no effect on collagen levels (**Figure 7B**).

H4CBD does not improve RAAS-mediated MetS-associated hypertension

Systolic blood pressure was monitored throughout the study via surgically implanted DSI radiotelemeters [HD-S10] to assess the effect of H4CBD on severe hypertension. No significant difference between treated and non-treated OLETF was detected though treated OLETF demonstrated an average 6.5mmHg (4%) increase after the 7th day of the daily dose regimen (**Figure 8A**). End of study plasma concentrations of Ang 1-7, AngII and aldosterone were measured to assess modulation of drivers of hypertension. Plasma AngII levels were 18% lower in OLETF control compared to LETO ($p<0.05$) and 24% higher in H4CBD ($p<0.05$) (**Figure 8B**). H4CBD tended to increase plasma Ang 1-7 levels by 26% ($p=0.097$) (**Figure 8C**) while plasma aldosterone tended to be 150% higher ($p=0.052$) (**Figure 8D**). Cardiac AT1 mRNA relative expression was assessed to determine the effect of H4CBD on RAAS tone. H4CBD tended to increase AT1 expression by 49% ($p=0.09$) (**Figure 8E**) but had no effect on cardiac counteractive non-classical Mas1 mRNA expression (**Figure 8F**).

Discussion

The prevalence of MetS has been estimated to be three times more pervasive than T2DM, which extrapolates to account for a population of over 1 billion people globally[4]. Since MetS is associated with development of lethal conditions, such as CVD and T2DM, intervention strategies that affect multiple cluster conditions of MetS are of critical interest. The effects of CBD compounds on metabolic syndrome pathophysiology have not been described though a few studies have shown beneficial[60,63] or null effects[72] of CBD on isolated MetS risk factors. However, these effects have not been explored in context as cluster factors. Therefore, the aim of this study was to preclinically assess the therapeutic effect of a cannabinoid compound on adiposity and elevated arterial pressure in the condition of advanced MetS, which is most frequently observed in older populations (>55 years of age). We chose for this purpose to evaluate the effects of the non-narcotic cannabinoid 1,2,8,9-tetrahydrocannabidiol (H4CBD), due to its synthetic accessibility in pure form and potential for greater adoption than CBD, which is subject to multiple regulatory restrictions worldwide.

H4CBD may reduce adiposity by driving energy expenditure rather than anorexigenesis

BM and food intake were consistently higher in OLETF compared to LETO as expected[92,93]. CBD has not been previously shown to reduce body mass *de novo* but rather inhibit body mass gain in rats without underlying metabolic dysfunction[63]. We establish here that H4CBD dosed daily for 1-week reduced OLETF BM to LETO control, which maintained for the duration of the study. Although relative food consumption was reduced in the treated group in the first week, by day 10, relative food consumption was higher than OLETF and LETO controls. High doses of CBD administered clinically have been noted to have anorexic and diarrheal effects indicative of gastric upset[133], which could account for the reductions noted in the first week. Daily dose was relaxed to every other day after the first week, which may have offset the anorexic effects of the terpene-rich (noxious) H4CBD, indicated by the increase in relative food consumption. However, all treated animals were observed to sporadically have soft stool at multiple points of the dosing window regardless of dose frequency relaxation. BM losses were not regained by increased food consumption, which indicated H4CBD did not exert anorexic effects when dosed every other day. Therefore, the energy balance (in/out) was thought to be enhanced through increased activity, which likely accounted for the loss in BM, as well as tendency to increased 3MH/Cr excretion. Indeed, activity scores were nearly doubled in treated OLETFs. However, the low sample size was

an unfortunate consequence of the unexpectedly high surgery attrition and loss of telemeter battery life which weakens this point. Nonetheless, there was an observed trend in increased energy expenditure, rather than caloric restriction, which is likely a significant contributing factor to BM loss overall.

H4CBD beneficially modulated lipid handling and adipose catabolism during advanced MetS

Catabolism of adipose tissue stores is mediated by lipase activity levels in multiple locations, which breaks down circulating and stored triglycerides (TGs) to NEFA for cellular uptake and metabolism[134]. Although consumption of excess NEFA is implicated in adipocyte proliferation[103,135], the pervasive insulin resistance of the older OLETF likely forces NEFA consumption in lieu of homeostatic glucose uptake and metabolism[136]. This is evidenced by the reduction in large adipocytes and abdominal fat masses overall in treated animals. Moreover, the implication of hyperlipidemia is a contribution to insulin resistance in peripheral tissue through an increase of NEFA uptake by skeletal muscle, thereby decreasing glucose uptake[136]. Indeed, obese, insulin-resistant OLETFs older than 25 weeks of age are hyperglycemic compared to age-matched LETO[93] and one of the most striking effects H4CBD had on OLETF was the complete attenuation of plasma TG levels, but not blood glucose, compared to OLETF control. Although none of the lipase activity measured reached statistical significance, there may be an implication of biological significance, which may not meet the statistical criteria. Lipase activity at the level of adipose tissue, liver and plasma were consistently higher than untreated OLETF, which was a likely contributor to abdominal adipose catabolism. Additionally, an increase in hepatic ApoB corroborates this assertion of enhanced lipid uptake for the purpose of FA oxidation. Hepatic lipid handling indicators measured here suggest H4CBD does not promote TG synthesis (GPAM and DGAT1) but rather promotes FA uptake (FATP2 and CD36). That said, hepatic protein expression of targets associated with β-oxidation (CPT1A, ACOX1 and PRDX6) were not beneficially increased, which may simply indicate β-oxidation is preferentially enhanced in the skeletal muscle rather than the liver in compensation for reduced glucose uptake. Regardless, the biological response to meet energetic demands is likely adipose catabolism to fuel skeletal muscle via NEFA consumption in the OLETF condition of severe insulin resistance and β-cell exhaustion[23].

H4CBD is not sufficient to attenuate hypertension during advanced MetS

Abdominal adiposity is a known risk factor for hypertension and even modest decreases in BM (5-10%) can normalize blood pressure in obese patients[137]. Although the latter is point is contentious, obesity is widely recognized a cluster factor of MetS but reduction of BM alone is not sufficient to reduce SBP in OLETF[96]. Elevated SBP observed in control OLETFs was consistent with strain phenotype at this age[123] but because LETO SBP data were not available, we utilized previously published, age-matched SBP data for lean-strain comparison. H4CBD treatment did not reduce hypertensive SBP in aged OLETF, which supports that the reduction of BM and/or abdominal adiposity is not sufficient to reduce arterial blood pressure. That said, the exact mechanisms promoting the strain-associated hypertension of OLETF are not well defined but elevated RAAS is thought to be a contributing factor[23,25,88]. On the other hand, the modest increase in plasma AngII may indicate a partial inhibition of AngII binding at the level of the AT_1, which is evidenced in ARB-treated OLETF[84]. Collectively, these findings support that the therapeutic effects of H4CBD are not sufficient to affect RAAS mediators at the level of ARBs in this condition of severe metabolic dysfunction.

Conclusion

The present study demonstrates that a long-term, high dose treatment of H4CBD reduces abdominal adiposity and dyslipidemia in the condition of advanced MetS. However, H4CBD treatment did not beneficially modulate RAAS and ultimately, did not reduce arterial blood pressure. Importantly, it is still unclear if cannabinoids interact with ARB and this point should be explored as recommendations for body mass reduction are typically made for individuals with clinical hypertension. Moreover, an intervention that reduces adiposity at the cost of muscle mass is a clear contraindication for conditions such as sarcopenic obesity. It is likely that intervention at an earlier stage of disease progression could have a more profound or potentially preventative effect on MetS cluster factors. Future pre-clinical research should determine not only the ideal timepoint to initiate a therapeutic intervention but minimum dose to effect, ideal dose duration, as well as the efficacy of intermittent dosing. Because both natural CBD, synthetic H4CBD and synthetic H2CBD compounds are tolerated similarly[73,138], there exists potential for similar therapeutic efficacy in other systems as they relate to amelioration of metabolic dysfunction. The benefits observed here are encouraging and contribute to establishing a foundation from which to inform future studies on the effects of CBD and other synthetic CBD analogues on metabolic disorders and associated cardiovascular dysfunction.

Limitations

A limitation may be the omission of isolation of specific CBD effects on tissue via cell culture studies (*ex vivo* or *in vitro*). Although tissue culture could provide insight to specific mechanisms mediated by CBD, cell culture distinctly omits complex *in vivo* contributions to MetS that are impossible to fully replicate *in vitro*. Because the relevance of this study is underscored by the *in vivo* complexity of MetS, cell culture falls outside the scope of this current study. Additionally, the low sample size was an unfortunate consequence of the unexpectedly high surgery attrition and loss of telemeter battery life, ultimately due to COVID-19-related delays.

Chapter 5: Pseudocannabinoid (H4CBD) improves glucose response during advanced metabolic syndrome in OLETF rats independent of increase in insulin signaling proteins

Abstract

Cannabidiol (CBD) use has grown exponentially more popular in the last two decades, particularly amongst older adults (>55 years), though very little is known about the effects of CBD use during age-associated metabolic dysfunction. Additionally, synthetic analogues have generated great interest because they can offer a chemically pure product, which is free of plant-associated contaminants. To assess the effects of a synthetic analogue of CBD (H4CBD) on advanced metabolic dysfunction, a cohort of 41-week-old Otsuka Long-Evans Tokushima Fatty (OLETF) rats were administered 200 mg H4CBD /kg by oral gavage for 4 weeks. Animals were fed ad libitum and monitored alongside vehicle-treated OLETF and Long-Evans Tokushima Otsuka (LETO) rats, the lean-strain controls. An oral glucose tolerance test (oGTT) was performed after 4 weeks of treatment. When compared to vehicle-treated OLETF rats, H4CBD decreased body mass (BM) by 15%, which was attributed to a significant loss in abdominal fat. H4CBD reduced glucose response ($AUC_{glucose}$) by 29% ($p<0.001$) and insulin resistance index (IRI) by 25% ($p<0.05$) compared to OLETF rats. However, H4CBD did not statically reduce fasting blood glucose or plasma insulin, despite compensatory increases in skeletal muscle native insulin receptor (IR) protein expression (54%; $p<0.05$). H4CBD reduced circulating adiponectin (40%; $p<0.05$) and leptin (47%; $p<0.05$) and increased ghrelin (75%; $p<0.01$) compared to OLETF. Taken together, a chronic, high dose of H4CBD may improve glucose response, independent of static changes in insulin signaling and these effects are likely a benefit of the profound loss of visceral adiposity.

Introduction

The popularity of cannabis use in older adults in the United States has more than doubled over the past two decades[75–78]. Sentiments toward cannabis as a viable treatment intervention continues to be well documented as a low risk alternative to pharmaceuticals[139,140]. Among the age-related ailments for which medicinal cannabis is sought, chronic pain, irritable bowel syndrome, and glaucoma are the most frequently cited[77–79]. However, a common consequence of aging is impaired substrate metabolism, which can result in the onset of metabolic conditions ranging in severity from risk factors for metabolic syndrome (MetS)[3] to frank Type II diabetes mellitus (T2DM)[5]. The prevalence of MetS is 54.9% among people ≥60 years of age[5] and the average age at diagnosis of T2DM is 46 with equal abundance across racial and ethnic groups[80]. Importantly, the effects of herbal cannabis and cannabis constituent use during conditions of metabolic dysfunction are vastly understudied[141] and the explosion of interest in cannabis amongst older adults, coupled with the relatively high incidence of MetS in this population, constitutes a critical intersection between cannabis users and advanced metabolic dysfunction.

Cannabidiol (CBD) is an abundant, non-intoxicating constituent of *Cannabis sativa* which is of particular interest for pharmacological investigation. One drawback of CBD is however its easy conversion to tetrahydrcannabinol (THC), the psychotropic constituent of cannabis. This, and the legislative ambiguity and increasing ease-of-access to unregulated cannabis constituents, have prompted endeavors to synthetize analogues (pseudocannabinoids) of natural cannabinoids. The use of these synthetic analogues circumvents the complex regulatory landscape surrounding the production of scheduled compounds while at the same time providing a chemically homogeneous compound free from undesired phytocannabinoids or pesticides. Synthetic analogues of CBD can offer similar therapeutic effects to that of natural CBD. For example, dihydrocannabidiol (H2CBD) was found to reduce seizure frequency and severity in rats with equal effectiveness to that of CBD[73]. Tetrahydrocannabidiol (H4CBD) is a compound that differs from CBD by the saturation of the two double bonds in the terpene fragment of the molecule. Like natural CBD, H4CBD has little affinity for the endocannabinoid receptors responsible for cannabis intoxication[74]. Although use of pseudocannabinoids *in vivo* has not been previously described in the context of metabolic dysfunction, *in vitro* use has yielded promising effects consistent with natural CBD[74,121,122]. Therefore, H4CBD is expected, like H2CBD, to exert similar effects to those of natural CBD.

Rigorous pre-clinical data are sparse concerning the effects of cannabinoids in models of metabolic dysfunction. Natural CBD reduced the incidence of diabetes in diet-induced obese (DIO) and non-obese diabetic mice[66,70], providing some evidence that synthetic analogues likewise have the potential to similarly ameliorate dysregulated metabolism. Therefore, the goals of this study were to assess the effect of H4CBD on MetS cluster factors such as glucose intolerance and insulin resistance during advanced metabolic dysfunction using the Otsuka Long-Evans Tokushima Fatty (OLETF) rat as a monogenic model of diet-induced obesity that slowly manifests into insulin resistance, MetS, and ultimately frank T2DM[92,93]. At >40 weeks of age, OLETF rats suffer from severe metabolic dysfunction[142] and therefore serve as a model of aged, acute MetS and T2DM common amongst older adults. We hypothesized that H4CBD would improve the glucose intolerance and insulin resistant condition characteristic of this model.

Methods

All animal procedures were reviewed and approved by the institutional animal care and use committees of the University of California, Merced.

Animals

Male lean Long Evan's Tokushima Otsuka (LETO) rats and obese Otsuka Long Evans Tokushima Fatty (OLETF) rats (Japan SLC Inc., Hamamatsu, Japan), both 14 weeks of age, were assigned to the following groups (n=8/group): **(1)** vehicle-dosed LETO (LETO), **(2)** vehicle-dosed OLETF (OLETF), and **(3)** H4CBD-treated (200mg/kg/day x 4 weeks) OLETF (H4CBD). LETO rats were purposefully not treated, as the use of cannabinoids in healthy individuals is either preventative or recreational, neither of which addresses our hypothesis that cannabinoids will serve a therapeutic purpose during a condition of deranged metabolism. Rats were maintained in a specific pathogen-free, climate-controlled facility at the University of California Merced on a 12-hour light:dark cycle (07:00-19:00 and 19:00-07:00, respectively). All animals had free access to water and were fed rat chow (Teklad Global; fat 9.0%; carbohydrate 44.9%; protein 19.0%) *ad libitum*. Treatment intervention was initiated at 41 weeks of age. Phenotypic data (i.e. body mass (BM), food intake) was collected daily for all animals.

Drug preparation & administration

H4CBD (1,2,8,9-tetrahydrocannabidiol, systematic name 2-(2-isopropyl-5-methylcyclohexyl)-5-pentylbenzene-1,3-diol), was prepared by reduction of synthetic H2CBD (8,9-tetrahydrocannabidiol) with hydrogen and a Pd/C catalyst in acetic acid solvent and was purified by distillation *in vacuo*. H2CBD was synthesized from olivetol and food-grade α-phellandrene according to the published procedure[73]. All chemicals were purchased from Millipore Sigma and used as received.

Purified H4CBD (>99%) was suspended in food grade sesame oil and administered by oral gavage at a dose of 200mg/kg/day. A volumetrically equivalent dose of vehicle was administered to LETO and OLETF control groups by oral gavage. This dose, administered via intraperitoneal injection, has been shown to be similarly effective for the mitigation of seizure frequency and severity in rats compared to natural CBD[73], which gave reasonable cause for efficacy of H4CBD at the same dose, which is well below documented toxicity of CBD (>600mg/kg) in rodents[94]. While this is a relatively high dose, the current study provides a basis to justify this dose as the highest needed.

Oral glucose tolerance test (oGTT)

At approximately 45 weeks of age and following an overnight fast, oGTTs were performed as previously described[96]. The positive incremental areas under the curve for glucose ($AUC_{glucose}$) and insulin ($AUC_{insulin}$) were calculated by the trapezoidal method[110]. Insulin resistance index (IRI), an indirect marker of peripheral insulin action, was calculated by $AUC_{glucose} x\ AUC_{insulin}/100$, as previously described[96,113,114]. QUICKI was also calculated as $1/\ [\log(I_o)+\log(G_o)]$ as previously described[114,143].

Tissue collection

After the 4-week study and 3 days following the oGTT, animals were fasted overnight and tissues were collected the following morning as previously described[23,87,96]. Briefly, trunk blood was

collected into prepared, chilled glass tubes containing either heparin and protease inhibitor cocktail or EDTA, protease inhibitor cocktail, and angiotensin converting enzyme inhibitor[82,87,96].

Western blot

Soleus was used to measure proteins involved in insulin signaling as previously described[23,96,114,144]. Densitometry values were quantified using ImageJ software (NIH) and normalized by correcting for densitometry values of representative protein bands below 37kDa stained with Ponceau S[145]. Results are reported as expression (%) compared to LETO.

Drug bioavailability detection

H4CBD bioavailability in heparinized plasma was determined via HPLC and MS/MS as described previously[95]. In brief, 100 µL heparinized rat plasma was diluted with 100 µL Mass Spec Gold human serum (Golden West Diagnostics, MSG3200). The samples were analyzed using a WATERS TQS-Micro. The first quadrupole was set to 319.16 m/z and the third quadrupole isolated fragments of 181.02 m/z. Any sample that had a peak area count of <1500 was considered negative for H4CBD.

Plasma analysis

Plasma concentrations of adiponectin (Millipore Sigma; EZRADP-62K), corticosterone (B; R&D Systems; KGE009), ghrelin (Sigma Aldrich; RAB0207), glucagon (Crystal Chemicals; 81519) and leptin (Millipore Sigma; EZRL-83K) were measured in fasted, end of study plasma samples. All samples were analyzed in duplicate and run in a single assay with intra-assay and percent CV of <10% for all assays.

Statistics

All values are represented as mean ± standard error mean (SEM) unless otherwise indicated. Means were compared by one-way ANOVA followed by Tukey's honest significant difference or unpaired, one-tailed t-test to assess significant differences among groups. Means and regressions were considered significant at $p<0.05$. Outliers were detected by ROUT (Q=1.0%) and removed; however, it should be noted that this was necessary for only 12 occurrences. All statistical procedures, including Pearson r correlations, were performed using GraphPad Prism 9.3.1 (GraphPad Software, Inc., San Diego, CA, USA).

Results

H4CBD was only detected in plasma from treated rats

Bioavailability of H4CBD compound was validated in end of study plasma and confirmed that only treated animals received drug (**Figure 1**). Due to the novelty of the compound, any sample that had a peak area count of <1500, was considered negative for H4CBD.

H4CBD reduced BM independent of changes in food consumption

BM and food consumption were measured daily and abdominal fat masses were weighed at dissection to determine the effect of H4CBD treatment on phenotypic indicators of metabolic dysfunction. H4CBD reduced BM to LETO levels (**Table 1**). BM of OLETF was 16% higher than LETO ($p<0.05$) and 15% higher ($p<0.05$) than the H4CBD group (**Table 1**). Relative

retroperitoneal fat, but not relative epididymal fat, was 267% more abundant in OLETF than LETO (p<0.0001) (**Table 1**). H4CBD reduced relative retroperitoneal fat by 24% (p<0.05) and relative epididymal fat by 35% (p<0.05) compared to OLETF (**Table 1**). Relative visceral (epi + retro) adipose was 149% higher in OLETF compared to LETO (p<0.0001), which tended to reduce by 25% with H4CBD treatment (p=0.057) (**Table 1**). Initial and final food intake between OLETF and H4CBD were comparable (**Table 1**).

H4CBD ameliorated dynamic glucose response but did not reverse diabetic phenotype

oGTTs were performed, wherein plasma insulin was measured at corresponding timepoints, to determine the effects of H4CBD on glucose intolerance. From these measurements, insulin resistance index (IRI) and QUICKI were calculated to determine the effect of H4CBD on peripheral insulin sensitivity during advanced MetS. LETO blood glucose response peaked at 10 minutes after glucose bolus while OLETF peaked 60 minutes after glucose bolus (**Figure 2A**). $AUC_{glucose}$ of discrete timepoints (T0-T60) were 32% lower for H4CBD compared OLETF (p<0.01) suggesting a potential delay of glucose absorption into the bloodstream (**Figure 2A**). Glucose response curve was lowest for H4CBD-treated animals overall (p<0.01) (**Figure 2A**). Overall $AUC_{glucose}$ was 49% higher in OLETF compared to LETO (p<0.001) and H4CBD reduced $AUC_{glucose}$ 29% from OLETF (p<0.001) (**Figure 2B**). Plasma insulin response was abolished in OLETF, which was not rescued by H4CBD treatment (**Figure 2C and D**). The calculated insulin resistance index (IRI) status was similar between aged LETO and OLETF rats, but IRI was reduced 23% in H4CBD compared to OLETF (p<0.001) (**Figure 2E**). H4CBD reduced QUICKI by 16% from OLETF (p<0.01), providing an additional measure of reduced insulin resistance (**Figure 2F**).

H4CBD did not improve static indicators of glucose tolerance

At 45 weeks of age, fasting blood glucose (FBG) was 44% (p<0.05) higher in OLETF than LETO while levels were 2.2-fold higher in H4CBD than LETO (p<0.0001) and 56% higher than OLETF (p<0.01) (**Table 2**). Fasting plasma insulin was 64% higher in OLETF compared to LETO (p<0.01) but similar to H4CBD (**Table 2**). Plasma glucagon was 63% lower in OLETF compared to LETO (p<0.05) but was not different from H4CBD (**Table 2**). H4CBD increased fasting glucose to insulin ratio by 47% (p<0.05) and reduced fasting insulin to glucagon ratio by 64% (p<0.05) compared to OLETF (**Table 2**).

H4CBD statically increased insulin receptor expression

Because skeletal muscle is the primary sink for glucose utilization, insulin signaling proteins were measured to determine effects to static tone of proteins in the insulin signaling cascade. No changes were detected in the abundance of static, phosphorylated insulin receptor (pIR) (**Figure 3A**). H4CBD increased native insulin receptor (IR) expression by 54% over OLETF (p<0.05) (**Figure 3B**) but pIR/IR was not changed (**Figure 3C**). Cytosolic pAkt and Akt expressions were comparable between LETO and OLETF but H4CBD reduced the expressions of pAkt, Akt and pAkt:Akt ratio by 58% (p<0.05), 32% (p<0.05), and 35% (p<0.01), respectively, compared to OLETF (**Figure 3D-F**). PI3K expression was reduced 49% (p<0.05) in OLETF compared to LETO and H4CBD tended (p=0.07) to further reduce PI3K expression by 47% compared to OLETF (**Figure 3G**). pAMPK was 34% (p<0.05) higher in OLETF compared to LETO and H4CBD had no effect (**Figure 3I**). Native AMPK was 32% (p<0.05) higher in OLETF compared to LETO but

H4CBD had no effect (**Figure 3J**). No changes were detected in the ratio of pAMPK/AMPK expression (**Figure 3K**).

Translocated GLUT4 may serve as a static indicator of increased insulin signaling and was measured by probing for its expression in the membrane fraction of skeletal muscle. Translocated GLUT4 was 46% ($p<0.05$) lower in OLETF compared to LETO, which H4CBD did not rescue (**Figure 3L**). Cytosolic GLUT4 was 39% lower in OLETF compared to LETO ($p<0.05$) and 58% ($p<0.01$) higher in H4CBD compared to OLETF (**Figure 3M**). The ratio of membrane to cytosolic GLUT4 was unchanged among the groups (**Figure 3N**).

H4CBD reduced circulating adiponectin and leptin and increased ghrelin

Fasting plasma adiponectin, corticosterone, ghrelin, and leptin were measured to assess the effect of H4CBD on levels of adipocytokines and other hormones associated with insulin resistance and obesity. While there was no strain difference observed, H4CBD reduced circulating adiponectin by 40% ($p<0.05$) compared to OLETF (**Figure 4A**). Leptin tended to be reduced in OLETF compared to LETO ($p=0.08$) and H4CBD reduced leptin by 47% ($p<0.05$) compared to OLETF (**Figure 4B**). The leptin:adiponectin ratio was comparable amongst groups (**Figure 4C**) along with plasma corticosterone (**Figure 4D**). Plasma ghrelin was 75% ($p<0.05$) greater in H4CBD compared to the other groups and levels were similar between LETO and OLETF (**Figure 4E**).

H4CBD-induced reduction of abdominal fat was positively correlated with adiponectin reduction

Pearson r correlations of end of study plasma leptin, adiponectin, food intake, epidydimal fat, retroperitoneal fat, visceral (combined) fat, and leptin:adiponectin ratio were conducted to determine significant interactions between fat mass and hormones associated with insulin resistance and obesity. LETO visceral fat was positively associated with plasma leptin (Pearson r 0.79; $p<0.05$) unlike OLETF (**Figure 5**). The H4CBD-induced reduction in visceral fat was positively correlated with plasma adiponectin (Pearson r 0.92; $p<0.01$) and negatively correlated with food intake (Pearson r -0.74; $p<0.05$) (**Figure 5**).

Discussion

As the prevalence of MetS continues to increase in the US and globally, identifying novel compounds to ameliorate the multiple morbidities that characterize MetS becomes increasingly more important. Addressing the many maladies that comprise MetS simultaneously is challenging, which contributes to the urgency to develop novel therapies. A review of the potential benefits of cannabinoids on a wide range of pathologies is encouraging; however, the effects on the various pathologies constituting MetS are largely unknown. A few studies have shown beneficial[60,63] or null effects[72] of CBD on isolated MetS risk factors, but these effects have not been explored in context of cluster factors. Therefore, the aim of this study was to assess the effects of a comparable pseudocannabinoid on glucose intolerance in the condition of advanced MetS, which is most frequently observed in older populations, while averting the use of a herbal cannabinoid. We chose for this purpose to evaluate the effects of the synthetic, non-narcotic cannabinoid analogue 1,2,8,9-tetrahydrocannabidiol (H4CBD), due to its synthetic accessibility in pure form and potential for greater adoption than CBD, which is subject to multiple regulatory restrictions worldwide.

A hallmark of MetS is sustained hyperglycemia, with or without insulin resistance, whereby blood glucose levels remain elevated. OLETF rats fed the same diet as their lean, strain-control, LETO rats develop later onset hyperglycemia by 15 weeks of age[23] which has been shown to be attenuated by caloric restriction at earlier stages of disease progression[96,146]. Although the primary etiologic event for hyperinsulinemia is not explicit, OLETF demonstrate pancreatic β-cell dysfunction by 16 weeks marked by insulin resistance and impaired insulin secretion[142]. We show that in older OLETF rats (>40 weeks of age) fed *ad libitum*, fasting blood glucose (FBG) was 43% higher than LETO and that OLETF rats struggled to clear glucose from circulation long after time-to-clearance in LETO. Both metrics are consistent with previously characterized hyperglycemia in OLETF rats at 40 weeks of age[142]. Moreover, the insulin response to oGTT was severely blunted at this age, though fasting insulin remained relatively high in response to sustained hyperglycemia, which is indicative of **(1)** insufficient β-cell insulin secretion[147] and **(2)** insulin resistance at the level of the receptor[148], respectively. Most importantly, treatment with H4CBD improved the dynamic, strain-associated glucose intolerance independent of static enhancements of proteins of the insulin signaling pathway.

Endocannabinoid signaling in the gut and peripherally has been shown to modulate nutrient absorption and uptake[119,120], which could confer a beneficial delay in the uptake of glucose into circulation. Thus, the improvement in glucose tolerance observed with H4CBD may have been accomplished via delayed absorption of glucose across the gut independent of static changes in insulin signaling. Because glucose-stimulated insulin secretion was blunted in both OLETF groups, the reduction in the IRI calculation in the H4CBD group was likely more of a function of reduced $AUC_{glucose}$ than insulin-dependent mechanisms. The reduction in visceral adiposity may have also contributed to the improvement in glucose tolerance. Regardless of the primary mechanisms, the result of 4 weeks of H4CBD treatment was an improvement in glucose tolerance subsequent to the insulin resistance status.

Interestingly, while H4CBD resulted in acute, dynamic improvements in glucose metabolism, the treatment did not appear to correct the chronically sustained hyperglycemia or hyperinsulinemia, suggesting that this dose and duration of treatment were not sufficient to reverse the diabetic phenotype.

Skeletal muscle is considered the primary sink for glucose metabolism, which is predominantly regulated by insulin receptor-mediated signaling[149]. At the cellular level, IR expression in skeletal muscle of H4CBD rats was higher than the other groups, although the phosphorylation of IR was not different, resulting in a reduced ratio and suggesting that IR activation was suppressed or at least not chronically and statically changed. The increase in native IR protein expression may be a compensatory response to the static hyperglycemia and hyperinsulinemia in this group, although humans and mice with insulin resistance have reduced IR expression[150] and the treatment of hyperglycemia with berberine (a natural alkaloid) has been shown to increase IR expression[151]. Alternatively, improved protein stability (reduced susceptibility to degradation) could contribute to the observed hyperinsulinemia and enhanced IR protein expression[152]. Regardless, the inherent limitation of this interpretation is the single time point at which these measurements were made. Similar analyses at various timepoints would reinforce this argument to interpret the direct or indirect dynamic effects of H4CBD on the insulin signaling cascade in skeletal muscle.

The loss of visceral adipose invariably affects circulating adipokines, which influence peripheral insulin resistance and modulate hunger[116,153]. The decreases in the visceral adipose depots in response to H4CBD treatment translated into reductions in plasma adiponectin and leptin and likely reflect the inability to restore hormone-driven insulin sensitization in skeletal muscle. While increased leptin may impair insulin sensitivity and adiponectin can counteract leptin to improve sensitivity, the simultaneous reductions in both likely contributed to the apparent insulin resistance of skeletal muscle in this model. Additionally, the reductions in both adipokines and their ratios suggest that H4CBD treatment may have shifted substrate metabolism, resulting in increased lipid catabolism and ultimately reduced adiposity. Preferential enhancement in lipid catabolism may compensate for the energy deficit conferred by insulin resistance but it is not clear if this compensation is sustainable or whether these effects are lost after cessation of treatment and warrants further investigation.

Though not statistically correlated, the increase in plasma ghrelin supports the observed increase in relative food intake in the presence of body mass loss. However, it is unclear if H4CBD directly stimulated ghrelin secretion or if it was compensatory in response to the loss in fat mass, and thus, responding to a shift in substrate metabolism to help maintain energetic homeostasis[117,154,155].

Limitations

In addition to the limitations of previous chapters, single time point analysis and dose to effect are outstanding limitations of the studies presented here. It is not clear if a lower dose would be sufficient to confer the effects observed here or if a shorter duration would be sufficient. And, similar to limitations of the study analyzed in chapter 2-3, accessibility to drug and perceived poor animal tolerance contributed to adjustment of dosing strategy to every other day. one could argue that a dose regimen that skips doses more accurately models human compliance but simultaneously begs the question around the sufficiency of every other day dosing to observe the same effects.

Conclusions

In summary, the H4CBD-mediated nuanced improvement in dynamic glucose tolerance observed here was biologically significant and likely attributed, at least in part, to the reduction in adiposity and/or modulation of nutrient absorption by the gut. While the improvement was modest, it is important to note that the conditions under which these benefits were realized are highly dysfunctional. The animals in this study were advanced in age with severe metabolic derangement, rendering any health benefits at this stage remarkable. It is likely that intervention at an earlier stage of disease progression could have a more profound or potentially preventative effect on MetS cluster factors. Moreover, careful consideration should be made for individuals diagnosed with sarcopenic obesity as this may preclude the use of CBD compounds for the purpose of adipose reduction due to the detrimental cost of lean tissue. Future preclinical research should determine not only the ideal timepoint to initiate a therapeutic intervention but minimum dose to effect and ideal dose duration as well as the efficacy of intermittent dosing. Because both natural phytocannabinoids and synthetic pseudocannabinoids are tolerated similarly[73,138], there exists potential for similar therapeutic efficacy in other systems as they relate to amelioration of metabolic dysfunction. The potential benefits on ameliorating glucose intolerance during advanced MetS observed here are encouraging and contribute to establishing a foundation from which to inform future studies on the effects of CBD and other synthetic CBD analogues on metabolic disorders.

Chapter 6: Discussion, limitations and future directions

CVD remains the leading cause of death in the US and is often a comorbidity of obesity and T2DM. The prevalence of these conditions, coupled with the precursory cluster of conditions that define MetS (e.g., hypertension, obesity, poor glucose tolerance, and dyslipidemia), underscores the deleterious implications of impaired substrate metabolism and the urgency for early intervention. *Cannabis* constituents, especially CBD, constitute an active area of research because of the potential application for the attenuation of cardiometabolic risk factors. Although the specific mechanism of action and pharmacology of CBD is not well-defined, a handful of clinical and preclinical studies substantiate the interest of the scientific community and public at large. Moreover, the paucity of literature on the subject prompt the studies herein that contribute to this dissertation, which attempt to better define the therapeutic potential for CBD in a translational model of MetS.

To this effect, in chapter 2, we demonstrated that CBD ameliorated hypertension during progressive but not advanced MetS. In both conditions, however, plasma AngII was elevated compared to OLETF, which could suggest that CBD compounds may impair AngII binding to AT1 for uptake and subsequent signal transduction (**Figure 23**). Indeed, this is the mechanism of action of ARB and previous studies have shown OLETF to be ARB-sensitive and plasma AngII levels respond similarly. However, the CBD-induced reduction of arterial blood pressure was not obviously classically nor non-classically RAAS-mediated. That said, measurement of reactive oxygen species and NADPH-oxidases (NOX1, 2, 4, 5) in the endothelium and sodium handling/excretion could provide insight to alternatives of BP modulation, as hypertension is primarily affected via third order arterial vasoconstriction.

In chapter 3, we demonstrated that CBD compounds prevented BM gain in the early MetS condition and reduced abdominal adipose accumulation overall. That said, both compounds had an initial anorexigenic effect, which may contribute in part to the observed reduction in mass accumulation. Although subtle improvements in glucose response to challenge manifested in treated animals, and insulin response reduced, the diabetic phenotype overall was not corrected. In the absence (or at least reduction) of carbohydrate metabolism, we looked to indicators of lipid catabolism to explain the improvement in the obese OLETF. We noted CBD compound reduced adipocyte morphology and plasma triglycerides but not plasma NEFAs, which suggests lipids and FFAs are being utilized rather than produced and/or stored. Targeted metabolomics analyses revealed the most significant metabolite shift in both the CBD- and (*top 5*) H2CBD-treated animals to be BAIBA, a metabolite implicated in the browning of white adipose tissue (**Figure 23**). Indeed, our crude assessment of BAT at dissection corroborated our metabolomics analysis and suggested a preservation of BAT or browning of WAT in animals treated with CBD compound.

Taken together, in early MetS, CBD compounds ameliorate multiple cluster conditions of MetS including hypertension, obesity and dyslipidemia. That said, we probed the effects of CBD compound on cluster conditions of MetS during severe disease in chapter 4. We found that CBD compound made similar improvements to reduce BM and abdominal adiposity but had no effect on hypertension, which suggests that the attenuation of hypertension observed in chapter 2 is not simply due to a reduction in BM or adiposity. However, the loss of lean tissue in the advanced condition suggests contraindication for use in sarcopenic obesity, which is more prevalent in older adults. Additionally, CBD compound did not correct the diabetic phenotype but did improve dynamic response to glucose at challenge. This may be a function of CBD compound activity at the level of glucose transporters in the gut (since there was also a delay in glucose response in the early condition) and this hypothesis constitutes a future direction of the work herein.

Notwithstanding, in chapter 5 we probed the insulin signaling cascade in the skeletal muscle of advanced MetS animals in an attempt to explain the improvement in dynamic glucose response. We found that the expression of insulin receptor statically increased but static phosphorylation and GLUT4 translocation were not affected. These findings contribute substantially to our understanding of CBD compound pharmacology in conditions of metabolic dysfunction and offer key insights into therapeutic applications for CBD.

Limitations

Although these results offer substantiating evidence for therapeutic application of CBD compounds, there are some limitations to this study design. First, the use of a single dose amount here does not address our outstanding question of dose to effect. Second, tissue samples, including plasma, were collected at end of study which only offers a single timepoint for consideration, thereby severely limiting the resolution of insights made from these samples and this study. Third, the sample size of the groups represented in the telemeter data of chapter 2 are limited (n=3/group). Although the lifetime of HD-S10 telemeters averages 6 months, the telemeters used in this study were sterilized and reused from a previous study. This served two purposes: (1) cost mitigation and (2) circumvention of prolonged lead times and slow delivery due to COVID-related delays in products. Fourth, every other day dosing after the first week was not planned but resulted from a mistake made by the only legally available source of CBD to academic institutions—DEA authorized distributor University of Mississippi. We were to receive an authorized amount of cannabis-extracted CBD to use for this study. However, we were sent the authorized quantity of extract at 55% concentration of CBD. This meant were actually supplied with 55% of the approved amount. Therefore, we took the necessary measures to maintain a consistent dosing routine while we applied for additional product and resumed daily dosing upon receipt. That said, one could argue that a dose regimen that skips doses more accurately models human compliance and begs the question around the sufficiency of every other day dosing to observe the same effects.

Future Directions

CBD may promote antioxidant and anti-inflammatory effects, which were implicated in the restoration of left ventricular function in a murine model of type I diabetes[60], vascular endothelial barrier function[61] and cisplatin-induced nephropathy[62]. Specifically, it was determined that CBD promoted antioxidant enzyme transcription events and nuclear factor-kappa-B (NFkB) inhibitor alpha (IkBα) sequestration of NFkB in the cytosol, which reduced inflammation and associated damage in cardiac tissue[60]. However, these effects are mitigated by upstream translocation of nuclear factor-erythroid factor 2-related factor 2 (Nrf2) to the nucleus[156–158]. Although similar antioxidative and ani-inflammatory benefits were realized in isolated hypertension model SHR, CBD (10mg/kg/day x 2 weeks) was not sufficient to attenuate elevated blood pressure[72]. The data here demonstrate CBD attenuates hypertension during MetS cluster factor conditions and, considering the literature findings, could implicate CBD may target RAAS components or Nrf2-associated signal transduction.

The Nrf2 signal cascade and NfkB-Nrf2 signal cascade crosstalk offer multiple points of analysis to determine the specific effect of CBD during MetS. Nrf2 is held in the cytosol by *Keap1* (Kelch-like ECH-associated protein 1), which is an adaptor subunit of Cullin 3-based E3 ubiquitin ligase[156,159] (**Figure 24**). In conditions of oxidative stress, Keap1 is deprotonated at thiol groups on cysteine residues, which releases Nrf2 to translocate to the nucleus and initiate transcription of antioxidant enzymes[158–160]. An alternative pathway of Nrf2 activation sequesters Keap1 to autophagic degradation, which frees Nrf2 for translocation[161]. Glycogen synthase kinase 3 (GSK3) is a Keap1-independent regulator of Nrf2, which facilitates the degradation of Nrf2[162].

GSK3 is known to be activated in conditions of insulin resistance[147]; although its role in the etiology of insulin resistance remains unclear[163]. In conditions of MetS, substrate metabolism is impaired due to poor glucose tolerance and insulin resistance, which can induce oxidation via mitochondrial oxidant production and subsequent inflammatory state[3]. Moreover, the hypertensive condition of MetS contributes to mechanical and oxidative damage to the vasculature[97,98,100]. In the altered (substrate metabolism) conditions of MetS, Nrf2 abundance is reduced, which limits the intrinsic anti-oxidative response[84,164–166] and Nrf2 deficiency induces IκBα phosphorylation and subsequent degradation, increasing NF-κB levels and inducing inflammation[167,168]. Activated Nrf2 transcription of downstream gene, heme oxygenase-1 (HO-1), has been implicated in the attenuation of NfkB transcription activation, which reduces production of interleukin-1 beta (IL-1β; inflammatory cytokine precursor), tumor necrosis factor alpha (TNFα; local inflammatory cytokine) and interleukin-6 (IL-6; pro-inflammatory cytokine)[169,170] (**Figure 24**). Also, NF-κB-DNA-binding activity was significantly inhibited in a murine model of type 1 diabetes with Nrf2 overexpression[171]. CBD has been shown to upregulate HO-1 expression *in vitro* in human umbilical artery smooth muscle cells (HUASMCs) and reduced phosphorylation of IkBα to inhibit NFkB translocation *in vivo* in murine type 1 diabetes[60] (**Figure 24**).

Because we hypothesize that CBD has a direct effect on Nrf2, we would measure Nrf2 abundance in the cytosolic and nuclear fraction of LV cardiomyocytes and dissected arterioles by Western blot. Increased abundance of Nrf2 in both the cytosol and nucleus of cardiomyocytes of CBD-treated animals will be indicative of **(A)** pro-transcriptional events of Nrf2 (relative mRNA and protein abundance compared to non-treated animals normalized to tissue mass and total protein normalization (ponceau S and/or actin for cytosolic fractions and histone for nuclear fractions) and **(B)** inhibited GSK3 degradation of Nrf2, which can be confirmed by lack of phosphorylated Nrf2 ratioed to non-phosphorylated Nrf2 in the cytosol. The increased abundance of downstream products will provide validation of CBD-induced enhancement of Nrf2 signal cascade.

Endocannabinoid signaling in the gut and peripherally has been shown to modulate nutrient absorption and uptake[119,120], which could confer a beneficial delay in the uptake of glucose into circulation. Thus, the improvement in glucose tolerance observed with H4CBD, and subtle improvements in the GTT curve of CBD-treated animals in chapter 3, may have been accomplished via delayed absorption of glucose across the gut independent of static changes in insulin signaling. This delay could be accomplished vi inhibition of SGLT1 on the intestinal lumen side and/or GLUT2 on the portal blood side (**Figure 23**). To better resolve the observed effects CBD compounds had on glucose and insulin response to challenge, we would conduct a similar study punctuated by both oGTT and intraperitoneal GTT to determine if CBD compounds functionally impair glucose transporters in the gut.

To better resolve our crude visual assessment of BAT abundance, we would employ proteomics to quantify uncoupling protein 1 and 2 (among other key protein identifiers associated with mitochondrial respiration) as a means of quantifying BAT abundance and activity, alongside dissection of the proteome of CBD-affected BAT, which is an impactful and novel endeavor. We would also seek to replicate the study and measure temperature, which is an indirect assessment of thermogenesis thought to be primarily mediated by BAT. That said, we have outstanding questions around the function of BAIBA in relation to BAT as well as energy expenditure. Increases in BAIBA have been suggested to be a function of increased energy expenditure or exercise alone. Therefore, CBD could be driving activity (**Figure 10E**), akin to exercise, which promotes the secretion of BAIBA from the skeletal muscle. Alternatively, CBD could be promoting the secretion of BAIBA directly to drive the BAIBA-associated benefits observed here. To dissect this out, we

would be interested in measuring plasma BAIBA levels in response to acute exercise versus an acute dose of CBD as well as chronic exercise versus a chronic dose of CBD. The addition of telemetry would afford a measured activity score to validate energetic expenditure conditions.

Above all, a dose-to-effect study would provide critical insight to the dose recommendation for the use of CBD compounds to attenuate MetS progression in a prophylactic capacity.

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Tables

Table 1. 3(β)-aminoisobutyric acid (BAIBA), metabolite associated with browning of white adipose tissue, amongst the most significantly increased metabolites in CBD-treated animals. Top shifted metabolites across group comparison ranked by lowest *p*-value (*student's t-test*) in 20-week-old LETO, OLETF, CBD-treated and H2CBD-treated OLETF.

	Top LETO_OLETF changes (lowest p-value)	Pathway	Direction
LETO/OLETF	linolenic acid	Lipid metabolism	Up
LETO/OLETF	isoleucine	Amino acid metabolism	Up
LETO/OLETF	glucose	Carbohydrate metabolism	Down
LETO/OLETF	leucine	Amino acid metabolism	Up
LETO/OLETF	succinic acid	Energy metabolism	Up

	Top OLETF_CBD Changes (lowest p-value)	Pathway	Direction
OLETF/CBD	3-aminoisobutyric acid	browning of WAT	Up
OLETF/CBD	hypotaurine	energy metabolism	Up
OLETF/CBD	glutamine	Amino acid metabolism	Up
OLETF/CBD	5-aminovaleric acid	amino acid metabolism	Up
OLETF/CBD	taurine	energy metabolism	Down

	Top OLETF_H2CBD Changes (lowest p-value)	Pathway	Direction
OLETF/H2CBD	linolenic acid	Lipid metabolism	Down
OLETF/H2CBD	isocitric acid	Carbohydrate metabolism	Down
OLETF/H2CBD	campesterol	Biosynthesis of terpenoids a	Down
OLETF/H2CBD	taurine	energy metabolism	Down
OLETF/H2CBD	3-aminoisobutyric acid	browning of WAT	Up

Table 2. Mean ± SEM calculations of fat loss versus lean tissue loss.

Strain	LETO			OLETF				H4CBD			
Body Mass (g)	508	±	10	611	±	41	*	476	±	18	†
Plasma Albumin (mg/mL)	55	±	2.7	56	±	2.8		54	±	2.6	
Heart Mass (g)	1.4	±	0.0	1.6	±	0.0	*	1.5	±	0.0	*
Liver Mass (g)	12	±	0.3	18	±	0.9	*	19	±	0.5	*
Epidydimal Fat Mass (g)	9	±	0.6	12	±	2		6	±	1	†
Retroperitoneal Fat Mass (g)	12	±	0.8	54	±	6	*	33	±	6	*, †
Kidney Mass (L+R) (g)	2	±	0.1	4	±	0.2	*	4	±	0.1	*, †
Adrenal Mass (g)	0.1	±	0.0	0.1	±	0.0		0.1	±	0.0	
Estimated Muscle Mass (g)	145	±	5	153	±	16		102	±	6	*, †
Estimated Total Body H_2O (g)	325	±	4	369	±	17	*	312	±	8	†
Lean Body Mass (g)	486	±	8.9	546	±	34		438	±	13	†
Fat Mass (%)	4.3	±	0.3	11	±	0.8	*	7.9	±	1.1	*#
ΔBM Compared to OLETF (g)								108	±	12	
ΔBM Compared to OLETF (%)								20	±	2	
ΔFM Compared to OLETF (g)								33	±	4	
ΔFM Compared to OLETF (%)								49	±	6	
ΔLM Compared to OLETF (g)								33	±	4	
ΔLM Compared to OLETF (%)								49	±	6	

*p<0.05 difference from LETO, †p<0.05 difference from OLETF by one-way ANOVA with Tukey's HSD or unpaired on-tailed t-test.

Table 3. Mean ±SEM morphometrical measurements in LETO, OLETF and H4CBD-treated OLETF male rats.

	LETO (n=8)	OLETF (n=8)	H4CBD (n=8)
Body Mass (g)	527 ± 10	640 ± 38	516 ± 21
Relative Retro Fat (g/100g BM)	2.4 ± 0.1	8.6 ± 0.6	6.6 ± 0.9
Relative Epi Fat (g/100g BM)	1.8 ± 0.1	1.8 ± 0.2	1.2 ± 0.1
Relative Total Fat (g/100g BM)	4.2 ± 0.2	10.5 ± 0.7	7.8 ± 1.1
FI pre-treatment (g)	25 ± 2	32 ± 1	34 ± 2
FI post-treatment (g)	21 ± 1	37 ± 2	36 ± 2
Relative FI (g/100g BM)	3.9 ± 0.1	6.1 ± 0.7	6.7 ± 0.4

FI = food intake (average of 3 days), Epi. = epidydimal, Retro. = retroperitoneal, BM = body mass $^{\$}p<0.05$ LETO vs. OLETF, $^{\wedge}p<0.05$ OLETF vs. H4CBD, $^{\#}p<0.05$ LETO vs. H4CBD one-way ANOVA with Tukey's HSD.

Table 4. Mean ±SEM end of study fasting plasma measurements.

Strain	LETO (n=6-8)	OLETF (n=6-8)	H4CBD (n=6-8)
Fasting BG (mmol/L)	7.0 ± 0.4	10.1 ± 1.0	15.8 ± 1.9†
Fasting Insulin (uIU/mL)	7.7 ± 0.7	12.6 ± 0.9*	13.9 ± 0.4
Fasting Glucagon (pg/mL)	21.7 ± 5.0	8.0 ± 1.3*	19.4 ± 9.4
Glucose:Insulin	0.9 ± 0.1	0.7 ± 0.1	1.1 ± 0.1†
Insulin:Glucagon	0.4 ± 0.0	1.6 ± 0.1*	0.6 ± 0.0†

*$p<0.05$ compared to LETO, and †$p<0.05$ compared to OLETF by one-way ANOVA with Tukey's HSD or unpaired one-tailed t-test.

Figures

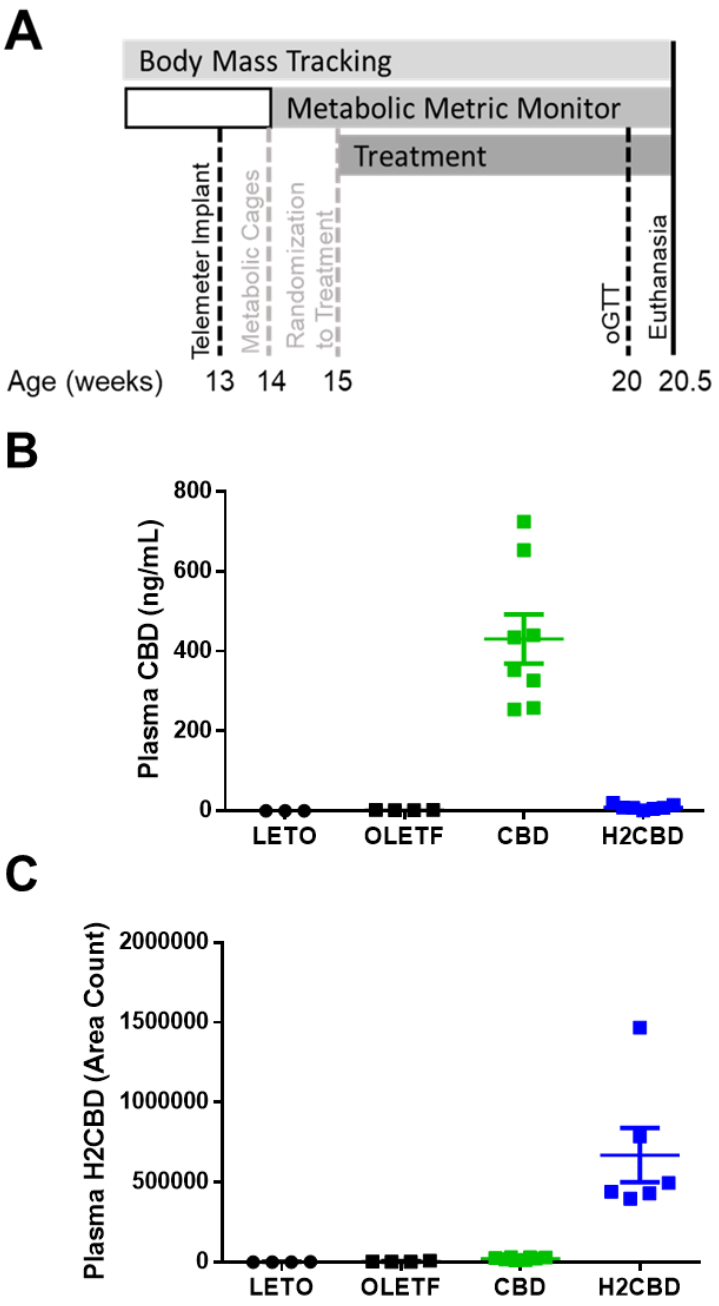

Figure 1. CBD compounds are uniquely bioavailable. (A) Timeline of study in younger (<20 weeks) animals. Bioavailability in end of study plasma of (B) CBD and (C) H2CBD in 20-week-old Long-Evans Tokushima Otsuka (LETO; n=3-4), Otsuka Long-Evans Tokushima Fatty (OLETF; n=4), OLETF+CBD (CBD; n=8) and OLETF+H2CBD (H2CBD; n=6-7).

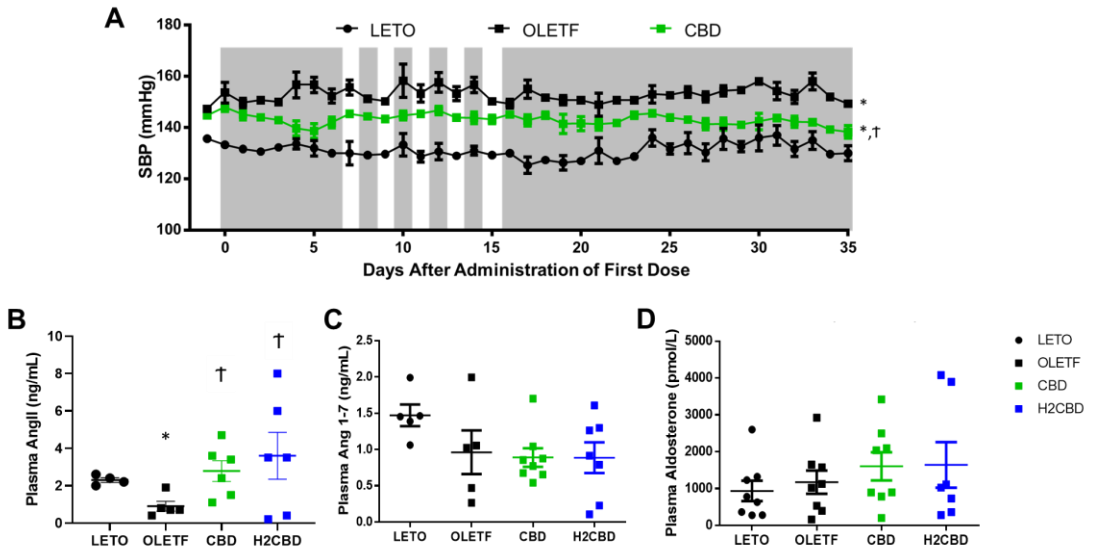

Figure 2. CBD ameliorates MetS-associated hypertension. Mean (±SEM) (A) systolic blood pressure (SBP; mmHg) of LETO (n=3), OLETF (n=3) and OLETF+CBD (CBD; n=3) rats >15 weeks of age over 5 weeks. Mean (±SEM) end of study plasma of (B) AngII, (C) Ang 1-7 and (D) aldosterone in 20-week-old LETO (n=4-8), OLETF (n=5-8), CBD-treated OLETF (CBD; n=6-8) and H2CBD-treated OLETF (H2CBD; n=6-7). ***p<0.05 vs. LETO and †p<0.05 vs. OLETF by 2-way ANOVA with Tukey's HSD.*

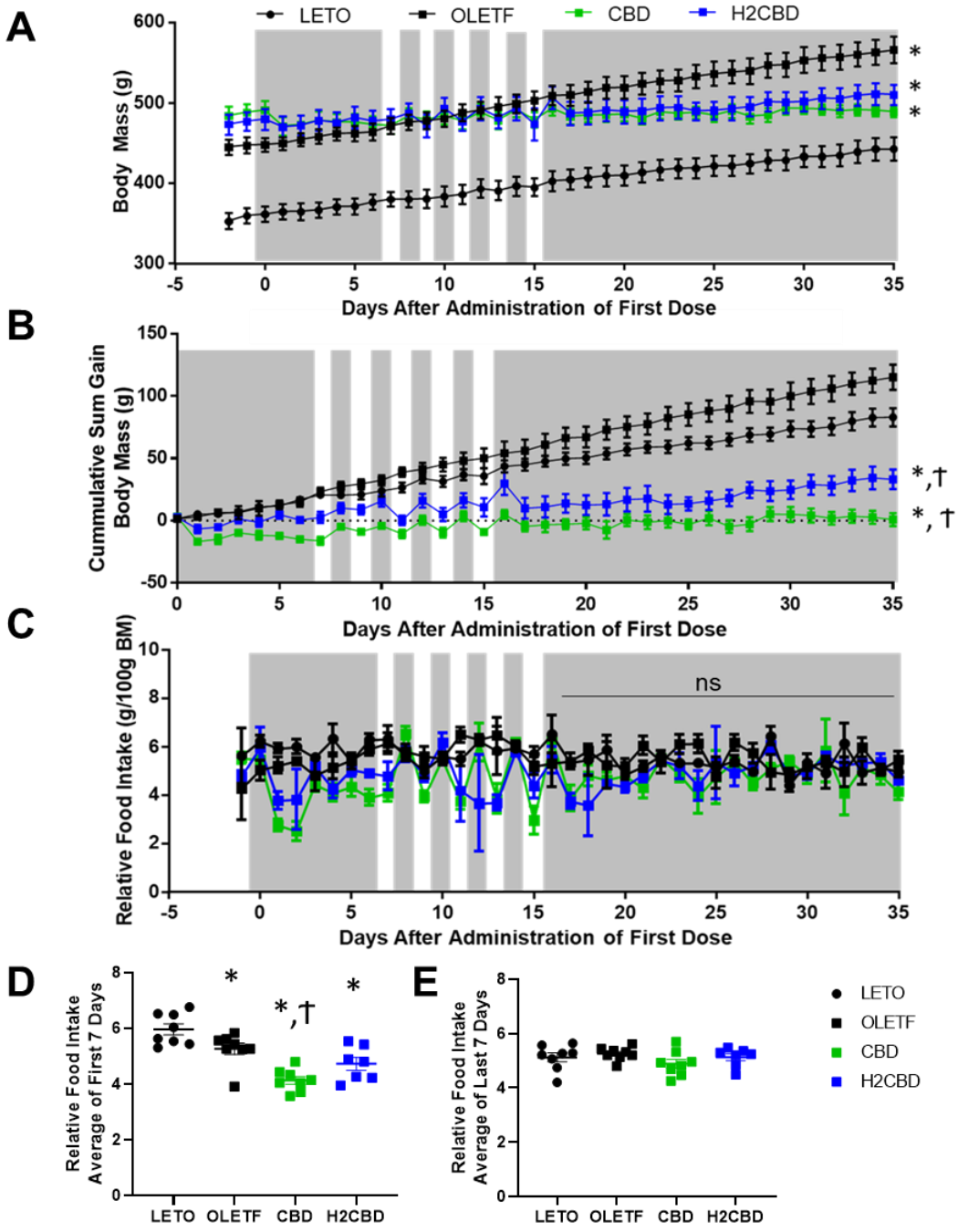

Figure 3. CBD compounds prevent body mass gain but are initially anorexigenic. Mean (±SEM) (A) daily body mass, (B) cumulative sum gain of body mass, (C) relative food intake (RFI) over time, (D) RFI average of first 7 days and (E) RFI average of last 7 days in 20-week-old LETO (n=8), OLETF (n=8), CBD (n=8) and H2CBD (n=7). **p<0.05 different from LETO, †p<0.05 different from OLETF by 1-way or 2-way ANOVA with Tukey's HSD or unpaired two-tailed t-test.*

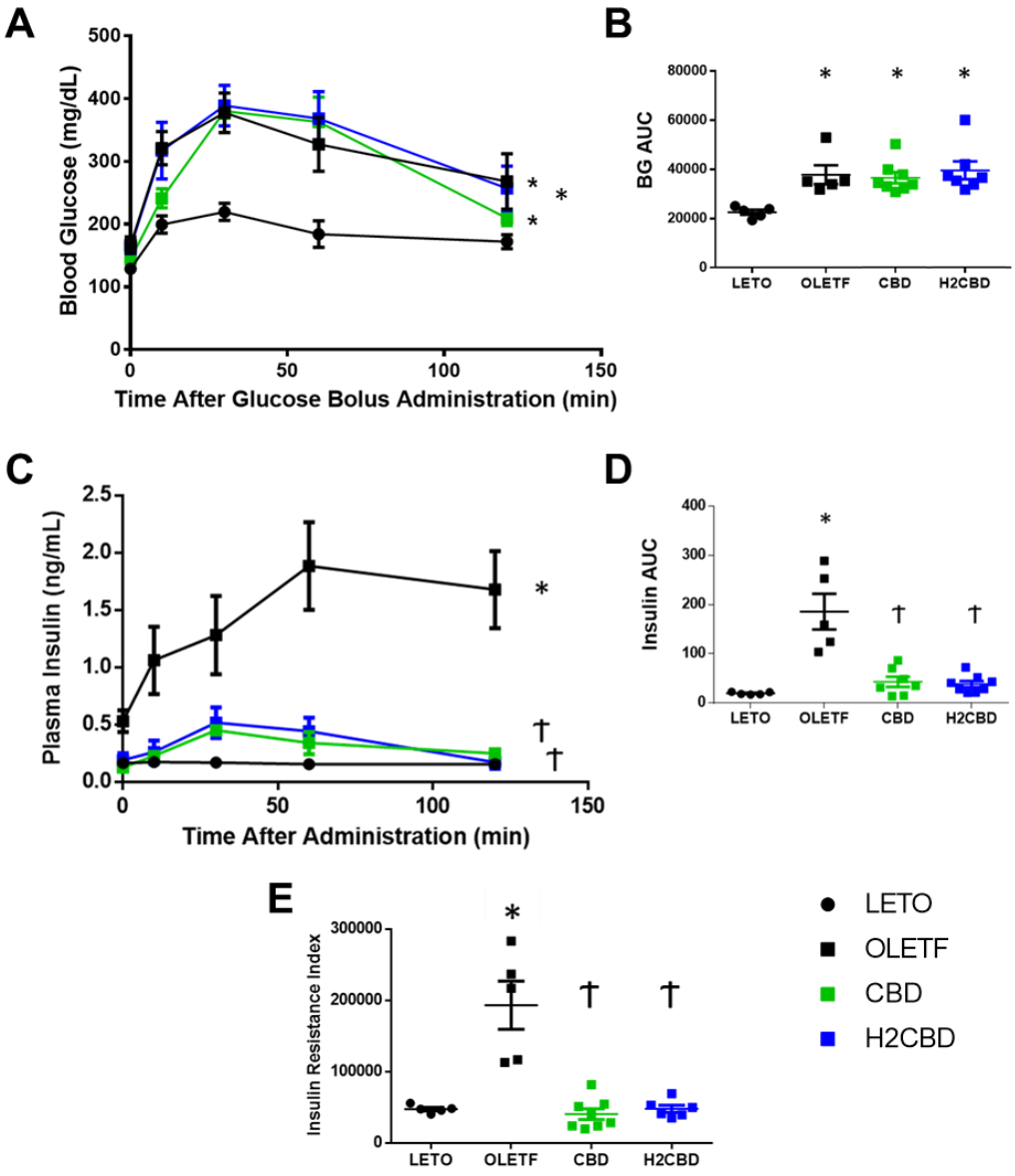

Figure 4. CBD compounds ameliorated insulin response to glucose challenge but did not reverse the diabetic phenotype. Mean (±SEM) (A) blood glucose (mg/dL) response to oGTT and (B) corresponding AUC (relative units; r.u.), (C) insulin response (ng/mL) to oGTT and (D) corresponding AUC (r.u.) and (E) insulin resistance index (IRI; r.u.) in 20-week-old LETO (n=5-8), OLETF (n=5-8), CBD (n=8) and H2CBD (n=7). *$p<0.05$ difference from LETO and †$p<0.05$ difference from OLETF by 1-way or 2-way (*for change over time data*) ANOVA with Tukey's HSD or by unpaired, one-tailed t-test.

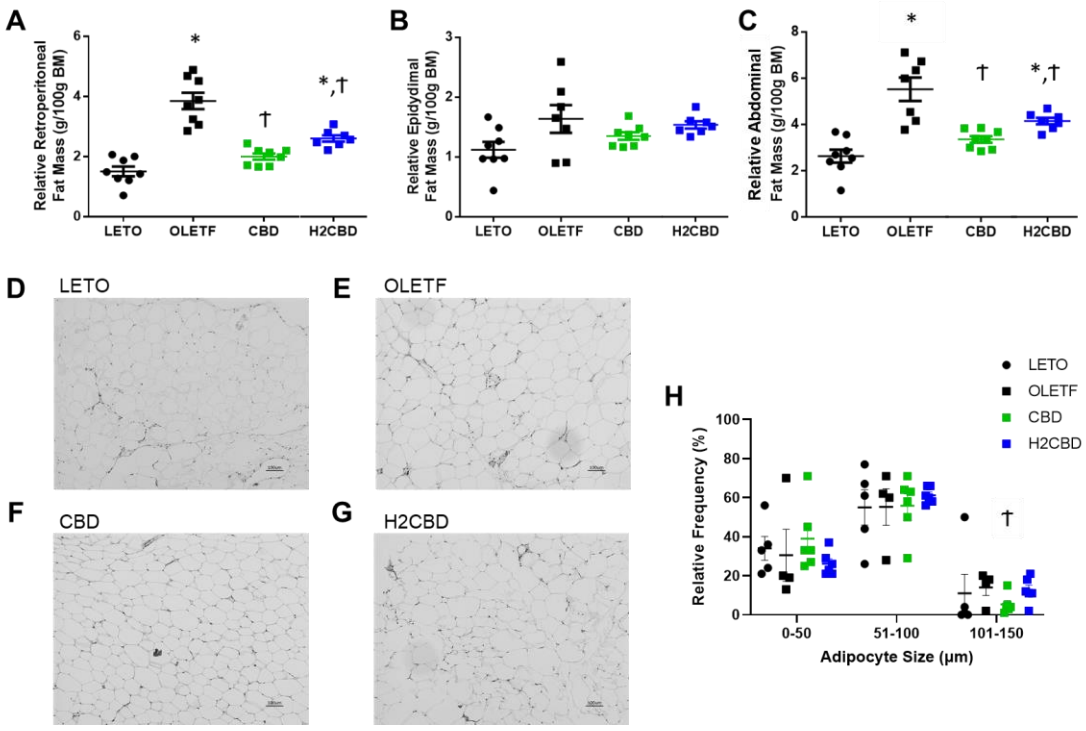

Figure 5. CBD reduced abdominal fat mass and adipocyte morphology in MetS. Mean ±SEM dissected relative (A) retroperitoneal fat mass, (B) epididymal fat mass and (C) abdominal (*retroperitoneal + epididymal*) fat mass. (D-G) Representative images of adipocytes from retroperitoneal adipose. (H) Adipocyte percent relative frequency distribution by size (µm) in 20-week-old LETO (n=5), OLETF (n=4), CBD-treated OLETF (CBD; n=6) and H2CBD-treated OLETF (H2CBD; n=6). $^{*}p<0.05$ *different from LETO,* $^{\dagger}p<0.05$ *different from OLETF* by 1-way ANOVA with Tukey's HSD or one-tailed unpaired t-test.

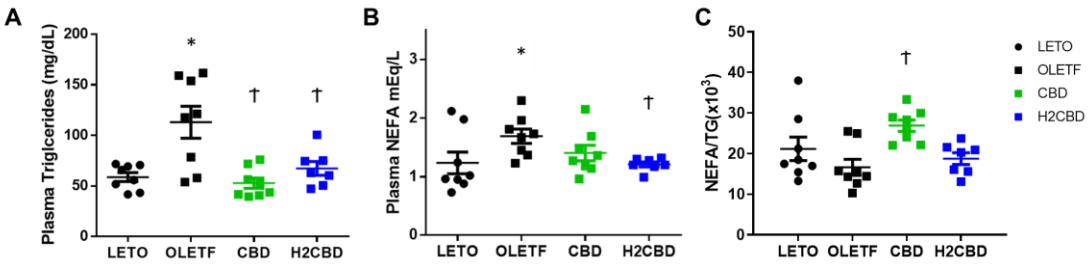

Figure 6. CBD compounds ameliorate dyslipidemia. Mean (±SEM) (A) plasma triglycerides (TG), (B) plasma non-esterified fatty acids (NEFA) and (C) NEFA/TG ratio in 20-week-old LETO (n=7-8), OLETF (n=8) CBD-treated OLETF (CBD; n=8) and H2CBD-treated OLETF (H2CBD; n=8). $^{*}p<0.05$ *different from LETO*, $^{\dagger}p<0.05$ *different from OLETF* by 1-way ANOVA with Tukey's HSD or one-tailed unpaired t-test.

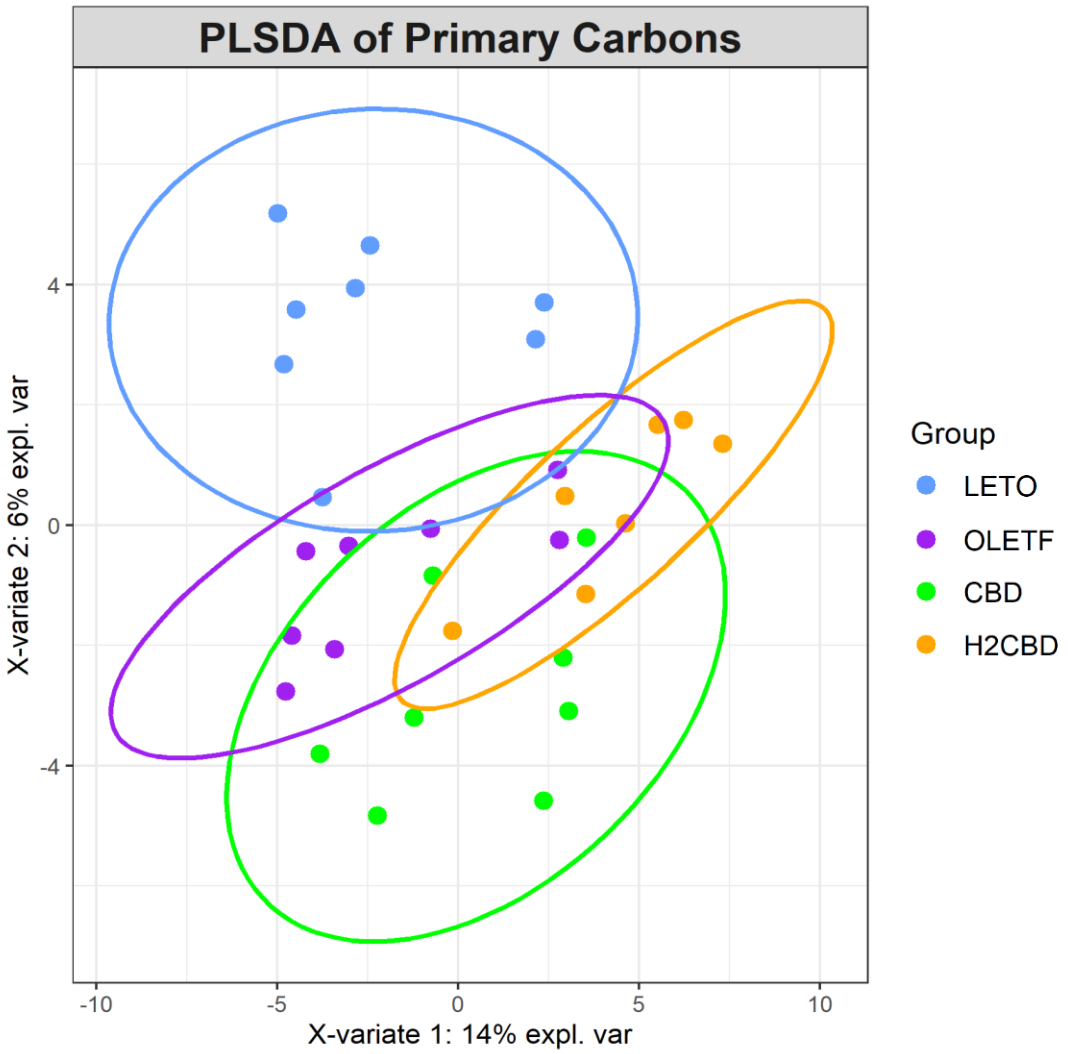

Figure 7. CBD compounds shift metabolomic profile. Partial Least Squares Discriminate Analysis (PLSDA) of metabolites in end of study plasma in 20-week-old LETO, OLETF, CBD-treated and H2CBD-treated OLETF.

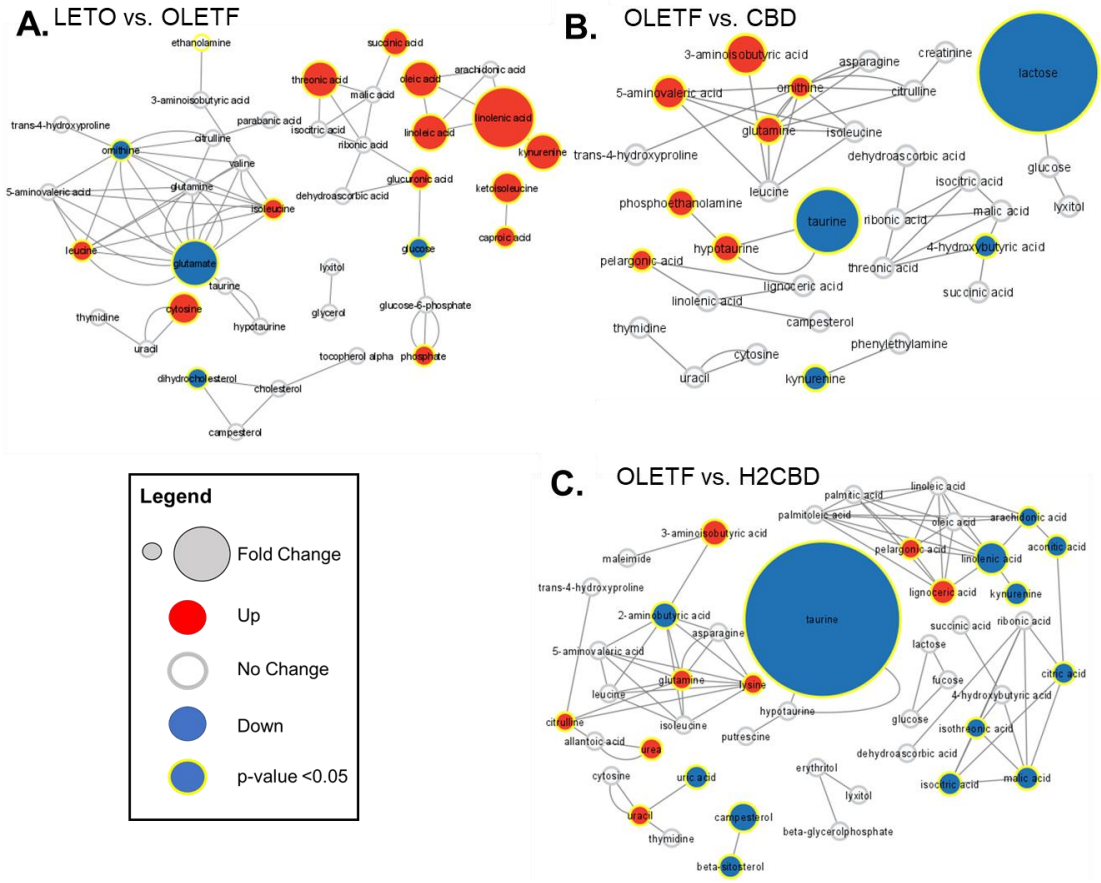

Figure 8. Metabolomic map showing metabolites with p<0.05 after Student's t-test, representing mean plasma peak intensity fold-changes between groups in metabolites with q<0.2, for (A) LETO vs. OLETF, (B) OLETF vs. CBD, (C) OLETF vs. H2CBD.

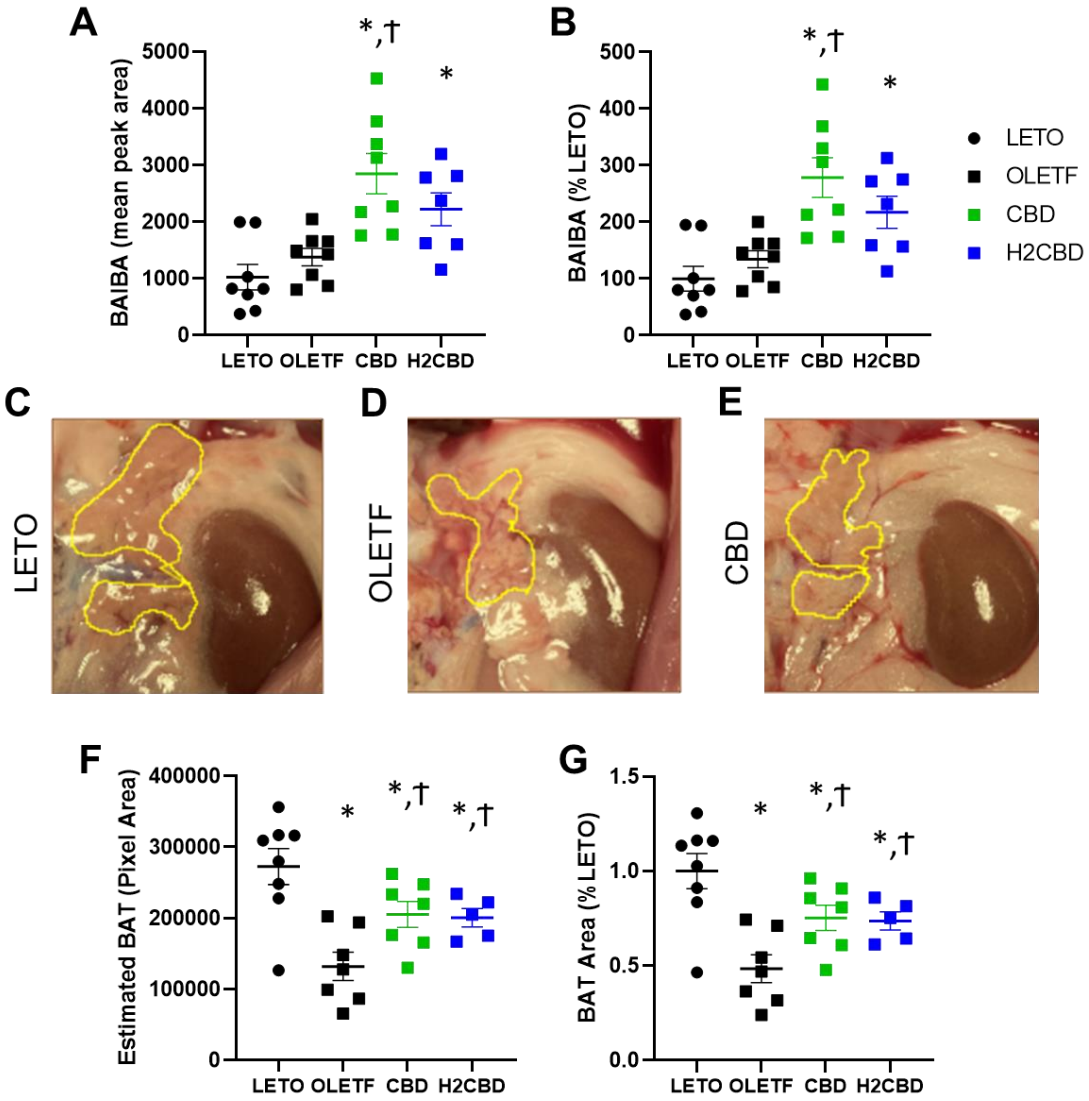

Figure 9. Increase in BAIBA corresponds with visual observation of increased BAT at dissection. Mean ±SEM (A) raw mean peak area of BAIBA and (B) BAIBA mean peak area normalized to LETO. (C-E) Representative images of outlined BAT. Mean ±SEM (F) estimated BAT pixel area and (G) estimated BAT pixel area normalized to LETO. *$p<0.05$ compared to LETO and †$p<0.05$ compared to OLETF by ANOVA with Tukey's HSD or student's t-test.

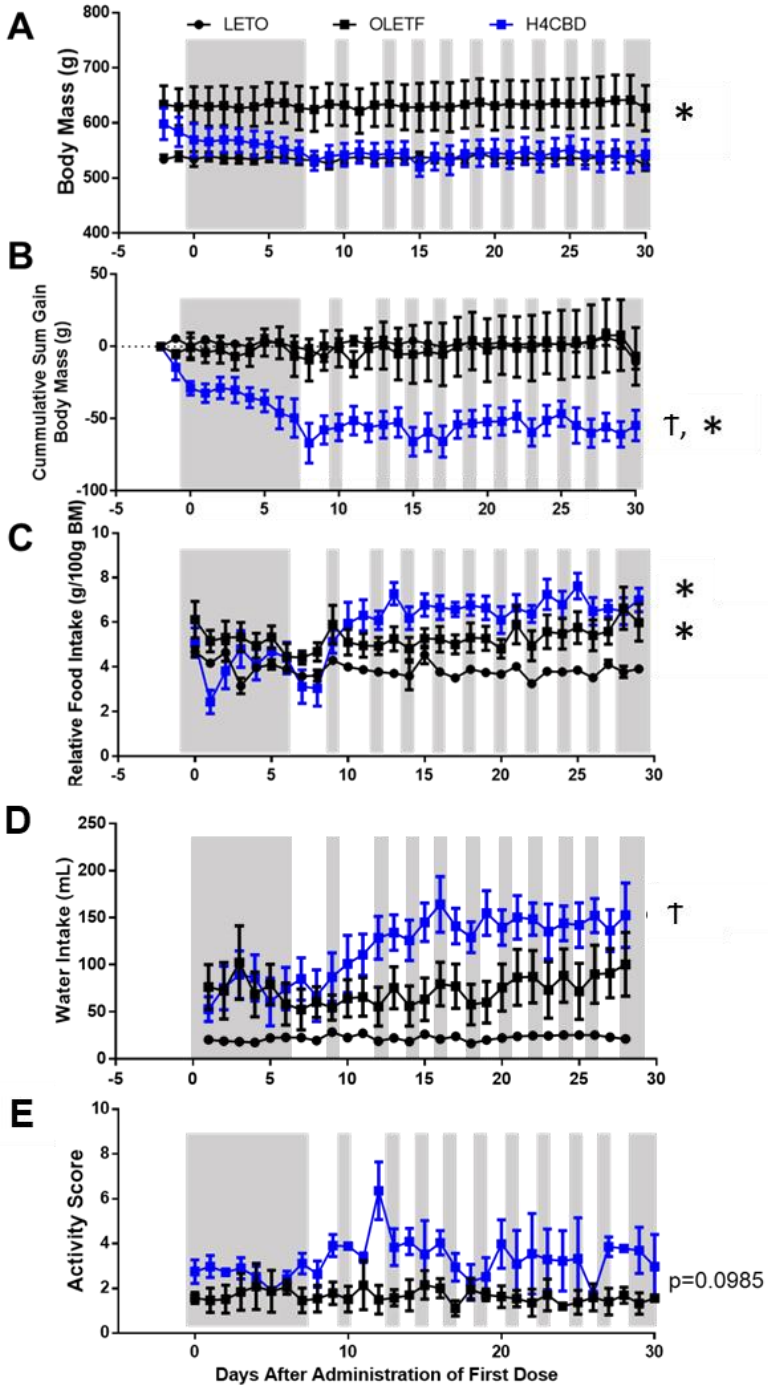

Figure 10. H4CBD decreased body mass. Mean (±SEM) (A) cumulative sum gain of body mass, (B) daily body mass, (C) relative food intake and (D) water intake in 41–45-week-old Long-Evans Tokushima Otsuka (LETO; n=8), Otsuka Long-Evans Tokushima Fatty (OLETF; n=8) and OLETF+H4CBD (H4CBD; n=8). (E) Activity score [DSI; HD-S10] of OLETF (OLETF; n=2) and OLETF+H4CBD (H4CBD; n=3). **p<0.05 different from LETO, †p<0.05 different from OLETF by 2-way ANOVA with Tukey's HSD.*

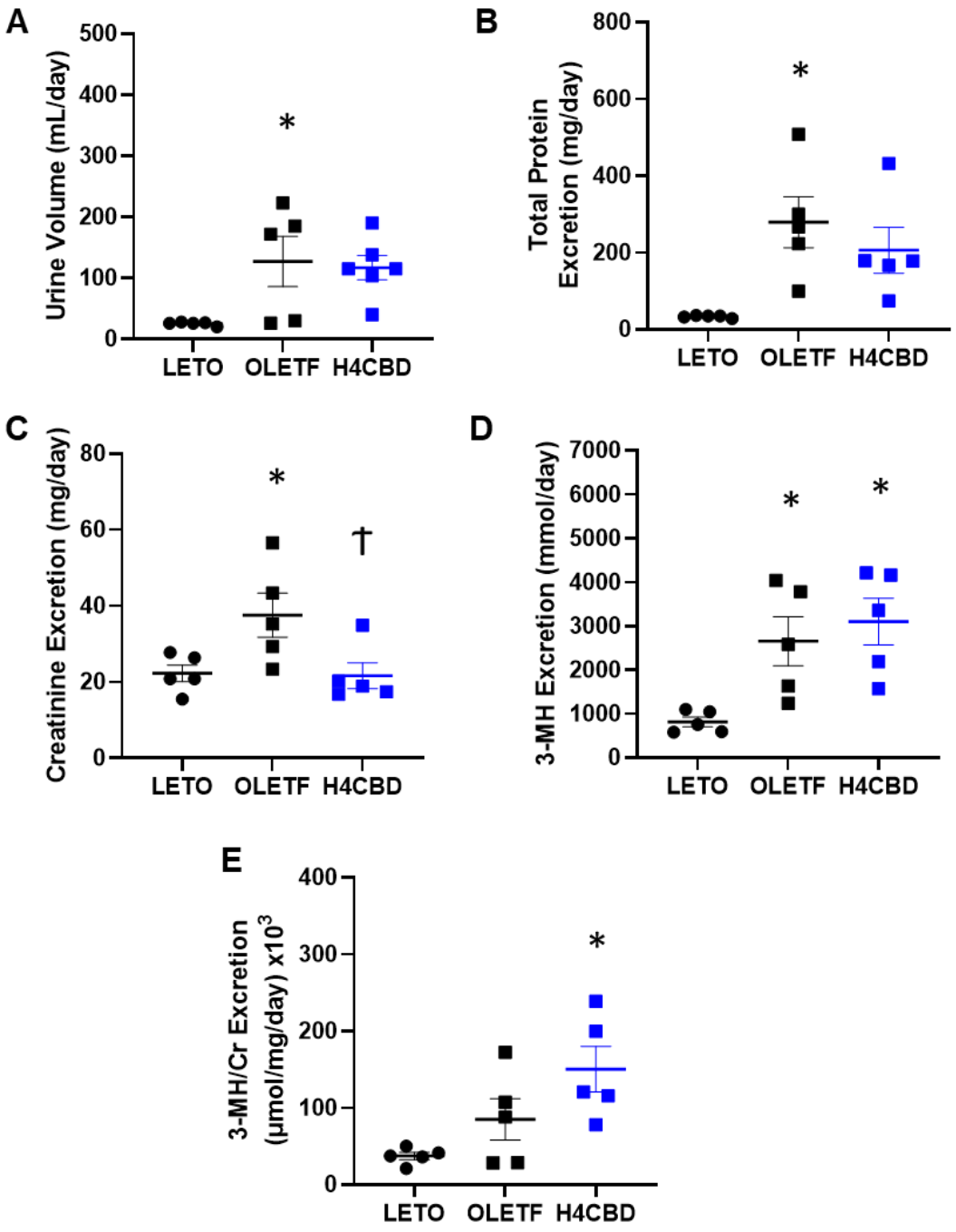

Figure 11. Increased 3MH/Cr in H4CBD-treated animals suggests lean tissue loss. Mean (±SEM) end of study (A) urine output (mL) (*n=8 all groups*), (B) urine total protein, (C) urine creatinine (Cr), (D) urine 3-methylhistidine (3MH) and (E) 3MH/Cr ratio in 45-week-old LETO (n=5), OLETF (n=5) and H4CBD-treated OLETF (H4CBD; n=5). $^{*}p<0.05$ *different from LETO,* $^{\dagger}p<0.05$ *different from OLETF* by 1-way ANOVA with Tukey's HSD or one-tailed unpaired t-test.

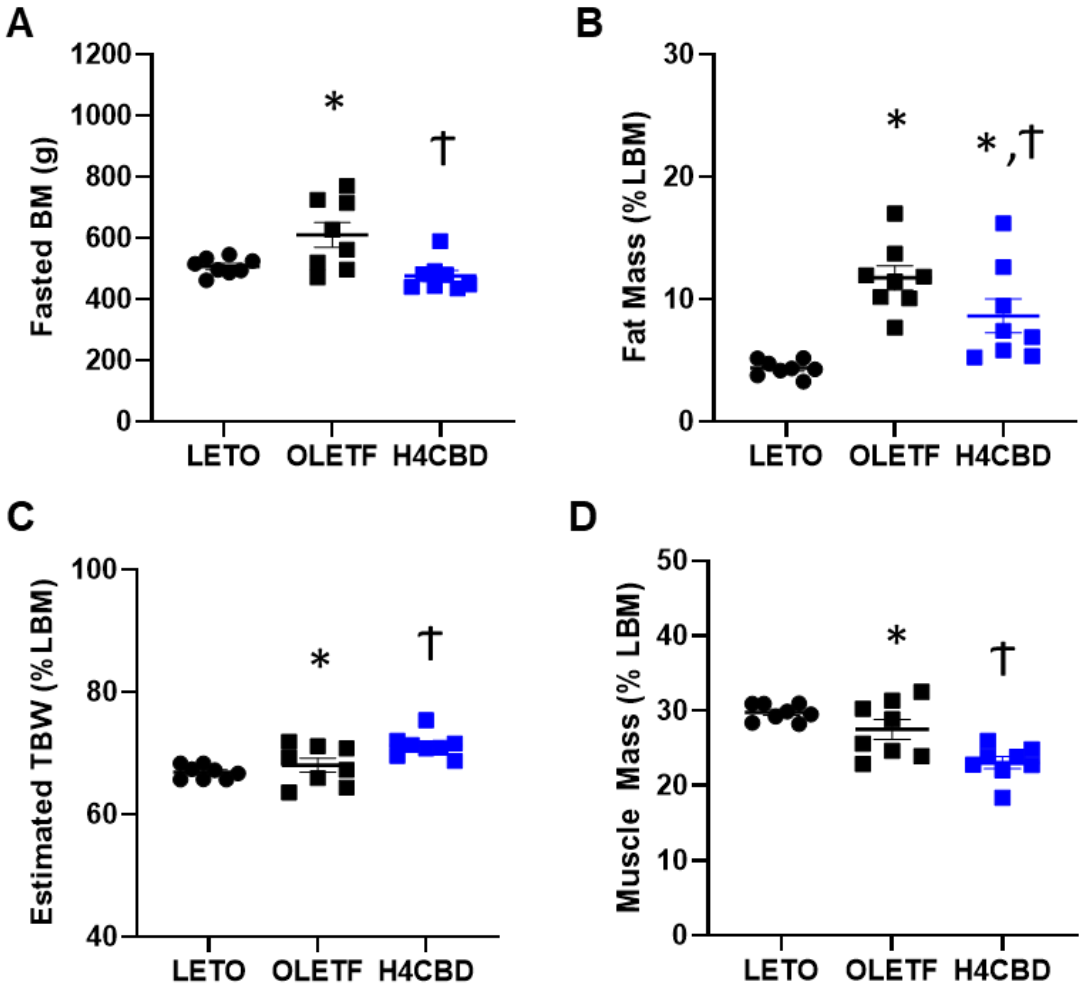

Figure 12. H4CBD reduced visceral fat mass at a modest cost to lean muscle in advanced MetS. Mean (±SEM) (A) fasted body mass (BM), (B) percent visceral fat mass, (C) estimated percent total body water (TBW) and (D) percent lean muscle in 45-week-old LETO (n=8), OLETF (8) and H4CBD-treated OLETF (H4CBD; n=8). $^{*}p<0.05$ *different from LETO,* $^{\dagger}p<0.05$ *different from OLETF* by 1-way ANOVA with Tukey's HSD or unpaired one-tailed t-test.

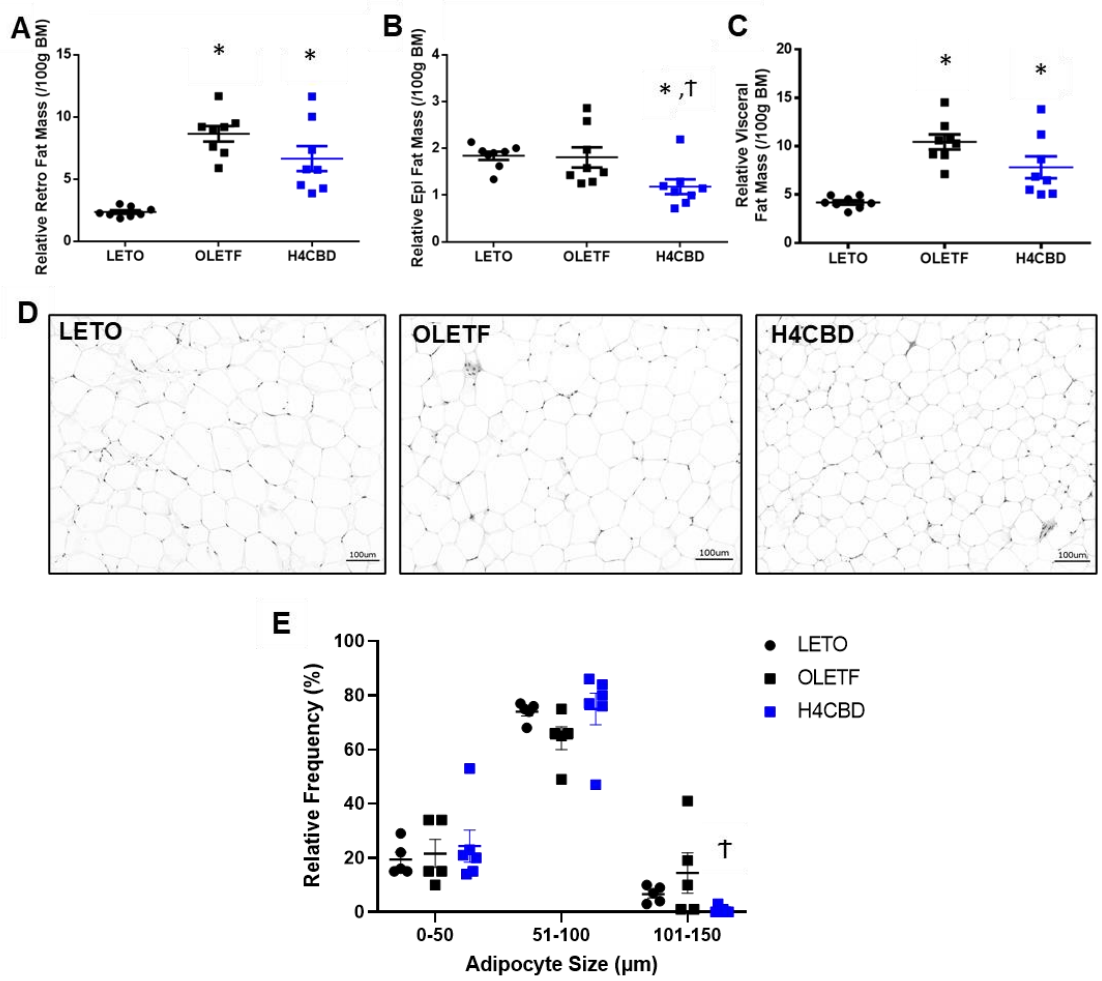

Figure 13. H4CBD reduced abdominal fat mass and adipocyte morphology in advanced MetS. Mean (±SEM) (A) relative retroperitoneal fat mass, (B) relative epididymal fat mass and (C) combined visceral adipose; (D) representative images of adipocytes from retroperitoneal adipose and (E) adipocyte percent relative frequency distribution by size (µm) in 45-week-old LETO (n=5-8), OLETF (n=5-8) and H4CBD-treated OLETF (H4CBD; n=6-8). $^{*}p<0.05$ *different from LETO,* $^{\dagger}p<0.05$ *different from OLETF* by 1-way ANOVA with Tukey's HSD or one-tailed unpaired t-test.

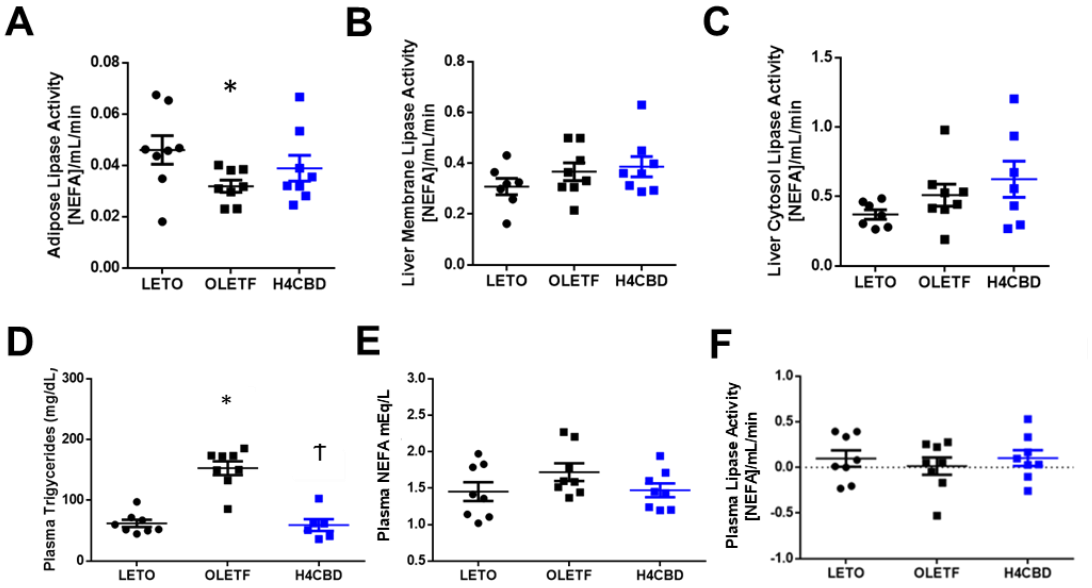

Figure 14. Synthetic CBD increased adipose lipase activity. Mean (±SEM) (A) adipose lipase activity, (B) liver membrane-bound lipase activity and (C) liver cytosolic lipase activity, (D) plasma triglycerides, (E) plasma non-esterified fatty acids (NEFA) and (F) plasma lipase activity in 45-week-old LETO (n=8), OLETF (n=8) and H4CBD-treated OLETF (H4CBD; n=8). $^{*}p<0.05$ *different from LETO*, $^{\dagger}p<0.05$ *different from OLETF* by 1-way ANOVA with Tukey's HSD or one-tailed unpaired t-test.

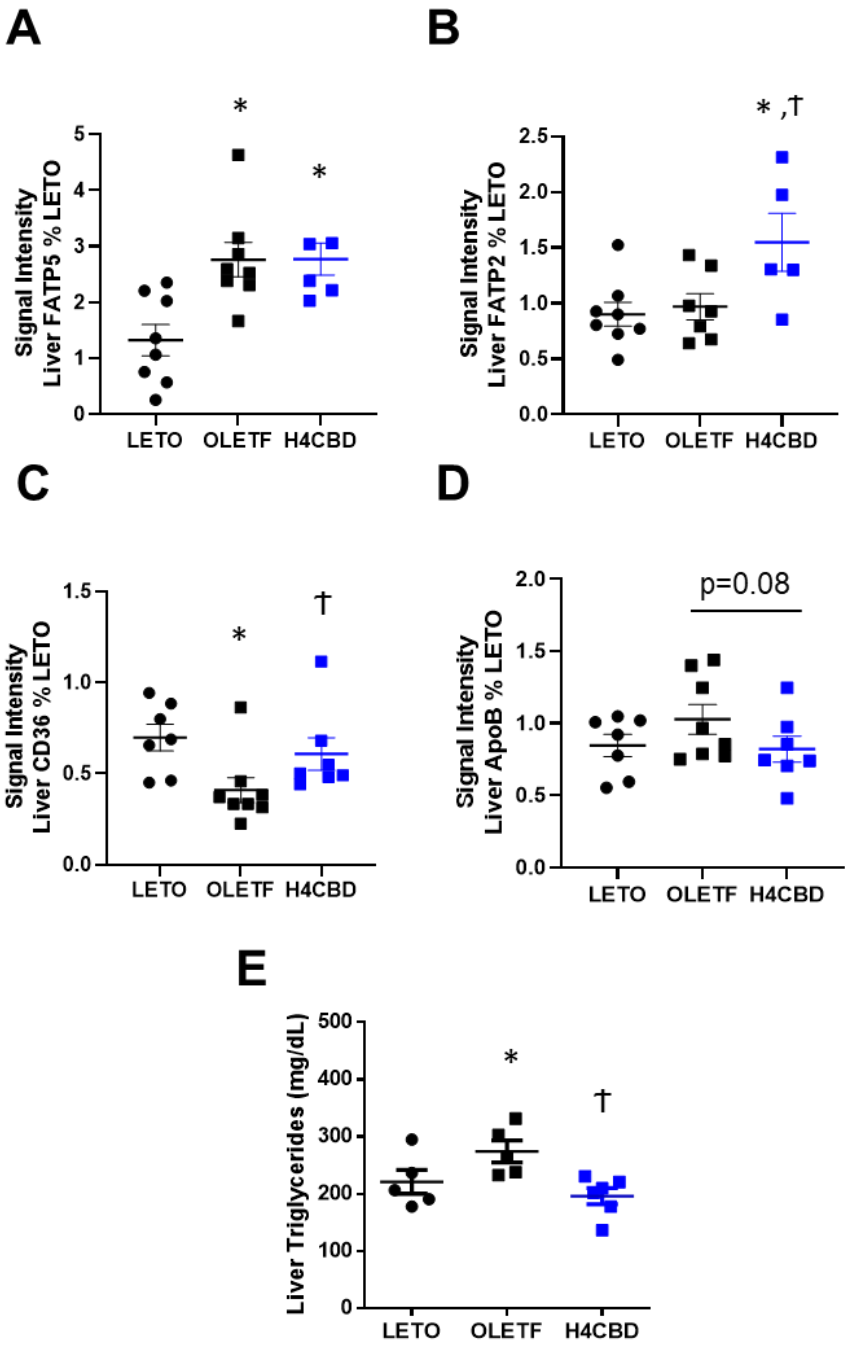

Figure 15. H4CBD increased hepatic CD36 and FATP2 expression but not lipid (TG) storage. Mean (±SEM) hepatic (A) FATP5 (B) FATP2, (C) CD36, (D) ApoB and (E) triglyceride content in 45-week-old LETO (n=5-8), OLETF (n=5-8) and H4CBD-treated OLETF (H4CBD; n=6-7). $^{*}p<0.05$ *different from LETO,* $^{\dagger}p<0.05$ *different from OLETF* by 1-way ANOVA with Tukey's HSD or one-tailed unpaired t-test.

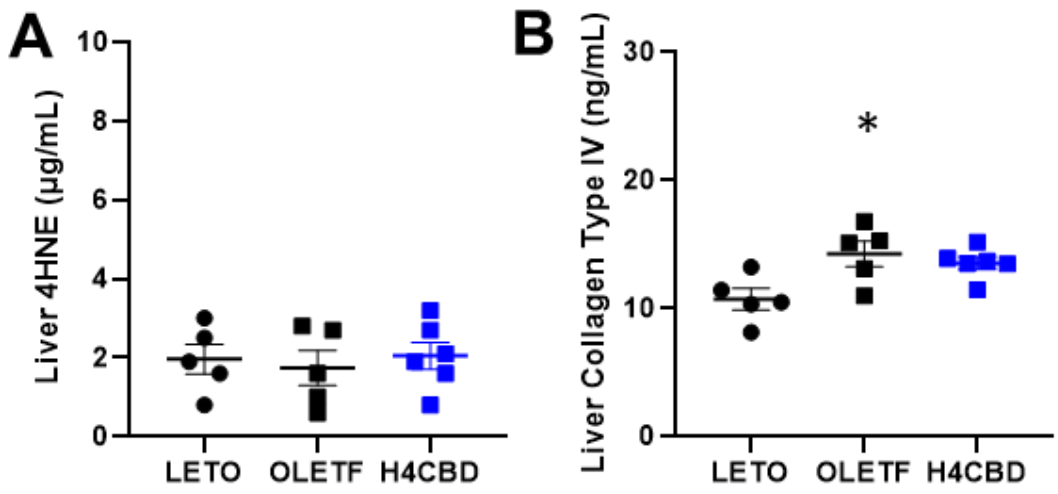

Figure 16. H4CBD did not contribute to liver injury. Mean (±SEM) hepatic (A) 4HNE and (B) Collagen Type IV in 45-week-old LETO (n=5), OLETF (n=5) and H4CBD-treated OLETF (H4CBD; n=6). *p<0.05 difference from LETO by one-way ANOVA w/ Tukey's HSD.

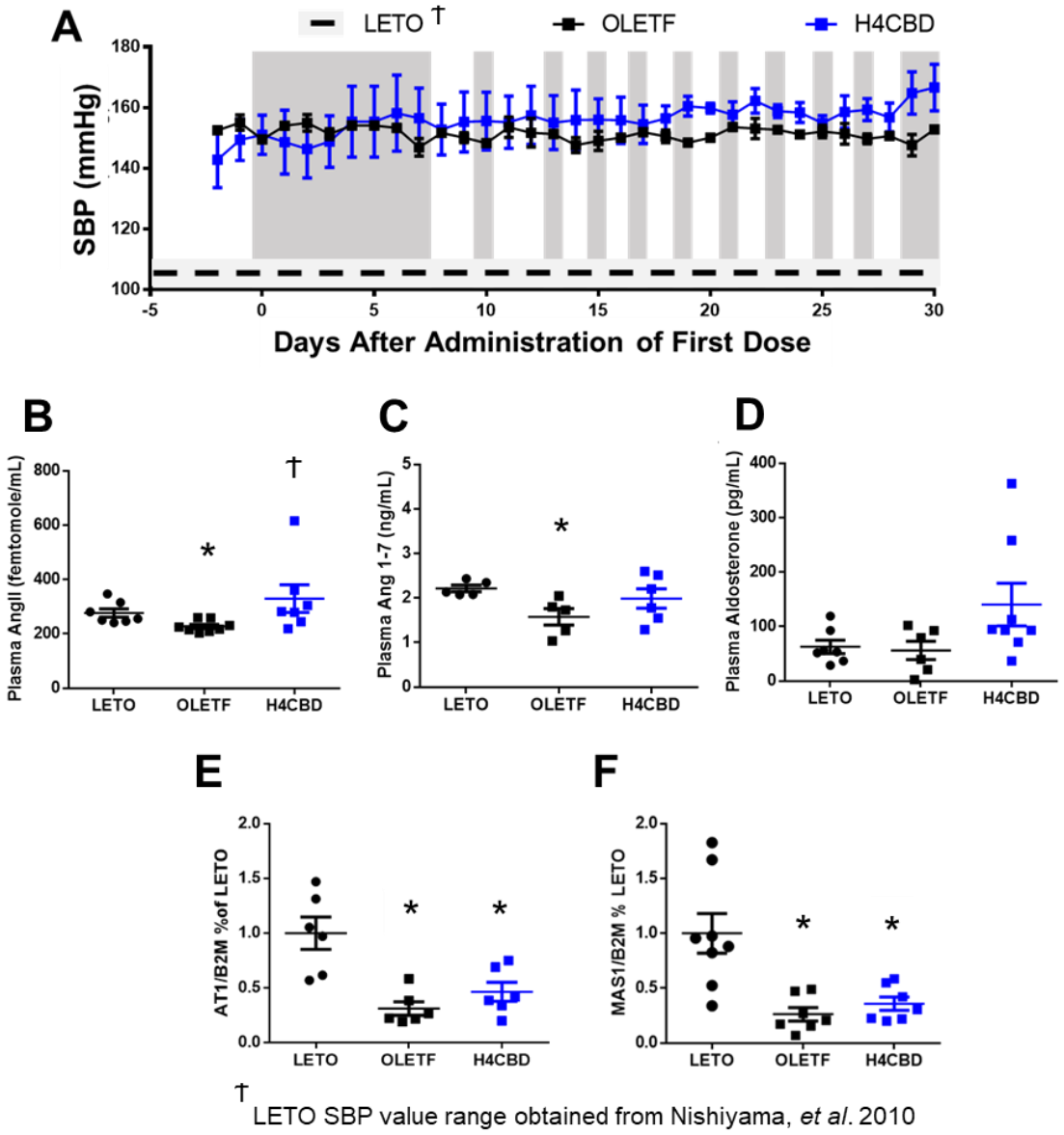

Figure 17. H4CBD does not reduce SBP or modulate hypertension drivers. Mean (±SEM) (A) systolic blood pressure (SBP; mmHg) of OLETF (n=2) and OLETF+H4CBD (n=3) rats >41 weeks of age over 4 weeks. Mean (±SEM) end of study plasma (B) angiotensin II and (C) angiotensin 1-7, (D) aldosterone in LETO (n=7), OLETF control (n=6-8) and H4CBD-treated OLETF (n=7-8). Cardiac mRNA relative expression of (E) At1 and (F) Mas1 in LETO (n=6-8), OLETF control (n=6-7) and H4CBD-treated OLETF (n=6-7). $^{*}p<0.05$ *different from LETO,* $^{\dagger}p<0.05$ *different from OLETF* by 1-way ANOVA with Tukey's HSD or one-tailed unpaired t-test.

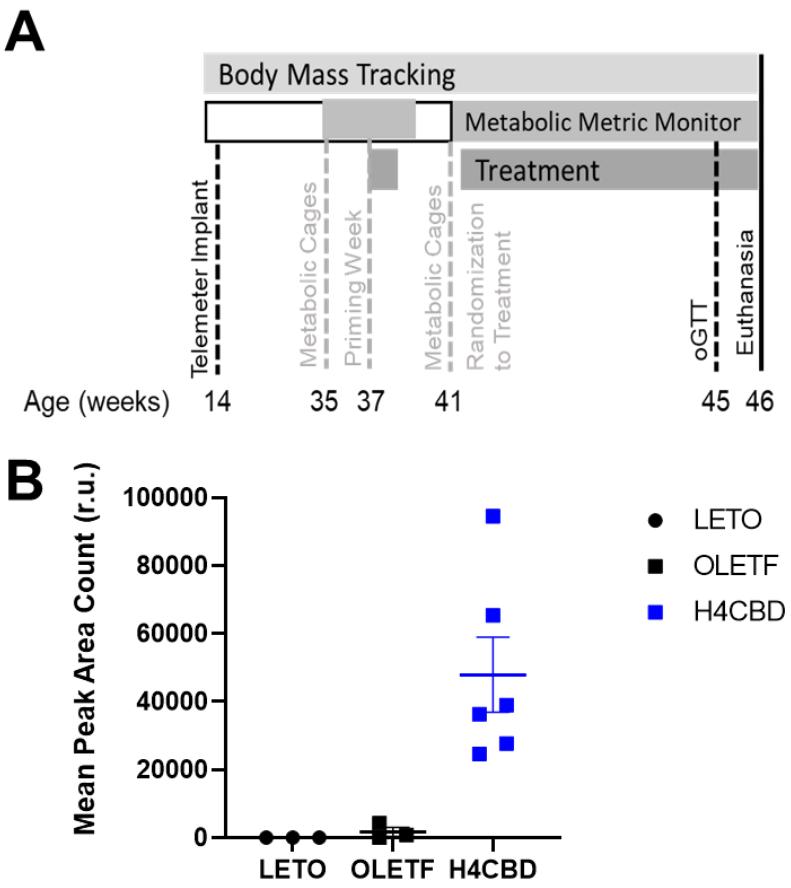

Figure 18. Experimental design and bioavailability of drug in end of study plasma. (A) Timeline of study in older (>40weeks) animals. (B) Mean (±SEM) Plasma AUC relative abundance of H4CBD in 45-week-old LETO (n=3), OLETF (n=3) and H4CBD-treated OLETF (H4CBD; n=6).

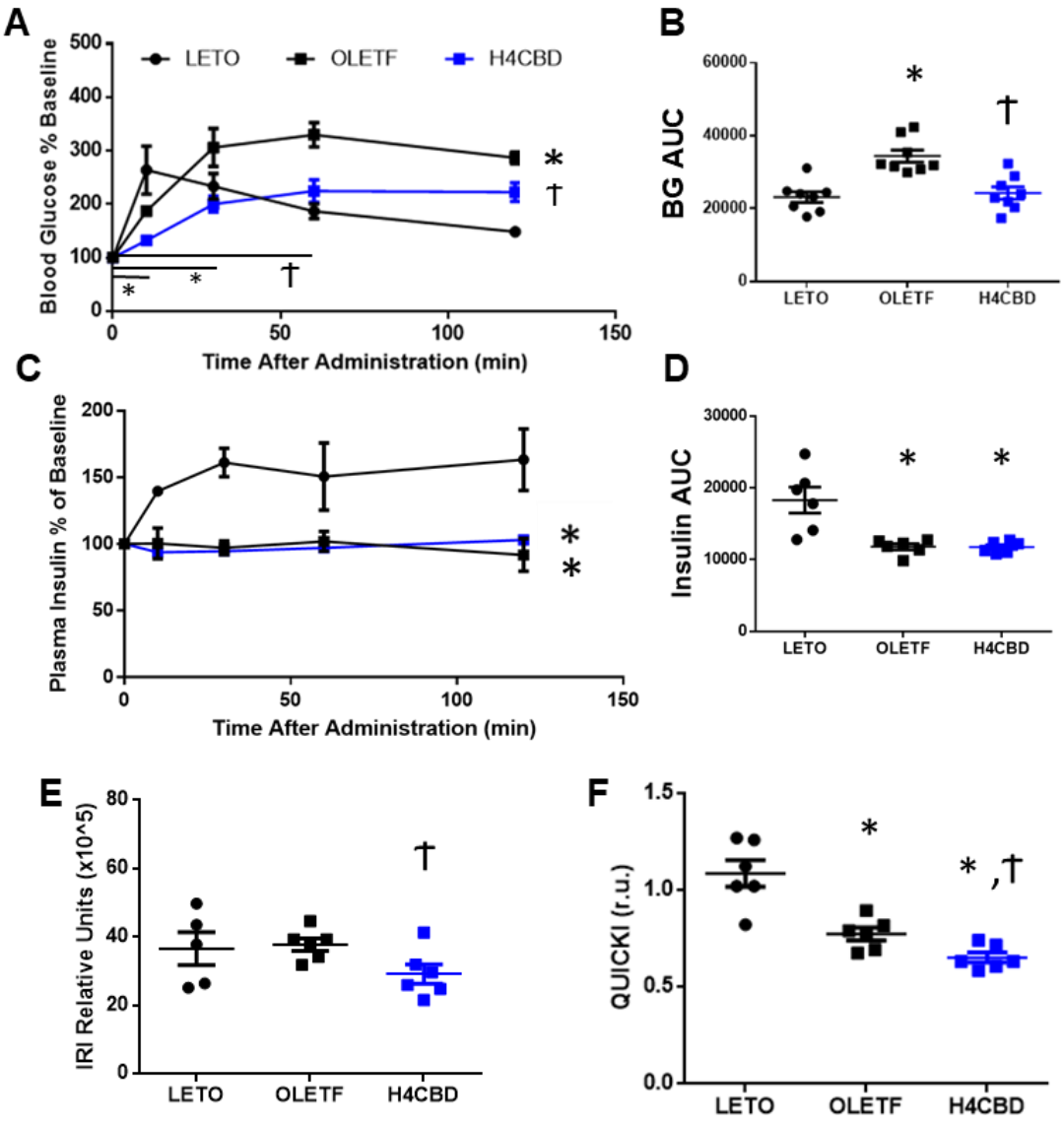

Figure 19. H4CBD ameliorated glucose response but not hyperglycemia in advanced MetS. Mean (±SEM) (A) blood glucose % baseline response to oGTT and (B) corresponding AUC (r.u.), (C) insulin response % baseline to oGTT and (D) corresponding AUC (r.u.), (E) insulin resistance index (IRI; relative units) and (F) QUICKI in 45-week-old LETO (n=8), OLETF (n=8) and H4CBD-treated OLETF (H4CBD; n=8). *$p<0.05$ difference from LETO and †$p<0.05$ difference from OLETF by 1-way or 2-way ANOVA with Tukey's HSD or by unpaired, one-tailed t-test.

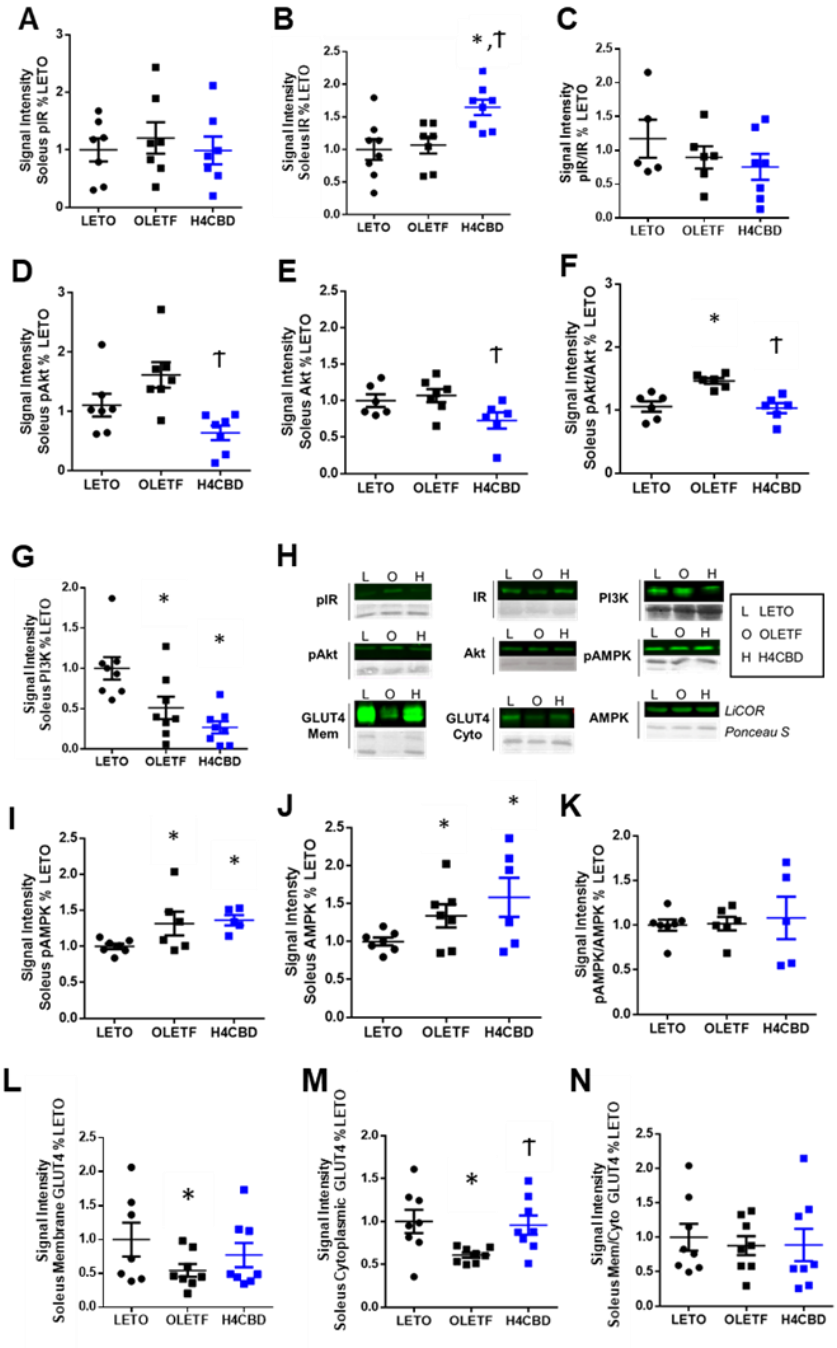

Figure 20. H4CBD induced compensatory increase in skeletal muscle IR expression. Mean (±SEM) (A) pIR, (B) IR, (C) pIR/IR, (D) pAkt, (E) Akt, (F) pAkt/Akt, (G) PI3K, (H) representative blots, (I) pAMPK, (J) AMPK, (K) pAMPK/AMPK, (L) membrane-bound GLUT4, (M) cytoplasmic GLUT4 and (N) Mem/cyto GLUT4 soleus protein expression in 45-week-old LETO (n=8), OLETF (n=8) and H4CBD-treated OLETF (H4CBD; n=8). *$p<0.05$ difference from LETO and $^{\dagger}p<0.05$ difference from OLETF by 1-way ANOVA with Tukey's HSD or by unpaired, one-tailed t-test.

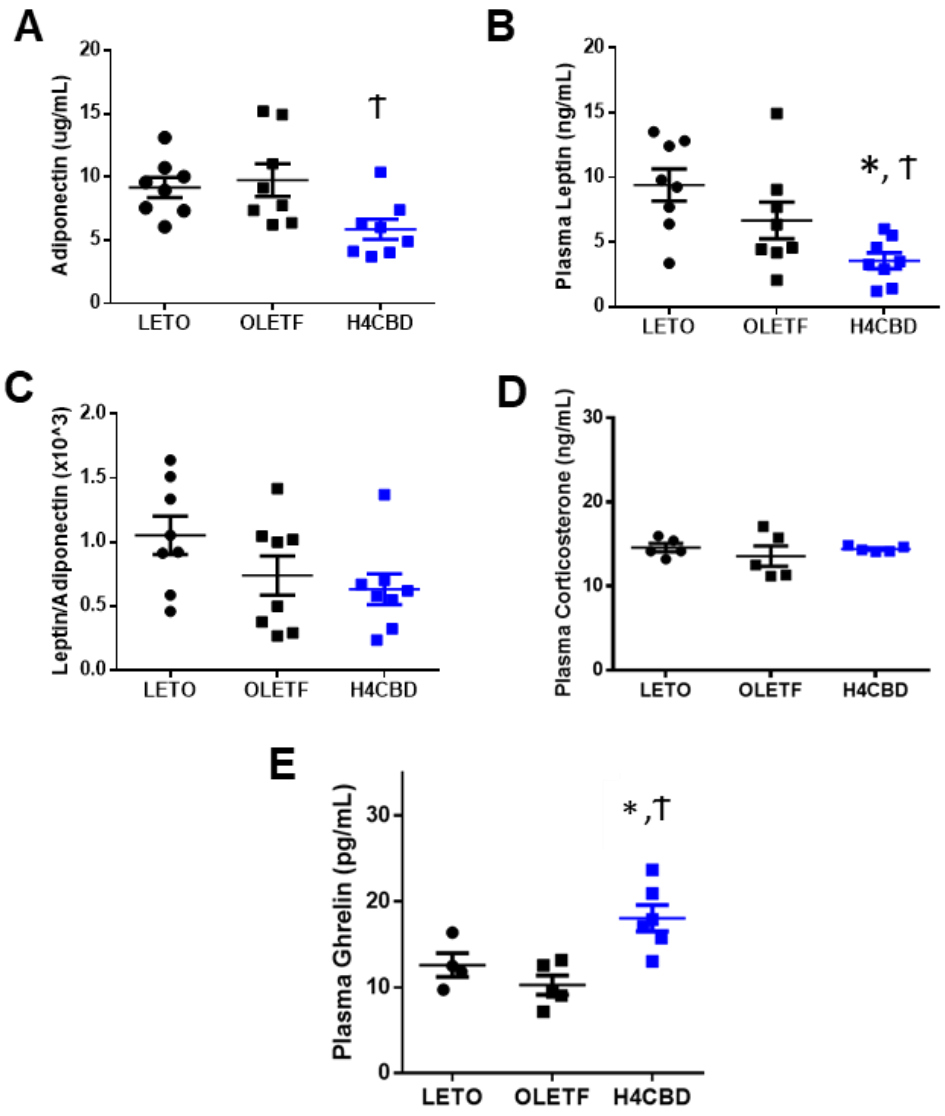

Figure 21. Synthetic CBD reduced circulating adipokines but increased hunger hormone ghrelin in advanced MetS. Mean (±SEM) (A) plasma leptin, (B) plasma adiponectin, (C) plasma leptin:adiponectin, (D) plasma corticosterone and (E) plasma ghrelin in 45-week-old LETO (n=8), OLETF (n=8) and H4CBD-treated OLETF (H4CBD; n=8). *$p<0.05$ difference from LETO and $^{\dagger}p<0.05$ difference from OLETF by 1-way ANOVA with Tukey's HSD or by unpaired, one-tailed t-test.

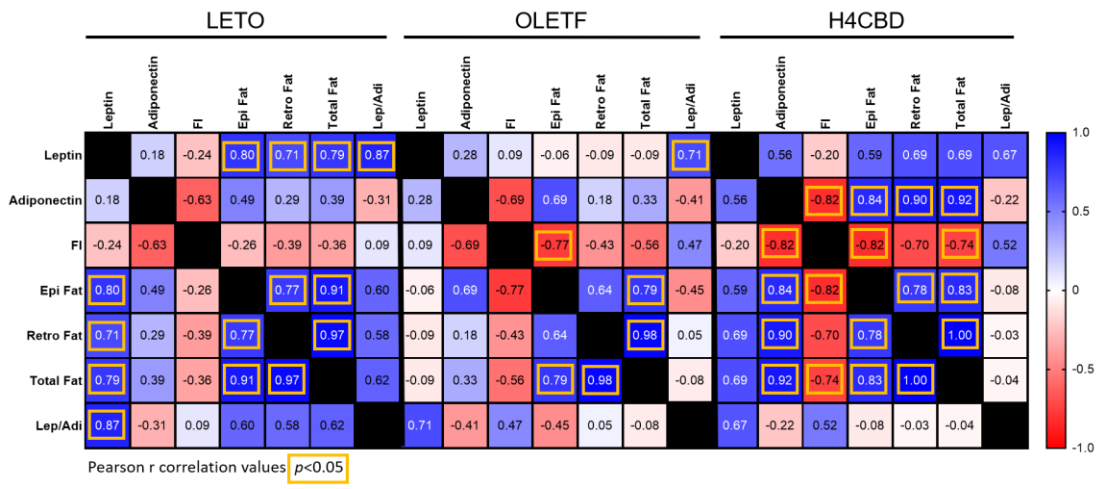

Figure 22. H4CBD-induced reductions of visceral fat and plasma adiponectin are positively correlated. Pearson r correlation values of end of study leptin, adiponectin, food intake (FI), epididymal (Epi) fat, retroperitoneal (Retro) fat, visceral (Epi + Retro) fat, leptin:adiponectin in LETO (n=8), OLETF (n=8) and H4CBD (n=8).

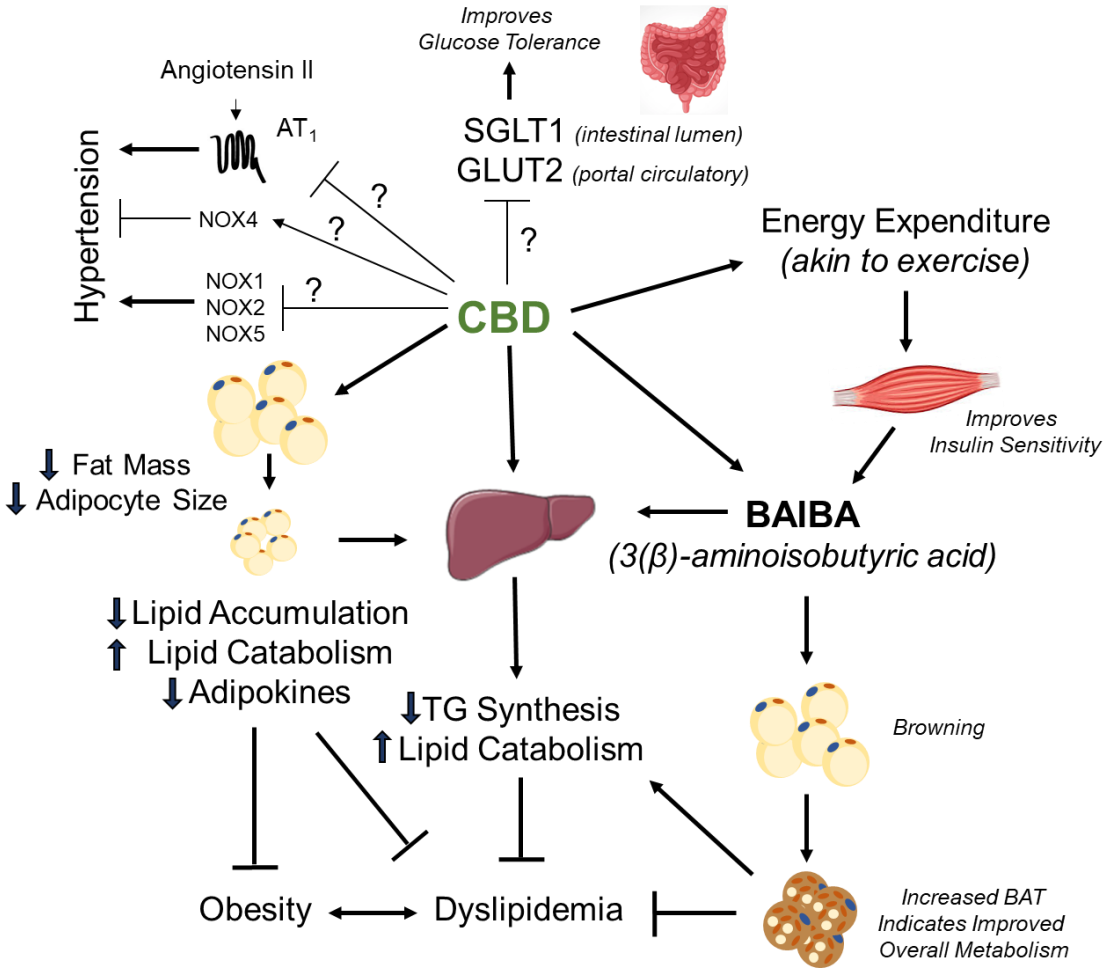

Figure 23. Schematic of CBD effects on MetS cluster factor conditions mediated by BAIBA.

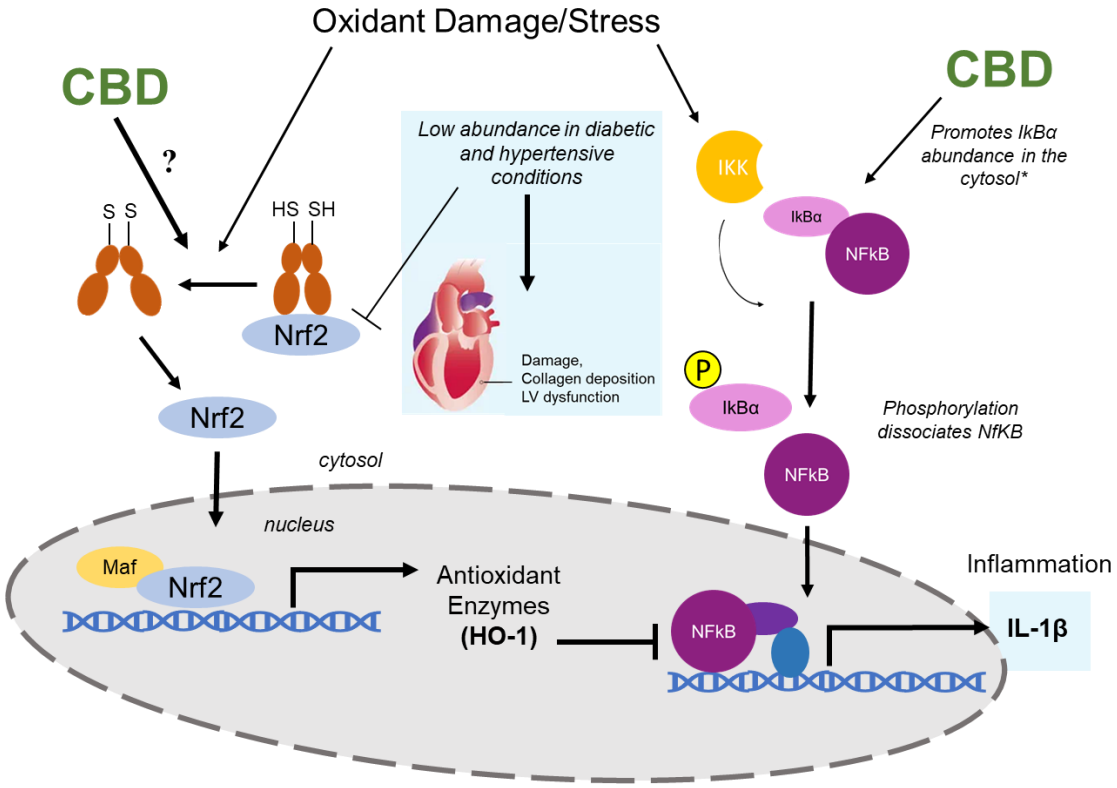

Figure 24. Schematic of CBD effect on Nrf2 hypothesis.