

The Emerging Potential of Zyflamend as a Caloric Restriction Mimetic

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Abstract

In the modern era, humankind seeks answers on how to live longer and healthier. Obesity and its associated co-morbidities present a major hurdle in achieving this goal. Unfortunately, obesity rates continue to rise and while significant discoveries have been made, the problem persists. However, reducing adiposity and increasing skeletal muscle mass and functions could drastically reduce the risk for a myriad of diseases and promote a healthier lifespan, particularly in obese populations. In addition, research targeting caloric restriction (CR) reveals vast potential in obesity related models. CR mimetics are compounds with the potential to mimic the desirable metabolic effects of CR. CR mimetics have demonstrated the capacity to extend the life span and increase metabolic functioning in a variety of experimental models. However, the effectiveness of natural CR mimetics varies greatly and often requires doses impractical for human equivalence. Through in-depth literature analysis, we realized that a gap in the knowledge exists and it left us with these questions: “could natural alternatives effectively reduce fat mass while simultaneously increasing skeletal muscle performance, alleviating the co-morbidities of obesity”? A polyherbal blend known as Zyflamend, has been shown to effectively activate adenosine monophosphate-activated protein kinase (AMPK) in numerous models. Considering that AMPK activation is a central component of CR, Zyflamend could pose significant promise as a novel CR mimetic. Understanding the potential for Zyflamend could lead to a transformative understanding of the overall scope of CR mimetics against obesity and metabolic disease. Therefore, I hypothesize that treatment with Zyflamend, will modulate AMPK signaling and lead to enhanced lipolysis in adipose tissue while simultaneously improving muscle performance. In order to test this hypothesis, we will develop a unique diet incorporated with Zyflamend utilizing human equivalent dosing to promote translation of our in vivo findings. Furthermore, we will utilize in-vitro models to uncover precisely how Zyflamend may target obesity leading to beneficial metabolic outcomes.

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CHAPTER 1
INTRODUCTION

Background: Aging Theories, Obesity and Caloric Restriction

Aging is ultimately the process through which the human body and its components begin to decline [1]. Aging has long been the insurmountable obstacle to humanities desire for life and longevity. The quest for the fountain of youth has been a long-lasting venture with little compensation allotted for our continued efforts. However, humankind has entered into a new age where we are living longer, and therefore, desiring a higher quality of life in the “golden years” [2]. Modern science and research have begun illuminating the integral processes and mechanisms through which we age. As these fundamental principles become more apparent, innovative and effective approaches to nutrition and clinical intervention strategies targeting age and age-related diseases may come to fruition [1, 3, 4].

CR can be simply defined as consuming calories below a given threshold without promoting malnutrition. Regarding aging, CR has been highly publicized for its life extending capacity in numerous scientific models such as mice, worms, yeast, flies, etc. [5]. Problematically, many hurdles arise when trying to translate these findings into practical application within humans [5]. However, through research on lower organisms science has made headway and begun understanding the generalized processes and functions through which living organisms age [5]. Subsequently, the development and advancement of theoretical principles centralizing around aging, have progressed significantly. Understanding the interactions and importance of adipose tissue and skeletal muscle in the processes of aging have proven a critical aspect of modern aging research. The obesity epidemic plaguing the western world has led to many populations and individuals burdened by excessive adipose tissue across the life span. This excessive adiposity can have systemic consequences and can substantially increase the risk of premature death.

Obesity and its co-morbidities pose a significant hurdle to the health and well-being of populations across the world [6, 7]. The effect of obesity on the metabolism is drastic and poses one of the more substantial risk factors for many age related diseases [7]. Comorbidities such as type 2 diabetes and the accumulation of radical oxidation species (ROS) are intrinsically connected to the metabolic processes [8-12]. The process through which humans and organisms age centralizes around the metabolism [9, 12]. The metabolism under normalized and healthy conditions thrives towards sustaining health and well-being [8, 9, 12]. However, the metabolic and cellular processes can be overwhelmed by exposure to a wide variety of internal and external

factors and conditions [9, 12-14]. The transition towards pathological conditions, disease and death is often directly related to the aging metabolism [9, 13, 14]. The nutritional intervention of CR has been identified in connection to improving metabolic functioning [15-17]. CR has also shown potential as a treatment option for obesity and its comorbidities [16, 18, 19]. However, significant problems arise when clinically implementing CR due to the undesirability of reduced caloric intake [20, 21]. Conversely, CR mimics have emerged as an area of vast interest of aging and nutritional research. Moreover, phytochemicals may prove capable of promoting healthier aging processes and acting as CR mimics. For instance, our research along with the investigations of others on the polyherbal supplement Zyflamend have identified this potential. Zyflamend's emerging potential as a novel CR mimetic will be thoroughly discussed. In addition, the following will explore the mechanistic and molecular interactions that drive the deleterious effects of aging. Furthermore, the analysis provided will centralize around the most prominent theories of aging. Such as, metabolic regulation and the specific metabolites along with the key enzymes and signaling molecules involved in aging. In addition, oxidative stress and mitochondrial dysfunction known to contribute to DNA damage, senescence, and overall genomic instability will also be reviewed [22]. Finally, epigenetics and factors such as histone modification, DNA methylation, and how the overall genetic profile contributes to aging will be closely analyzed [22].

Aging Theories

Metabolism and Aging

Currently, the theories of aging revolve around fundamental concepts essential to the aging process including the effects on the overall metabolism, oxidative stress, mitochondrial dysfunction and epigenetic modification. Within the modern era, technological and agricultural advancements have resulted in unparalleled food surpluses across industrialized nations. This surplus along with other factors has resulted in a rise in metabolic disease rates exhibiting a direct relation to increasing age and weight [1]. Obesity, age, increased weight, and fat mass have also shown a direct correlation to increased cancer rates [23]. The systemic effect of aging, increased fat mass and caloric intake often leads to heightened levels of metabolic distress [1]. As a result, efforts to uncover treatments capable of aiding in metabolic regulation and alleviating metabolic stress have been ongoing [3]. There are many different components and factors capable of influencing metabolic functions and regulation [1]. Details regarding metabolic and growth regulating proteins such as insulin like growth factor (IGF), mechanistic target of rapamycin

complex (mTOR), sirtuins, AMPK, p53 and peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1a) have taken center stage in the current literature [3, 4]. It has been well established, that these proteins play critical roles in maintaining and promoting metabolic homeostasis in various aging organisms [24-26]. With significant consideration for their relationship with aging and restricted caloric intake, the modification and interactions of these proteins within tissues such as adipose and muscle, will be a central concept within this analysis.

Targeting and modifying the metabolism has emerged as one of the essential theories regarding the aging process. CR is the process by which an organism consumes a significant reduction in calories with regards to its normalized caloric requirements and is a fundamental approach in targeting the metabolism [3]. Through reducing caloric intake in various organisms, a substantial correlation between increased lifespan and caloric reduction in multiple organisms (mice, primates, insects, etc.) has been discovered [3, 5, 27]. However, implementing this strategy within a clinical setting has proven rather problematic for a myriad of reasons [3, 5]. Metabolic modulation through CR and other means may attenuate or cure the symptoms of aging related diseases while increasing the life-span [3]. CR has shown the capability to deliver many benefits including but not limited to; increased glucose homeostasis, decreased radical oxygen species (ROS), positive changes in gene expression, and increased cell proliferation [28]. Conversely, the pursuit of CR mimetics has ensued due to the difficulties involved in implementing CR in humans [28]. Rapamycin has been introduced as a CR mimetic which acts through mTOR inhibition leading to a downstream effect on protein translation and autophagy [28]. Compounds like this could become integral to the pursuit of implementing the benefits of CR in a more practical way [28]. Moreover, targeting the metabolism shows invaluable potential in decreasing the negative consequences associated with age.

Oxidative Stress and Mitochondrial Dysfunction

Within living organisms, cells are subjected to the constant bombardment of damaging redox and oxidation reactions [1, 4]. It is believed that prolonged ROS exposure can interfere with many integral biological processes including the synthesis of ATP [1]. Increased levels of ROS within the body is regarded as a biological hallmark of aging [4]. ROS are perceived to promote oxidative stress through increasing the number of free radicals within circulation, which ultimately damage cell structures, lipids, nucleic acids and proteins within the cells [1, 4]. This damage can

result in impaired functioning of the organism and ultimately death. To varying degrees, CR has proven capable of countering the negative effects of ROS and could protect the organism at the cellular level.

The process through which CR results in a reduction of oxidative stress and ROS accumulation seems to closely resemble the combustion process involved within a mechanical engine. The mitochondria in many ways is the vehicle while the reactions such as electron transport chain and oxidative phosphorylation are similar to the engine [29]. Every combustion engine produces exhaust and accumulates mileage, wear and tear, similarly to the reactions and outcomes related to the mitochondrial generation of ROS as a byproduct [29]. Through lowering the mileage (oxidative stress) it could be possible to maintain the engine (organism) and potentially extend the life span.

The body contains many endogenous antioxidant systems to counter the effects of generated ROS, such as catalase, glutathione derivatives and superoxide dismutase [29]. Many investigations have targeted the interrelationship between CR and aging processes. For instance, amplified expression of mitochondrial catalase could prolong the life span of C57Bl6 mice [30]. As a nutritional intervention, targeting oxidative stress often centralizes around the idea that antioxidants may have the ability to scavenge free radicals [29]. However, the current literature shows mixed results on the actual capability of antioxidants to reduce oxidative stress [1, 29]. For example, 68 randomized trials including 232,606 participants were analyzed for changes in mortality risk in response to antioxidant supplementation [31]. The results showed little significance and actually identified an increase in mortality rate in those supplemented with beta carotene and vitamin A and E [31]. Continued investigation of the disease and tissue specific effects of antioxidants may allow for an improved understanding of aging processes and how they could protect organisms from the consequences of age. Beyond antioxidants, many other phytochemicals and natural compounds have been demonstrated to elicit similar antioxidant effects and will be explored in detail within latter portions of this review.

Epigenetics

The human genome harbors several genes that could exhibit major implications on the overall lifespan of the species [2]. These highly specific profiles consist of different genotype and phenotypes within the DNA construct [2]. The overall homeostasis of the epigenome is a complex process dependent on multiple mechanisms [22]. One key aspect of epigenetic modulation is the

hyper and hypo methylation of the CpG dinucleotides and their islands along with the subsequent age-related effects. The loss of methylation with age can result in the modification of the chromatin domains and eventual alteration to DNA transcription, binding and gene silencing [32-34]. The overall effect of these alterations can vary in response to age and the specific tissue in which the epigenetic modifications occur [35, 36]. Over time this process of epigenetic modification can have severe consequences including cellular senescence, disease, and eventually death. Once again CR has shown the ability to positively interact with these processes and could have protective capabilities [37].

The mechanisms through which CR interacts with the genome often involve histone modification and DNA methylation. DNA methyltransferases (DNMT) are responsible for mediating DNA methylation and decreased methylation plays a critical role in the phenotypical changes complementary to aging [37, 38]. The specific role of hyper and hypomethylation has become the target of many aging studies [2, 37]. CR can lead to hypermethylation and gene silencing of key role players in age-related disease progression such as H-Ras [39] and p16INK4a [34] [38]. Moreover, histone modification through deacetylation can activate SIRT1 and HDAC which causes deviations in age related gene expression such as p53 [38, 40]. However, studies have demonstrated conflicting findings when reporting on the effect of sirtuins and CR in various models [27, 41]. Supportively, it has been shown that CR could have an impact on the activity of tumor suppressor expression genes [37, 38]. Overall, CR appears to impact the aging organism at the genetic level. However, epigenetics is a fairly new field and further advancements in genome science and technology will be integral to uncovering how these findings might be translated [38].

Obesity

Epidemiology and Statistics

Obesity along with its co-morbidities represent one of the most significant health epidemics facing the modern world [7, 42]. The effect of obesity on metabolism is drastic, in lieu of this, the condition represents a primary risk factor for developing numerous age related diseases [7]. Comorbidities such as cardiovascular disease (CVD) and type 2 diabetes (T2D) are intrinsically connected to the metabolic processes [8, 10, 12, 43, 44]. While obesity is not directly related to age, the comorbidities of obesity, such as type II diabetes, CVD, and metabolic syndrome become increasingly apparent with age [7].

Obesity has become a full-blown epidemic that effects nearly 37% of Americans [7]. In many cases, obesity directly correlates with disproportionate calorie intake [7, 45]. Excess adipose tissue can burden and impair metabolic function and processes important for maintaining homeostasis [7, 12, 45]. Furthermore, obesity has been systematically linked to organ damage and failure through a variety of proposed mechanisms. Moreover, obesity has been correlated with dysfunction in tissues such as the kidney, liver, pancreas, and heart [42, 46-48]. In order to treat these comorbidities you must reduce obesity and varying forms of CR have emerged as one of the key nutritional intervention strategies [18, 45]. Caloric Restriction (CR) is an essential nutrition recommendation and intervention that can be difficult to implement. CR is defined by the NIH (National Institute of Aging, 2018) as any reduction in calories below a given threshold represented by the daily energy intake requirements, without inducing micronutrient deficiencies or malnutrition. Furthermore, there are many ways that CR can be implemented such as, intermittent fasting, alternative day fasting, and varying degrees of calorie reduction (approximately 15-30%). For instance, the nutritional intervention of calorie counting to achieve CR has been identified in connection to improving metabolic functioning [16, 17, 49]. Additionally, CR has shown potential in reducing weight in obese subjects and a subsequent alleviation of the negative outcomes associated with the comorbidities of obesity [16, 18, 19]. Moreover, 20-30% caloric intake reduction has been correlated to weight loss in obese subjects [18, 45]. Importantly, as a nutrition intervention CR has shown vast potential in age related research [16-18, 45, 49]. The benefits of CR may closely relate to its impact on metabolic function and disease.

Metabolic Disorders

Type II Diabetes

The pancreas is comprised of intricate and diversified cellular components that must function harmoniously in order to promote and maintain biological homeostasis. Physiologically, the pancreas contains endocrine and exocrine portions. The exocrine portion includes the acinar cells and ductal networks essential for the secretion of digestive enzymes such as lipases, amylases and proteases. On the other hand, the endocrine portion also known as the islets of Langerhans, is comprised primarily of the alpha and beta islets responsible for the secretion of hormones responsible for maintaining blood glucose homeostasis. T2D has emerged as a foremost global health related epidemic. Developmentally, T2D can be multifaceted with the primary components revolving around β cell dysfunction and subsequently reduced insulin secretion, synonymous with

insulin resistance which promotes reduced glucose uptake and clearance [50-52]. Critical for maintaining glucose homeostasis, Pancreatic β cells primary function is to release insulin. Functionally, the capacity of β cells is often measured by the level of insulin secreted and/or urinary excretion of C-peptide [50, 53]. Insulin secreted from β cells is responsible for a complex array of interconnected biological factors and outcomes [54, 55]. Typically, in the postprandial state, glucose levels rise resulting in increased uptake into the pancreatic β cells. As a result, levels of Ca^{2+} rise within the β cells and when functioning properly, insulin is secreted [56]. This process is known as glucose-stimulated insulin secretion (GSIS), and is dependent on KATP channel depolarization in response to elevated intracellular levels of ATP/ADP [56, 57]. Over time, hyperglycemia can detrimentally impact the function of β cells resulting in reduced cell mass, decreased insulin secretion, and increased inflammation [58]. Insulin resistance and hyperlipidemia both occur frequently and simultaneously within obese populations [7]. T2D often occurs due to insulin resistance and an influx in glucose levels causing strain to be placed on pancreatic β cells leading to impaired pancreatic function [7, 12, 42]. Compounding the problem, the inflammation associated with obesity could lead to the diverse activation and recruitment of cytokines and stromal cells, promoting the microenvironment changes necessary for initiating acute pancreatitis [59]. In the US alone, acute pancreatitis resulted in the hospitalization of 245,023 patients and 1,571 deaths in 2014 [60]. Obesity has been shown to potentially contribute to acute pancreatitis through increased incidence of biliary disease, hypertriglyceridemia and T2D [59]. The damage that can occur in response to acute pancreatitis can result in impaired endocrine and exocrine pancreatic function [59]. Specifically, abdominal visceral adiposity represents of the most significant risk factors for acute pancreatitis [61]. Amplifying the deleterious effects of obesity, the burden of impaired pancreatic function can have systemic effects on the gastrointestinal system [59].

Beyond the Pancreas: Gastrointestinal Diseases

Increased adiposity as observed in obesity can promote the development of gastrointestinal diseases such as, inflammatory bowel disorders (IBD), non-alcoholic fatty liver disease (NAFLD), diverticulosis, gastro-esophageal reflux disease (GERD), and gastric cancer of many varieties [62]. Liver diseases encompass any issue related to the function of the liver to either aid in metabolism or breakdown toxins within the body. While there are many forms of liver disease, the two commonly studied in research are liver fibrosis and non-alcoholic fatty liver disease (NAFLD)

[63, 64]. Similar to T2DM association with obesity, NAFLD shows similar trends [65]. NAFLD is a metabolic disease, primarily characterized by an accumulation of lipids within the liver. During steatosis and in the obese state excessive free fatty acids (FFAs) are delivered to the liver, often exceeding the level of FFAs being utilized and excreted, thus accumulating [66]. NAFLD can vary in severity and often develops slowly over time. Left uncorrected, NAFLD can progress into the life-threatening form known as steatohepatitis that can result in liver failure [66]. The pathology of this disease differs from most other metabolic illnesses due to the nature of its progression as described by the “multiple hit” hypothesis [67]. The hypothesis divides the progression of NAFLD into multiple parts: the first occurs in response to insulin resistance and triglyceride accumulation promoting elevated levels of oxidative stress, ER stress, and pro-inflammatory cytokine release. Recently, the flora and role of the gut microbiome have been identified as another factor that can affect the progression of NAFLD [67]. Adipose tissue can contribute to NAFLD through its actions as an endocrine organ and release pro-inflammatory cytokines such as IL-6 and TNF- α [67]. Overall, the diverse consequences of obesity on metabolic function and the gastrointestinal system represents a major health concern. Furthermore, substantial resources are required for proper care and treatment of gastrointestinal diseases increasing the economic strain on the healthcare system. Therefore, the need for solutions has never been higher and innovative approaches targeting lesser known aspects of obesity are warranted.

The Importance of Muscle and Adipose

Biologically, the human organism relies on an intricate network of organs and tissue to maintain the delicate balance required to promote and maintain homeostasis. In general, the muscle and adipose tissue play critical roles in either storing or utilizing the nutrients absorbed from carbohydrates, lipids and proteins. As previously mentioned, rising levels of glucose result in the pancreatic β cells secreting insulin that enters circulation where it can stimulate the uptake of glucose by insulin responsive tissue. The muscle and adipose tissue are critical to the clearance of accumulating and excessive glucose within circulatory system. In harmony, these tissues work together to maintain and promote metabolic homeostasis, keeping blood glucose levels from rising to dangerous and damaging levels. However, across the life span these essential processes can be compromised by the destructive effects of aging and circumstances such as obesity.

Impact of Caloric Restriction on Age-Related Diseases

As presented previously, CR could potentially target obesity, aging, epigenetics, mitochondrial dysfunction, inflammation and the overall metabolic function systematically. CR has been proven to increase longevity within many different scientific models [68]. Various complications arise when attempting to verify the effects and implement CR within humans and long-lived organisms [69-71]. Time, cost, and adherence issues remain the major hurdles to objectively analyzing CR in a clinical setting [71]. Observational studies such as the study conducted on Okinawans have proven the most effective in understanding the potential of CR within humans thus far [72]. CR can be used as a nutritional intervention for targeting not only obesity, but a wide array of systemic health issues. Recently, CALERIE 2 an RCT including 218 middle age men and women of healthy BMI, where the variable group was prescribed a 25% CR (while only achieving an 11.9% CR) over 24 months [20]. The CR group exhibited drastic weight loss at an average of 16.7 pounds compared to almost no change amongst the control. In addition, questionnaire response analyses of the same CR group revealed them as having self-reported decreased stress and anxiety, improved sexual function, health, mood, and sleep quality [73]. In addition, the CALERIE 2 findings demonstrated that while only a mere 11.9% CR was achieved there were still substantial decreases in fat mass and weight with a concomitant increase in fat free mass [20]. CR shows vast potential as a recommendation in elderly and aging populations for a myriad of reasons [74-77]. The effects of 30% CR relative to the previous diet over 3 months, has been investigated in fifty healthy elderly subjects at a mean age of 60.5 years and of normal to overweight status [74]. The findings demonstrated that CR led to enhanced verbal memory capacity and this directly correlated with a decrease in fasting levels of insulin in the plasma and increased C-reactive protein [74]. In a follow up study in 2016, the group investigated the neural correlates of CR [78]. They discovered that in response to weight loss from CR, their participants experienced improvements in recognition memory and cognitive function, along with decreased serum glucose concentrations. Furthermore, they identified that CR resulted in positively associated structural and functional alterations in brain activity [78]. CR has also been linked to potentially reversing autonomic function decline in elderly subjects [79]. In addition, CR has been correlated to weight loss and improved metabolic profile while simultaneously reducing chronic inflammation [75, 76].

Overall, the potential benefits of CR be may achieved primarily through creating a negative energy balance that promotes downstream changes in energy sensing pathways such as AMP/ATP and NAD⁺/NAD [80]. As previously mentioned, numerous systemic consequences and metabolic interactions often centralizing around the mTOR, AMPK, IGF-1 and sirtuin pathways have been identified [24-26]. Modification of these signaling pathways and their respective intracellular targets can have profound effects on the organism. Overall, the pathways impacted by CR play critical roles within adipose tissue. The following will explore the detrimental impact of aging on adipose tissue and its relationship with obesity.

A Double Edge Sword: Aging and Obesity

Adipose Tissue and Aging

Adipose tissue can consist of either white adipose tissue (WAT) or brown adipose tissue (BAT). However, the majority of human fat is present in the form of WAT and plays the critical role of mobilizing and storing energy supplies in the form of triacylglycerol. Additionally, adipose tissue is responsible for vital endocrine and paracrine functions, along with mediating inflammatory responses, insulin signaling, angiogenesis, appetite control and other metabolic processes [81, 82]. These critical functions have led to adipose tissue being identified as a dynamic organ. The physiological functions of WAT are carried out predominantly by adipocytes. The cellular maturation of adipocytes through adipogenesis, is dependent upon the accumulation of an internal fat droplet and displacement of surrounding cytoplasm and organelles [83]. The accumulation of mature adipocytes is ultimately what forms adipose tissue. In opposition to accumulating glycerol BAT predominantly releases energy through thermogenesis by way of mitochondrial uncoupling protein 1 (UCP1) [84]. This uncoupling response has made WAT to BAT conversion the target of many obesity related studies [85-87]. Most recently, researchers showed that the KO of regulator for G protein signaling 14 (RGS14) and subsequent increase of BAT, showed an increased lifespan in mice [88]. Inversely, it has been shown in rats that the effectiveness of thermogenesis [89] and the overall functionality of BAT [90] could decrease with age.

WAT plays an essential part in preserving energetic homeostasis through the storage of TAG's and esterification and release of fatty acids. WAT also plays critical roles in endocrine function and immune response. WAT accumulation and distribution change in response to the aging process. Markedly increased visceral WAT depots and altered WAT distribution [91, 92]

along with an overall increase in total WAT mass throughout middle age. The location, ratio and type of fat reservoir often dictates the biological impact and cellular functions [93]. Visceral WAT accumulation has been related to impaired metabolic function, insulin resistance, increased risk for CVD, increased inflammatory response and the development of T2DM [94, 95]. Conversely, subcutaneous WAT could be beneficial and has been shown to prevent visceral fat and ectopic lipid accumulation [96]. TAG accumulation in non-fatty tissues (such as pancreas, liver and muscle), is known as ectopic lipid accumulation. Furthermore, this accumulation can lead to many undesired systemic metabolic effects and has been shown to increase throughout the aging process [97-99]. Potentially impeding normal lipid distribution, subcutaneous WAT storage and expansion has been shown to potentially decrease with age in rats [100, 101]. The ability of WAT to differentiate and accumulate is predominantly dependent on preadipocytes, stem cells, and adipocytes [102].

The expansion and accumulation of adipose tissue occurs in response to cellular differentiation processes where naïve pre-adipocytes mature and accumulate lipids. Known as adipogenesis, the cellular progression and maturation of adipocytes is critical for maintaining homeostasis through acting as a reservoir for excessive glucose and lipids within the blood stream. Adipogenesis relies on intricate and interwoven signaling that is heavily influenced by nutritional and hormonal factors. Mechanistically, pre-adipocyte cell cycle regulation, reentry and mitotic clonal expansion have been identified as key intracellular components of adipocyte differentiation [103, 104]. In origin, preadipocytes are thought to be produced by pluripotent stem cells and progenitors in the surrounding vascular endothelium [105, 106]. The process of preadipocyte differentiation is regulated by peroxisome proliferator-activated receptor gamma (PPAR γ) [107]. CCAAT/enhancer-binding proteins (C/EBP) are responsible for mediating the expression of PPAR γ , and together they regulate adipocyte differentiation and lipid accumulation [108, 109]. Throughout the lifespan, it has been shown that preadipocyte functioning can be altered in response to aging [110-112]. Aging decreases adipogenesis through modulation of PPAR γ and C/EBP α expression [113, 114]. C/EBP homologous protein (Chop) and C/EBP β liver-inhibitory protein (C/EBP β -LIP) have been identified in the dysregulation of adipogenesis [112, 115] and their expression was shown to increase with age [116, 117]. The overall inflammatory response that occurs in response to endoplasmic reticulum stress (ER) has also been linked to an increase in CHOP [118]. These events are often interconnected and could lead to the detrimental consequence

of deteriorating preadipocyte function in response to age [119, 120]. Additionally, both inflammation and ER stress may lead to ectopic lipid accumulation and disruption in metabolic homeostasis that eventually damages organs and tissues thus increasing overall risk of mortality [121].

Once mature, adipocytes can become hypertrophic, hyperplastic or exhibit a combination of the two states. *In vivo* experimental models have shown that as adipocytes accumulate lipids they begin to reach maximum capacity and this could initiate adipogenesis and lead to hyperplasia [122, 123]. However, it has been reported that the adipocyte number remains approximately the same during the course of adulthood in both obese and lean individuals [124]. Moreover, weight loss and obesity may not change the baseline amount of adipocytes significantly in adulthood [124]. Hypertrophy is the enlargement of the adipocyte and can lead to increased fatty acid release, decreased adiponectin, increased pro-inflammatory cytokines, ectopic lipid accumulation, increased insulin resistance and increased immune cell recruitment [125-129]. These complex interrelated events can contribute to the overall inflammatory event that often occurs in response to obesity and aging. The mechanisms through which CR might mitigate the decline in adipose tissue function will now be explored.

Caloric Restriction; Aging and Adipogenesis

Many of the pathways effected by CR play important roles regarding adipose tissue function and development. One metabolite, SIRT1 may be capable of protecting against age-related metabolic diseases, as evidenced within animal studies [130]. Within adipose tissue, SIRT1 appears to be upregulated in response to caloric restriction [131]. Adipose tissue specific investigations regarding SIRT1 have demonstrated its capacity to increase insulin sensitivity and lipolysis while decreasing adipocyte lipid accumulation [132]. Furthermore, adipose tissue might increase adiponectin synthesis and fatty acid mobilization in response to SIRT1 activation [133]. Picard F et al., identified that starvation resulted in SIRT1 binding to PPAR- γ -DNA cofactor docking sites and subsequent reduction in fat storage and concomitant increase in FFA release from murine WAT [133]. Conversely, *ad libitum* feeding did not initiate SIRT1 to PPAR γ binding within the mice [133]. Additionally, it has been revealed that calorie restriction could enhance SIRT 1 activity through reducing the level of biotin in adipose tissue [134]. Specifically, mice overexpressing either wild type SIRT1 (Adipo-SIRT1) or decreased SIRT1 (Adipo-H363Y) in adipose tissue were either fed *ad libitum* or exposed to a 60% CR accompanied by biotin

supplementation [134]. The 60% CR improved the metabolic profile of the mice and reduced histone biotinylation, a mechanism potentially mediated through SIRT1 activation [134]. Collectively, these results support the importance of the SIRT1 pathway in relation to CR and adipaging. Beyond SIRT1, other potential avenues and signaling pathways through which CR may impact adipose tissue have been explored.

For instance, Dogan et al., utilized a novel study design where mice were exposed to either chronic calorie restriction (CCR) (75% calorie restriction), intermittent calorie restriction (ICR) (50% restriction 3 week time frame), or control [135]. CCR at 73/74 weeks revealed that AMPK activation in the mammary fat pads was significantly increased, while decreasing the number of mammary tumors [135]. The CCR fed animals exhibited increased mTOR, p70S6K, and HIF-1 within the mammary fat pads [135]. These elevated signaling responses occurred in direct relation to increased age and or the duration of the caloric restriction [135]. The findings also supported that caloric restriction could interfere with mammary tumor development through its interaction with the IGF-1 and mTOR signaling pathways [135]. However, the findings contribute to the understanding of how aging, caloric restriction, fat tissue and cancer could be related. Furthermore, the benefits of CR could directly relate to the cascade of events that occur in response to reduced fat mass and the subsequent impact on the inflammatory pathways. Collectively, the high level of CR utilized in these investigations could impede translation into practical application within clinical settings. However, furthering our understanding of how CR interacts with fat metabolism and their inflammatory pathways will provide a clearer picture of how CR can benefit humans.

Caloric Restriction and Adipogenesis (White and Brown)

The effects of CR on adipogenesis and the conversion of WAT into BAT have recently been explored. In a recent study, researchers exposed C57BL/6J (WT) mice to either 40% CR or ad libitum (AL) conditions [136]. They discovered that CR could have promoted browning of WAT in the perigonadal visceral adipose tissue (pgVAT) and inguinal subcutaneous adipose tissue (ingSAT). Both pgVAT and ingSAT exhibited increased adipocyte density, with less large adipocytes and increased Ucp-1 expression in comparison to the AL control [136]. Supportively, Ucp-1 expression was increased by 150-fold in the CR mice in comparison to only a 60-fold increase in the AL [136]. Furthermore, Seahorse analysis and Clark electrode of the CR and AL adipocytes revealed that the oxygen consumption rate (OCR) and glycerol release were

significantly elevated in the adipose of CR mice [136]. Furthermore, CR mice exposed to cold exposure maintained their body temperature, inversely the AL mice experienced a decrease in body temperature [136]. These findings show strong support for the potential of CR to promote browning in adipose tissue.

Caloric restriction and inflammation of adipose tissue

Adipose tissue plays a complex role as an endocrine organ capable of promoting the generation of various inflammatory factors. Left uncontrolled, inflammation can lead to systemic metabolic consequences in obese organisms [137]. Therefore, reducing adiposity through CR could in theory, be effective at reducing inflammation. Demonstrating support of this idea, murine investigations have targeted the relationship between CR, inflammation and adipose tissue [138, 139]. Park et al., explored this association in vivo, where C57BL/6 N mice were broken into three groups CR (15%), control (AL) and HFD [138]. The findings showed that in comparison to HFD, a mere 15% CR resulted in a substantial reduction in markers of inflammation IL-1 β , M α p-1, IL-6, and Tnf- α , within the epididymal adipose tissue [138]. They also identified that the CR group displayed elevated levels of adiponectin and postulated that this increase may have been responsible for the suppression of Tnf- α and IL-6. The fact that these findings were shown in response to a 15% CR strengthens the results and promotes the potential for translation. In a previously mentioned study by Nuñez et al., evidence was identified that supports these findings in humans and mice [139]. Serum analysis revealed that CR and subsequent BMI reduction initiated by Roux-en-Y gastric bypass surgery (RYGB) resulted in significantly elevated levels of Tnf- α and IL-1 β in circulation [139]. Furthermore, another clinical trial reported similar findings in obese females with and without T2DM [140]. As evidenced, both obese groups displayed elevated Tnf- α , IL-6, IL-8, and inflammatory chemokines in the serum [140]. In addition, two week exposure to very low-calorie diet (2500 kJ/d) resulted in an improved metabolic profile and decreased expression of chemokines [140]. Collectively, the findings promote CR as an effective approach to reducing adiposity and lowering the inflammatory response. However, the previously mentioned barriers impeding the therapeutic application of CR such as lack of desirability and intolerance, warrant continued efforts to uncover alternative approaches. Beyond adipose tissue, skeletal muscle is vital for maintaining metabolic homeostasis and similar to adipose tissue can functionally decline with age. In the subsequent section, the biological and metabolic roles of skeletal muscle will be highlighted.

Muscle

Intro

The various movements, contractions, and motions required in exercise, strength training, and daily activities depend on eccentric action of skeletal muscle. The highly regenerative skeletal muscle stem cells, known as (satellite cells), have the uncanny ability to quickly and effectively restore, repair and maintain skeletal muscle. Under normal conditions, activated satellite cells proliferate and fuse into myotubes allowing for muscle fiber regeneration, a process commonly referred to as myogenesis. Problematically, the rate and availability of satellite cell's for myogenic differentiation can decrease with age, in response to accumulating cellular and metabolic stress from internal and external stimuli [141, 142]. Investigations aimed at improving skeletal muscle cell differentiation have revealed encouraging findings. For instance, the active form of vitamin D (1,25-D3) [143], l-glutamine [144], and other compounds such as dehydrocorydaline (naturally derived from *Corydalis* tuber) [145], and IBS004735 [146], have all been shown to promote and improve varying aspects of myogenic differentiation in C2C12 mouse skeletal muscle myoblasts. Moreover, transforming growth factor- β (TGF- β) family member myostatin has been shown to promote loss of skeletal muscle mass. Intriguingly, antibody blockade of myostatin has demonstrated potential to increase muscle mass and anabolic activity in murine and non-human primate models [147]. However, research efforts towards uncovering the in vivo effects of natural and synthetic compounds on improving skeletal muscle differentiation have been rather limited.

In the postprandial state, skeletal muscle plays a predominant role in the uptake, utilization, storage and clearance of glucose through oxidative (glycolytic) and non-oxidative (glycogenesis) pathways. The pathological manifestation of insulin resistance is often initiated by reduced skeletal muscle mass and functionality [148], [149]. Under normal conditions, insulin binds and initiates the autophosphorylation of the transmembrane tyrosine kinase receptor and subsequent activation of the receptor's kinase domain. Resultantly, Insulin Receptor Substrates 1 and 2 (IRS1, IRS2) are phosphorylated leading to the activation of signaling cascades that promote glycolysis, glycogen synthesis, protein synthesis, skeletal muscle cell growth and differentiation. Mechanistically, glucose uptake is initiated independently by AMPK and the IRS-1/Phosphatidylinositol-3 kinase/Akt insulin signaling pathway and initiates the formation and translocation of glucose transporter 4 (GLUT4) to the surface of the cell membrane [150]. GLUT4 is the primary glucose

transporter, and defective GLUT4 can promote insulin resistance in individuals with type 2 diabetes and impair glycogen synthesis in those with familial history of type 2 diabetes [151]. Insulin signaling pathway inactivation is attributed to fatty acid accumulation within skeletal muscle. Elevated levels of fatty acids within the skeletal muscle can result from excessive consumption of high-fat, high-carbohydrate food, which overwhelms the capacity of adipocytes to properly store triglycerides [152]. Furthermore, excessive fatty acids can burden skeletal muscle mitochondria leading to oxidative stress and subsequent ROS accumulation [153]. Problematically, ROS can damage the mitochondria generating defects in fatty acid oxidation giving rise to toxic lipid metabolites [153]. These intermediate metabolites such as ceramides, acyl CoA, and diacylglycerol are responsible for activation of protein kinase C (PKC), which interrupts tyrosine phosphorylation of IRS-1 by the insulin tyrosine kinase receptor and suppresses IRS-1 activity [154]. Considering these deleterious and systemic effects, investigating novel approaches to improve the insulin responsiveness of skeletal muscle could result in new and improved clinical approaches to MetS. Furthermore, sarcopenia and skeletal muscle loss in response to disease and malnutrition can impede the tissue's functionality. Therefore, efforts to understand and enhance skeletal muscle differentiation and myogenesis have been ongoing [155].

Muscle Recovery, Aging and CR

Repeated or sustained eccentric muscle actions can result in an accumulation of ROS, inflammation and ultimately muscle tissue damage [156]. Novel strategies to effectively reduce recovery times and pain from eccentric muscle damage and muscle weakness could have significant implications for athletes, and aging populations. Compounding matters, sarcopenia from normal aging processes can diminish insulin signaling and sensitivity in skeletal muscle [157]. As thoroughly evidenced, declining mitochondrial function in aging organisms may play a role in the progression and onset of sarcopenia [158-160]. While the findings vary to some degree, CR has proven capable of improving mitochondrial function and attenuating the deleterious effects of sarcopenia, in rodents [161]. For instance, Lanza et al., identified that within the skeletal muscle of mice, chronic CR promoted antioxidant defense mechanisms and reduced the emission of mitochondrial oxidants [162]. Their findings showed that CR did not effectively promote mitochondrial biogenesis or prevent age related reductions in mitochondrial abundance in murine skeletal muscle. However, CR did preserve the functional integrity of aging mitochondria through enhanced conservation of cellular components [162]. Beyond mitochondria, diminished AMPK

activity with age may play an integral role in the development and progression of sarcopenia [163, 164]. The role of AMPK will be discussed thoroughly in a latter section.

While CR shows therapeutic promise against age-related decline of skeletal muscle, it has been shown that it can lead to reductions in lean body mass (LBM) in elderly subjects [165]. While loss of LBM poses a substantial hurdle to therapeutic application of CR, a solution to this problem has been proposed. Researchers demonstrated that incorporating resistance training with approximately 22% CR led to a near complete halt of LBM loss in obese elderly. They also revealed that CR combined with resistance training led to reductions in fat body mass and total body mass [77]. However, the relative risk between BMI and age is not clear and excessive weight loss has been associated with worsened clinical outcomes [166, 167]. Moreover, CR can lead to bone deterioration and increased incidence of fractures in aging populations [168]. Importantly, it is a well-known fact that resistance training can reduce the risk for age-related bone fractures. Therefore, it seems important to incorporate resistance training with any level of proposed CR nutritional intervention. In general, these findings suggest that the benefit of CR as a nutritional intervention is recommendable for some elderly individuals. However, it seems integral that CR be accompanied by a steady resistance training regimen to avoid detrimental effects and preserve LBM. Furthermore, careful consideration must be made when implementing CR in the elderly and all aspects of nutritional well-being should be accounted for.

Overall, the benefits of CR appear substantial and warrant continued efforts, particularly in obesity and age-related research. While the benefits of CR appear significant, adhering to CR can be undesirable for many. The difficulties faced when implementing CR in clinical settings, has generated vast pursuit and interest in potential compounds both natural and synthetic capable of mimicking the benefits of CR.

CR Mimics

Intro

CR mimics both natural and pharmaceutical have been heavily investigated and targeted for their impact on adipose, muscle tissue and beyond. However, translation of findings from the various experimental models (worms, mice, yeast, etc.) into human application can be challenging [68, 69]. Progressive studies have continued to characterize and isolate the specific mechanisms of CR. As a result, the diversified nature and untapped potential of CR mimics is becoming more

apparent. Novel ideas and innovative perspectives may be the keys to uncovering the secrets to CR and its impact on longevity and metabolic function. In the following some of the most recent research and newest developments in CR mimics will be reviewed.

CR Mimics Targeting Adipose

The polyphenolic compound Resveratrol (RS) has been one of the most heavily researched CR mimics. One angle of RS research has targeted the effects of RS on adipose tissue [169, 170]. Gómez-Zorita et al., identified that RS could act as a CR mimic through its ability to decrease plasma cytokine levels, mediate lipolysis and fatty acid uptake, and potentially inhibit the NF- κ B pathway in Zucker (fa/fa) rats [170]. The findings showed a reduction in BW in the RS group accompanied by a reduction in Tnf- α , MCP-1, and IL-6 biomarkers in the adipose tissue [170]. They also showed that RS could protect against adipose tissue macrophage infiltration while also inhibiting the NF- κ B pathway. In another double-blind crossover study, human subjects were exposed to a 30 day placebo treatment followed by 4 week washout and then 30 days of 150mg of resVida (trans-resveratrol) per day [171]. SQ adipose tissue was then collected for biopsies and analysis [169]. They identified a reduction in abdominal SQ adipocyte size in response to RS treatment [169]. They also conducted a gene expression analysis and saw a potential increase in adipogenesis and altered autophagy response [169]. Another study with a larger sample size could provide more concrete verification of these findings. Beyond RS, other CR mimics such as rapamycin and SRT1720 have been investigated for their effects on adipose tissue. While these findings are promising, CR mimicking compounds have also been shown to potentially impact WAT browning.

CR Mimics and WAT to BAT Conversion

The PI3-kinase/AKT/TOR signaling pathway has been associated with the regulation of lipid biosynthesis [172]. mTORC1 is a potential regulator of adiposity and lipid synthesis and has recently begun to draw attention [173]. It has previously been reported that obese mice exhibit increased mTORC1 in adipose tissue and that S6K1 deficiency could prevent diet induced obesity while also promoting insulin sensitivity [174]. Interestingly, mTORC1 inhibition through rapamycin (CR mimic) targeting has been shown to potentially increase adipose tissue UCP-1 content in raptor^{ad-/-} mice [175]. Additionally, mTORC1 inhibition resulted in reduced adipocyte lipid accumulation attributed to decreased PPAR γ and lipoprotein lipase (LPL) activity in Sprague-Dawley rats [176]. Most recently, Magdalon et al., generated tuberous sclerosis complex (Tsc)1

KO mice and showed that this KO led to increased mTORC1 activation in adipocytes [177]. The Tsc1 deletion and subsequent mTORC1 activation could have also promoted visceral WAT browning through the regulation of $ERR\alpha$ and potentially increased UCP-1 expression [177, 178]. The findings also suggested that mTORC1 activation could potentially reduce adiposity [177]. While the findings in adipose are significant, CR mimics may also have the capacity to improve various aspects of muscle function and development.

CR Mimics Targeting Muscle

As previously discussed, enhancing skeletal muscle function, differentiation and growth, can potentially promote metabolic homeostasis. Similar to the findings in adipose, CR mimics such as artemisinin [179], RS [180], metformin [181] and others [182], may be capable of preferentially augmenting the molecular nature of skeletal muscle. Wang et al., showed that L-arginine, sodium nitroprusside, L-arginine and artemisinin, compounds all capable of generating nitric oxide, acted as CR mimics and proved capable of initiating ROS scavenging antioxidant networks when injected into murine skeletal muscle. Individually, CR and all three compounds effectively up-regulated mitochondrial superoxide dismutase (Mn-SOD), glutathione (GSH), and catalase (CAT) within the skeletal muscle cells. Furthermore, the CR mimics also increased the expression of protein kinases p-AMPK and p-Akt accompanied by increased phosphorylation of eNOS. Finally, they showed that the CR mimics might enhance mitochondrial biogenesis through increasing the level of skeletal muscle ATP and nitric oxide. In another clinical study, the CR mimetic metformin was investigated for its therapeutic potential against type 2 diabetes. Analysis of skeletal muscle biopsies revealed that metformin significantly increased AMPK $\alpha 2$ activity, a finding that was correlated with increase glycogen levels and enhanced glucose clearance [181]. This increase in AMPK $\alpha 2$ was associated with reduced acetyl CoA-carboxylase-2 activity and increased phosphorylation of AMPK at Thr172. Mechanistically, AMPK $\alpha 2$ is an isoform and catalytic subunit of AMPK which is expressed in the muscle [181]. These clinical results confirmed their previous in vitro findings where metformin upregulated AMPK activity and promoted glucose uptake in rat muscle cells [183]. Within another study, a novel micronutrient blend proved extended the lifespan and promoting vitality of *C. elegans*. Moreover, this same blend elicited transcriptomic alterations in the skeletal muscle of mice similar to the changes invoked by CR. Regarding gene expression changes, RS has been shown to at least partially mimic

the transcriptomic effects of CR in skeletal muscle [180]. As evidenced, CR mimics appear capable of at least partially emulating the valuable effects of CR on skeletal muscle through increasing antioxidant activity, protein kinase activity, mitochondrial biogenesis, and transcriptomic regulation. However, the impact of CR mimetics on skeletal muscle remains relatively limited.

CR Mimics Targeting Obesity and its Co-Morbidities

While demonstrating varying degrees of success, many of the current clinical and pharmacological approaches to obesity fail to produce long term results and can lead to significant side effects [184, 185]. Recent advancements have explored new potential for nutrition related CR mimics targeting the detrimental side effects of obesity and aging. Nonalcoholic fatty liver disease (NAFLD) shows a direct relation to impaired metabolic function and obesity [186]. NAFLD could also occur in direct relation to WAT dysfunction and prolonged ectopic lipid accumulation. Pharmacological treatment for NAFLD is currently ineffective and antioxidants have emerged as a potential treatment option due to oxidative stress being a critical factor to the development of NAFLD [186, 187]. Moreover, a recent human RCT focused on the effect of RS found intriguing results [186]. The double blind, placebo trial found that daily RS supplementation was able to reduce inflammatory markers and hepatocyte cell death [186]. The results also identified that RS could be more effective than lifestyle modifications as an intervention strategy [186].

SRT1720 has been recognized as a potential SIRT1 activator, a protein capable of modifying metabolic outcomes that has proven effective against diabetes in animal models [188]. The effect of SRT1720 on glucosamine induced ER stress has also been targeted [189]. The findings identified that SRT1720 decreased the level of ER stress significantly. Furthermore, the study identified SRT1720 in a potential novel mechanism where it acted as a deacetylase targeting mTORC1. This interaction with the mTOR pathway could be integral to the unfolded protein response, particularly under stressful conditions [189]. In addition, the impact of SRT1720 on preadipocytes has recently been investigated. The findings demonstrated that SRT1720 induced SIRT1 activation and led to increased expression of angiogenic genes Vegfa, and Fgf2, in 3T3-L1 preadipocytes in both ex-vivo and in-vitro models [190]. Furthermore, their findings demonstrated that the increase in angiogenic genes resulted in decreased fibrosis and inflammation leading to increased insulin sensitivity in WAT. Impaired vascularization is a serious problem in obese adipose tissue and improving this can have diverse beneficial metabolic outcomes, including improved glucose homeostasis [190, 191]. In addition, their findings show that SRT1720 can

significantly reduce the expression of genes important to inflammation including TNF- α , MCP-1, and IL-6 [190]. However, this study used a very high dose at (100 mg/kg/day) that would likely lack human translation. The research on this experimental pharmaceutical has been limited but appears to possess potential as a CR mimetic. Please see Figure 2, for a theoretical model of how CR mimetics might be combined to synergistically target adipose tissue function and metabolism. While mimicking CR has shown promise, clinical verification can prove problematic as CR impacts the tissues and organs of the human organism in a systemic and intricate fashion. However, natural approaches to mimicking CR centralizing around herbal remedies and their constituent phytochemicals continue to find growing support for their array of CR like benefits. Relying on a combination of 10 distinctive herbs, Zyflamend has shown promise as an alternative to conventional medicine. The following will explore the potential for the individual and combined ingredients within Zyflamend as CR mimics.

Zyflamend

Introduction

New Chapter, Incorporated, first promoted Zyflamend with the fundamental theoretical concept that it could act as a natural anti-inflammatory supplement. Regarding Zyflamend's anti-inflammatory properties, numerous investigations across various models have found substantial evidence supporting this claim. Initially, the research centralized around the effectiveness of Zyflamend as a Cox inhibitor. Since then, this theory has proven true as research shows that Zyflamend acts as a Cox inhibitor in models such as: melanoma [192], prostate [193], and oral cancer models [194]. Beyond its effects on COX, Zyflamend has been shown to modify many other cellular pathways. The mechanistic and signaling interactions of Zyflamend include: AMPK [195, 196], NF- κ B [197-199], mTORC1 [196], apoptosis, cell growth [193, 200], ER stress [198, 201, 202] and autophagy [192]. Taken together, Zyflamend appears to possess significant potential against a wide variety of diseases and metabolic disturbances. Furthermore, a large tome of literature has suggested that the individual components of Zyflamend might exert CR like benefits.

Recipe

As a combination supplement, Zyflamend (New Chapter, Inc., Brattleboro, VT) makes use of 10 herbal extracts including the following in a two capsule once-daily dose; turmeric (110 mg), rosemary (150 mg), holy basil (100 mg), ginger (100 mg), organic green tea (100 mg), hu zhang

(80 mg), organic oregano (40 mg), barberry (40 mg), chinese goldthread (40 mg), and Chinese skullcap (20 mg). Zyflamend has been highly regarded as an anti-inflammatory agent. In comparison to individual herbal supplementation, the poly-herbal encapsulation within Zyflamend revolves around the idea of generating an additive or a synergistic response promoting superior benefits [203]. One central avenue of Zyflamend research has related to its potential against various forms of cancer, a topic that will be covered briefly within the following. [203].

Zyflamends as an Agent Against Cancer

As thoroughly evidenced, many of the individual ingredients within Zyflamend possess a wide degree of anti-tumorigenic capabilities. Representing a significant obstacle, the excessive doses utilized within these studies are in many cases, unrealistic options for most patients [204-206]. The combination supplement of 10 herbal extracts, has been implicated for its therapeutic potential in a plethora of cancer research platforms [192-194, 197-199, 207]. A growing body of literature indicate that Zyflamend exhibits anti-survival and pro-apoptotic effects on several cancer models [198, 200, 208-210]. Multiple pathways have been proposed through which Zyflamed could exert its effect including, reduced NF- κ B signaling in bone cancer [198], decreased cyclooxygenase Cox-2 in melanoma [192] and arachidonic pathway inhibition through targeting 12-Lox and 5-Lox in prostate cancer [207]. In addition, human equivalent dosing of Zyflamend in a xenograft prostate cancer cell model, resulted in decreased *in vivo* tumor growth [211]. In addition, Zyflamend treatment resulted in the decreased expression of histone deacetylases, protein kinase B (Pkb) phospho-Akt, prostate specific antigen and androgen receptor, all biomarkers associated with prostate cancer [211]. Furthermore, Zyflamend has proven to decrease the overall viability of various prostate cancer cell lines [193, 207, 212]. Beyond prostate cancer, Zyflamend demonstrates anti-tumor potential in other areas of cancer research targeting the skin, mouth, bone, kidney, colon and pancreas [198, 200, 208, 209]. Within our lab, we showed that Zylfamend was capable of dose-dependently initiating apoptosis, G2/M phase cell cycle arrest, and subsequent reductions in beta-TC-6 pancreatic insulinoma cell (β -TC6) proliferation [213]. Mechanistically, Zyflamend activated the c-Jun N-terminal kinase (JNK) pathway, accompanied by significant induction of autophagy, and ER stress. Beyond cancer, a large plethora of clinical and experimental research have found evidence supporting the individual components of Zyflamend as potential CR mimetics.

The Potential of Zyflamends Individual Components as CR Mimetics

Promoting the allure of Zyflamend as a CR mimetic candidate, all ten of its unique components proven to possess diversified potential against various age and obesity related diseases and conditions. For instance, most of Zyflamends ingredients including, turmeric polyphenol, curcumin [214], ginger [215], rosemary [216], green tea polyphenol, Epigallocatechin-3-gallate (EGCG) [217], holy basil [218], barberry [219], and Chinese gold thread [220] have all been shown to individually impact varying aspects of glucose homeostasis and/or insulin resistance. Beyond glucose homeostasis, chronic inflammation is another critical factor in the pathological progression and onset of most diseases. Conceptually, a sustained mild inflammatory response across the lifespan can have systemic consequences that left uncorrected can aggravate and promote the development of numerous diseases [221]. However, in many cases, CR and CR mimetics such as RS, metformin, and rapamycin have proven capable of reversing the negative consequences associated with age-related inflammation [221]. Similarly, the compounds within the Zyflamend blend have been shown to possess diverse anti-inflammatory properties such as ginger [222], and curcumin [223]. The intricate nature through which these various herbs and their constituents can beneficially impact metabolic function, appears limitless. Therefore, combining these compounds could theoretically synergistically amplify or promote these benefits providing a highly promising approach to metabolic syndrome and other obesity related diseases. With consideration for the ingredients both alone and in combination, the following sections will focus on the effects of Zyflamend on muscle and adipose tissue, with particular regard for AMPK.

Modulation of AMPK Activity and the Subsequent Effects on Metabolism

As previously mentioned, AMPK is a critical intracellular pathway impacted by CR and CR mimetics. Acting as a primary energy sensor within the cell, the AMPK pathway is activated when intracellular ATP levels are low and inactivated by increased ATP generated by catabolic metabolism. Moreover, AMPK activation has been shown to reduce insulin resistance in the adipose tissue and skeletal muscle [150, 224]. The impact of AMPK on insulin signaling occurs in response to the inhibition and activation of numerous intracellular cascades. Directly, AMPK modulates signal transductions capable of promoting the translocation of GLUT4. Furthermore, AMPK plays a fundamental role in alleviating oxidative stress and indirectly upregulating DNA repair mechanisms that recuperate and maintain mitochondrial function [225]. Following strenuous exercise or activity, AMPK responds to decreased intracellular energy stores and

stimulates glucose uptake for replenishment [226]. AMPK is a primary mediator of phytochemical induced insulin sensitivity. AMPK is phosphorylated and activated as a result of phytochemical treatment and is responsible for increased glucose uptake by stimulating translocation of GLUT4 or other insulin-independent glucose transport pathways [227]. In addition to increased glucose uptake, AMPK also reduces insulin resistance by improving mitochondrial function and discouraging the storage of triglycerides in skeletal muscle [227]. Phytochemical stimulation of the AMPK pathway can lead to improved insulin sensitivity and helps to reduce mitochondrial defects related to the development of insulin resistance in skeletal muscle [227]. It has been well described that Zyflamend's effectiveness is in large part due to the AMPK signaling cascade. Through activating AMPK, Zyflamend is capable of synergistically triggering a dose-dependent anti-lipogenic response in prostate cancer cells [195]. Furthermore, studies have shown that Zyflamend effectively activates AMPK in several cell lines [195] and within a murine model mimicking U.S. diet-induced metabolic disorder [228]. As first evidenced by MacDonald et al., Zyflamend appears to regulate this pathway through phosphorylation of PKC ζ , and subsequent phosphorylation and activation of Liver Kinase 1 (LKB1)[195]. Once Activated, LKB1 phosphorylates AMPK, initiating a signaling cascade that promotes the inhibition of anabolic metabolism and stimulation of catabolic pathways. Considering the diverse regulatory functions of AMPK, Zyflamend appears to possess significant promise as a CR mimetic. In order to better understand this potential, the impact of Zyflamend and its individual components on adipose and skeletal muscle function, will be explored further.

Zyflamend and Adipose

As previously discussed, prolonged high calorie consumption can promote dysregulated expansion of the adipose reservoir and ultimately, obesity [229]. Encouragingly, a wide variety of dietary factors, herbal extracts and botanical compounds have shown significant anti-obesogenic potential [230]. Regarding the compounds contained within Zyflamend, curcumin [231, 232], EGCg and green tea [233-237], berberine from Chinese goldthread [238-240] and *Rosemary officinalis* and carnosic acid [241, 242] have all been investigated for their diversified anti-adipogenic effects. As previously discussed, PPAR γ and C/EBP α act as key regulators of adipogenesis primarily through their impact on lipolysis and pre-adipocyte differentiation. Within both in vitro and in vivo models, components of Zyflamend such as curcumin [232], EGCg [236] and berberine [239] have all been shown to significantly downregulate both PPAR γ and C/EBP α .

Specifically, both curcumin [232], and berberine [239] effectively reduce the expression of PPAR γ and C/EBP α in 3T3-L1 adipocytes. Moreover, ECGg [236] and berberine [240], significantly lowered the expression of PPAR γ , a finding accompanied by reduce adiposity and improved metabolic profile in HFD fed mice, respectively. Beyond this, the individual components of Zyflamend have been shown to impact genes involved in the cell cycle [242], WNT/ β -catenin pathway [234], thermogenesis and beta oxidation [236]. Furthermore, ECGg treatment has been shown to upregulate uncoupling protein 2 (UCP2) expression in 3T3-L1 cells [235] and in HFD fed mice [236]. UCP2 is located within the inner membrane of mitochondria and responds to and protects against the accumulation of ROS [243]. Overall, the diverse nature through which the Zyflamend's ingredients impact adipose tissue and cells, appears substantial. Furthermore, many of the findings support the potential of Zyflamend as a novel CR mimetic. In order to further confirm this, past, present and future research regarding Zyflamend will have to be carefully considered and contrived. In recent times, the role of Zyflamend in adipose has been investigated in murine and cellular models.

Tague et al., demonstrated that Zyflamend incorporation into a specialized U.S. diet led to reduced adiposity in a murine investigation. Within their model, the U.S. diet fed to the animals was allometrically scaled and comprised to simulate the average nutritional makeup of the Westernized diet with or without Zyflamend. Intriguingly, their findings demonstrated that Zyflamend treatment resulted in reduced adiposity attributed to its capacity to regulate lipogenesis. As identified, the regulatory functions mediated by Zyflamend were likely potentiated through AMPK activation and acetyl CoA-carboxylase (ACC) inhibition within the adipose tissue [228]. Based on these findings, our lab investigated the cellular mechanisms through which Zyflamend might disrupt adipogenesis and lipolysis [244]. Under normal conditions, adipogenesis plays an essential biological role that allows for the expansion of adipose tissue and allows for long term storage of energy. We discovered, that within 3T3-MBX pre-adipocytes, Zyflamend treatment reduced proliferation, disrupted cell cycle progression and promoted apoptosis [244]. In differentiating cells, Zyflamend effectively increased lipolysis, evidenced by reduced lipid accumulation and concomitant reductions in lipogenic gene expression. Cell signaling investigations revealed that Zyflamends capacity to disrupt adipogenic differentiation was potentiated through activation of c-jun N-terminal kinase (JNK) and Protein kinase A (PKA) and was partially mediated by AMPK [244]. In fully differentiated adipocytes, Zyflamend treatment

dose dependently reduced the phosphorylation of hormone-sensitive lipase (HSL), providing a potential explanation for the increased lipolysis.

Zyflamend and Muscle Individual Extracts and Potential

Intriguingly, phytochemicals from dietary sources such as ginger [245], EGCg [246] and pomegranate juice [247] have been shown to exert potential ergogenic effects on muscle. In further support of phytochemicals, investigations targeting their effects on aspects of muscle insulin signaling and responsiveness have revealed promising findings [248]. Functionally, the mechanisms through which phytochemicals may improve skeletal muscle insulin sensitivity and function, could relate to their effects on signaling pathways involving insulin receptors (IR), the IRS family, PI3K, AKT and AMPK [248]. Within skeletal muscle, insulin-stimulated glucose transport by GLUT4 accounts for a majority of glucose uptake. Therefore, increasing the level of GLUT4 is an attractive therapeutic target for overcoming insulin resistance in muscle [248]. As presented by Sayem et al., incorporating various dietary phytochemicals, may to varying degrees, increase GLUT4 activity and alleviate insulin resistance in skeletal muscle [248].

Regarding the individual components and polyphenolic extracts of Zyflamend such as, curcumin [214], ginger, *Zingiber officinale* [249], and rosemary, [250], have all been independently investigated for their impact on insulin signaling and/or glucose uptake within skeletal muscle. Mechanistically, the improved metabolic capacity of skeletal muscle could be explained by an increase in the phosphorylation and activation of AMPK in L6 skeletal muscle cells, as demonstrated by all three autonomous studies. Furthermore, curcumin [214], and *Zingiber officinale* [249], treatment resulted in significant improvements to insulin resistance in two separate rat models emulating diabetes and MetS, respectively. While phytochemical targeting of the various components of insulin signaling appears integral to the potential of these bioactive compounds, their effects on AMPK signaling further promotes the extent by which these compounds might improve muscle function. While these findings show promise, Zyflamend's role in skeletal muscle has yet to be investigated. Displaying a high degree of potential crossover with CR, targeting Zyflamend's impact on AMPK within skeletal muscle appears to be a valid and intriguing target.

Conclusion

Understanding the downstream process through which obesity and aging result in impaired metabolism and eventual death, remains integral to unlocking the secrets to longevity. The quest for extending the healthy life span seems to encircle extending our understanding of the metabolic processes. Through further experimentation and better understanding of CR it seems likely that better treatment options will follow. In the future, it will be integral to isolate the roles and interactions of CR and the systemic metabolism. Moreover, identifying the specific mechanisms of interaction between obesity, metabolic syndrome, and mitochondrial dysfunction will be crucial to better understanding the metabolism. The development of better CR mimics and nutritional intervention strategies will rely on the innovation of future research. Based on the diverse impact and high degree of crossover with the effects of CR, Zyflamend appears to possess substantiated promise as a CR mimetic. However, further efforts to uncover its tissue specific effects may help clarify how it might be utilized in clinical and therapeutic settings.

APPENDIX

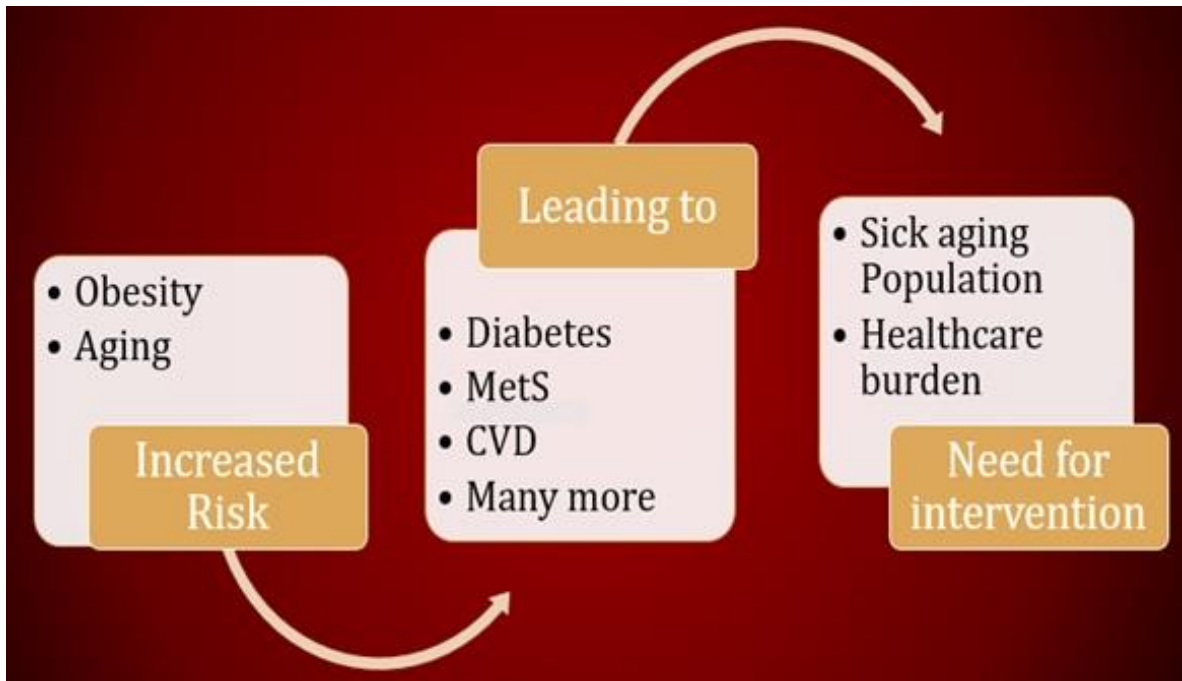


Figure 1. Obesity and Age-Related Outcomes

Showing the logical flow of how obesity and aging directly relate to metabolic disease and the need for intervention.

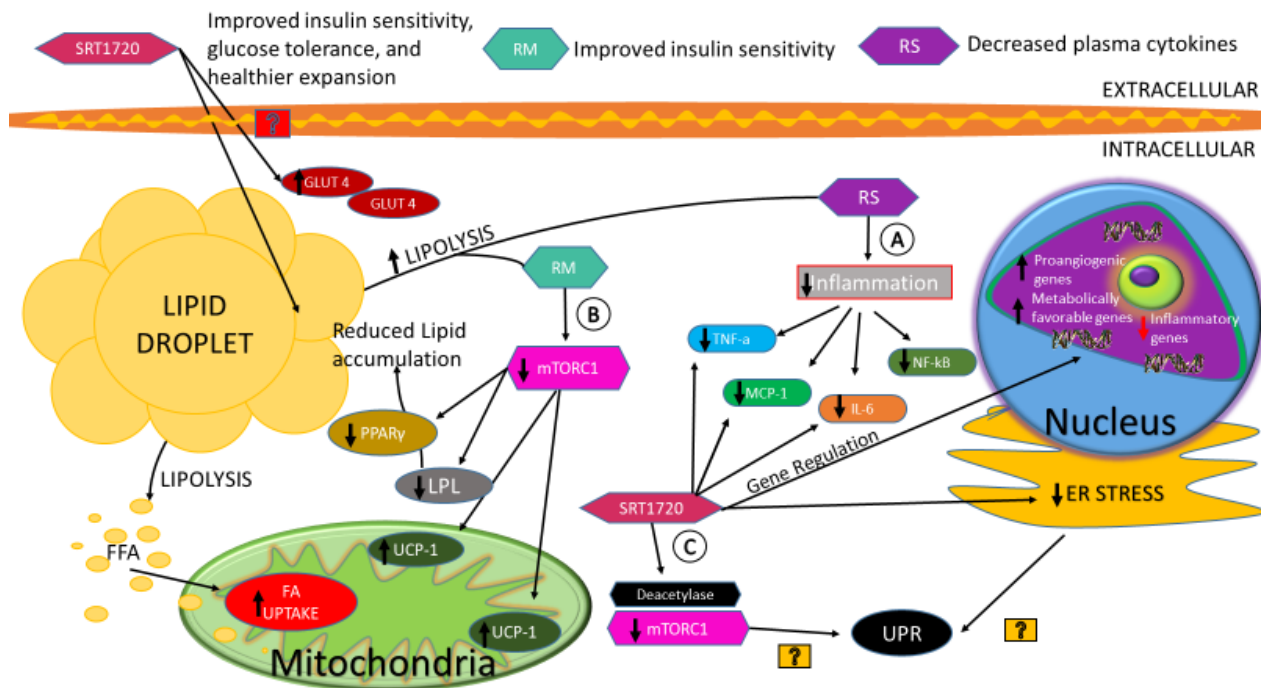


Figure 2. Overview of Combination Potential for Combining CR Mimetics

Model providing evidence for how combining various CR mimetic compounds could prove synergistically viable at improving adipose tissue function and metabolism

CHAPTER 2
RESEARCH PROJECT I

Zyflamend, a Unique Herbal Blend, Induces Cell Death and Inhibits Adipogenesis through the Coordinated Regulation of PKA and JNK

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Running title: Zyflamend inhibits white adipocytes differentiation

Keyword: Zyflamend, Adipogenesis, Lipolysis, AMPK, PKA, JNK

Conflict of interest: The authors declare that there is no conflict of interest regarding the publication of this article.

Abstract

Objective: The prevalence of obesity and its comorbidities has sparked a worldwide concern to address rates of adipose tissue accrual. Recent studies have demonstrated a novel role of Zyflamend, a blend of natural herbal extracts, in regulating lipid metabolism in several cancer cell lines through the activation of the AMPK signaling pathway. Yet, the role of Zyflamend in adipogenic differentiation and lipid metabolism remains largely unexplored. The objective of this study is to investigate the effects of Zyflamend on white 3T3-MBX pre-adipocyte differentiation and elucidate the molecular mechanisms.

Methods: 3T3-MBX pre-adipocytes were treated with Zyflamend, and the expression of various key adipogenic and lipolytic regulators was examined. We also investigated the effects of Zyflamend on pre-adipocyte survival, proliferation, and cell cycle.

Results: Zyflamend treatment altered cell cycle progression, attenuated proliferation, and increased cell death of 3T3-MBX pre-adipocytes. In addition, treatment with Zyflamend inhibited lipid accumulation during the differentiation of 3T3-MBX cells, consistent with decreased expression of lipogenic genes and increased lipolysis. Mechanistically, Zyflamend-induced alterations in adipogenesis were mediated, at least in part, through the activation of AMPK, PKA, and JNK. Inhibition of AMPK partially reversed Zyflamend-induced inhibition of differentiation, whereas the inhibition of either JNK or PKA fully restored adipocyte differentiation and decreased lipolysis.

Conclusion: Taken together, the present study demonstrates that Zyflamend, as a novel anti-adipogenic bioactive mix, inhibits adipocyte differentiation through the activation of PKA and JNK pathways.

General Significance: Our findings suggest that Zyflamend supplementation might help in the development of novel anti-obesity therapeutic strategies.

Significance: We identified novel properties of Zyflamend as a potential anti-adipogenic, anti-lipogenic, and pro-lipolytic herbal mix. We firmly believe that these novel and unexpected findings have significant translational implications and will be of major interest to a wide spectrum of scientists. Additionally, while we believe that Zyflamend may have potential therapeutic implications for obesity and its complications, we do not suggest the use of this combination as primary treatment for obesity. However, there is a possibility that it could be used either as an adjuvant with medically-approved standard therapies or in a preventative manner.

Introduction

Obesity and its comorbidities have emerged as an epidemic of substantial proportions [6, 7]. Obesity affects 36.5% of all Americans [7] and often shows a direct correlation to excessive calories intake [7, 45]. The burden of excess adipose tissue on metabolic homeostasis can be drastic and remains a major risk factor for the development of numerous diseases [7]. Moreover, obesity has been associated with the impaired functioning of critical organs including the kidney, liver, pancreas, and heart [6, 47, 251, 252]. As a result, there is a high demand for innovative and effective approaches, targeting adipose tissue and obesity in both a therapeutic and preventative manner.

Adipogenesis, the process in which an immature pre-adipocyte differentiates and matures leading to increased lipid accumulation, is an important component in the development of obesity. Once mature, these cells act as energy storage sites and can modulate processes essential to the homeostasis and function of a variety of other tissues. Disruption of this process offers an alluring target in both the prevention and treatment of obesity. Thus, targeting varying aspects of adipogenesis has been at the forefront of obesity research. Although current anti-obesity medications promoting fat loss do exist, the safety of these medications is often disputed [184, 253] and dedication to new prevention and treatment strategies must be continued. A growing body of evidence suggests that phytochemicals and natural herbal extracts, when used at pharmacological doses, could be capable of delaying the onset and progression of obesity through a variety of mechanisms including differentiation, cell death, and lipolysis.

Zyflamend, a unique blend of ten herbal extracts, was proven to have anti-inflammatory and anti-tumorigenic properties at physiologically relevant doses [254]. However, Zyflamend's biological roles beyond cancer metabolism have been relatively unexplored. Recently, we demonstrated that Zyflamend activates AMPK *in vitro* across various cell lines [195] and *in vivo* in a preclinical experimental model of U.S. diet-induced metabolic disorder [228]. Given the critical role for AMPK in regulating adipogenesis, glucose uptake, fatty acid metabolism, and mitochondrial function, it is likely that Zyflamend may also influence body mass and glycemic control. We also reported that Zyflamend supplementation for 4 weeks significantly reduced adiposity, improved insulin sensitivity, and reduced plasma levels of non-essential fatty acids [228]. These changes were concomitant with increased AMPK phosphorylation and inhibition of

ACC in adipose tissue [228] suggesting that Zyflamend may exhibit anti-adipogenic and/or lipolytic properties. In this study, we examined the *in vitro* effects of Zyflamend on the differentiation of white adipocytes and investigated the potential molecular mechanisms mediating its actions.

Material and Methods

Chemicals and Reagents: Trypsin, media, and sera utilized in cell culture experiments were purchased and acquired from Gibco (Thermo Fisher Scientific, Waltham, MA). Zyflamend (**Table S1**) was purchased from New Chapter Inc. (Brattleboro, VT). Antibodies, both primary and secondary, were obtained from numerous sources [244]. Chemical reagents including digitonin, protease inhibitors cocktail, dithiothreitol (DTT), propidium iodide, percoll, phenylmethylsulfonyl fluoride (PMSF), sodium fluoride (NaF), sodium deoxycholate, ethylene glycol-bis-(2-aminoethyl)-N,N,N',N'-tetraacetic acid (EGTA), Triton X-100, PKA inhibitor (H89), and JNK inhibitor (SP600125) were obtained from Millipore-Sigma (Burlington, MA). AMPK inhibitor (BML275) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). General caspases inhibitor (Z-VAD.fmk) was obtained from Calbiochem (La Jolla, CA).

Cell Culture: 3T3-L1 MBX (ATCC ® CRL-3242) white pre-adipocytes were purchased from ATCC. Brown precursor and 3T3-L1 MBX cells were cultured in DMEM containing 25 mM glucose, 10% FBS, 50 U/ml penicillin, and 50 µg/ml streptomycin. To induce cell differentiation, pre-adipocytes were grown to 100% confluency in culture medium containing 10% FBS, then switched to differentiation media containing 10% FBS, 20 nM insulin and 1 nM triiodothyronine [T3] for 48 hr. Adipocyte differentiation was induced by switching to a fresh differentiation media supplemented with 5 µM dexamethasone, 0.5 mM isobutylmethylxanthine, and 0.125 mM indomethacin for 48 hr (induction media). Cells were then returned to differentiation medium until they exhibited a fully differentiated phenotype characterized by the accumulation of fat droplets.

Zyflamend Treatment: Zyflamend was dissolved in DMSO at a concentration of 800 mg/ml for the stock solution. Cells cultured in 60 mm dishes were treated with Zyflamend for the indicated duration and at the indicated concentration. Treatments were stopped with two washes with ice-cold phosphate buffer saline (PBS). Finally, plates were flash frozen in liquid nitrogen and stored at -80°C until further analyses.

Cytotoxicity Assay: Cytotoxicity assays were performed using sulforhodamine B (Millipore-Sigma) as previously described [255] with modification. Briefly, 3T3-MBX pre-adipocytes were treated with Zyflamend at 37°C in an atmosphere of 10% CO₂ for the indicated time. After treatment, cells were fixed with 17% trichloroacetic acid in PBS, and cellular protein was stained for 10 min at room temperature with 0.4% sulforhodamine B in 1% acetic acid solution. The plates were then washed with water, dried, and the stain was dissolved in 10 mM Tris (pH 9). Quantification of sulforhodamine B stain was carried out using the Synergy™ HTX Multi-Mode microplate reader (BioTek Instruments, Inc. Winooski, VT) at a wavelength of 540 nm. The relative survival rates of cells were determined by dividing the absorbance observed for a given treatment by the absorbance detected in the control cells treated with DMSO and expressed as a fold change. In some experiments, the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) cytotoxicity assay was performed as previously described with modification [256]. Briefly, pre- (20,000 cells/well) or fully differentiated 3T3-L1-MBX adipocytes (5,000 cells/well) were plated in a 96-well plate for 24 hr, then starved in 200 µl DMEM culture medium containing 0.1% FBS for 12 hr prior to an additional 24 hr treatment with Zyflamend (200 µg/ml). At the end of the experiment, 40 µl of the MTT solution (5 mg/ml) was added to each well for 4 hr, then cell culture was removed and the dye was dissolved in 100 µl 10% SDS solution overnight at 37 °C. Relative cytotoxicity was determined by measuring the absorbance at 570 nm using the Synergy™ HTX Multi-Mode microplate reader.

Quantification of lipid accumulation and Oil Red O staining: Quantification of lipid accumulation was performed as previously described [257]. Briefly, on day 12 of differentiation, cells were fixed with 10% PBS buffered formalin for at least 12 hr at 4°C. Cells were stained for one hour with filtered Oil Red O solution (5 g/L in isopropyl alcohol), washed with distilled water, and examined using the Leica DMI8 inverted microscope (Leica Microsystems Inc. Buffalo Grove, IL).

Caspase activity: Following Zyflamend treatment, caspase-3 activity was determined as previously described with modification [258]. Briefly, 2 x 10⁶ cells were lysed in 50 µl cell lysis buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% CHAPS, 1 mM DTT, 0.1 mM EDTA) in a 96 well plate and incubated at -20 °C for 30 min. Next, 50 µl of the caspases assay buffer solution (50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% CHAPS, 10 mM DTT, 0.1 mM EDTA and 10% glycerol) containing 50 µM of the caspase-3 substrate (Ac-DEVD-pNA; Calbiochem, La Jolla,

CA) was added to each well and incubated at 37°C for 30 min. Caspase-3 activity was determined by measuring the absorbance at 405 nm using the Synergy™ HTX Multi-Mode microplate reader and was presented as caspase-3 activity relative to control cells.

Western Blotting Analysis: Cells were lysed in radio-immunoprecipitation assay (RIPA: 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% sodium dodecyl sulfate [SDS], 1% Triton X-100, 1% sodium deoxycholate, 5 mM EDTA, 1 mM NaF, 1 mM sodium orthovanadate and protease inhibitors) buffer as we previously described [259]. Clarification of lysates occurred through centrifugation at 15,000 g for 10 min, and the level of protein concentration was quantified using the bicinchoninic acid assay kit (Thermo Scientific™ Pierce™ BCA Protein Assay, Thermo Fisher Scientific, Waltham, MA). Proteins (10-30 µg) were resolved by SDS-PAGE and then transferred on polyvinylidene fluoride (PVDF) membrane. Immunoblotting of lysates was performed with primary antibodies, and following incubation with secondary antibodies, proteins were detected using Luminata™ Western Chemiluminescent HRP Substrate (Millipore-Sigma). Immunoreactive bands were quantified based on pixel intensity using FluorChem Q Imaging software (Alpha Innotech Corp, San Leandro, CA). Data is represented as being either normalized to the respective unphosphorylated form or to β -actin as loading controls for each respective condition.

RNA extraction and real-time PCR: RNA was extracted from cells using TRIzol Plus RNA Isolation System (Invitrogen, Carlsbad, CA). The concentration of RNAs was determined using the NanoDrop® ND-1000 spectrophotometer (Thermo Scientific). Reverse transcription was performed on equal RNA samples using the High-Capacity cDNA Synthesis Kit (Applied Biosystems, Austin, TX). Expression of different genes was assessed by quantitative real-time PCR using SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad Laboratories, Inc., Hercules, CA) and the Bio-Rad CFX96™ system. Primer sets used in these experiments are described in [244]. The relative abundance of target gene mRNA was measured using the $\Delta\Delta$ CT method and normalized to 18S ribosomal RNA.

Annexin V Staining: Annexin V staining was performed as previously described [260] with modification. Briefly, 3T3-MBX pre-adipocytes were exposed to Zyflamend for the indicated duration, then washed with PBS and resuspended in 0.5 ml of PBS containing 10% FBS. Equal volumes of the Guava Nexin reagents were added, and cells were incubated for 10 min at room temperature in the dark. Cells (5,000) were then analyzed using the Guava® easyCyte™ Flow

Cytometer (Millipore-Sigma). Intensities of fluorescence emitted by annexin V-fluorescein isothiocyanate (FITC) and 7-amino-actinomycin D (7-AAD) were collected on PM1 and PM2 channels, respectively. Both viable (negative for both annexin V-FITC and 7-AAD) and apoptotic cells (early apoptosis: positive for annexin V-FITC, but negative for 7-AAD, and late apoptosis positive for both: annexin V-FITC and 7-AAD) were quantified using the InCyte™ and Guava Suite Software Package (Luminex Corp., Austin, TX).

Cell cycle analysis: Cell cycle analysis was performed in observation of the cellular DNA content and level of propidium iodide stain as previously described [261] with minor alterations. Briefly, 3T3-MBX pre-adipocytes were treated with Zyflamend for the designated duration, then washed with PBS and fixed in 70% ethanol for 12 hr at 4°C. Cells were then washed twice with cold PBS and incubated in RNase solution (100 U/ml) in PBS for 15 min at 37°C. Cells were then incubated in propidium iodide solution (10 µg/ml in PBS) overnight at 4°C. A total number of 5000 cells was acquired using the Guava® easyCyte™ Flow Cytometer. Cells that are labeled with propidium iodide are then plotted as a function of cells at each intensity level (Y axis). Fluorescence intensity of propidium iodide (relative to DNA content and shown as the X-axis on the representative histogram) was quantified using the InCyte™ and Guava Suite Software package.

2-Deoxyglucose uptake assay: 2-deoxyglucose uptake was determined using the colorimetric glucose uptake assay kit (Biovision Inc., Milpitas, CA). Briefly, fully differentiated 3T3-MBX adipocytes were starved overnight in low glucose (1 mM) and 0.1% FBS media, then treated with insulin for 30 min in the presence of 2-deoxyglucose. Cells were then washed with ice-cold PBS, and the accumulated 2-deoxyglucose was quantitated using the Synergy™ HTX Multi-Mode microplate reader.

Statistical analysis: Data analysis relied on the JMP Pro 13.2 program (SAS Institute, Chapel Hill, NC) and was represented as means ± standard error of the mean (SEM). For all statistical analyses, unpaired heteroscedastic two-tail Student's t-test was used, and differences were considered highly significant at $p < 0.01$ and significant at $p < 0.05$. Single symbol (such as *) corresponds to $p < 0.05$, while double symbols (such as **) corresponds to $p < 0.01$.

Results

Zyflamend inhibits adipocyte differentiation in a dose dependent manner. We previously reported that Zyflamend is a polyherbal supplement composed of 10 botanical extracts (**Table S1**), significantly reduced adiposity and activated AMPK signaling [228]. Based on these *in vivo* effects and the established role of AMPK in regulating lipid metabolism and adipogenesis, we tested the effects of Zyflamend on multiple aspects of adipocyte differentiation *in vitro*. 3T3-MBX white pre-adipocytes were induced to differentiate into fully mature adipocytes and simultaneously treated with increasing concentrations of Zyflamend as outlined in [244]. Cells were then stained using the fat-specific dye Oil Red O to monitor the effects of treatment with increasing concentrations of Zyflamend on lipid accumulation and differentiation. Adipocytes treated with Zyflamend showed a dose-dependent decrease in lipid accumulation [244]. Quantification of lipid accumulation with Oil Red O staining indicated a significant reduction in cellular triglyceride content with Zyflamend doses as low as 50 µg/ml in comparison to vehicle (DMSO) controls [244]. Notably, cells treated with 1 mg/ml of Zyflamend exhibited blunted differentiation on day 12 and were comparable to the non-differentiated cells on day 1

To sequentially monitor the effect of Zyflamend on lipid accumulation, cells were induced to differentiate in the presence or absence of Zyflamend (200 µg/ml) and stained with Oil Red O at 1, 3, 6, 8 and 12 days post-induction [244]. This dose was chosen as a physiologically relevant level representative of the maximum plasma concentration of 2 µM of its constituent curcumin that was reported in humans upon supplementation [262]. At this human-equivalent dose, Zyflamend significantly attenuated adipogenesis and accumulation of lipid droplets [244]. Additionally, microscopic analysis revealed a drastic difference between control and Zyflamend treated cells. As shown in [244], control cells accumulated fat droplets and exhibited a fully differentiated phenotype on day 12 of differentiation. However, only a small percentage of cells accumulated fat droplets in the Zyflamend-treated group, confirming the anti-adipogenic effects of this herbal blend [244]. However, the precise mechanism and stage in which Zyflamend repressed differentiation were yet to be determined.

A physiologically relevant dose of Zyflamend causes cell cycle arrest and induces apoptosis in 3T3-MBX pre-adipocytes. Adipocyte differentiation is a complex process that requires the coordinated actions of several hormonal and nutritional factors, and the integration of multiple signaling pathways [263-266]. Based on findings from cancer studies, Zyflamend-

induced alteration in adipogenesis can be caused by a plethora of signaling pathways, including, but not limited to those involved in the regulation of cell cycle, proliferation and/or death, lipolysis, lipogenesis, contact inhibition, clonal expansion, and/or trans-differentiation into brown-like adipocytes. Thus, we conducted a detailed analysis of the effects of Zyflamend on pre-adipocyte proliferation, cell cycle, cell death, and differentiation.

First, we used the sulforhodamine B cytotoxicity assay, also used as an indirect measure of cell proliferation [267], to examine whether Zyflamend exhibits cytotoxic effects on 3T3-MBX pre-adipocytes, and whether it alters cell proliferation. Proliferating pre-adipocytes were cultured in the presence of Zyflamend (200 $\mu\text{g/ml}$) or DMSO (0.25 $\mu\text{l/ml}$) as vehicle control for up to 48 hr. Consistent with cancer studies [192, 207, 211], Zyflamend significantly reduced cell proliferation [244]. Next, we examined changes in the cell cycle in proliferating 3T3-MBX pre-adipocytes in response to Zyflamend treatment. Accordingly, cells at 50% confluency were treated with increasing concentrations of Zyflamend, and changes in cell cycle progression were examined. Analysis of cell distribution among the different phases of the cell cycle according to their DNA contents revealed that Zyflamend caused a dose-dependent cell cycle arrest at G2/M phase. The percentages of cells in the G2/M cell cycle phase were significantly higher in Zyflamend-treated cells and increased from $1.3 \pm 0.7\%$ in DMSO-treated control cells to $17.1 \pm 3.4\%$, $23.4 \pm 4.1\%$, and $32.6 \pm 5.7\%$ in cells exposed to 200, 400, or 500 μM of Zyflamend for 24 hr, respectively [244]. Additionally, we observed remarkable changes in response to Zyflamend treatment in the expression of D-type cyclins [244] which play a critical role in coordinating cell proliferation, survival, and differentiation, and are the core of cell cycle machinery. Indeed, the expression of cyclins D1 and D2 was significantly attenuated in Zyflamend treated cells compared to controls, whereas, the expression of cyclin D3 was high on day 1 of differentiation then decreased gradually during differentiation [244]. Next, the levels of early and late apoptosis in 3T3-MBX pre-adipocytes treated with DMSO or Zyflamend were measured using annexin V- FITC and 7-AAD as markers of early and late cellular apoptosis, respectively [244]. Zyflamend-treated pre-adipocytes exhibited a dose-dependent increase in both early and late apoptosis as judged by the elevated number of cells that stained positive for annexin V (x-axis), and 7-AAD (y-axis) [244]. As the dose of Zyflamend increased, so did the percentages of cells in late apoptosis [244]. Taken together, our data show that Zyflamend elicited strong effects toward the suppression of pre-adipocyte growth and proliferation, while simultaneously inducing cell death.

Patterns of differentiation correlate with the expression of adipogenic markers. To further investigate the effects of Zyflamend on adipogenesis, we determined the expression levels of several key adipogenic markers; fatty acid synthase (FASN), pyruvate carboxylase (PCB), perilipin, peroxisome proliferator-activated receptor gamma (PPAR γ), CCAAT-enhancer binding protein alpha (C/EBP α), adiponectin, and GLUT4. Consistent with previous reports [268, 269], FASN, PCB, C/EBP, adiponectin, PPAR γ exhibited a progressive increase in expression during adipocyte differentiation in control cells treated with the vehicle DMSO [244]. However, cells treated with Zyflamend (200 μ g/ml) failed to exhibit increased expression of these proteins that characterize the progression of adipocyte differentiation [244], with only modest expression of some proteins detectable in the late stages of differentiation. In contrast, the expression of pre-adipocyte factor 1 (PREF-1), a marker of pre-adipocytes, persisted at comparable levels through day 12 in Zyflamend-treated cells, compared to the expected decline as observed in control cells. Levels of other proteins associated with the non-differentiated state of white adipocytes, such as α 5 integrin, fibronectin, and suppressor of cytokine signaling 9 (SOX9), also declined with the progression of adipogenesis in control cells, but persisted longer with Zyflamend treatment [244]. In addition, we examined changes in mRNA levels of genes associated with the non-differentiated state of white adipocytes, such as *Delta like non-canonical notch ligand 1* (*Dlk1*; a.k.a. *pre-adipocyte factor 1*), and genes involved in the differentiation of white adipocytes, including fatty acid synthase (*Fasn*), resistin (*Retn*), *Fatty acid-binding protein* (a.k.a. *Adipocyte protein P2*; *Fabp4/Ap2*), *adiponectin* (*Adipoq*), and CCAAT/enhancer-binding protein alpha (*c/ebp1a*). As shown in [244], while control cells exhibited a marked increase in *Fas*, resistin, *c/ebp1a*, and *adiponectin*, the expression of these genes was significantly abolished in Zyflamend-treated cells. In contrast, *pref1* mRNA levels are significantly reduced in differentiated adipocytes compared to pre-adipocytes but remained higher in Zyflamend-treated cells compared to controls [244], further confirming our Western blotting data and the anti-adipogenic effects of Zyflamend.

To further assess the effects of Zyflamend on differentiation, and confirm that cells treated with Zyflamend remain undifferentiated, we evaluated basal and insulin-stimulated 2-deoxy-glucose uptake. In agreement with the reduced expression of Glut 4 in response to Zyflamend [244], cells treated with Zyflamend (200 μ g/ml) exhibited a robust and significant decrease in insulin-stimulated glucose uptake compared with control cells treated with the vehicle DMSO [244]. Collectively, these findings demonstrate that Zyflamend attenuates differentiation of white

adipocytes, as established by Oil Red O staining, lipid accumulation, protein and mRNA expression of differentiation markers, and glucose uptake.

Zyflamend dose-dependently regulates lipogenic and lipolytic proteins in mature adipocytes. In an effort to determine the effects of Zyflamend on mature adipocytes, we treated fully differentiated 3T3-MBX cells with Zyflamend and assessed the effects on markers of de novo lipogenesis and lipolysis. Additionally, we examined whether Zyflamend could alter AMPK phosphorylation [270] at threonine 172 (T172), a phosphorylation site that is required for AMPK activation and is associated with lipolysis and inhibition of lipogenesis in adipose tissue and adipocytes [271]. Zyflamend treatment induced the phosphorylation and activation of AMPK in a dose dependent manner [244]. ACC is rate-limiting enzyme in de novo lipogenesis, and phosphorylation by AMPK on serine 79 inhibits ACC activity. Zyflamend dose-dependently increased phosphorylation of ACC, in parallel with activation of AMPK [244, 272]. In addition, Zyflamend significantly decreased the abundance of fatty acid synthase (FAS), which, along with ACC, regulates the synthesis of fatty acids from glucose [244].

Next, we evaluated changes in the activity of HSL, a rate-limiting enzyme that regulates lipolysis in human and rodent adipose tissue, to determine if Zyflamend also promotes lipolysis in mature adipocytes. HSL is phosphorylated at multiple sites, where each modification regulates both the translocation and activity of the enzyme. PKA phosphorylates HSL at serine site 660 (S660) which promotes HSL translocation to lipid droplets and triacylglycerol hydrolysis [273]. Treatment of fully-differentiated 3T3-MBX adipocytes with Zyflamend resulted in a dose dependent increase in the phosphorylation of HSL at S660 [244].

Zyflamend inhibits the maturation of adipocytes through the coordinated regulation of PKA and JNK. A number of studies have shown the requirement of crosstalk between cAMP-PKA and MAP kinase signaling pathways in orchestrating adipocyte differentiation and functions [274]. While the role of Zyflamend in activating PKA has not been previously addressed, a number of studies have shown that Zyflamend activates both JNK and the MAP kinase P38 in various cell types [201, 254]. Thus, to begin to determine the mechanism underlying the anti-adipogenic and pro-lipolytic effects of Zyflamend, and the possible involvement of MAP kinases in mediating these effects, we examined the activation of PKA and MAP kinases during the course of differentiation of 3T3-MBX cells. Our data show that Zyflamend (200 µg/ml) had no effects on

changes in extracellular signal-regulated kinases 1/2 (ERK1/2) and protein kinase B (AKT) phosphorylation during differentiation (data not shown). However, a marked decrease in P38 phosphorylation, concomitant with a significant increase in JNK phosphorylation, particularly on day 6 of differentiation, was observed in Zyflamend-treated cells compared to controls [244]. Zyflamend also increased the phosphorylation and activation of the JNK canonical downstream effectors, namely c-FOS and c-JUN [244]. Furthermore, Zyflamend treatment led to a higher PKA activity across the differentiation process, in comparison to the control cells as judged by the phosphorylation of PKA substrates [244]. In addition, PKA-mediated phosphorylation of HSL at S660 was significantly higher in Zyflamend-treated cells compared to controls [244]. Collectively, our results suggest that PKA, P38, and/or JNK signaling pathways are mechanisms that potentially mediate the block in adipocyte differentiation induced by Zyflamend. Therefore, we investigated whether inhibition of PKA, P38, or JNK could abolish the effects of Zyflamend on 3T3-MBX differentiation. BML275 (a.k.a. compound C) was also used to inhibit AMPK specifically. As shown in [244], inhibition of JNK or PKA with SP600125 and H89, respectively, lessened the effect of Zyflamend on differentiation as judged by Oil Red O staining and triglyceride levels. However, the inhibition of P38 using SB203580 did not rescue the effects of Zyflamend on differentiation (data not shown). On the other hand, inhibition of AMPK using BML275 only resulted in a moderate rescue of Zyflamend-induced attenuation of adipocyte differentiation and accumulation of lipid droplets [244].

It has been proposed that the activation of PKA or JNK during adipocyte differentiation can lead to lipolysis and abolish adipogenesis [275, 276]. However, little is known about the crosstalk between the two proteins in coordinating the hydrolysis of triglycerides and how this affects the overall differentiation process. To address this question, we used specific inhibitors for PKA (H89) and JNK (SP600125) and examined changes in PKA and JNK activity and phosphorylation in response to the combined treatment with Zyflamend and SP600125, or Zyflamend and H89, respectively. We also evaluated the effects of Zyflamend and H89 on the overall differentiation process by accessing changes in the protein expression of several adipocyte differentiation markers; FASN, PCB, Perilipin, and PREF-1. Inhibition of PKA markedly attenuated the phosphorylation and activation of JNK while rescuing the effects of Zyflamend on the expression of the differentiation markers [244]. On the other hand, while inhibition of JNK restored the differentiation of 3T3-MBX cells, Zyflamend-induced PKA activation remained

unchanged [244]. These data suggest that Zyflamend-induced activation of both JNK and PKA attenuates 3T3-MBX differentiation through lipolytic induction.

Next, because treatment with Zyflamend induced apoptosis in pre-adipocytes [244], we sought to investigate whether inhibition of apoptosis using carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone (Z-VAD.fmk) could reverse the anti-adipogenic effects of Zyflamend. Z-VAD.fmk has been previously shown to relieve the anti-adipogenic effects of various stressors and restore pre-adipocytes differentiation [277-279]. Thus, we co-treated pre-adipocytes with Zyflamend and Z-VAD.fmk throughout the differentiation process and monitored adipocytes differentiation using Oil Red O. As shown in [244], although treatment with the pan-caspase inhibitor Z-VAD.fmk was able to reduce Zyflamend-induced cell toxicity and caspase-3 activation [244], it failed to alleviate the anti-adipogenic effects of Zyflamend, suggesting that apoptosis is not the main molecular mechanism that mediates the anti-adipogenic effects of Zyflamend. Conversely, inhibition of JNK and PKA using SP600125 and H89, respectively, attenuated Zyflamend-induced cytotoxicity and caspase-3 activity [244] and partially restored adipocytes differentiation [244]. Furthermore, both the MTT and caspase 3 activity assays revealed that the pro-apoptotic and cytotoxic effects of Zyflamend were significantly lower in fully differentiated adipocytes compared to pre-adipocytes [244]. Additionally, Z-VAD.fmk-mediated inhibition of cell death did not prevent Zyflamend-induced phosphorylation of HSL at S660 on day 8 of differentiation [244].

Discussion

Zyflamend is a polyherbal mix, whose efficacy as an anti-inflammatory and anti-cancer agent has been well characterized in a number of studies [193, 197, 202]. Interest in its use as an adjuvant with medically-approved standard cancer therapies or in a preventative manner gained traction due to its ability to modulate energy metabolism in cancer cells and its effectiveness at relatively low levels due to the synergistic effects of its components [262]. Nevertheless, its effects on metabolism and other homeostatic functions in non-cancer cells remain largely unexplored. We recently reported that Zyflamend supplementation to C57Bl6/J mice fed a western diet resulted in a significant reduction in fat mass, but had no effects on overall body weight or growth rates [228]. Effects on adiposity were accompanied by significant changes in tissue purine metabolism, concomitant with increased AMPK phosphorylation in epididymal white adipose tissue.

Accordingly, we sought to investigate the basis for these effects by analyzing the effects of Zyflamend treatment on pre-adipocyte proliferation, differentiation, and metabolism *in vitro*.

This study is the first to explore the potential effects of Zyflamend treatment on white adipocyte differentiation and lipid accumulation and to elucidate the molecular mechanisms. In this study, we report the anti-adipogenic properties of Zyflamend, which attenuated pre-adipocyte proliferation and increased cell death. In addition, treatment with Zyflamend inhibited lipid accumulation during the differentiation of 3T3-MBX cells, consistent with a decreased expression of PPAR γ and rate-limiting genes in *de novo* lipogenesis. These effects were mediated, at least in part, through the upregulation of AMPK, PKA and JNK activities as inhibition of AMPK partially reversed Zyflamend-induced inhibition of differentiation, while the inhibition of either JNK or PKA fully restored adipocyte differentiation and decreased lipolysis. Taken together, these findings identify Zyflamend as a novel regulator of adipogenesis and suggest that Zyflamend supplementation could prove valuable for the prevention of obesity and its complications.

Adipocyte differentiation is a complex process that requires the integration of multiple signals and is regulated by several hormonal and nutritional factors [263-266]. Synchronous cell cycle reentry and mitotic clonal expansion of committed pre-adipocytes are among the first steps leading to adipogenesis [244]. However, previous research has been inconclusive on whether this is essential for optimal differentiation [280-282]. Studies using experimental models of obesity have further underscored the coordinated functions of cell cycle regulators in adipogenesis and whole body metabolic homeostasis [103, 104]. Alterations in cell cycle reentry and mitotic clonal expansion may result in a notable impact on adipogenic differentiation. Here within, we demonstrate that Zyflamend treatment attenuated the proliferation of 3T3 MBX pre-adipocytes. Additionally, treatment with Zyflamend resulted in a dose dependent increase in both early and late apoptosis in pre-adipocytes. Furthermore, Zyflamend treatment interfered with the cell cycle machinery by reducing the levels of cyclins D1 and D2 and causing a G2/M cell cycle arrest in pre-adipocytes. In support of our findings, numerous phytochemicals have been shown to act as cell cycle regulators through altering cyclin protein expression [283]. Individual components of Zyflamend, such as curcumin [284] and rosemary [285], were both shown to alter the progression of the cell cycle. Pre-adipocytes treated with rosemary extracts exhibited reduced expression of cyclin-D1 and other cell cycle regulators such as cyclin-dependent kinase 4 and cyclin-dependent kinase inhibitor 1A (p21, Cip1) [242]. However, the use of single dietary bioactive compounds

for both treatment and prevention of obesity has resulted in a lack of clinical success. Conversely, the combination of several phytochemicals and the subsequent effects of targeting multiple signal transduction pathways simultaneously through additive or synergistic actions has been proposed to be a better approach to combat obesity [283, 286, 287].

At the molecular level, Zyflamend abolished the differentiation of white adipocytes, at least in large part, through up-regulating PKA and JNK activities. Here, we report inhibition of PKA eliminated the effects of Zyflamend on differentiation and resulted in fully differentiated adipocytes in comparison to cells treated with Zyflamend alone. It is worth noting that the PKA's precise contribution to adipogenesis remains controversial and warrants additional investigation. PKA has four regulatory (RI α , RII α , RI β , RII β) and four catalytic (C α , C β , C γ , Prkx) subunit isoforms that are differentially expressed and regulated between different mammalian tissues and species. Previous reports have shown that RII β is the primary PKA regulatory subunit in the adipose tissue of both human and rodents [288] and that mice lacking RII β exhibited resistance to high fat diet-induced obesity and associated metabolic alterations [288, 289]. However, recent studies have demonstrated that the disruption of the ubiquitously expressed PKA-RII α subunit in mice is also protective against diet-induced obesity, glucose intolerance, and hepatic steatosis [290]. Conversely, deletion of the *Prkarc* gene, which encodes the catalytic subunit C β in mice exhibited increased fat mass and decreased fat-free mass compared with controls when fed a regular chow diet. However, when animals were fed a high fat diet, PKA-C β deficiency protected male, but not female, mice against diet-induced obesity [291]. Additionally, recent studies have shown that both the expression of PKA-RII β and stimulated PKA activity are differentially regulated between different fat depots in obese versus lean humans [292], further adding to the complexity of the role of PKA in adipogenesis and obesity. Here, we report that inhibition of PKA using H89 alleviated the anti-adipogenic effects of Zyflamend and restored lipid accumulation and the expression of adipogenic genes. These findings are in line with previous reports demonstrating that H89 enhances adipocytes differentiation [293]. Notably, treatment of 3T3-L1 cells with epidermal growth factor (EGF) activated PKA and blocked adipogenesis. Additionally, in a study by Li and colleagues, stimulation of PKA activity was suggested to inhibit the differentiation of 3T3-L1 pre-adipocytes, possibly through the modulation of the insulin signaling pathway and the down regulation of the phosphorylation of insulin receptor substrate 1 (IRS-1) [276]. In the same study, the authors demonstrated that, while forskolin-mediated activation of PKA inhibited

adipogenesis in a dose-dependent manner, inhibition of PKA activity using H89 abolished the anti-adipogenic effects of forskolin [276]. Furthermore, activation of PKA by vinpocetine, a phosphodiesterase (PDE) type-1 inhibitor that increases cAMP and cGMP levels, resulted in the suppression of adipogenesis and lipid accumulation in 3T3-L1 cells [294]. These findings are consistent with the study by Li and colleagues showing that, reducing cAMP levels using a specific adenylate cyclase inhibitor (SQ22536; which would prevent PKA activation) promoted adipogenesis [276]. Nevertheless, and despite the controversies around PKA's role in adipogenesis, it is well established that activation of PKA via phosphorylation is a critical step for the phosphorylation of perilipin and HSL, both of which are highly expressed in adipose tissue. The phosphorylation and activation of HSL trigger the recruitment of HSL to lipid droplets, where it mediates the hydrolysis of stored lipids in concert with adipose triglyceride lipase (ATGL) [295]. The combined action of the neutral lipases ATGL, HSL, and monoacylglycerol lipase (MGL), is essential for the hydrolysis of TAGs and the release of glycerol and NEFAs [296]. HSL can be phosphorylated and activated by PKA in response to catabolic hormone induction of the cAMP signaling cascade [296]. Other protein kinases including AMPK, ERK, glycogen synthase kinase-4, and Ca^{2+} /calmodulin-dependent kinase II have been shown to regulate HSL enzymatic activity through phosphorylation [296]. The regulation of HSL activation by phosphorylation is a critical and reversible process. Five critical phosphorylation sites on serine residues have been revealed including S563, S659, and S660 that are phosphorylated by PKA and the basal site (S565) that is phosphorylated by AMPK and Ca^{2+} /calmodulin-dependent kinase II. Phosphorylation of HSL at the latter site prevents its activation by PKA [297]. Here, we report increased phosphorylation of HSL at the PKA regulatory site S660 by Zyflamend. However, the mechanism as to how Zyflamend activates the PKA/HSL signaling cascade is unknown, although many of its constituents have been shown to independently regulate PKA and HSL activities in several cell types including differentiating adipocytes [298-302].

Similar to PKA research, studies looking at the role of JNK in adipogenesis are inconclusive and produced mixed results with some studies supporting the anti-adipogenic and pro-lipolysis functions of JNK, while other reports arguing for a pro-adipogenic role. In a study by Kusuyama and colleagues, a differential role of the different JNK isoforms in regulating adipogenesis has been proposed [303]. The overexpression of the p46, but not p54 isoform increased adipogenesis while the inhibition of both isoforms suppressed adipogenesis [303].

Conversely, shRNA mediated knockdown of JNK1 and/or JNK2 had no impact on 3T3-L1 pre-adipocyte differentiation but drastically accelerated basal lipolysis [304]. Furthermore, the inhibition of JNK using phloretin, a glucose transporter (GLUT) inhibitor, induced adipogenesis in marrow stromal cells [275]. In addition, treatment with the JNK inhibitor SP600125 significantly increased lipid accumulation in ST2 cells [275]. Other studies, however, argue that JNK is not directly involved in the differentiation process of adipocytes [305]. While the role of JNK in adipogenesis is yet to be resolved, our study demonstrates an anti-adipogenic role of JNK in 3T3-MBX cells because SP600125-mediated inhibition of JNK fully rescued the effects of Zyflamend on adipogenesis. It is noteworthy to point out that inhibition of JNK activity reduces the phosphorylation of IRS1 on serine 307, a phosphorylation site that disrupts the interaction between the insulin receptor (IR) and IRS1 [306] which alter the transduction of insulin signaling and glucose uptake in white adipocytes [307]. JNK also inhibits insulin receptor substrate 2 (IRS2) through a similar mechanism [308]. Both IRS-1 and IRS-2 play a critical role in adipocyte differentiation through regulating glucose uptake as well as the expression of adipogenic proteins C/EBP α and PPAR γ [309]. Altering the function of IRS-1 and IRS-2 was reported to impair the adipogenesis and differentiation processes of both white [310] and brown [311] adipocytes, supporting the anti-adipogenic function of JNK. However, additional studies are necessary to determine the precise mechanisms underlying its role in adipocytes differentiation. In addition, future research aimed at elucidating the crosstalk mechanisms between PKA and JNK in halting adipogenesis and promoting lipolysis is warranted.

In conclusion, we report the anti-adipogenic properties of Zyflamend, which caused a G2/M cell cycle arrest in white 3T3-MBX pre-adipocytes, attenuated pre-adipocyte proliferation, and increased cell death. In addition, treatment with Zyflamend inhibited lipid accumulation during the differentiation of 3T3-MBX cells, with a concomitant decrease in the expression of lipogenic genes and increased lipolysis [244]. These effects were mediated, at least in part, through the upregulation of AMPK, PKA and JNK activities. Inhibition of either JNK or PKA fully restored adipocyte differentiation and decreased lipolysis [244]. However, AMPK inhibition using compound C partially rescued adipogenesis. Taken together, these findings identify Zyflamend as a novel regulator of pre-adipocytes survival and adipogenesis, and suggest that Zyflamend supplementation could prove valuable for the prevention of obesity and its complications.

However, it is important to note that many of the potentially desirable effects may have been mediated by Zyflamend's cytotoxic effects and future studies targeting its efficacy are warranted.

CHAPTER 3 RESEARCH PROJECT II

Zyflamend Supplementation Alleviates High-Fat Diet-Induced Obesity and Impairment of Skeletal Muscle Insulin Sensitivity

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Running title: Zyflamend Improves Metabolic Homeostasis

Abstract

Obesity represents a progressive epidemic that continues to pose substantial health challenges across the globe. Problematically, obese individuals have a much higher risk for developing chronic diseases such as cardiovascular disease, MetS, type 2 diabetes and NAFLD. As a unique poly-herbal supplement, Zyflamend is comprised of ten herbal extracts known to individually exert a wide variety of potential benefits. These include but are not limited to, its ability to activate AMPK in numerous cancer cell lines. Upon activation, AMPK has been shown to effectively inhibit lipogenesis along with upregulating fatty acid oxidation and other catabolic pathways that generate ATP. Additionally, the scope and impact of Zyflamend on skeletal muscle has yet to be investigated. Individually, Zyflamends constituents and other polyphenols have been shown to exert ergogenic and insulin sensitizing potential within skeletal muscle. Considering these implications, Zyflamends capacity to improve the function of adipose tissue and skeletal muscle warrants investigation. Therefore, the objective of our in vitro and in vivo study, was to identify the impact of Zyflamend on skeletal muscle, adipose tissue and glucose homeostasis. To accomplish this, for 24 weeks C57BL/6 mice were fed one of four dietary combinations including chow or high fat diet (HFD) incorporated with or without Zyflamend. Consumption of HFD supplemented with Zyflamend resulted in reductions in both weight gain and WAT fat pad mass, along with ameliorated HFD-induced glucose intolerance. Mechanistic investigations revealed, Zyflamend enhanced insulin signaling in the skeletal muscle evidenced by upregulated tyrosine phosphorylation of the IR, IRS1, and enhanced downstream signaling component activation. In addition, Zyflamend effectively reduced markers of HFD induced ER stress and inflammation within the skeletal muscle. Within WAT and BAT, Zyflamend induced AMPK phosphorylation in both chow and HFD fed animals. In WAT and BAT, Zyflamend treatment initiated changes to the thermogenic program evidenced by elevated expression of brown and/or beige fat markers, immunostaining for UCP1, and improved cold tolerance. Providing in vitro confirmation, Zyflamend treatment in fully differentiated mouse primary and HIB1b brown adipocytes, upregulated the expression of brown fat markers. When combined with metformin, Zyflamend synergistic upregulated expression of brown fat markers (UCP1 and PRDM16) in both cell lines. Taken together, our findings demonstrate the potential for Zylamend as a preventive and adjunct approach to alleviating the metabolic complications associated with obesity.

Background

Impacting the lives of nearly 37% of Americans [7], obesity along with its co-morbidities represent one of the most significant health epidemics facing the modern world [7, 42]. The metabolic consequences of obesity can be drastic, in lieu of this, the condition represents a primary risk factor for the development of numerous age related diseases [7]. In many cases, obesity directly correlates with disproportionate calorie intake and expansion of the adipose reservoir [7, 45]. Accrual of excessive adipose tissue can burden and impair metabolic function and processes important for maintaining homeostasis [7, 12, 45]. The systemic damage and inflammation caused by obesity can promote organ failure and damage through a wide variety of mechanisms [312]. Furthermore, obesity can promote insulin resistance and impede glucose uptake and clearance, within tissues such as the skeletal muscle and liver. Prolonged hyperglycemia can result in the progression of MetS and left untreated, the development of T2DM. Central obesity, elevated blood pressure, hyperglycemia, and high triglycerides are the primary risk factors associated with the development of MetS. MetS represents a significant global health concern, with an approximated one billion people afflicted with the disease [313]. Reducing the burden of obesity requires long term weight loss strategies [314] and/or alleviation of its systemic impact on tissues and metabolic function. While pharmaceutical, surgical and dietary approaches have shown varying degrees of success [315], the problem remains and obesity rates have continually risen over the past several decades [316].

In metabolic function, skeletal muscle is critical for maintaining glucose homeostasis when properly responding to insulin signaling. Problematically, factors of obesity and MetS can disrupt the skeletal muscle's capacity to effectively respond to insulin, impairing glucose uptake. Insulin signaling pathway inactivation is attributed to fatty acid accumulation within skeletal muscle. Elevated levels of fatty acids within the skeletal muscle can result from excessive consumption of high-fat, high-carbohydrate food, which overwhelms the capacity of adipocytes to properly store triglycerides [152]. Elevated levels of fatty acids within the skeletal muscle can result from excessive consumption of high-fat, high-carbohydrate food, which overwhelms the capacity of adipocytes to properly store triglycerides [152]. Furthermore, excessive fatty acids can burden skeletal muscle mitochondria leading to oxidative stress and subsequent ROS accumulation [153]. Problematically, ROS can damage the mitochondria generating defects in fatty acid oxidation giving rise to toxic lipid metabolites [153]. These intermediate metabolites such as ceramides, acyl

CoA, and diacylglycerol are responsible for activation of PKC, which interrupts tyrosine phosphorylation of IRS-1 by the insulin tyrosine kinase receptor and suppresses IRS-1 activity [154]. Considering these deleterious and systemic effects, investigating novel approaches to improve the insulin responsiveness of skeletal muscle could result in new and improved clinical approaches to MetS. Beyond skeletal muscle, adipose tissue represents another significant target relating to obesity and MetS.

Adipose tissue can consist of either WAT or BAT. However, the majority of human fat is present in the form of WAT and plays the critical role of mobilizing and storing energy supplies in the form of triacylglycerol. Additionally, adipose tissue is responsible for vital endocrine and paracrine functions, along with mediating inflammatory responses, insulin signaling, angiogenesis, appetite control and other metabolic processes [81, 82]. These critical functions have led to adipose tissue being identified as a dynamic organ. The physiological functions of WAT are carried out predominantly by adipocytes. The cellular maturation of adipocytes through adipogenesis, is dependent upon the accumulation of an internal fat droplet and displacement of surrounding cytoplasm and organelles [83]. The accumulation of mature adipocytes is ultimately what forms adipose tissue. In opposition to accumulating glycerol, BAT predominantly releases energy through thermogenesis by way of mitochondrial UCP1 [84]. This uncoupling response has made WAT to BAT conversion, also known as browning, the target of many obesity related studies [85-87]. Targeting both obesity and MetS, a wide variety of dietary factors, herbal extracts and botanical compounds have shown significant anti-obesogenic [230] and insulin responsiveness promoting potential. For instance, a large volume of plants and their derivatives from garlic, curcumin, fenugreek, grapes, and cinnamon, have shown promise in metabolic research through their capacity to increase insulin sensitivity and/or reduce insulin resistance [317].

Intriguingly, phytochemicals from dietary sources such as ginger [245], EGCg [246] and pomegranate juice [247] have been shown to exert potential ergogenic effects on muscle. In further support of phytochemicals, investigations targeting their effects on aspects of muscle insulin signaling and responsiveness have revealed promising findings [248]. Functionally, the mechanisms through which phytochemicals may improve skeletal muscle insulin sensitivity and function, could relate to their effects on signaling pathways involving IR, the IRS family, PI3K, AKT and AMPK [248]. As evidenced, phytochemical stimulation of the AMPK pathway can lead

to improved insulin sensitivity and helps to reduce mitochondrial defects related to the development of insulin resistance in skeletal muscle [227]. In addition, a wide variety of phytochemicals have been shown to effectively target varying aspects of adipogenesis [318], and browning [319].

As a combination supplement, Zyflamend (New Chapter, Inc., Brattleboro, VT) makes use of 10 herbal extracts including the following in a two capsule once-daily dose; turmeric (110 mg), rosemary (150 mg), holy basil (100 mg), ginger (100 mg), organic green tea (100 mg), hu zhang (80 mg), organic oregano (40 mg), barberry (40 mg), chinese goldthread (40 mg), and Chinese skullcap (20 mg). Zyflamend has been highly regarded as an anti-inflammatory agent. In comparison to individual herbal supplementation, the poly-herbal encapsulation within Zyflamend revolves around the idea of generating an additive or a synergistic response promoting superior benefits [203]. Individually, curcumin [214], and *Zingiber officinale* [249], treatment resulted in significant improvements to insulin resistance in two separate rat models emulating diabetes and MetS, respectively. In addition, many of the compounds and polyphenols encapsulated within Zyflamend have been shown to affect skeletal muscle and adipose tissue function. Considering the combined potential of these individual ingredients, Zyflamend could prove to be an effective approach to MetS through its impact on adipose and skeletal muscle.

Materials and Methods

Murine studies: Initially, C57BL/6 mice were age-grouped and administered standard lab chow (Purina lab chow, # 5001) for five weeks. Between week 5 and 6 age, the animals were provided with a chow diet containing olive oil as vehicle control (Ctrl) or Zyflamend (1mg/kg/day). Then, on week 6, animals were transferred to a high fat diet (HFD; 60% kcal from fat, Research Diets) integrated with either olive oil as vehicle control (Ctrl) or Zyflamend (1mg/kg/day) for 16-36 additional weeks. The murine investigations adhered to federal regulations and received approval from the Institutional Animal Care and Use Committee at University of Tennessee, Knoxville

Metabolic measurements. Glucose measurements were performed on either fed or fasted mice (12hrs) between 7-9 am. Regarding insulin tolerance tests (ITTs), animals were fasted for 4 hrs and regular human insulin (HumulinR; Eli Lilly Corp., Indianapolis, IN) was intraperitoneally

administered at a dose of 0.75-1.0 mU/g, with measurements of blood glucose prior and post injection (15, 30, 45, 60, 90, and 120 min). Regarding glucose tolerance test, animals were fasted overnight pre-injection glucose levels were measured and then 20% D-glucose (2 mg/g body weight) was injected intraperitoneally, and blood glucose was measured before and 15, 30, 60, and 120 min following injection.

Assessment of food intake and feed efficiency: Food intake was measured in the fed state as previously described [320]. Briefly, the overall twenty-four-hour food intake was calculated as the difference in weight between the food put into the cage and that which remains at the end of 24 hours. Feed efficiency was calculated as the average of the amount of weight gained (in grams) per the amount of food consumed in grams for three consecutive days on weeks 9, 16, and 22 of the feeding study.

Cold exposure: Mice were acclimated for 5 consecutive days for 6 hours at 4°C. Then, on day 6, mice were housed at 4°C for 24 hours and animals were euthanized. Rectal temperature was recorded 2 hours prior euthanasia.

Isolation of adipocytes. Three grams of tissue from different fat depots were incubated with ml of isolation buffer {0.1 M HEPES, pH 7.4, 0.12 M NaCl, 0.05 M KCl, 1.2 mM CaCl₂, 0.6 mM, MgSO₄·7H₂O and 1.5% (w/v) bovine serum albumin Fraction V (Fisher) containing 0.002% (w/v) collagenase (Worthington, NJ) in 50 ml siliconized tubes. Incubations were performed in a water bath at 37°C degrees for 60 minutes. Tissue remnants were then removed by filtration through a nylon screen (Tetko, NY) with a pore size of 250 µm into a siliconized test tube. For 5 mins, fat cells were left suspended on the surface, then a siliconized injection needle was used for the aspiration of the infranatant. Then by adding 5ml of isolation buffer and 2 ml of dinonylphthalate oil (Fluka) the adipocytes were further purified. Cells were then centrifuged at 1,000g for 5 min. Supernatant was then transferred to Eppendorf tubes, with RIPA buffer used to pellet and lyse cells for final processing.

Cell culture. Precursor cells and brown adipocytes were isolated from brown fat pads of newborn wild type mouse by a previously described method of collagenase digestion [321, 322]. Cells were kept in 10% NBSC, DMEM containing 25 mM glucose, 50 µg/ml streptomycin and 50 U/ml penicillin. Once confluent, cells were transferred to differentiation media comprised of 20% FBS,

1 nM triiodothyronine [T3] and 20 nM insulin for 48 hours. Differentiation induction occurred by treating cells with the same differentiation media supplemented with 0.125 mM indomethacin (induction media) and 5 μ M dexamethasone for an additional 48 hours. Following this induction period, cells were then reintroduced to differentiation medium and allowed to reach a fully differentiated phenotype, as evidenced by the accumulation of triglyceride.

Biochemical analysis. For insulin signaling experiments, following an overnight fast, mice were sacrificed 10 minutes after injection with i.p. with insulin (10 mU/g body weight). Tissues were immediately dissected and snap frozen in liquid nitrogen. Tissue samples were then ground and lysed using radio-immunoprecipitation assay (RIPA) buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% sodium dodecyl sulfate, 1% TritonX-100, 1% sodium deoxycholate, 1 mM NaF, 5 mM EDTA, 1 mM sodium orthovanadate and protease inhibitors). Lysates clarification was achieved through centrifugation of samples at 13,000 rpm for 10 min and bicinchoninic acid protein assay kit (Pierce Chemical, IL) was used to determine protein concentrations. Proteins (500-1000 μ g) were immunoprecipitated with anti-IR, Dr. R. Kahn, Joslin Diabetes Center) and anti-IRS1 antibodies (Millipore, MA). G-Sepharose magnetic beads (GE Healthcare) were utilized to collect immune complexes and washed three times with lysis buffer. Proteins were resolved with SDS-polyacrylamide gel electrophoresis and then transferred to nitrocellulose membranes. Immunoblots were performed with anti-phosphotyrosine monoclonal antibodies 4G10 (Upstate Biotechnology), or antibodies against IR, IRS1, PTP1B (Upstate), pAkt, Akt, pErk (Cell Signaling), anti-phosphotyrosine PY99 and Erk (Santa Cruz). Visualization of proteins was accomplished using enhanced chemiluminescence (ECL, Amersham Biosciences) and FluorChem 9900 (Alpha Innotech, CA) was used to quantify pixel intensities of immuno-reactive bands.

Protein extraction and Immunoblots: Newly prepared solutions of RIPA buffer containing phosphatase and protease inhibitors were used to prepare lysates from muscle tissue samples harvested from Zyflamend treated and control animals. In sequence, freshly prepared lysates were kept on ice and sonicated in two rounds (10 secs each) separated by 10 min delay, followed by centrifugation at 15,000 g at 4°C. Bicinchoninic acid assay kit (Pierce Chemical, Dallas, TX, USA), was used to determine the total concentration of proteins within samples. Electrophoresis was used to resolve 10-50 μ g of proteins followed by transfer to PVDF membranes and then the membranes were blocked for 45-60 min at room temperature in TBST containing 5% BSA and

0.1% tween, pH: 7.4. Sequentially, the membranes were then incubated for 1 hr at room temperature with primary antibodies listed in (Table 1). Following three 5 min wash cycles in TBST, the membranes were incubated for 1 hr at room temperature with corresponding secondary antibodies. After four washes in TBST for 15 min each, protein band intensity was observed by coating the membranes with Luminata™ Western Chemiluminescent HRP Substrate (Millipore Corp., Billerica, MA, USA), and Flourochem software (Alpha Innotech Corp., San Jose, CA, USA) was used to quantify band density.

Immunohistological Staining. For Immunohistochemistry experiments, freshly harvested adipose tissue from controls and Zyflamend-treated mice were fixed in 10% formalin and embedded in paraffin. 5 μ M sections were deparaffinized by three washes in freshly prepared 100% xylene (3 min each) followed by three washes in freshly prepared decreasing concentrations of ethanol (95, 90, 75, and 50%; 3 min each wash). Sections were then stained with anti-UCP1 antibodies, counterstained with hematoxylin, and then mounted using Aqueous Mounting Medium (AbCam; Waltham, MA). Bright-field microscopy imaging was conducted using the Leica DMI8 inverted fluorescent microscope.

Statistical analysis. Data has been expressed as means $\pm$ standard error of the mean (SEM). Statistical analyses were conducted using JMP program (SAS Institute, NC). Repeated measures analysis of variance (ANOVA) was utilized to analyze ITTs and GTTs. Post hoc differences were considered significant at $P \leq 0.05$ and highly significant at $P \leq 0.01$ using Tukey test. A symbol (such as *) indicates $P \leq 0.05$, whereas a duplicate symbol (such as **) indicates $P \leq 0.01$.

Results

Wild type C57BL/6 mice were age-matched and administered standard lab chow (Purina lab chow, # 5001) for 6 weeks, then transferred to high fat diet (HFD; 60% kcal from fat, Research Diets) containing either vehicular control (Ctrl/olive oil) or Zyflamend (1mg/kg/day) for a final 16 week period. In comparison to the HFD alone, Zyflamend incorporation into the diet resulted in significant reduction in body weight gain ($p < 0.05$) across the final four bi-weekly time points collected from 16 weeks to 22 weeks of age. In contrast, mice administered the Zyflamend-chow or control-chow diet displayed no significant differences in body weight between the two (**Fig 3.A**). Glucose tolerance test (GTT) revealed that Zyf-HFD fed animals had significantly lower glucose levels after 30 ($p < 0.05$) 60 and 120 min ($p < 0.01$) (**Fig 3.B**). Further suggesting improved

insulin sensitivity, insulin tolerance test (ITT) identified that the Zyf-HFD mice had significantly lower levels of plasma glucose across all post injection time points, relative to HFD control (**Fig 3.C**). In order to address any potential for preference bias between the diets, food intake was analyzed. Adjusting for body weight, no significant differences in food intake or feed efficiency were observed between Zyflamend and Ctrl HFD mice (**Fig 3.D**). To better understand the effects of Zyflamend on adiposity, fat pads from the (subcutaneous (SQ), BAT, epididymal (Epi), and visceral (Visc)) depots were weighed. Zyflamend treatment significantly reduced the weight of all depots except BAT, while no significant differences in organ tissue weight was observed (**Fig 3.E**). While non statistically significant, Zyflamend HFD feeding resulted in slight elevation of BAT (**Fig 3.E**). Collectively, these findings suggest Zyflamend may exert metabolic changes that may protect against the deleterious effects of HFD-induced obesity.

Considering the important role skeletal muscle plays in maintaining metabolic homeostasis, we set out to investigate how Zyflamend might impact insulin signaling within the tissue. HFD has been shown to effectively induce insulin resistance in murine skeletal muscle [249], therefore, we utilized HFD fed mice with or without Zyflamend incorporated in the diet. Animals were fasted overnight (12 hr) (-) followed by 10 mins of insulin stimulation (+) allowing for direct assessment of the kinetics of tyrosine phosphorylation and activation of key regulators in this signaling pathway (**Fig 4**). Tyrosine phosphorylation of individual components in insulin signaling such as insulin receptor (IR) and insulin receptor substrate 1 (IRS1) proteins was also determined by immuno-precipitating them with specific antibodies then probing with anti-phosphotyrosine. Our findings demonstrate that Zyflamend effectively induced tyrosine phosphorylation of IR and IRS1 in response to both fasting and insulin stimulation, in comparison to HFD alone (**Fig 4. A-B**). Furthermore, we observed increased phosphorylation of IRS1 at the Tyr608 site, in the insulin stimulated Zyflamend group (**Fig 4. C**). In addition, Zyflamend treatment significantly increased the level of insulin signaling components p85 and pERK under both fasted and insulin stimulated conditions (**Fig 4. D-F**). Finally, Zyflamend increased the level of pAKT in response to fasting but did not significantly impact its signaling in response to insulin stimulation (**Fig 4. E**). In further exploration of Zyflamend's impact on skeletal muscle function, we investigated its diet specific effects on ER stress and inflammatory signaling pathways (**Fig 5**).

It has been well established, HFD feeding promotes ER stress, inflammation and insulin resistance in murine skeletal muscle. Furthermore, accumulating ER stress and inflammation within skeletal muscle, has been identified as a key promoter of insulin resistance in aging populations [157]. Therefore, we investigated how Zyflamend treatment might impact these signaling pathways in both chow and HFD in vivo models (**Fig 5.**). Overall, we observed no significant changes to either ER stress or inflammatory signaling pathways between the chow and chow-Zyflamend groups (**Fig 5.**). However, significant differences were observed between the HFD ctrl and Zyflamend groups. Zyflamend significantly reduced HFD induced ER stress as evidenced by the inactivation of ER transmembrane sensors, inositol-requiring transmembrane kinase/endoribonuclease 1 α (IRE1 α), and protein kinase RNA-like endoplasmic reticulum kinase (PERK) with subsequent reductions in their respective downstream targets X-Box Binding Protein 1 (XBP1) and eukaryotic translation initiation factor 2 alpha (EIF2 α) (**Fig 5.**). In addition, Zyflamend reduced the cleavage of the ER stress sensing activating Transcription Factor 6 (ATF6) and reduced the phosphorylation of inflammatory promoter's JNK and p38 MAPK in the HFD model. These findings provide evidence for the intrinsic mechanisms by which Zyflamend might promote insulin responsiveness in skeletal muscle. In order to better understand the scope of Zyflamend's metabolic implications, we next investigated its impact on white and brown adipose tissue.

As evidenced by Tague et al., the actions of Zyflamend within adipose tissue appear to be mediated at least in part through AMPK activation [228]. In support of this finding, incorporation of Zyflamend into both chow and HFD effectively induced AMPK phosphorylation and activation in murine WAT and BAT (**Fig 6.A**). In addition, concomitant activation of thermogenesis evidenced by elevated expression of brown and/or beige (UCP1, PRDM16, CIDEA CD137, BMP7, and ELOVL3) markers was observed in both tissues (**Fig 6.B**). Moreover, Zyflamend reduced the level of white fat marker (HOXC8) in WAT. However, it is worth noting that HOXC8 was slightly increased in BAT. This was further confirmed through tissue immunostaining for UCP1 in BAT and subcutaneous WAT of mice fed chow and HFD (**Fig 6.C**). Further promoting thermogenic reprogramming, Zyflamend supplemented chow (**Fig 6.D**) and HFD (**Fig 6.E**) fed animals, maintained significantly higher body temperatures when exposed to the cold.

Collectively, our data suggest Zyflamend might possess significant anti-obesogenic potential and warrants further investigation.

The anti-obesogenic effects of metformin remain controversial [323, 324]. However, since both Zyflamend and metformin activate AMPK, we sought to test the hypothesis that Zyflamend synergistically enhances metformin-induced brown adipogenesis. This hypothesis is based on studies showing that metformin alleviates HFD-induced obesity by inducing FGF21 expression and brown adipocyte differentiation [325]. As shown in **Figure 7**, treatment of murine primary (**Fig 7.A**) and established (**Fig 7.B**) brown adipocytes with Zyflamend and metformin synergistically increased the expression of brown fat markers (UCP1 and PRDM16). Moreover, Zyflamend and metformin synergistically enhanced the expression of PGC1 α , a master regulator of mitochondrial biogenesis and respiration, and adaptive thermogenesis. Notably, similar levels of UCP1 expression were observed when cells were treated with Zyflamend and 0.5-1 μ M of metformin compared to metformin alone. These data validate our hypothesis and the potential use of Zyflamend as an agent against MetS and obesity.

Discussion

As evidenced, the polyherbal encapsulation known as Zyflamend has proven experimentally efficacious against numerous forms of cancer [192, 211, 326]. Zyflamend is a commercially available well-defined poly-herbal blend, with each herb included at a relatively lower dose in comparison to standard single-nutrient supplements and compounds. We recently investigated the anti-obesogenic potential of Zyflamend, mainly because it robustly activates AMPK [195, 228]. Using doses that are equivalent to those recommended by the manufacturer for humans, we demonstrated that oral administration of Zyflamend reduced fat mass and plasma levels of non-essential fatty acids in mice [13]. These changes were concomitant with inhibition of ACC and increased AMPK phosphorylation in the liver and adipose tissues [13]. In this study, we examined the potential effects and molecular mechanisms mediating Zyflamend's impact on high fat diet-induced metabolic dysregulation, muscle insulin sensitivity, and adipose tissue.

In order to investigate the impact on weight gain, metabolic parameters, glucose homeostasis and insulin tolerance, mice were administered either chow or HFD with or without Zyflamend. Promoting its anti-obesogenic potential, Zyflamend ameliorated HFD induced weight

gain. Regarding adiposity, Zyflamend treatment reduced the weight of all WAT depots analyzed. In addition, the Zyflamend HFD fed mice were more effective at clearing plasma glucose following either glucose or insulin injections. Moreover, Zyflamend exerted protective effects against the negative metabolic consequences of obesity as evidenced by decreased FFA and increased HDL-C. While significant, the mechanisms and tissue specific effects through which these findings were propagated, remained undetermined.

Long considered synonymous with MetS, insulin resistance is an intricate, and progressive disease that can substantially burden physiological systems. At the cellular level, the monosaccharide glucose is the preferred source of energy within most tissues and its uptake into the cell is reliant on insulin signaling. For a myriad of reasons, insulin sensitivity and signaling can be reduced, impeding the cellular uptake and utilization of glucose, particularly by the skeletal muscle and liver. Consequently, this can result in hyperglycemia, and if left uncorrected can lead to the manifestation of MetS and ultimately T2DM. This is arguably most apparent within the insulin secreting pancreatic beta islets where progressive autoimmune destruction or dysfunction of these cells can be exacerbated by both insulin resistance and hyperglycemic conditions [327]. Overall, improving insulin responsiveness and promoting glucose uptake in adipose tissue and skeletal muscle is a central component of managing or treating MetS.

When properly functioning and healthy, skeletal muscle tissue is highly effective at clearing plasma glucose in response to insulin signaling. However, the insulin responsiveness and glucose uptake of skeletal muscle can be impeded by a variety of interconnected factors of MetS and obesity. Investigations aimed at improving skeletal muscle insulin sensitivity using natural compounds have revealed promising findings. For instance, a large volume of plants and derivatives from garlic, curcumin, fenugreek, grapes, and cinnamon, have shown the capacity to increase insulin sensitivity and/or reduce insulin resistance [317]. In addition, phytochemicals and flavonoids extracted from plants such as black ginseng (GBG05-FF) [328], *Gymnema sylvestre* leaves (dihydroxy gymnemic triacetate) [329], olive leaf [330] and various citrus species (sudachitin [331], and quercetin [332]) have all shown potential to ameliorate insulin resistance, at least in part, through their beneficial effects on skeletal muscle. Individually the components and polyphenolic extracts of Zyflamend such as, curcumin [214], ginger (*Zingiber officinale*) [249], and rosemary, [250], have all been independently investigated for their impact on insulin

signaling and/or glucose uptake within skeletal muscle. The mechanisms through which phytochemicals improve skeletal muscle insulin sensitivity and function may relate to their anti-inflammatory and insulin signaling regulatory properties [248, 317, 333].

Within skeletal muscle, insulin mediated tyrosine phosphorylation of IR and subsequent phosphorylation of IRS-1 initiates the downstream signaling cascade required for the uptake of glucose [334]. Under both fasting and insulin stimulated conditions, Zyflamend enhanced skeletal muscle insulin signaling as evidenced by increased tyrosine phosphorylation of IR, IRS-1 and elevated levels of downstream signaling components p85 and pERK. Zyflamend treatment also increased the level of pAKT under fasting conditions. Taken together, these findings show that Zyflamend treatment may ameliorate the deleterious effects of HFD on insulin signaling within skeletal muscle. Considering that ER stress and inflammation have been shown to impair skeletal muscle insulin sensitivity [157], Zyflamend's anti-inflammatory properties could potentially explain these findings. In order to further assess this possible mechanism, we next investigated Zyflamend's impact on ER stress and inflammation signaling components.

It is well known that disease states and conditions associated with MetS often promote metabolic dysfunction and hyperglycemia. In addition, the abnormal conditions promoted by MetS can result in elevated levels of oxidative stress, ER stress and inflammation [335]. Critical to maintaining homeostasis within the tissue, skeletal muscle cells contain a unique ER known as the sarcoplasmic reticulum (SR) [336]. Under normal conditions, a tightly regulated intracellular process known as the unfolded protein response (UPR) relies on ER-associated protein degradation (ERAD), to properly detect and dispose of unfolded or misfolded proteins. However, disease states can promote an excessive accumulation of misfolded proteins that can ultimately exceed the capacity of the ER stress response. Left uncorrected, this accumulation of ER stress can result in significant consequences to the SR and skeletal muscle homeostasis [336]. In comparison to the control diet, Zyflamend chow incorporation had no significant impact on inflammatory or ER stress signaling pathways. Within the skeletal muscle of HFD Zyflamend treated animals, we observed reduced activation of PERK and IRE1 α , both ER transmembrane sensors responsible for initiating independent signaling branches of the UPR. Furthermore, this was accompanied by inactivation of their respective downstream targets EIF2 α and XBP1. In addition, Zyflamend

reduced the cleavage of ATF6, another independent ER transmembrane signaling protein. Diversified crosstalk between aspects of ER stress response, the UPR and inflammatory signaling components has been documented [337]. For instance, IRE1 α phosphorylation has been shown to indirectly initiate the activation of JNK, a key inflammatory signaling promoter [338]. In addition, IRE1 α , PERK and ATF6 have all been shown to promote p38 induced inflammation [337]. HFD Zyflamend treatment decreased the phosphorylation of both JNK and p38. Taken together, these findings may provide a mechanistic explanation for how Zyflamend may enhance skeletal muscle insulin responsiveness in HFD mice. Beyond the impact on skeletal muscle, the metabolic implications of Zyflamend treatment may also relate to its effect on adipose tissue.

Regarding the compounds contained within Zyflamend, curcumin [231, 232], EGCg and green tea [233-237], berberine from Chinese goldthread [238-240] and Rosemary officinalis and carnosic acid [241, 242] have all been investigated for their diversified anti-adipogenic effects. As previously demonstrated, Zyflamend treatment resulted in reduced adiposity attributed to its regulation of lipogenesis in a murine model. As identified, the regulatory functions mediated by Zyflamend were likely potentiated through AMPK activation and ACC inhibition within the adipose tissue [228]. Based on these findings, our lab investigated the cellular mechanisms through which Zyflamend might disrupt adipogenesis and lipolysis [244]. Under normal conditions, adipogenesis plays an essential biological role that allows for the expansion of adipose tissue and allows for long term storage of energy. We discovered, that within 3T3-MBX pre-adipocytes, Zyflamend treatment reduced proliferation, disrupted cell cycle progression and promoted apoptosis [244]. In differentiating cells, Zyflamend effectively increased lipolysis, evidenced by reduced lipid accumulation and concomitant reductions in lipogenic gene expression. Cell signaling investigations revealed that Zyflamend's capacity to disrupt adipogenic differentiation was potentiated through activation of JNK and PKA and was partially mediated by AMPK [244]. As evidenced herewith, Zyflamend effectively induced the phosphorylation of AMPK in the WAT and BAT of both chow and HFD fed mice. This finding is in direct correspondence with previous reports. Acting as a primary energy sensor within the cell, the AMPK pathway is activated when intracellular ATP levels are low and inactivated by increased ATP generated by catabolic metabolism. Moreover, AMPK activation has been shown to reduce insulin resistance in the skeletal muscle and adipose tissue [150, 224]. AMPK is phosphorylated and activated as a result of phytochemical treatment and is responsible for increased glucose uptake by stimulating

translocation of GLUT4 or other insulin-independent glucose transport pathways [227]. In addition to increased glucose uptake, AMPK also reduces insulin resistance by improving mitochondrial function and discouraging the storage of triglycerides in skeletal muscle [227]. While the influence of Zyflamend on AMPK signaling within adipose is substantial, it also demonstrated the capacity to modify the thermogenic program of adipose tissue.

Thermogenesis within BAT allows for the generation of heat through a biochemical mechanism heavily dependent on the uncoupling of the mitochondrial electron transport chain by UCPs. Contrary to the role of white fat, This uncoupling reaction allows for the dissipation of energy and limits triglyceride storage. Beiging, is the process where WAT is stimulated to express a more thermogenic profile that is typically associated with brown fat [339]. ECGg has been shown to impact genes involved in thermogenesis and beta oxidation [236]. Furthermore, ECGg treatment has been shown to upregulate UCP2 expression in 3T3-L1 cells [235] and in HFD fed mice [236]. Within our study, Zyflamend treatment elevated the expression of brown and/or beige markers in both the WAT and BAT of HFD fed mice. Interestingly, both chow and HFD Zyflamend treated animals proved capable of sustaining higher body temperatures in response to cold exposure. Beyond this evidence of thermogenic reprogramming, we also found that in combination with metformin, Zyflamend synergistically increased the expression of brown fat markers (UCP1 and PRDM16). Furthermore, the synergistic upregulation of PGC1 α observed in response to combination treatment provides even more evidence of Zyflamend's adipogenic regulatory potential. The diverse nature through which Zyflamend's ingredients impact adipose tissue and cells, appears substantial.

In the modern era, humankind seeks answers on how to live longer and healthier. Obesity and its associated co-morbidities present a major hurdle in achieving this goal. Unfortunately, obesity rates continue to rise and while significant discoveries have been made, the problem persists. However, reducing adiposity and increasing skeletal muscle mass and function could drastically reduce the risk for a myriad of diseases and promote a healthier lifespan, particularly in obese populations. Biologically, the human organism relies on an intricate network of organs and tissue to maintain the delicate balance required to promote and maintain homeostasis. The muscle and adipose tissue are critical to the clearance of accumulating and excessive glucose within circulatory system. In harmony, these tissues work together to maintain and promote metabolic homeostasis,

keeping blood glucose levels from rising to dangerous and damaging levels. However, these essential processes can be compromised by the destructive consequences of MetS and obesity. As a natural alternative or adjunct to conventional therapy, Zyflamend's potential to improve the functionality of skeletal muscle and adipose tissue could help alleviate the burdens of metabolic dysfunction.

APPENDIX

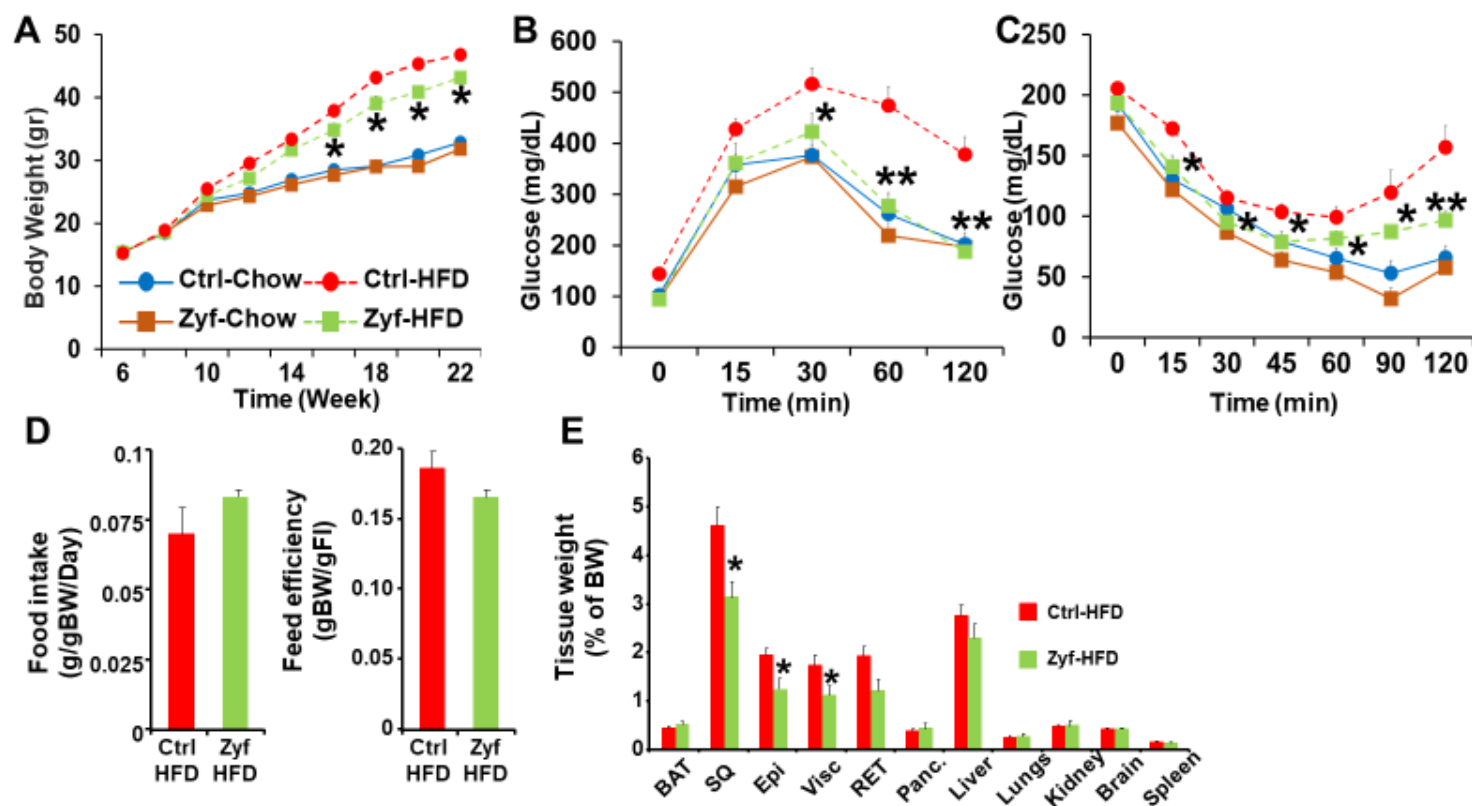


Figure 3. Effects of Zyflamend on Body weight, adiposity, food intake and glucose homeostasis

(A) Body weight of male mice fed chow or HFD for 16 weeks with and without Zyflamend treatment. (B) Glucose tolerance test (GTT) taken from all treatment groups including (Ctrl-Chow, Zyf-Chow, Ctrl-HFD, Zyf-HFD) (C), Insulin tolerance test (ITT) taken from all treatment groups including (Ctrl-Chow, Zyf-Chow, Ctrl-HFD, Zyf-HFD). (D) Adjusting for body weight, food intake and feed efficiency are compared between the Zyflamend and Ctrl HFDs. (E), Relative tissue weights of male control and Zyf-treated mice fed HFD for 16 weeks **p<0.01 and *p<0.05 to varying degrees of confidence, indicate statistical significantly different outcomes between control and Zyf-treated mice.

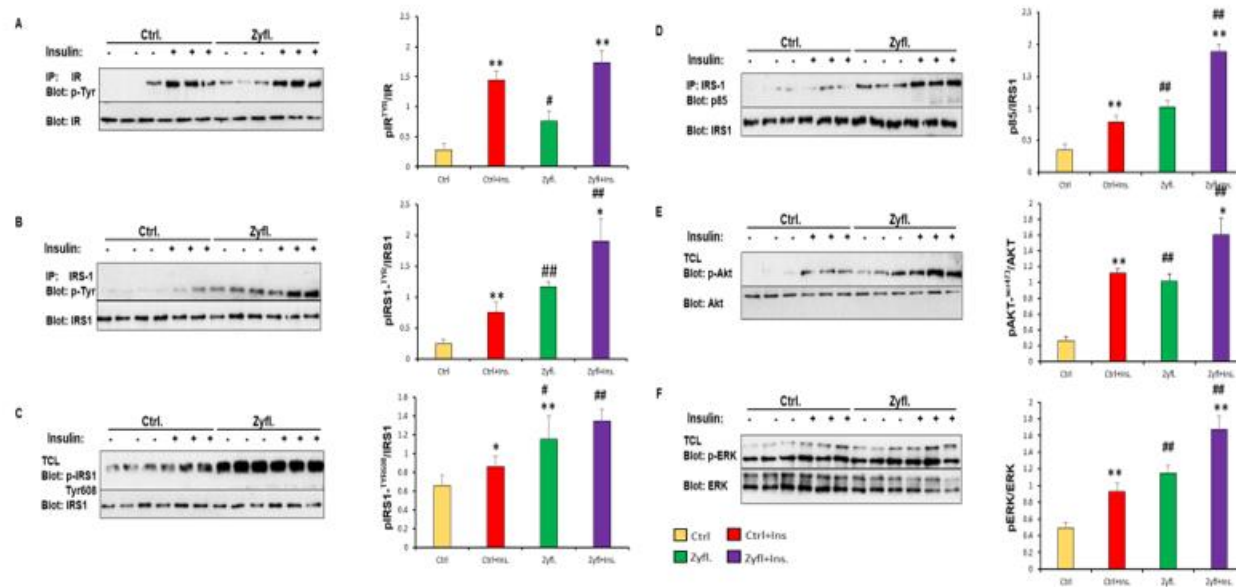


Figure 4. Zyflamend promotes skeletal muscle insulin signaling/responsiveness in a HFD model of insulin resistance.

A-F Following an overnight fast (12 hr), and exposure to HFD with/without Zyflamend and with/without insulin stimulation, skeletal muscle tissue samples were harvested independently from all experimental groups and lysates were prepared. **A-B** Level of tyrosine phosphorylation of individual components in insulin signaling IR and IRS1 proteins determined by immuno-precipitation and with respective unphosphorylated proteins as control. **C-F** Immunoblots representing components of insulin signaling p-IRS1 (Tyr608)/IRS1, p85/IRS1, pAKT/AKT, and pERK/ERK. **A-F** Bar graphs represent respective blots as means + SEM with * $p < 0.05$, ** $p < 0.01$ indicating a significant difference between HFD (12 hr) fast with or without Zyflamend, while # $p < 0.05$, ## $p < 0.01$ indicates a significant difference between the HFD insulin stimulated groups with or without Zyflamend.

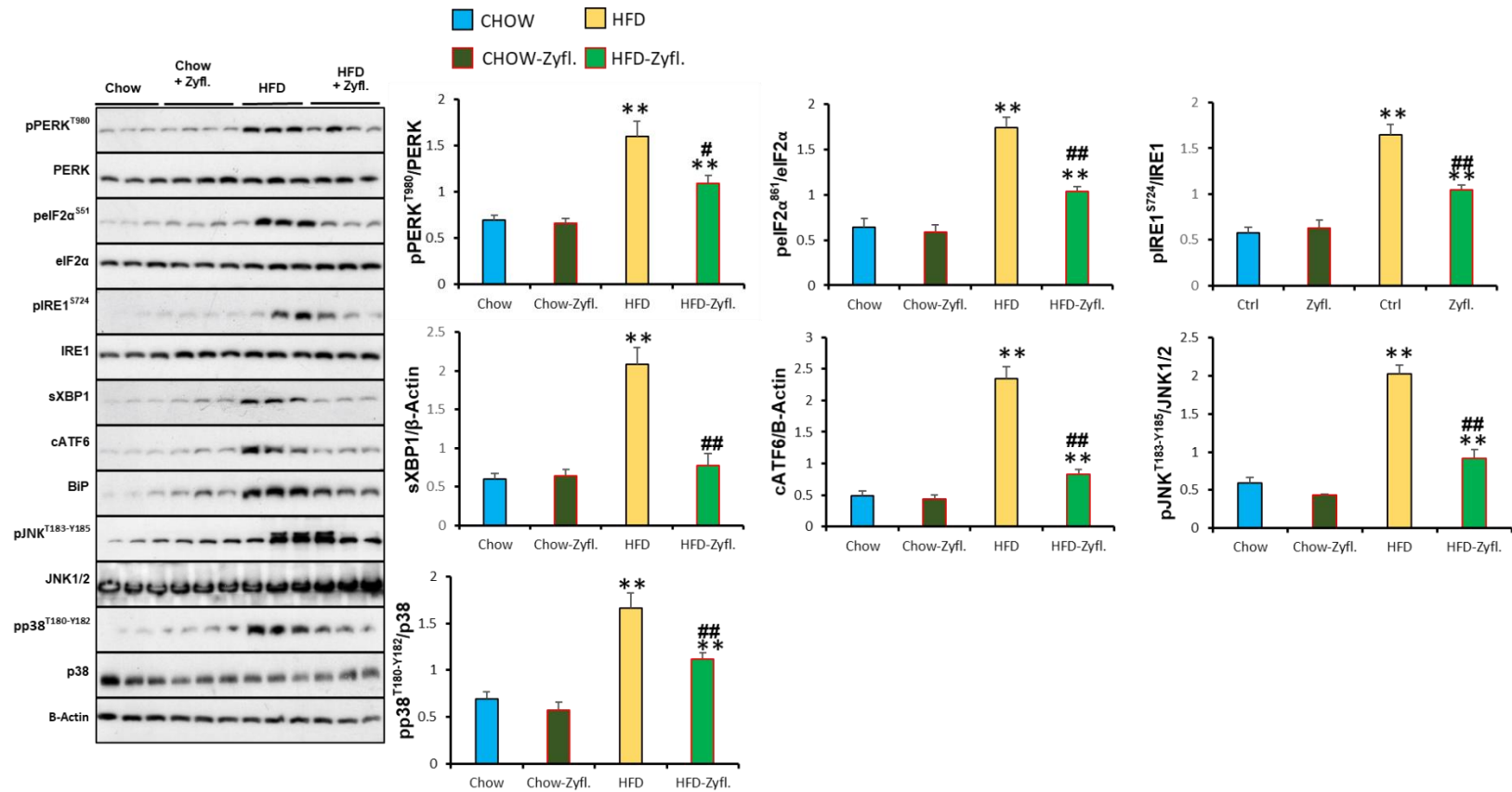


Figure 5. The diet specific effects of Zyflamend on ER stress and inflammatory signaling pathways within murine skeletal muscle tissue.

A-B. Lysates prepared from skeletal muscle tissue samples harvested independently from all diet groups (Chow, Chow-Zyf, HFD and HFD-Zyf,) were immunoblotted for ER stress markers: pPERK/PERK, pEIF2α/EIF2α, pIRE1α/IRE1, sXBP1 and ATF6 with β-actin as loading control and inflammatory markers: pJNK/JNK1/2, and pp38/p38. **B.** Bar graphs represent pPERK/PERK, pEIF2α/EIF2α, pIRE1/IRE1, sXBP1/β-actin, cATF6/β-actin, pJNK/JNK1/2 and pp38/p38, as means ± SEM. * $p < 0.05$, ** $p < 0.01$ indicate a significant difference between Chow Vs HFD for both, ctrl and Zyflamend treated mice while # $p < 0.05$, ## $p < 0.01$ indicates a significant difference between Zyflamend treated vs non-treated mice regardless of the diet.

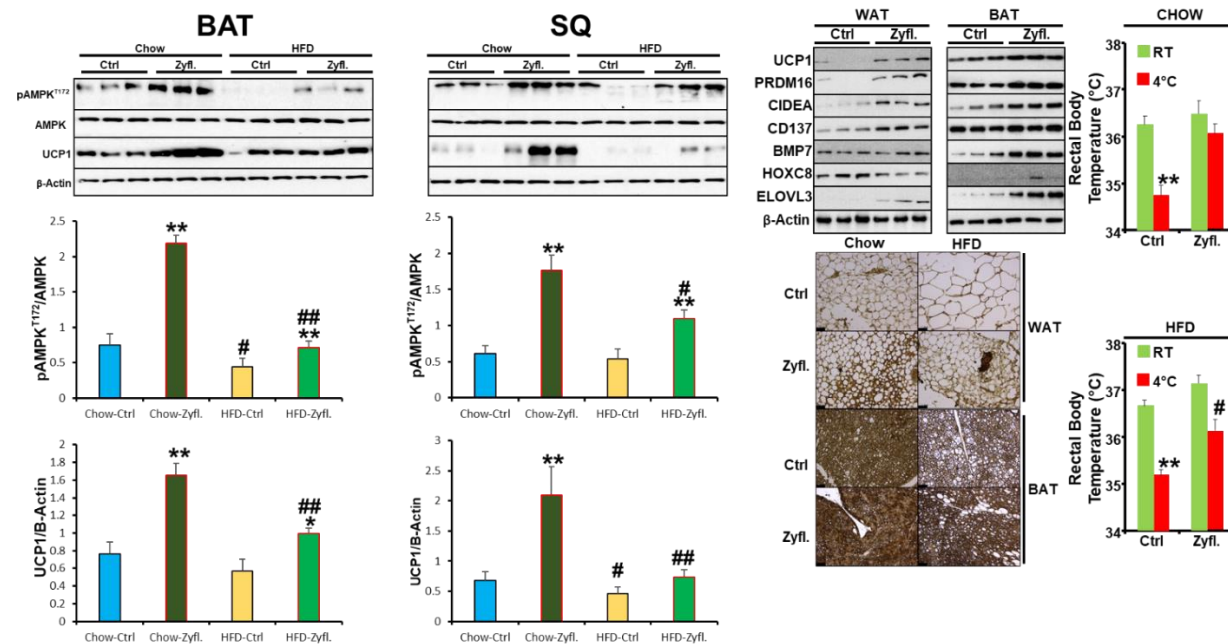


Figure 6. Diet specific effects of Zyflamend on adipose tissue and thermogenic reprogramming

A. Lysates prepared from brown (BAT) and white (SQ) adipose tissue samples harvested independently from all diet groups (Chow, Chow-Zyf, HFD and HFD-Zyf,) were immunoblotted for ER stress markers: pAMPK/AMPK, and UCP1 with β -actin as loading control and for evidence of thermogenic reprogramming (**B**) with UCP1, PRDM16, CIDEA CD137, BMP7, ELOVL3 and white fat marker HOXC8, all with β -actin as loading control. **A.** Bar graphs represent pAMPK/AMPK, and UCP-1/ β -actin, and means + SEM. * $p < 0.05$, ** $p < 0.01$ indicate a significant difference between Chow vs HFD for both, ctrl and Zyflamend treated mice while # $p < 0.05$, ## $p < 0.01$ indicates a significant difference between Zyflamend treated vs non-treated mice regardless of the diet. **C.** Subcutaneous WAT and BAT samples from all groups were immunostained for the presence of UCP-1. Either with or without Zyflamend, mice from both chow (**D**) and HFD (**E**) groups were exposed to 4°C and rectal body temperatures were measured after 24 hrs of cold exposure. **D-E.** Again, bar graphs represent significant difference in body temperature, and means + SEM. * $p < 0.05$, ** $p < 0.01$ indicate a significant difference between between Chow vs HFD for both, ctrl and Zyflamend treated mice while # $p < 0.05$, ## $p < 0.01$ indicates a significant difference between Zyflamend treated vs non-treated mice regardless of the diet.

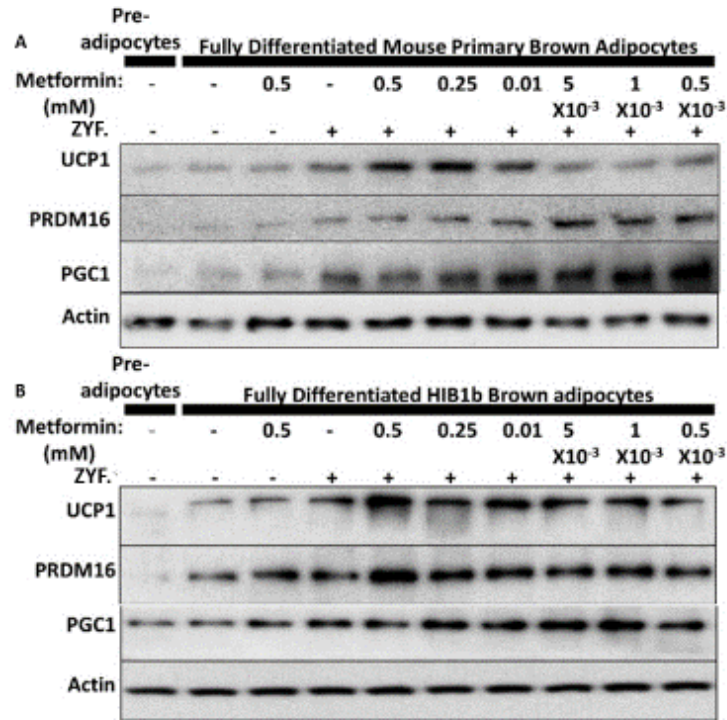


Figure 7. Zyflamend enhances metformin-induced brown adipogenesis

A-B. With untreated pre-adipocyte lysates as control for differentiation, cell lysates from mouse primary and HIB1b brown adipocytes were analysed in response to metformin and Zyflamend treatment both alone and in combination. Fully differentiated adipocytes from both cell lines were independently treated either with and without metformin at 0.5 mM, and with or without Zyflamend at (200 μ g/ml). In addition, fully differentiated cells were treated with decreasing concentrations of metformin (0.5, 0.25, 0.01, 5×10^{-3} , 1×10^{-3} , and 0.5×10^{-3} in combination with Zyflamend (200 μ g/ml).

CHAPTER 4

CONCLUSION AND FUTURE PERSPECTIVES

Conclusion

In the modern era, humankind seeks answers on how to live longer and healthier. Obesity and its associated co-morbidities present a major hurdle in achieving this goal. Unfortunately, obesity rates continue to rise and while significant discoveries have been made, the problem persists. However, reducing adiposity and increasing skeletal muscle mass and function could drastically reduce the risk for a myriad of diseases and promote a healthier lifespan, particularly in obese populations. Biologically, the human organism relies on an intricate network of organs and tissue to maintain the delicate balance required to promote and maintain homeostasis. The muscle and adipose tissue are critical to the clearance of accumulating and excessive glucose within circulatory system. In harmony, these tissues work together to maintain and promote metabolic homeostasis, keeping blood glucose levels from rising to dangerous and damaging levels. However, these essential processes can be compromised by the destructive consequences of MetS and obesity. As a natural alternative or adjunct to conventional therapy, Zyflamends potential to improve the functionality of skeletal muscle and adipose tissue could help alleviate the burdens of metabolic dysfunction. Furthermore, Zyflamend appears to possess significant promise as a CR mimetic through its impact on signaling pathways central to the effects and benefits of CR.

Future Perspectives

As we have demonstrated, Zyflamend ameliorated HFD induced weight gain, improved skeletal muscle insulin signaling, and promoted thermogenic reprogramming in adipose tissue. While these findings were encouraging, future studies targeting Zyflamends efficacy and safety are warranted. Furthermore, future mechanistic studies investigated the cell signaling impact of Zyflamend on skeletal muscle could substantiate its role in the tissue. Finally, clinical studies involving obese and metabolically compromised patients could help solidify the translational potential for our findings.

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VITA

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