Effects of Cafestol and Enterolactone on Glucose Uptake in 3T3-L1 Differentiated Adipocytes

Sierra Hall

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Acceptance of Senior Honors Thesis

This Senior Honors Thesis is accepted in partial
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William Moore, Ph.D.
Thesis Chair

Jeremiah Winter, Ph.D.
Committee Member

Morgan Roth, Ph.D.
Assistant Honors Director

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Date

Abstract

A review of current literature has revealed that moderate habitual coffee consumption is inversely associated with the risk of T2D development. Cafestol (CF) and enterolactone (ENL), two bioactive compounds found naturally in coffee, have been shown to promote glucose disposal in skeletal muscle and basal glucose uptake in L6 myotubes, respectively. However, the anti-diabetic effects of these compounds on adipose tissue and the mechanisms they utilize have not yet been characterized. Thus, in this study, the effects of CF and ENL on adipose tissue were determined by differentiating 3T3-L1 fibroblasts into adipocytes and treating them with either compound in a concentration-dependent manner. The results of this investigation suggest that both compounds stimulate glucose disposal in adipocytes via a GLUT4-dependent mechanism.

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Background and Rationale

Epidemiology of Type 2 Diabetes

Type 2 diabetes (T2D) is a chronic metabolic disease that has increased in prevalence at an alarming rate over the past few decades and has become a major public health concern especially in developed nations (Khan et al., 2019). According to the International Diabetes Federation, 4.2 million deaths were attributed to diabetes in 2019, and 463 million adults aged between 20 and 79 years old were reported to be living with the disease—a number predicted to escalate to 700 million by 2045 (Galicia-Garcia et al., 2020). From 1990 to 2017, T2D swiftly advanced from the eighteenth leading cause of mortality in the United States to the ninth leading cause of mortality (Khan et al., 2019). The rising worldwide prevalence of T2D has also been accompanied by concern for the markedly increased incidence of the metabolic disorder in younger generations (Rao & Jensen, 2020). Furthermore, diabetes currently stands as the most expensive chronic condition in the United States, with one out of every four dollars spent in healthcare being used to care for people with diabetes(Dieleman et al., 2016). From the outrageous health expenditures, which surmounted $720 billion USD in 2019, to the significantly decreased quality of life for enormous populations of people, the emergence of T2D has overwhelmed countless healthcare systems and the communities they serve, and no countermeasures have yet been successful in curbing its continually rising prevalence (Dieleman et al., 2016).

Health Complications and Risk Factors for T2D

Galicia-Garcia et al., 2020 noted that the primary agents behind the sudden surge in T2D cases are the global rise in obesity, sedentary lifestyles, high caloric diets, and population aging. Of these, obesity and lack of physical activity have been identified as the strongest risk factors for T2D. Physical activity plays a significant role in delaying the onset of T2D by stimulating blood flow through skeletal muscle contraction, by improving insulin sensitivity and glucose uptake from plasma, and by reversing many T2D predisposing factors such as chronic metabolic inflammation, oxidative stress, and abdominal fat accumulation, which are known to contribute to insulin resistance (Galicia-Garcia et al., 2020). The second prominent risk factor for T2D is chronic over-nutrition. While the habitual, excessive consumption of a variety of different foods can lead to the development of predisposing factors like hyperglycemia and obesity, previous studies have specifically implicated the consumption of highly processed foods and soft drinks in the sudden rise of T2D. This is because these consumables contain large quantities of high fructose corn syrup and glycated chemicals which have been shown to steadily increase BMI and augment insulin resistance (Sami et al., 2017). It is worth noting that fructose itself is not likely harmful, but because it does not inhibit ghrelin nor promote leptin, the feeling of society may not be as extant in means consuming large percentages of these foods. This can result in overeating, which is the primary culprit as chronic positive energy balance will necessarily translate to increased adiposity. Furthermore, diets consisting predominantly of fats and carbohydrates not only elevate blood glucose levels, but also promote increased circulation of very-low-density lipoproteins (VLDLs), chylomicrons (CMs), and their remnants (CMRs) that are rich in triglycerides (TG) that can induce spikes in reactive oxygen species (ROS) concentrations and can lead to an abnormal generation of pro-inflammatory mediators (Galicia-Garcia et al., 2020).

While poor dietary habits and sedentary lifestyles are both examples of reversible predisposing factors, there are also a number of irreversible predisposing factors that increase the risk of developing T2D including age, genetics, race, and ethnicity. According to a previously conducted survey, the prevalence of both T2D and prediabetes increases with advancing age, with the prevalence of diabetes and prediabetes among people aged 40–49 being 11.1% and 40.3%, respectively, while the prevalence of diabetes and prediabetes among people aged 60–69 is an increased 23.9% and 47.6%, respectively (Yan et al., 2023). These statistics reflect the gradual deterioration of the tightly regulated mechanisms responsible for maintaining glucose homeostasis over time in response to age-related factors such as diminished insulin secretion and the gradual progression of sarcopenia (Mordarska & Godziejewska-Zawada, 2017).

While age has been established as a dependable indicator of diabetes risk, genetic predisposing factors, which have also been implicated in previous studies, are not quite as reliable. A few previous studies have proposed that individuals from African American, Hispanic, and Native American ethnic origins may have an increased predisposition to clusters of cardiovascular risk factors, including hypertension, insulin resistance, and dyslipidemia (Galicia-Garcia et al., 2020). However, the existing literature in this area is currently inadequate to conclusively determine the relationship between certain genetic factors and the risk of developing T2D. Thus, risk evaluations for T2D using traditional risk factors such as BMI, family history, age, sex, and HDL/triglyceride levels still consistently outperform the risk scores determined by any set of genetic markers (Ali, 2013). To better understand the inherited risk of this disease and better inform current treatment and prevention methods, more research into specific genetic markers for T2D and the associated gene-gene and gene-environment interactions involved in T2D pathophysiology is needed. Overall, the variety and steadily

increasing ubiquity of the described risk factors for T2D in developed nations has been foundational to the proliferation of the T2D epidemic.

Current Treatment Options for T2D

Despite the severity of the T2D public health crisis and all of the existing literature describing the predisposing factors and pathophysiology for the disease, the attempts to spread awareness about this increasingly common metabolic disorder and the various available treatment methods have both failed in significantly improving the incidence, prevalence, and burden of T2D. Rather, the response of existing public health systems to the spread of T2D over the last few decades has culminated in the implementation of a number of robust diet and exercise programs as well as the development of effective pharmacological agents that have positively impacted the management of T2D symptoms and overall quality of life, but have had minimal effect on the continually rising incidence and prevalence of T2D (Shah et al., 2021). Additionally, obstacles such as cost, availability, metabolic memory, and apprehension towards medication in populations where diabetes is most prevalent have further dampened the impact of existing T2D treatment options (Galicia-Garcia et al., 2020). One of the most familiar treatments associated with T2D is a controlled regimen of insulin injections, which can be especially effective in individuals who have sustained severe pancreatic beta cell damage. Unfortunately, insulin is extremely expensive, especially for patients who are uninsured, and since more than 80% of patients with T2D come from low-to-middle-income socioeconomic backgrounds, insulin is financially unattainable for a large percentage of individuals with T2D(Galicia-Garcia et al., 2020). Additionally, while medications such as Metformin and Ozempic have been approved by the FDA and have shown promise in improving the quality of life for people with T2D, these drugs are only capable of managing T2D symptoms and do not improve the

prevalence nor incidence of T2D. Furthermore, there have been shortages in these medications that have caused prescriptions to be delayed, leaving many people with diabetes in need and exacerbating problems with drug adherence and control of symptoms.

A more recently identified issue concerning the treatment of T2D is metabolic memory, which refers to the persistence of diabetic complications even after glycemic control is established. This concept arose from the results of multiple large-scale clinical trials, which showed that diabetes complications continued to persist and progress even when glucose homeostasis had been restored through pharmaceutical intervention (Galicia-Garcia et al., 2020). Although metabolic memory has been linked to epigenetics, oxidative stress, non-enzymatic glycation of proteins, and chronic inflammation, more research into the persisting influence of T2D on these areas is needed for novel therapeutics to succeed in curbing the rise of T2D. Finally, distress surrounding T2D has caused many people with diabetes to struggle to adhere to a prescribed medication schedule. Socioeconomic status, occupation, and food environment as well as feelings of frustration or defeat concerning health have also been shown to deter people with T2D and those at risk of developing T2D from adhering to a diet and exercise routine that could help improve, delay, or even prevent the onset of the disease (Pandit et al., 2014). Thus, more affordable, readily available, and less intimidating therapies for the treatment and prevention of T2D are desperately needed. However, to begin the process of developing a novel therapy that meets these conditions, a thorough understanding of the molecular mechanisms responsible for the pathogenesis of T2D must first be obtained.

Tissues Implicated in T2D Pathogenesis

With respect to T2D pathogenesis, the liver, skeletal muscle, and adipose tissue are generally considered to be the primary insulin-responsive tissues that pertain to the progression

of the disease. Considering their profound roles in the maintenance of glucose homeostasis, these three tissues hold the greatest potential as targets for anti-diabetic treatment. The liver is responsible for maintaining blood glucose homeostasis by releasing stored glucose during hypoglycemia and is a prominent target for studies aimed at preventing hyperglycemic gluconeogenesis and glycogenolysis due to insulin-resistance. However, in terms of reducing chronic postprandial hyperglycemia by serving as a sink for glucose disposal, skeletal muscle and adipose tissue are more suitable targets for pharmacological intervention. Furthermore, since skeletal muscle is responsible for the greatest percentage of postprandial glucose uptake of the three tissues, most current literature is comprised of studies exploring avenues of intervention in skeletal muscle. Thus, despite being responsible for approximately 15% of postprandial glucose disposal (Kahn, 1996) and serving as a crucial endocrine mediator by secreting multiple anti-diabetic hormones (Rosen & Spiegelman, 2006), adipose tissue has frequently been overlooked as a target for novel T2D treatments. One of the hormones secreted by adipocytes is adiponectin, which has previously been shown to have insulin-sensitizing, anti-atherogenic, and anti-inflammatory effects in both humans and rodents, indicating versatile therapeutic potential for the treatment of many common predisposing factors for T2D (Achari & Jain, 2017). Additionally, a large quantity of endocrine-based treatment approaches to T2D have sought to stimulate secretion of the hormone leptin from adipose tissue, as previous literature has revealed that the hormone markedly improves glycemic control, insulin sensitivity, and plasma triglycerides in patients with severe insulin resistance (Coppari & Bjørbæk, 2012). Due to the substantial role adipose tissue plays as an endocrine mediator of glucose homeostasis, novel treatments capable of stimulating glucose uptake into adipose tissue hold great potential to

attenuate T2D pathogenesis. Considering the gap in knowledge regarding its ability to serve as a target of pharmacological intervention, adipose tissue was the primary focus of the present study.

Pathogenesis of T2D

T2D is a complex metabolic disorder characterized by chronic hyperglycemia that leads to the development of insulin resistance. There is a variety of molecular interactions as well as genetic, metabolic, and environmental factors that, when combined, can subsequently result in T2D. However, two specific mechanisms related to glucose homeostasis have been identified as the most commonly disrupted biological activities in individuals with T2D. These two mechanisms are insulin secretion from pancreatic β-cells and the sensing of insulin by tissues of the body.

Normal Physiology of Insulin Secretion

In normoglycemic individuals, postprandial glucose disposal is promoted by insulin, which is synthesized and released by pancreatic β-cells. Briefly, when the concentration of glucose in the blood is high, glucose is transported into pancreatic β-cells by GLUT2, which has a uniquely high K_m compared to other glucose transporters. This ensures that the amount of glycolytic activity in pancreatic β-cells and the associated insulin response are proportional to the levels of glucose in the blood. Once blood glucose levels are elevated enough to overcome the high K_m of GLUT2, the glucose is transported into the β-cells. Once the glucose reaches a sufficient intracellular concentration to reach the Km of hexokinase IV, it is metabolized, consequently increasing the ATP/ADP ratio within the cell. The increased ATP/ADP ratio causes ATP-sensitive K^+ channels to close, resulting in the depolarization of the cell membrane and the opening of voltage-sensitive Ca^{2+} channels, allowing for an influx of Ca^{2+} ions. The calcium ions then stimulate increased production of cAMP via activation of soluble adenylyl

cyclases (sACs), one of the two adenylyl cyclase families found in mammals (Ramos et al., 2008). Unlike transmembrane adenylyl cyclases, which are directly regulated by heterotrimeric G proteins and are primarily responsible for basal cAMP production, sACs are insensitive to G proteins and are instead activated synergistically by calcium and bicarbonate ions. (Kamenetsky et al., 2006). Calcium-stimulated production of cAMP from sACs results in an accumulation of intracellular cAMP, which in turn stimulates vesicles containing insulin granules to fuse to the cell membrane and release insulin into circulation (Petersen & Shulman, 2018).

Normal Physiology of Insulin Signaling

Insulin from the pancreatic β-cells binds to the transmembrane insulin receptor (IR) expressed on adipocytes. This results in the dimerization of the alpha and beta subunits of the IR, and the subsequent autophosphorylation of tyrosine residues within the transmembrane portion of the beta subunits. Once the IR is phosphorylated, the insulin receptor substrates 1 and 2 (IRS1 and IRS2) are recruited to the IR, initiating an intracellular phosphorylation signaling cascade that leads to the phosphorylation of a number of differnet substrates, including Akt. Phosphorylated Akt then carries out the pivotal task of phosphorylating several downstream proteins that function, among other things, the translocation of GLUT4 storage vesicles (GSVs) to the plasma membrane to facilitate the diffusion of glucose into adipocytes. Once inside the cell, the glucose is either used as an energy substrate, stored as triglycerides, or converted to lactate and secreted to help sustain the basic energy needs of the body (Cignarelli et al., 2019).

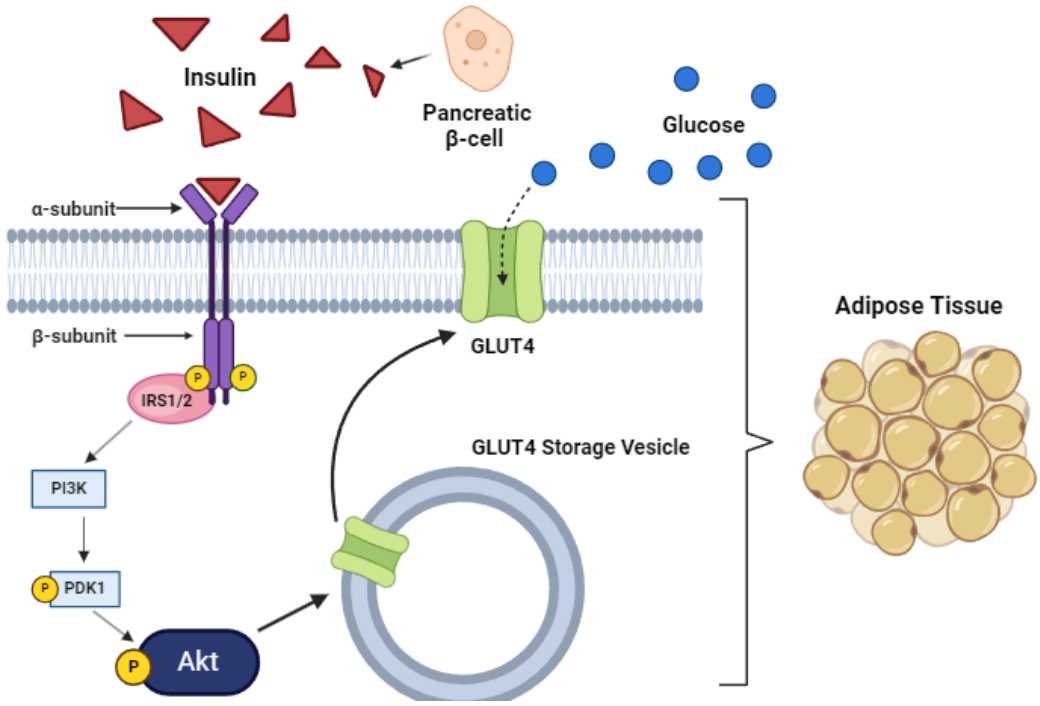

Figure 1. Schematic representing the cellular mechanism of glucose uptake in adipocytes (BioRender).

T2D Pathophysiology

As previously stated, glucose homeostasis is a complex, tightly regulated system, and any disturbance that alters the biological activities of the two previously highlighted mechanisms or any of the numerous other involved regulatory agents can result in an imbalance and the subsequent development of insulin resistance. With respect to the function of pancreatic β-cells, chronic hyperglycemia presents a particularly difficult challenge to the maintenance of glucose homeostasis. Since insulin is a signaling molecule released in response to elevated blood glucose levels, individuals with chronic hyperglycemia are perpetually able to overcome the high K_m of GLUT2 on pancreatic β-cells. Thus, the regulatory mechanisms that are responsible for ensuring that secreted insulin responses are proportional to the levels of glucose in the blood become

overwhelmed, resulting in the constant stimulation of insulin release. This often results in severe damage to pancreatic β-cells and loss of β-cell function. Furthermore, the consistent presence of insulin causes previously insulin-responsive tissues to lose their insulin sensitivity over time (Petersen & Shulman, 2018). The resultant lack of glucose disposal further exacerbates the hyperglycemia, leading to an endless destructive cycle that, if not treated, can result in severe damage to the eyes, kidneys, nerves, heart, and peripheral vascular system as well as a significantly increased risk of heart attack, stroke, and kidney failure(Shah et al., 2021). Overall, patients with T2D have a 15% increased risk of all-cause mortality compared to people without diabetes, with cardiovascular disease being the greatest cause of morbidity and mortality resulting from T2D (Galicia-Garcia et al., 2020).

Relationship between Coffee Consumption and Risk of T2D Pathogenesis

Moderate, habitual coffee consumption has been shown to provide potentially therapeutic and chemopreventive effects, such as anti-oxidant, anti-inflammatory, anti-mutagenic and anti-carcinogenic activities (Lee et al., 2012). Notably, previous research exploring this relationship suggests that a daily intake of 2–5 cups of coffee significantly reduces the risk of T2D development, with the association between coffee consumption and T2D risk appearing to be linear up to about 8–10 cups per day (Carlström & Larsson, 2018). Among the hundreds of bioactive compounds present in coffee, the most distinguished and comprehensively characterized compound is caffeine. Due to its well-characterized molecular mechanism, caffeine has been thoroughly studied in the context of T2D management and the resulting data from such studies have indicated that both caffeinated and decaffeinated coffee exert approximately the same effect on most of the biological processes implicated in T2D pathogenesis (Kolb et al., 2021). Further, a separate study found that consumption of decaffeinated coffee significantly

increased insulin sensitivity in healthy men compared to caffeinated coffee, which had little effect on glucose homeostasis (Reis et al., 2018). Because decaffeinated coffee has demonstrated similar if not better anti-diabetic activity than caffeinated coffee, it is unlikely that caffeine is responsible for the observed inverse epidemiological relationship.

Alternatively, there has recently been a growing interest in the potential medicinal properties of small, naturally occurring, bioactive compounds, in the context of T2D management. Particularly, two compounds, cafestol (CF) and enterolactone (ENL), have been identified as potentially influential in the observed anti-diabetic effects of coffee. CF is an *ent*-kaurene diterpenoid (Fig. 2) that is characterized by the possession of a unique furan ring and can be found naturally in coffee drinks at concentrations of approximately 0.25–0.3 mg/100 mL in filtered coffee and up to 4 mg/100 mL in unfiltered coffee (Farias-Pereira et al., 2019). CF has been shown to impact a variety of biological processes, with previous research demonstrating the ability of the compound to stimulate anti-inflammatory effects, neuroprotective effects, hepatoprotective effects, and certain anti-tumor effects, such as induction of apoptosis through regulation of specificity protein 1 (Sp1) expression in human malignant pleural mesothelioma (Ding et al., 2017). Conversely, ENL (Fig. 3) is a gut microbial metabolite of matairesinol, a bioactive compound found naturally in coffee, and it has been reported to exert various therapeutic biological activities including lowering the risk of breast cancer development (Saarinen et al., 2007) and acute coronary episodes (Vanharanta et al., 1999). These discoveries prompted investigations into the potential of CF and ENL to act as anti-diabetic agents. More recent studies seeking to explore this potential were able to successfully demonstrate that CF is capable of significantly stimulating both increased insulin secretion from pancreatic β-cells and increased glucose disposal in skeletal muscle compared to controls using in vitro models

consisting of a clonal rat insulinoma cell line and a human skeletal muscle cell line, respectively (Mellbye et al., 2015). Additionally, further research has shown that ENL promotes glucose disposal in skeletal muscle and basal glucose uptake in L6 myotubes (Zhou et al., 2017).

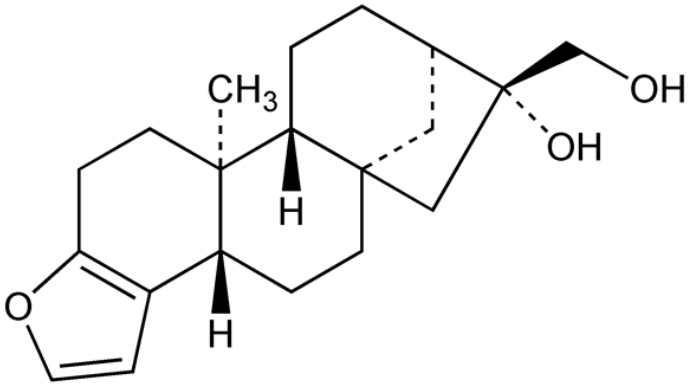

Figure 2: The molecular structure of CF (Merck Index Online)

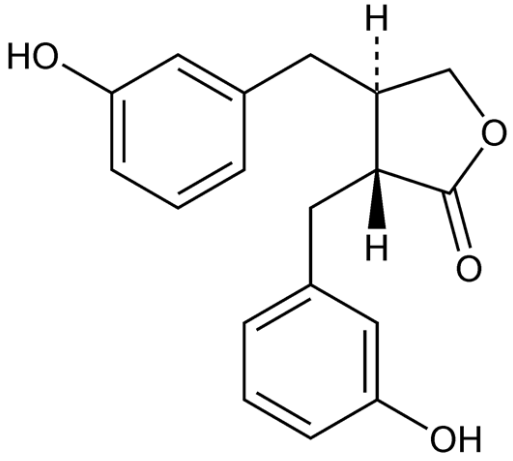

Figure 3: The molecular structure of ENL (Merck Index Online)

While these studies contribute to the vast reservoir of existing literature that describes the effects of CF and ENL on glucose disposal in skeletal muscle, whether these compounds stimulate similar effects on glucose disposal in adipose tissue as well as the molecular mechanisms they utilize have yet to be determined. Thus, the primary objective of the present

study was to use an in vitro model to determine the effects of CF and ENL on glucose uptake in adipocytes as well as to begin characterizing the molecular mechanism by which these compounds accomplish their respective activities.

Methods

Cell Line and Media Compositions

3T3-L1 embryonic mouse fibroblasts were purchased from the American Type Culture Collection (ATCC). The cells were cultured in T75 culture-treated flasks (Corning; cat. #431464) by using a Complete Growth Medium (CGM) consisting of DMEM high glucose with L-Glutamine (ATCC; cat. #30-2002) and 10% fetal calf serum (ATCC; cat. #30-2030). The cells were differentiated in a medium consisting of CGM with the addition of 0.5 mM isobutlymethylxanthine (IBMX) (Caymen Chemical; cat. #13347), 1 μM dexamethasone (Caymen Chemical ; cat. #11015), and 10 μg/mL insulin solution from bovine pancreas (MilliporeSigma; cat. #I0516). The subsequent post-differentiation medium consisted of CGM with a final insulin solution concentration of 10 μg/mL.

Cell Culture Maintenance and Subculture Protocol

Recommended optimal values for seeding and confluency from Thermo Fischer Scientific (Waltham, MA) were used to culture and subculture the cells. The 3T3-L1 fibroblasts were reanimated from cryopreservation, seeded into a T75 flask with 10 ml of CGM, and incubated at 37 ºC in 5% CO_2. Just before the culture reached 70-80% confluency (approximately 48-72 hours), the cells were subcultured utilizing 5 mL of a Trypsin-EDTA (ATCC; cat. #30-2101) solution to detatch the cells from the bottom of the flask. To neutralize the Trypsin-EDTA, 5 mL of CGM were added to the T75 flask. The suspended cells were centrifuged for 7 minutes at 130 RCF. The cell pellet was resuspended in 2 ml of CGM and

counted using an inverted microscope and a hemocytometer. Resuspended cells were diluted in CGM and seeded into either a new T75 flask or tissue-treated assay plates for experimentation.

Measuring Fluoresence and Absorbance Data

Using a Tecan Infinite 200 Pro Plate Reader (Tecan Trading AG, Switzerland), fluorsescence (excitation/emission = 485/535 nm) was measured for the glucose uptake and the BAY-876 inhibitor assays, and absorbance at 450 nm was measured for the Akt and adiponectin ELISAs.

2-NBDG Fluorescent Glucose Uptake Assay

Fibroblast to Adipocyte Differentiaion Protocol

For the glucose uptake and inhibitor assays, cells were seeded into GenClone Flat Black Clear Bottom 96-well plates (Genesee; cat. #91-420TB) at $1x10^4$ cells/well in a final volume of 100 µL per well. For the Akt and hormone ELISAs, fibroblasts were seeded into GenClone Flat Bottom 6-well cell culture plates (Genesee; cat. #25-209) at $3x10^5$ cells/well in a final volume of 2 mL per well. The cells were incubated until 70% confluency was obtained (24-36 hours) (Fig. 4A). At confluency, the CGM was replaced with differentiation media at the same respective volumes for each plate. The cells were incubated for an additional three days, or until differentiation from fibroblasts to adipocytes was observed via microscopy (Figure 4B). The differentiation media was then replaced with post-differentiation media in the same volumes. The plates were incubated for three more days before being replaced with CGM in which cells were maintained until experiments were conducted.

Glucose Uptake Assay

On day 6, the differentiated adipocytes were treated for 30 minutes at 37 ºC with either vehicle, insulin (10 nM), CF (0.01, 0.1, 1.0, 10, and 20 µM) (Caymen Chemical; cat. #13999), or

ENL (1.0, 10, 20, 50, and 100 μM) (Caymen Chemical; cat. #10112) prepared in 1X PBS with 100 μg/mL 2-NBDG. After incubation, the plate was centrifuged at 400 RCF for 5 minutes at room temperature. The treatments were aspirated and the wells were washed with 200 μL of 1X PBS, then the plate was centrifuged again at the same settings. The PBS was discarded and replaced with 100 μL of 1X PBS, then the fluorescence was measured.

BAY-876 Inhibitor Assay

Adipocytes that had been differentiated in 96-well plates were treated with 100 μL of either vehicle, insulin (10 nM), insulin plus BAY-876 (290 nM) (Cayman Chemical; cat. #19961), CF (10 μM), CF plus BAY-876 (2 nM or 290 nM), ENL (1.0 or 10 μM), or ENL plus BAY-876 (2 nM or 290 nM) prepared in 1X PBS with 100 μg/mL 2-NBDG for 30 minutes at 37ºC. BAY-876 has been shown to inhibit GLUT1 and GLUT4 at 2 and 290 nM, respectively (Reckzeh, & Waldmann, 2019). Following incubation, the plate was centrifuged at 400 RCF for 5 minutes at room temperature, then the treatments were aspirated and replaced with 200 μL of 1x PBS solution. The plate was centrifuged again at the same settings, aspirated, and replaced with 100 μL of 1x PBS solution before fluorescence was measured.

Protein Quantification

Cells were seeded and differentiated in 6-well plates. For this procedure, cells were treated the same as they were in the glucose uptake assays (vehicle, insulin (10 nM), CF 10 and 20 μM, and ENL 1 or 10 μM). After incubating at 37 ºC for either 30 minutes (Akt assays) or 24 hours (adiponectin assay), treatments were aspirated and the cells were lysed by adding 200 μL of 1X diluted lysis solution containing protease and phosphatase inhibitors (ThermoFisher; cat. #A32961) to each well. Cell lysate protein was quantified by using a BCA assay kit according to

the manufacturer protocol (Prometheus, cat. #18-440). Using a prepared 1X Assay Diluent, quantified cellular proteins were reconstituted to 45 µg/µL.

ELISA (pAkt, Akt, and Adiponectin)

ELISAs were conducted according to the manufacturer protocols (RayBiotech, Norcross, GA). *Adiponectin* was measured by adding 45 µg lysate, in a final volume of 100 µL, to a 96-well plate that was pretreated with anti-Mouse adiponectin, covered, and incubated at room temperature for 2.5 hours with gentle shaking. The solutions were then discarded and the wells were washed four times with 300 µL of 1X wash solution per well. 100 µl of 1X biotinylated antibody were subsequently added to each well. The plate was incubated for 1 hour at room temperature with gentle shaking. The solutions were then discarded and the previous wash steps were repeated. To each well, 100 µl of Streptavidin solution were added, then the plate was incubated at room temperature for 45 minutes with gente shaking. After incubation, the solutions were discarded and the previous wash step was repeated. Then, 100 µl of TMB One-Step Substrate Reagent were added to each well. The plate was incubated for 30 minutes at room temperature in the dark with gentle shaking. 50 µl of stop solution were added to each well and the absorbance at 450 nm was promptly measured (RayBiotech; cat. #ELM-Adiponectin).

pAkt and Akt were measured by adding 45 µg of cell lysate, in a final volume of 100 µL, to a 96-well plate that was pre-treated with anti-pan Akt antibody and incubated at room temperature for 2.5 hours with gentle shaking. Absorbance was assessed and displayed as a ratio of pAkt:Akt. The solutions were then discarded and the wells were washed four times with 300 µL of 1X wash solution per well. 100 µl of prepared 1X rabbit anti-phospho-Akt (Ser473) antibody were added to each well, then the plate was incubated for 1 hour at room temperature with gentle shaking. The wash step was repeated, then 100 µl of prepared HRP-conjugated anti-

rabbit IgG solution was added to each well and the plate was incubated for 1 hour at room temperature with gentle shaking. The wells were washed and then filled with 100 µl of TMB One-Step Substrate Reagent. The plate was incubated for 30 minutes at room temperature in the dark with gentle shaking. 50 µl of Stop Solution was added to each well then absorbance was assessed and displayed as a ratio of pAkt:Akt (RayBiotech, cat. #PEL-Akt-S473-T).

Statistical Data Analysis

All quantitative data were tested for normal distribution and equal variance and subsequenly analyzed by one-way ANOVA. Tukey's multiple pairwise comparison test was used if differences ($p < 0.05$) were observed.

Results

3T3-L1 Fibroblast Differentiation

3T3-L1 fibroblasts were differentiated into adipocytes over the course of six days. After seeding the cells into T75 flasks, approximately three days of incubation in CGM were required for the cells to reach 70% confluency (Fig. 4A). While incubating in differentiation media from day 4 to day 6, the cultures grew at a slower rate and the morphology of the cells changed from the flattened, spindle-like morphology of fibroblasts (Fig. 4A) to the more spherical morphology of adipocytes (Fig. 4B).

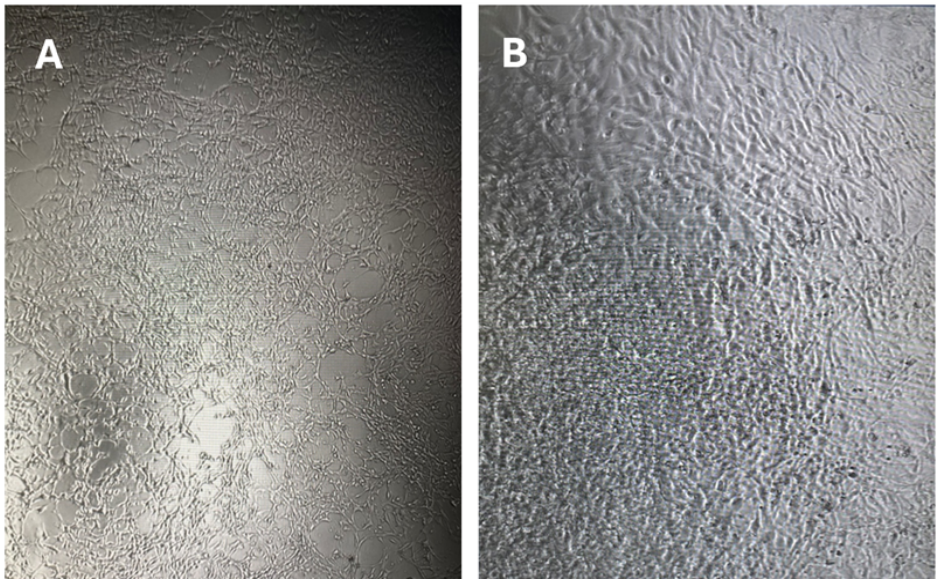

Figure 4: Characteristics of fibroblast to adipocyte differentiation in 3T3-L1 cell cultures. (A) 3T3-L1 fibroblasts on day 3 in Complete Growth Media (CGM) compared to **(B)** day 6 in differentiation medium.

CF and ENL both Significantly Stimulate Glucose Uptake in 3T3-L1 Adipocytes

A 2-NBDG fluorescent glucose uptake assay was used to measure glucose uptake in 3T3-L1 adipocytes that had been treated with either CF or ENL in a concentration-dependent manner using a positive control of insulin and a negative control of ethanol. The data indicate that CF significantly stimulates increased glucose uptake in 3T3-L1 adipocytes compared to the negative control, with 10 and 20 μM concentrations demonstrating the greatest increase in glucose uptake (Fig. 5). ENL-stimulated glucose uptake was also observed to be significantly greater than that of the negative control, with the greatest increases in glucose uptake occurring at the 1 and 10 μM concentrations (Fig. 6). Additionally, the glucose uptake stimulated by both compounds were not significantly different form the glucose uptake stimulated by insulin, suggesting that both compounds may be utilizing a molecular mechanism similar to the well-characterized glucose uptake mechanism of insulin.

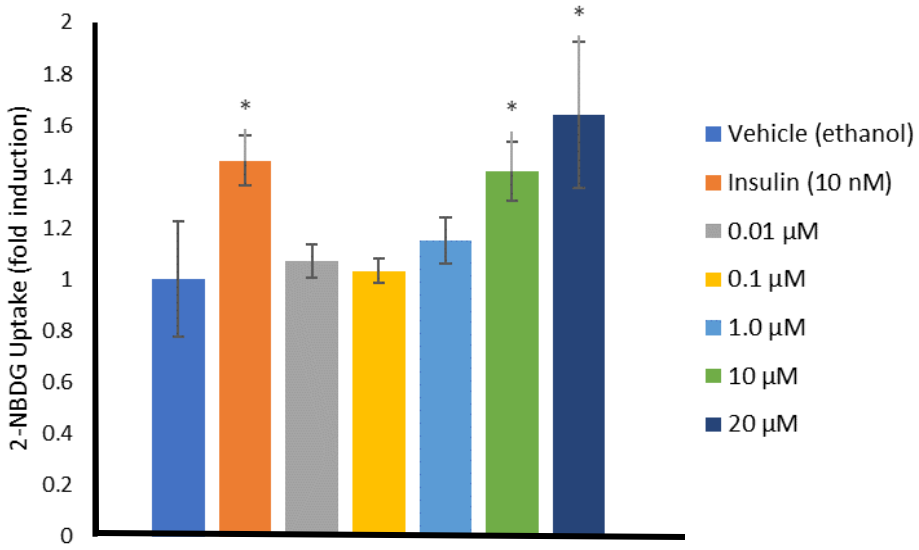

Figure 5. CF-stimulated glucose uptake is significantly greater than the negative control but not statistically different from the positive control. Cells were treated with either CF (0.01, 0.1, 1, 10, or 20 µM), insulin (10 nM) or the vehicle (99% ethanol). The 10 and 20 µM concentrations of CF significantly increased glucose uptake relative to the negative control ($p < 0.05$) with 20 µM eliciting the greatest effect (n=2).

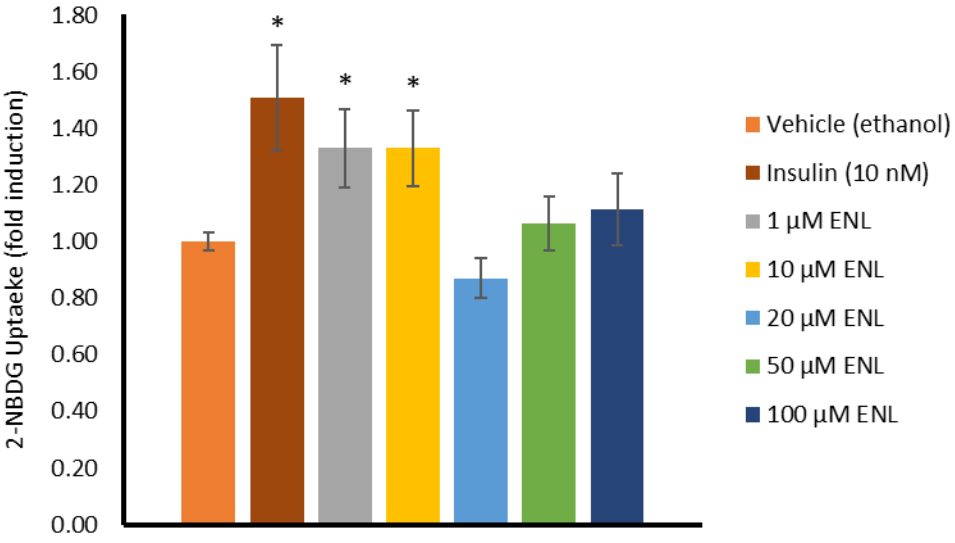

Figure 6. ENL significantly increased glucose uptake at 1 µM and 10 µM compared to the negative control. ENL (1, 10 µM) increased 2-NBDG uptake compared to the vehicle ($p < 0.05$) and in a similar manner as the positive control (n=3). The data was normalized to the negative control (ethanol), and insulin (10 nM) was used as the positive control.

BAY-876 Inhibition of GLUT 4 Reduces Compound-Stimulated Glucose Uptake

After the 2-NBDG fluorescent glucose uptake assays were conducted to determine the optimal concentrations of each compound for stimulating glucose disposal, further glucose uptake assays were run with the addition of BAY-876, a chemical inhibitor of GLUT1 and GLUT4 when added at concentrations of 2 nM and 290 nM, respectively. The results of this assay indicate that glucose uptake at the 10 µM CF was significantly reduced by the inhibition of GLUT4 (Fig. 7). Additionally, the glucose uptake stimulated by the 1 and 10 µM ENL were significantly blunted by GLUT4 inhibition (Fig. 8). These data indicate that CF and ENL may be utilizing GLUT4-dependent mechanisms of glucose transport.

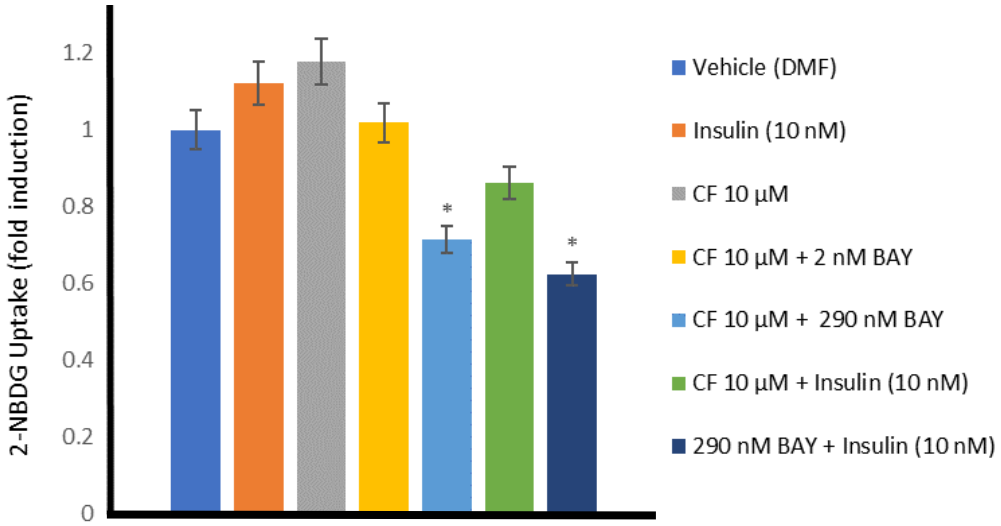

Figure 7. Inhibition of GLUT4 significantly reduces CF-stimulated glucose uptake in 3T3-L1 Adipocytes. Treatment with both 2 nM and 290 nM BAY-876 reduced glucose uptake in CF-treated adipocytes at the 10 µM concentration, with 290 nM BAY-876 resulting in the greatest inhibition of glucose uptake (n=3). Assay results were normalized to the negative control (DMF).

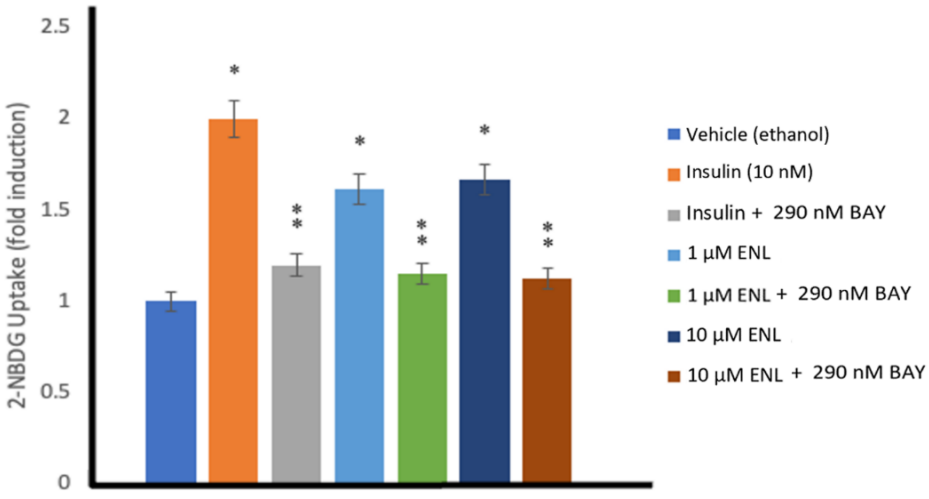

Figure 8. GLUT4 inhibition significantly suppresses ENL-stimulated 2-NBDG uptake in differentiated 3T3-L1 adipocytes. The cells were treated with either vehicle (ethanol), ENL (1 or 10 µM), insulin (10 nM) or ENL/insulin and BAY876 (290 µM). The 1 and 10 µM concentrations of ENL along with insulin significantly increased 2-NBDG uptake (*$p < 0.05$) relative to the negative control. Further, BAY876 (290 µM) significantly blunted the effect of the insulin, 1 µM ENL, and 10 µM ENL (** $p < 0.05$). Two biological replicates of the assay were performed.

ENL Stimulates Increased Akt Phosphorylation in 3T3-L1 Adipocytes

To further characterize the molecular mechanism of ENL, an ELISA was used to examine the ability of the compound to promote Akt phosphorylation. The results demonstrated a significant increase in the ratio of phosphorylated to non-phosphorylated Akt in cells treated with either 1 µM ENL, 10 µM ENL, or insulin when compared to the vehicle (Fig. 9). These findings further suggest that the effect of ENL on glucose uptake is dependent on GLUT4, as Akt promotes the translocation of GLUT4 containing vesicles to the plasma membrane.

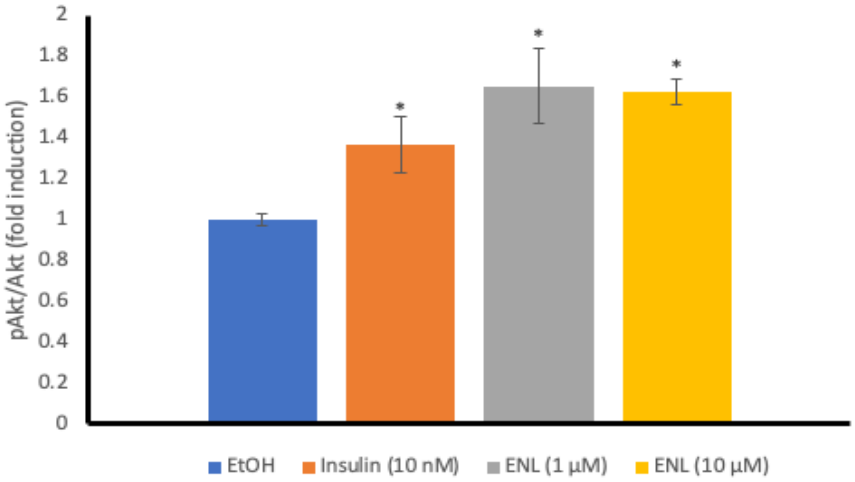

Figure 9. Enterolactone (ENL) significantly increased Akt phosphorylation in differentiated 3T3-L1 adipocytes. Cells were treated with either vehicle (ethanol), insulin (10 nM), or ENL (1 µM or 10 µM). pAkt and Akt were measured and expressed as a ratio of pAkt/Akt. Insulin, 1 µM ENL, and 10 µM ENL significantly increased Akt phosphorylation relative to the negative control (n=3).

CF Stimulates Increased Secretion of Adiponectin in 3T3-L1 Adipocytes

To evaluate the effect of CF on the secretion of anti-diabetic hormone, an ELISA kit was used to measure adiponectin secretion after treatment with 10 and 20 µM concentrations of CF. The preliminary data suggest that CF stimulates increased adiponectin secretion from 3T3-L1 adipocytes, with the 20 µM concentration of CF stimulating the greatest amount of adiponectin production (Fig. 10).

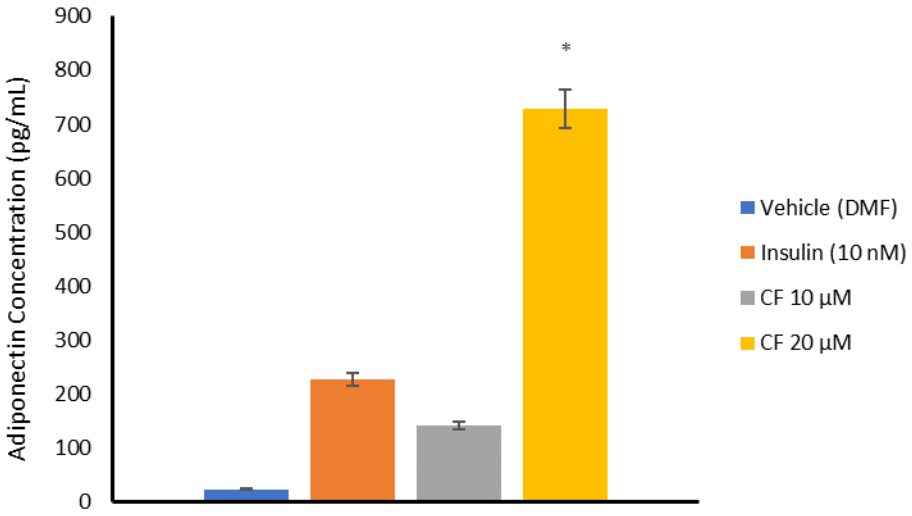

Figure 10. CF stimulates increased secretion of adiponectin in 3T3-L1 differentiated adipocytes. 3T3-L1 adipocyte cultures were treated with either insulin, DMF, 10 µM CF, or 20 µM CF, then cell lysates collected were collected and assessed via an ELISA to quantify adiponectin secretion. The 20 µM concentration of CF significantly increased adiponectin production compared to the negative control (n=1).

Discussion

This study evaluated the effects of CF and ENL on glucose disposal in 3T3-L1 adipocytes and provides evidence that both compounds promote glucose disposal in adipocytes to a level that is statistically indistinguishable from that of insulin. These findings are consistent with the results of previous studies that conducted similar experiments with CF and ENL in skeletal muscle (Mellbye et al., 2015; Zhou et al., 2017). Furthermore, using immunocytochemistry, a study conducted by Zhou et al. was able to visualize and confirm ENL-stimulated GLUT4 translocation to plasma membrane This finding is also in agreement with results of the present study, as treatment with the GLUT4 inhibitor BAY-876 significantly blunted the effects of ENL on glucose disposal. Additionally, GLUT4 inhibition via BAY-876

also significantly reduced CF-stimulated glucose uptake in 3T3-L1 adipocytes. Although there is a gap in the literature regarding the molecular mechanism utilized in CF-stimulated glucose disposal for both skeletal muscle and adipose tissue, the results of the present study suggest that the CF molecular mechanism is mediated by GLUT4 as well. However, further research must be conducted in both tissues in order to confirm the results of this study and elucidate the remainder of each mechanism.

Additionally, although the overall effects of ENL were consistent in both studies, it is important to note that Zhou et al. tested 25, 50, and 100 μM concentrations ENL and found that glucose uptake in L6 myotubes increased in a dose-dependent manner (2017), while the 1 and 10 μM concentrations of ENL in the present study outperformed the 20, 50, and 100 μM in terms of increasing glucose disposal in adipocytes. Thus, although ENL appears to promote glucose disposal in both skeletal muscle and adipose tissue, the data suggests that the concentration of the compound capable of eliciting the greatest effect is variable between the tissues.

The effects of ENL on Akt phosphorylation were also evaluated in this study, with the gathered data indicating that ENL stimulates increased Akt phosphorylation in 3T3-L1 adipocytes. This result contradicts some of the existing literature, as previous studies have reported data that suggests ENL stimulates a variety of anti-proliferative effects, including the inhibition of Akt phosphorylation in prostate cancer cell lines (Chen et al., 2009; Mcann et al., 2014; Xiong et al., 2014).

Lastly, preliminary data from the evaluation of CF-stimulated effects on adiponectin secretion in 3T3-L1 adipocytes revealed that treatment with 20 μM CF significantly increased adiponectin secretion compared to the negative control. While the existing literature indicates that habitual coffee consumption is associated with higher levels of serum adiponectin (Williams

et al., 2008; Yamashita et al., 2012), this effect has not yet been directly linked to CF. Although further investigation and experimental replicates are necessary in order to verify the significance of the data, this result still provides further evidence for the potential of CF to act as an anti-diabetic agent.

Limitations of the Current Study

Insulin was used as the positive control for the glucose uptake and Akt assays, however, insulin was also a component of the differentiation and post-differentiation media used for the cell cultures. Using a different compound as the positive control or experimentally determining the amount of time necessary for the 3T3-L1 adipocytes return to their normal physiological state after responding to the insulin in the media could help ensure that the insulin does not impact the results of the glucose uptake assays. Additionally, further investigation into the optimal incubation period would likely allow for the observation of a more realistic distinction between the positive and negative controls and would convey a more accurate depiction of the glucose disposal stimulated by CF and ENL. For the dilutions of CF, ethanol was originally used as the solvent but was changed to dimethyl formamide prior to the inhibitor assays to achieve more optimal solubility. Finally, the glucose uptake assays were time sensitive for both the overall treatment incubation time and the speed of the individual treatments. Based on previous literature, only incubation times of 15-30 minutes were tested, but there is potential for the optimal concentrations of CF for glucose uptake to reach higher values with different incubation times.

Future Aims

Future aims of this project include using the optimal concentrations of these compounds, to further characterize the molecular mechanism(s) by which they exert their effects. Because of

previous studies showing that ENL uses an AMPK-dependent mechanism in skeletal muscle (Zhou et al., 2017), this regulator will be assessed in response to both compounds. Furthermore, the ability of both compounds to stimulate the secretion of the anti-diabetic endocrine signaling hormones leptin, omentin, and visfatin should also be assayed. Finally, bioavailability studies may be conducted to determine what concentration of each compound is needed to replicate the observed in vitro effects in vivo.

Conclusion

Cafestol (CF), a diterpene, and enterolactone (ENL), a polyphenol metabolite, were experimentally examined to determine their effects on glucose uptake in 3T3-L1 adipocytes and to begin characterizing the molecular mechanisms they utilize, specifically looking at their impact on GLUT4 translocation, Akt phosphorylation, and adiponectin production. The results of this study indicate that both CF and ENL significantly stimulate glucose disposal in adipocytes compared to controls via a GLUT4-depedent pathway, perhaps similar to that of insulin. Additionally, the data indicates that ENL induces Akt phosphorylation, and that CF stimulates the production of the anti-diabetic hormone adiponectin in 3T3-L1 adipocytes. Furthermore, the abundance of these compounds in coffee as well as other fruits and vegetables shows promise in addressing both the availability and apprehension concerns towards diabetes medications, as many people already consume CF and ENL on a regular basis, just in smaller quantities than those used in these in vitro analyses. Overall, these results support the rationale for further research that might affirm the potential of CF and ENL as anti-diabetic agents.

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