12-2019

Zyflamend Inhibits Adipogenesis: A mechanistic study evaluating the impact of phytonutrients on adipocyte differentiation

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To the Graduate Council:

I am submitting herewith a thesis written by Victoria Frankel entitled "Zyflamend Inhibits Adipogenesis: A mechanistic study evaluating the impact of phytonutrients on adipocyte differentiation." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Nutritional Sciences.

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Zyflamend Inhibits Adipogenesis: A mechanistic study evaluating the impact of phytonutrients on adipocyte differentiation

A Thesis Presented for the
Master of Science
Degree
The University of Tennessee, Knoxville

Victoria Danielle Frankel
December 2019

ACKNOWLEDGEMENTS

First and foremost, I would like to thank my sister (future Dr.) Sarah Elizabeth Frankel for her unending support through my academic career. She has been my biggest fan and her love, encouragement, and analytical mind have been essential for my growth as an individual and as a researcher.

I would also like to thank my committee; Dr. Ahmed Bettaieb, Dr. Jay Whelan, and Dr. Dallas Donohoe for their continual support for the project. Dr. Bettaieb in particular helped me develop my scientific research and critical thinking skills.

A particularly special thank you to Dr. Whelan and Ms. Lee Murphy for providing me multiple opportunities to focus more on my academic development through funding opportunities. Their support provided me an opportunity to finish the master's program at the University of Tennessee within a reasonable time frame; something I do not believe would have happened without the financial flexibility afforded to me by my teaching assistantship.

And to other nutrition faculty who have supported my research development: Dr. Ling Zhao, whose encouragement to pursue new research opportunities has led me down exciting paths.

I would also like to thank my mentor, Dr. Michael Zemel, for introducing me to the nutrition program at the University of Tennessee and captivating my mind with tales of science and discovery, inspiring greatness in me. Dr. Zemel's

insight has shifted my perception of personal weaknesses and helped to identify my strengths in my professional development.

The emotional support given to me by my inspirational and motivational colleagues within the Nutrition department, specifically Haley Overby, Emily Simon, and Dexter Puckett, have shown me how strong and budding scientific minds can impact your success as a researcher and as a member of society.

I would also like to extend a special thanks to additional graduate students and dear friends outside of the nutrition department for their encouragement, support, love, and belief in my success: Leslie Cuevas, Anna Jaggers, and Jacob Kowalski.

In addition, I would like to thank my parents Dr. Jay I Frankel and Ms. Valerie Frankel for their profound acceptance of all the various paths I've taken to arrive here in this program. They have had profound patience as I've found my way. There are many individuals who are not nearly as lucky as I am to have such caring and enthusiastic parents who support my sincere curiosity and passion for nutrition and science. Despite the difficulties I have raised as an inquisitive daughter, they have done their best to answer all my questions and direct me down a quest for knowledge.

Last but not least, to my beloved fur-daughter Saki Frankel, for whom I do everything. Your love has held me through all the obstacles I have encountered. If not for you, I would be lost.

ABSTRACT

Zyflamend, a polyphenol herbal supplement, includes numerous different extracts. This botanical complex has been used as a natural anti-inflammatory due to its impact on COX-2. More recently, research has exhibited its potential as an anti-cancer agent. Currently, the effects of Zyflamend in non-cancer cells, specifically adipocytes, has yet to be explored. Due to Zyflamend's ability to promote activation of AMPK, it may prove relevant to study the potential interactions with cells treated with Zyflamend during differentiation, a metabolically involved process. This study sought to determine the impact of Zyflamend on non-cancer cells such as 3T3-L1 adipocytes during differentiation and identify the mechanisms involved. Zyflamend was shown to effectively and significantly attenuate adipogenesis by downregulating markers of lipogenesis, decreasing glucose uptake by downregulating GLUT4, and upregulating hormone-sensitive lipases. These effects halted differentiation of 3T3-L1 cells and altered their metabolic dynamic. As evidenced in previous research, Zyflamend was found to increase phosphorylation AMPK, affecting fatty acid metabolism. Furthermore, an upregulation of PKA-regulated lipolysis through pHSLS660 was unexpectedly observed. In addition, Zyflamend drastically increased activity of JNK. Through investigation of these effects, partial rescue of adipogenesis was observed through treatments with PKA inhibitor H89 and AMPK inhibitor BML275; however, the presence of JNK inhibitor SP600125 showed the strongest rescue over the effects of Zyflamend on differentiation. These results suggest Zyflamend's effect on

attenuating adipogenesis may be modulated in part by upregulation of JNK and PKA. The ability of Zyflamend to attenuate the adipogenesis process suggest a potential to target cell differentiation mechanisms relevant to energy metabolism.

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CHAPTER ONE

INTRODUCTION

1. Obesity

Obesity affects over two thirds of the nation and is associated with increased risk of a multitude of comorbidities including cardiovascular disease, diabetes, and cancer. Understanding how adipose tissue develops and matures is essential to the development of dynamic intervention strategies to counter its prevalence. Previous literature has comprehensively explored adipose tissue's ability to store excess energy and regulate metabolic requirements. Addressing the mechanisms associated with adipocyte differentiation has made adipogenesis a viable target for a variety of therapeutic approaches. While current pharmaceuticals tend to combat symptoms of obesity, the safety of these medications is often disputed. On the other hand, a growing body of evidence suggests that a new era of nutraceuticals may provide health specialists new means of treating the disease through the use of phytochemicals.

Overview

Over the last few decades, the prominent trend in increasing body weight has sparked concern for a worldwide obesity epidemic. Obesity has been correlated with the prevalence of many other diseases such as cancer, metabolic disorders, and other illnesses[1]. Epidemiological studies during this time have sought to identify the relationship obesity may play in these occurrences[2]. This has resulted in an international scientific movement exploring causes and symptoms of obesity and investigating its pathogenesis.

"Obesity" is a term coined through the use of the body mass index screening tool with a designated value of 30.0 or greater. Although not a clinical diagnosis, many health professionals continue to use BMI as a means of determining weight status and health risk. It is assessed by calculating weight in relation to height, but can be further supported through other direct measurements determining overall fat density. These methods may range through the use of skin calipers, measuring hydration levels, fatness density via densiometers, or through dual energy x-ray absorptiometry (DXA scan).

In the last forty years, researchers have identified a distinct pathology of obesity increasing the interest in potential therapeutic interventions. Improving activity levels and decreasing dietary intake have shown the most promising evidence in reducing symptoms of obesity, but as cases become more extreme, other more severe methods have been considered. These cases have ranged from surgical procedures to reduce stomach size, to pharmaceutical alternatives that mediate symptoms or alter nutrient absorption. Instead, efforts to find alternative effective therapeutic interventions that rely less on pharmaceuticals may counter disease-based physiological changes.

2. Obesity and Health

Epidemiology and Current Statistics

The National Health and Nutrition Examination Survey, through the NCHS, has conducted a stratified, multistage probability sample of the civilian noninstitutionalized population of the United States[1]. This data focused on the prevalence of overweight, obesity, and severe obesity among adults age 20 and over between the years of 1960-2016. According to their findings, 39.8% of adults in the United States are currently obese, with 70.1% as overweight or obese. Variances in BMI were accounted to include differences among genders, ethnicity, demographic, and age. Designated a chronic medical disease state, obesity is responsible for a number of comorbidities including cardiovascular disease, diabetes, kidney disease, cancer, as well as many other disease related deaths and continues to be a spreading epidemic[2, 3].

Etiology and Pathophysiology

The development of obesity can result from behavioral, social, cultural, genetic, and physiological factors[4]. This exposes the pathology of obesity to be of complex, multidimensional origins. Occurrence of obesity is dependent on an imbalance between dietary intake and energy expenditure. As intake supersedes energy requirements, physiological mechanisms occur to store excess energy in case energy needs shift (such as during starvation or activity). The development

of adipose tissue is the body's means of providing energy storage sites in cells called adipocytes. Among many other functions, adipocytes are responsible for excess energy storage in the form of triglycerides and have the ability to increase their volume up to several thousandfold to accommodate excess lipid storage[5].

Many institutes, such as The World Health Organization Consultation on Obesity, have focused predominantly on behavioral and environmental factors responsible for dramatic increases in dietary intake[4]. An increase in sedentary lifestyles coupled with excessive energy intake has been suggested to be primarily responsible for changes in adiposity[6-8]. Changes in dietary patterns contributing to preferences towards sugar-sweetened beverages, high fat, and high nutrient dense foods appear to be the culprit in many studies[9, 10]. Economic changes in fast-food and convenience food institutions providing budget-friendly, high calorie foods have also contributed to the ease of access of obesity-promoting foods[10]. Despite substantial evidence suggesting environmental and behavioral influences increase risk of obesity, additional confounding evidence has been unable to explain every occurrence. This has supported the paradigm shift of research efforts to begin investigating additional genetic, epigenetic, and physiologic influences.

As adipose tissue increases, changes in metabolic function and health have been observed in a multitude of studies[2]. Obesity is a major contributor to metabolic dysfunction, resulting in changes to glucose intolerance and insulin resistance[11]. In addition to adipocytes' ability to store excess energy, adipose

tissue exhibits endocrine system functions by regulating fat metabolism through secreted hormones and growth factors[12]. In cases of obesity, many studies have suggested that dysregulation of hormones may contribute to symptoms that perpetuate the disease and increase the risk of comorbidities[13]. Also known to influence organ dysfunction, obesity has been shown to increase prevalence of cardiac, liver, intestinal, pulmonary, endocrine and reproductive diseases[13].

In some cases, as increases in adipose deposits occur the body reaches a degree of capacity with lipid storage and resorts to adipose deposits in other sites. This contributes to diseases such as non-alcoholic fatty liver disease (or NAFLD) and other diseases where adipose deposits affect organ health[14, 15]. Exhausted storage of triglycerides in adipocytes prevents adequate clearance of circulating fatty acid levels resulting in hypertriglyceridemia and lipotoxicity[13]. The production of inflammatory adipokines, or cytokines secreted by adipocytes, is associated with the development of hypertension and changes in endocrine secretion. Changes in inflammatory response have displayed coordinated direct effects on immune dysfunction, heightening risks of cancer, including hepatocellular, esophageal, and colon cancers[15, 16]. The cumulative and accelerating effects of obesity have been profound on modern society and its resolution of equally profound significance in improving health worldwide.

Biology of Adipose Tissue

In mammals, adipose tissue sites are formed and support excess energy stores. White adipose tissue (WAT) and brown adipose tissue (BAT) can function

in similar ways: through storage of energy. However, they vary in phenotype, primary function, and metabolic capacity. The principal role of WAT is as an endocrine organ and energy storage site; regulating glucose uptake and lipogenesis/lipolysis pathways. WAT's ability to mobilize lipid deposits makes it instrumental in a body system's metabolic capacity. BAT varies in phenotype by exhibiting multiple, smaller lipid droplets and increased numbers of mitochondria with uncoupling protein-1 (UCP-1), facilitating heat production by bypassing the respiratory chain to ATP synthetase[16]. The unique metabolic variances between the two types has developed a strong interest in the development and regulation of BAT due to its potential role in protection against obesity and other metabolic disorders.

Brown and white adipocytes share the ability to expand substantially to encapsulate large levels of lipids. The ability of mature adipocytes to swell previously lead scientists to believe adipose tissue consisted exclusively of adipocytes, however recent studies have shown that adipose tissue has a large variability of cell type. Many different types of cells can form vascular stroma cells, including pre-adipocytes and macrophages; accounting for 10% of WAT in obese individuals[17]. Other cell types include stem cells, endothelial cells, and the presence of neutrophils and lymphocytes. Adipose depots may additionally be influenced by the level of inflammation produced from surrounding tissue, causing each depot site to be unique in its makeup[17].

As the state of obesity progresses, adipose tissue undergoes various cellular and structural modelling processes, such as tissue expansion by adipocyte hypertrophy and hyperplasia[18]. Adipocytes can facilitate rapid remodeling of the extracellular matrix to allow for tissue expansion improving their level of plasticity to retain normal lipid storage[19]. Research has suggested that appropriate WAT plasticity seems to act as a safeguard against metabolic disorders[20]. Improving the plasticity of adipose tissue by increasing its anabolic function has been a strong focus for therapeutic strategies. Other focuses have investigated “browning”, or the conversion of white adipocytes to acquire more phenotypic similarities to brown adipose tissue. This term describes the process of increasing the thermogenesis function through recruitment of pre-existing BAT activation or BAT differentiation factors[21, 22]

White Adipose Tissue

Development Origin of WAT

White adipose tissue (WAT) coordinates systemic metabolism through energy storage and hormone regulation[23]. The developmental pathway of WAT is a dynamic force that responds to homeostatic and external cues. Changes to this process have been associated with the development of various disease states resulting in hyperglycemia, hyperlipidemia, hypertension, diabetes, liver disease, increased carcinogenesis, cardiovascular disease, and obesity[24].

The formation of adipose tissue begins *in utero* during the peripartum period and continues throughout life[25]. In humans, the first "fat lobules" seem to be formed at the head level, around the 14–24th week of gestation[26]. These lobules then progressively appear in the trunk, then in the limbs, and by 28 weeks they can be detected in all presumptive visceral and subcutaneous WAT locations. In rodents, WAT cannot be macroscopically detected during embryogenesis and develops mainly after birth, first in the perigonadal and subcutaneous depots, and only later in the omental depot[27]. In humans, adipocytes are postulated to arise from mesodermal stem cells typical to the adipose tissue stroma[28]. These adipose mesenchymal stem cells form pre-adipocytes. This initial phase known as "determination" is characterized by their loss of ability to differentiate into other mesenchymal paths; committing to their adipocytic lineage[28]. Terminal differentiation is then initiated, whereby pre-adipocytes take on adipocyte characteristics and differentiate into mature white adipocytes through cascade events triggered by IGF-1, macrophage colony stimulating factor, fatty acids, prostaglandins, and glucocorticoids[29]. These positive stimulators work to activate transcription factors PPARγ and CCAAT/enhancer binding protein (C/EBP) known to promote adipogenesis[30].

Although typically the cellular content of adipose tissue is made of adipocytes, stromal vasculature fraction of fibroblasts, endothelial cells, macrophages and pre-adipocytes, WAT in humans expresses a unique depot-specific genetic profile depending on site or origination[31]. This has been well

evidenced in human and mice studies, suggesting a varying developmental program adding to the unique heterogeneity of the tissue[32]. Depot-specific adipocyte compositions account for the gene and developmental differences found in subcutaneous (SAT) and visceral (VAT) WAT locations. The function of VAT is genetically unique to account for different metabolic functions and activity in comparison to SAT and even peripheral adipose tissue. VAT is comprised of intraperitoneal (including omental, mesenteric, and umbilical), extraperitoneal (peripancreatic and perirenal), and intrapelvic (gonadal, urogenital) adipose tissue. These anatomic locations are associated with increased metabolic activity, compared to subcutaneous (truncal, mammary, gluteofemoral) adipose tissue depots[73].

In addition, a preference for depot sites has been reported between genders; women tend to accumulate fat around peripheral and subcutaneous depots resulting in a “gynoid” distribution. Conversely, men have increased visceral depot deposits representing an “android” distribution[33]. Although women typically present a higher fat mass by roughly 10% in comparison to men, the distribution of the fat mass seems to alleviate the influence of the risk in associated diseases[34]. Sex hormones are responsible in modulating this distribution with estrogen levels supportive of fat mass accumulation in the femoral-gluteal subcutaneous depots. Additionally, stress hormones such as glucocorticoids have been found to induce fat accumulation in neck and upper back depots over prolonged exposure[35]. Several reviews have been written

linking glucocorticoids to Cushing's syndrome, impaired peripheral glucose uptake, dysregulated blood pressure and obesity[34, 36].

Distinctive consequences are associated with each adiposity distribution between depots. It is important to note that there is clinical evidence supporting that increased visceral adipose tissue depots relate to increased risk factors of type 2 diabetes, hypertension, and lipidemia[32]. This may account for the increased prevalence of men exhibiting symptoms of metabolic and cardiovascular disease in comparison to women of similar age. Alternatively, increased SAT accumulation has been shown to intensify coronary heart disease (CHD) and peripheral vascular disease risk[37].

Varying levels of bioactive molecules and their influence on enzymatic processes associated with fat metabolism have also been reported between SAT and VAT. Intrinsic differences in various gene expression levels, such as increased expression of Shox2, En1, HoxC9 and Gpc4 have been found in subcutaneous depots[31]. In addition, visceral depots expressed higher transcription of genes Sfrp2, Nr2f1, Thbd, HoxA5, HoxC8 and Tbx15[31]. These differences are not concentrated to variances solely between subcutaneous and visceral locations, but seem to continue across other anatomical locations, although the relationship between VAT and SAT has been heavily investigated due to their varying associations with metabolic disease.

Further distinctions in characteristics include the preference of WAT depots to adjust growth type to favor adipocyte cell number (hyperplasia) over

cell size (hypertrophy) and vice versa. As suggested, type of stem/progenitor cells expressed in each depot can account for the variances between proliferative and adipogenic properties[25]. Expansion over proliferation can have impressive connotations depending on the depot of origination. Adipocyte hypertrophy has been shown to have a lesser impact on an altered lipid profile in women over adipocyte hyperplasia[38]. Although these cases may be specific to each depot, it has been suggested that a 10% increase in omental adipocytes (as part of VAT type depots) increases hyperlipidemia risk 4-fold from baseline, whereas enlarged subcutaneous adipocytes failed to significantly alter hyperlipidemia risk[38].

To influence this balance between hypertrophy and hyperplasia, growth factors in adipocytes are dependent on a strong cross-talk relationship between adipose cells to support energy storage demands. Clonal pre-adipocytes, a subtype typical of subcutaneous WAT depots, undergo extensive lipid accumulation, and express strong replicative and adipogenic characteristics resistant to TNF-alpha induced apoptosis[39]. This tissue seems to express low-density lipoprotein-related protein (LRP)-5 in the WNT signaling cascade as a major regulator for its distribution[40]. However, visceral depots have markedly weaker characteristics in both inheritable and cell-autonomous manners despite confirmation of same pre-adipocyte lineage[41]. These indicators contribute to the extent of hypertrophy/hyperplasia represented in the anatomic depot.

Genetic and Epigenetic Control of WAT

As discussed, WAT's difference in phenotype from BAT originates with its own distinct genotype. The differentiation of white adipocytes requires a myriad of transcription factors elegantly working together coordinating processes that often work with each other. When the conditions are ripe for adipogenesis, the sequence is activated through the transcription factor activating protein-1 (AP-1). AP-1 triggers the induction of PPARγ; which is essential for trans-differentiation of mesenchymal stem cells, or MSC's, to initiate pro-adipogenic transcription factors. To shift toward mature adipocyte differentiation, other transcription factors are employed to stimulate, activate, and regulate the process toward growth and proliferation.

AP-1

AP-1 consists of a family of transcription factors including v-Jun, JunB, JunD, c-Jun, v-Fos, Fos B, c-Fos, ATF2/3, B-ATF, and Fra1/2[42]. The role of these transcription factors is to employ direct interaction with DNA to regulate gene expression involved in differentiation and proliferation. Evidence supporting the role of AP-1 members and their role in adipocyte differentiation originated from research investigating the effects of transgenic mice generated to interrupt DNA binding with C/EBP and Jun family transcription factors[42]. This produced

mice lacking white adipose tissue and elucidated the necessity of AP-1 transcription factors in adipogenesis[43].

WNT

The wingless-type MMTV integration site family members, or WNT family, is responsible for the regulation of adipogenesis and is comprised of 19 secreted glycoproteins that mediate adult tissue homeostasis through multiple pathways[44]. These proteins act locally on autocrine and paracrine growth factors and mediate cell to cell communication essential for adipocyte maintenance and remodeling by repressing adipogenesis when needed[45]. In experiments disrupting the canonical WNT signaling pathway, preventing the convergence of receptors Frizzled(Fzd)/LRP-5/6 in stimulating the transcriptional factor ß-Catenin pathway, spontaneous adipogenesis occurs, suggesting a strong correlation with WNTs in restraining pre-adipocyte differentiation by blocking induction of PPARγ and CEBPA[46, 47]. Adipocyte fate determination and stem/progenitor cell self-renewal is associated with this pathway, though conversely, a noncanonical WNT pathway can also occur[47]. The ß-catenin independent noncanonical pathway is associated more with regulating crucial events associated with embryonic development. It differs from its sister pathway by circumventing its reliance on ß-catenin and triggering other receptors outside of Fzd; however, little is known about how the non-canonical receptor is activated[47].

KLF

Other transcription factor families interact with zinc-finger DNA domains, such as the Kruppel-like family. KLF's have been implicated in cell development, differentiation, and proliferation and display critical roles of gene activation and suppression[48]. To date, 17 members have been identified; in adipogenesis, however, KLFs 4, 5, 6, and 15 have proven to be stimulators of adipogenesis[49]. Several studies have identified these factors to be highly expressed in WAT pre-adipocytes during differentiation and may also contribute to lipid accumulation and insulin sensitivity[49-51].

C/EBP

Additionally, consider the role of "leucine zipper" transcription factors in the family of CCAAT/enhancer-binding proteins (C/EBPs). Each of the six isoforms of C/EBPs serve as an interaction point between sequence-specific DNA and bZIP domains connecting other transcription factor families[52]. Ample research has supported the observation that C/EBPs promote adipogenesis. In vivo studies have compared the relationship of the various isoforms and their role in adipose tissue development. In general, C/EBP deficient mice are marked by low levels of lipid accumulation and defective adipocyte proliferation[53, 54].

SREBP-1

During an investigatory study by the Speigelman group examining basic helix-loop-helix DNA binding proteins, a novel member of these bHLH-leuzine zipper transcriptions factors was identified[55]. Originally termed “adipocyte determination and differentiation factor-1” or ADD-1 in mice studies, the human homolog was renamed as the sterol response element-binding protein-1, or SREBP-1[56]. SREBP’s contain three isoforms (SREBP-1a, -1c, and -2) that function as critical factors of adipocyte development that binds to the sterol response element in the promoter region of low-density lipoproteins receptors[57]. In vitro studies have shown that point-mutations inhibiting the expression of ADD-1/SREBP-1 repressed adipocyte marker genes and prevented differentiation[58]. Efforts to mirror results in *in vivo* studies have been less consistent; transgenic mice with SREBP-1 knockout expression developed adipose tissue with limited inhibition of adipocyte markers lipoprotein lipase and adipocyte lipid binding protein[59]. Despite other studies conferring that SREBP-1 may not be required for adipogenesis, further analysis is necessary to determine its full role in adipose tissue proliferation and development[60].

Epigenetic modifications through DNA methylation and histone modifications can also result in influential changes to regulating gene expressions and shifts in phenotypes influencing states of disease. Gene repression has historically been associated with epigenetic changes in the context of CG dinucleotides (CpG islands) through changes in body mass index,

physical activity, and type 2 diabetes[61]. These changes have been associated with altered cellular composition of WAT and not necessarily linked to true genetic changes to specific cell types[62]. Typical environmental factors known to influence the epigenetics of WAT include decreased/increased physical activity, increased/decreased dietary intake, and storage. The epigenetic influence of obesity is an expanding topic that may yield insight into disease development and prevention. In a study comparing post-obese and never-obese women, a series of genes were mapped and compared exposing 8504 demethylation sites. Nearly 30% of the genes linked to adipogenesis displayed genetic modifications due to DNA methylation[63]. Other pathways shown to be over-represented included pathways linked to cell differentiation (i.e. WNT signaling), apoptosis, and autophagy. In this particular study, researchers elucidated 18 adipogenesis genes differentially expressed due to increased body fat: IGF1, NCOR2, RARA, MEF2D, SREBF1, NRIP1, LIFR, EBF1, GDF10, KLF5, AHR and PLIN2[63]. Other genes linked to lipid turnover such as ABCG1, AGPAT4, and ACAD9 were shown to have differential gene expression, suggesting common epigenetic changes resulted in increases of lipid accumulation and lipid synthesis[63]. Other variances were noted in genes specific to obesity, such as the MRAP2 and RCAN2 gene. Interestingly, some epigenetic changes can be reversed upon weight loss, although adipocyte cell numbers are not affected. In conclusion, epigenetic marks and chromatin-modifying proteins increase adipogenesis and control metabolism through maintenance of mature adipocytes[64].

WAT Functions

The traditional role of adipose tissue is well understood; the storage of energy and the release of fatty acids when fuel is required. The extent of the role WAT plays in metabolism has proven to be more complex. An evolving concept of adipose tissue as an endocrine organ has continued to gain footing, expanding on previous interpretations. This includes adipocytes' ability to sense, manage, and send signals to adjust energy equilibrium within the body. This metabolically active tissue regulates through endocrine, paracrine, and autocrine signals that extends to other organs involved in metabolism regulation (i.e. brain, liver, muscles, and pancreas)[65].

WAT's contributes to lipid metabolism through opposing processes of lipogenesis and lipolysis. Lipogenesis is the synthesis of esterified fatty acids to form triglycerides from energy sources consumed in the diet. In humans, the liver is predominantly responsible for the process in response to a postprandial state and the process is inhibited in fasting conditions. Once released, these fatty acids are entered into circulation through chylomicrons in their non-esterified form and become reliant on adipose tissue uptake for re-esterification and eventual lipid storage[66]. Insulin released during feeding stimulates enzymes involved in fatty acid biosynthesis and uptake, including sterol regulating element-binding protein (SREBP). Once in the cell, transcription factors regulating lipolysis work to hydrolyze the triglycerides in preparation for storage[66].

Perilipin A acts to surround the triglycerides, preventing access of lipases to degrade the lipids and ß-adrenergic stimulation of adipocytes induces downstream effectors to move and increase lipid droplet accumulation in white adipocytes. Lipogenesis is required for proper energy packaging and storage, allowing for its counterpart, lipolysis, to have the proper components available for degradation. Dysregulation of lipogenesis has been linked to obesity, type 2 diabetes, and cardiovascular disease. Thus, proper regulation is necessary for signaling factors to appropriately balance the flux of lipogenesis and lipolysis pathways.

At times, when mammals experience an increased need of energy requirements, adipose tissue responds by appropriating stored fatty acids through lipolysis. The innervation of white adipose tissue from the sympathetic nervous system works to activate lipolysis through ß-adrenergic factors (i.e. epinephrine) to stimulate adipose triglyceride lipase (ATGL), hormone sensitive lipase (HSL), and perilipin A phosphorylation[67]. Epinephrine stimulates cyclic-AMP-mediated activation of protein kinase A (PKA). PKA phosphorylates ATGL as the first responder to hydrolyze triglycerides into diglycerides and fatty acids[68]. Activation of accessory proteins and HSL further hydrolyzes to produce monoglycerides and fatty acids. Monoglyceride lipase ends the chain by generating glycerol and a third fatty acid[69]. These key contributors of stored triacylglycerol breakdown produce non-esterified fatty acids to be delegated to peripheral tissues as energy to be oxidized[70].

Studies have shown that lipolytic enzymes may be directly inhibited by the presence of insulin, suggesting that insulin directly mediates nutritional regulation. As with all metabolic processes, relative balance is required to prevent dysregulation. Understanding the mechanisms and influences of all metabolic systems and their harmonizing relationships with WAT is essential to understanding the development and pathophysiology of obesity, diabetes, and other metabolic disorders.

In 1994, Jeffrey Friedman and Douglas Coleman discovered leptin, the adipocyte cell-produced hormone that signals to the brain the energy status of peripheral tissues[71]. This discovery seemed to illuminate researchers to the potential of a physiological feedback loop controlling energy homeostasis and improve the understanding of the genetic and neuroendocrinological potential of adipose tissue. As these investigations continued, the ability of adipocytes to influence physiological processes through the secretion of adipokines became evident. Adipokines, comprised of hormones, cytokines, and other proteins with specific biological function, are unique to adipocytes and are involved in energy homeostasis, extracellular matrix dissolution and reformation, angiogenesis, steroid metabolism, immune response, and hemostasis[72, 73]. As researchers have continued to elucidate the potential of adipokines, adipose tissue has expanded its role from excess energy storage to a fundamental participant in energy metabolism control.

In recent years, the list of adipokines has expanded rapidly to include resistin, adiponectin, apelin, visfatin, retinol-binding protein-4 (RBP-4), serpin, lipcalin-2, vaspin, omentin, in addition to inflammatory cytokines TNF-alpha, interleukin-6 (IL-6), and monocyte chemoattractant protein-1 (MCP-1). In order to understand some of these functions, a few of these adipokines should be discussed.

Certain adipokines have developed a role in modulating glucose intolerance, such as resistin and RBP-4. Resistin acts to induce insulin resistance in insulin sensitive tissues such as muscle and adipose tissue and may be related to fasting adaptation[74]. In animal studies, RBP-4 has also been shown to decrease insulin sensitivity and seems to be highly expressed in insulin resistant obese individuals[75]. Conversely, adiponectin enhances insulin sensitivity and decreases influx of non-esterified fatty acids, increasing oxidation in the liver and muscle[76]. Of interest is adiponectin's ability to increase uncoupling protein 2 (UCP-2) and acyl coenzyme oxidase expression in muscle and improving PPARγ expression in adipose tissue[76]. These genes improve lipid catabolism and decrease lipid storage, improving metabolic parameters. Apelin is also known to influence obesity by improving cardiovascular function through increasing cardiac contractility and lowering blood pressure[77]. Interestingly, the distribution and activation of certain adipokines is dependent upon the location and effect of the energy intake of the individual.

Major Signaling Pathways Involved in WAT Adipogenesis

The differentiation process that supports proliferation is controlled by stimuli that can influence adipocyte growth. As mentioned, a variety of ß-adrenergic factors can activate this process; however, multiple pathways can be stimulated. The culmination of these signaling pathways represents regulators that can promote and inhibit adipogenesis. For example, the WNT signaling pathway has been previously described to be a strong regulator of adipocyte differentiation. As a highly conserved signal transduction pathway that plays a role in proliferation and differentiation during embryonic development, activation of this pathway is essential to communicating stop-signals. In this section, other pathways that stimulate this process and key regulators will be explored.

BMP's

As described, MSC's have the ability to differentiate into a variety of cell types. The formation into pre-adipocytes can be triggered by members of the bone morphogenic protein (BMP) family, or specifically BMP4 and BMP2. The presence of BMP4 has been shown to commit pluripotent C2H10T1/2 cells to an adipose lineage, confirming the development into the white adipocyte phenotype[78]. Although BMP2 itself has little influence over adipogenesis, it does interact with other factors such as TGF-ß and insulin to promote differentiation,

although in some in vitro studies, the TGF-ß signaling cascade has also been shown to inhibit adipogenesis by repressing C/EBP's transcriptional activity[78, 79].

MAPK's

Mitogen activated protein kinases or MAPK's represent a family of signaling pathways well investigated to have the capacity to influence various cell signaling cascades affecting adipogenesis[80, 81]. A subsection of this family is comprised of extracellular signal-regulated kinases (ERKs), which function to control cell cycle and regulation of proliferative factors. Other members of this family include c-Jun amino-terminal kinases (JNKs) and p38 mitogen activated kinases, which have proven to be associated with stress response and apoptotic regulation.

In response to cytokines, osmotic stress, heat shock, fatty acids, and growth factors, JNKs can interfere with insulin signaling pathways, promote inflammation, induce apoptosis, and effect rates of proliferation[82, 83]. The direction of the cascade events triggered by JNKs is dependent upon the specific stimuli and cell-type associated with its activation. Although obesity and metabolic disease research has illuminated a complex relationship between apoptotic events and inflammation, a pathway illuminating a direct correspondence with adipogenic factors has yet to be evidenced.

Research on p38 has proven to be even more convoluted with studies determining a complex dynamic between activation and inhibition of proliferative

properties. While p38 is well evidenced to be supportive of UCP1 expression in BAT, it's role in differentiation and glucose uptake in WAT has yielded controversial results[84]. For example, considering p38 MAPK inhibitor interactions during adipocyte differentiation, results have shown that inhibitors, such as SB20350, during pre-adipocyte differentiation decreased the formation of adipocytes and downregulated C/EBPß phosphorylation[85]. Conversely, there is evidence that p38 may indirectly inhibit differentiation by phosphorylating CHOP10, a member of the C/EBP family. P38 has also been shown to phosphorylate nuclear factor of activated T-cells (NFATc4), binding with PPARγ to inhibit its expression[86]. As studies examining the relationship of MAPKs with adipogenesis continue to be explored, the development of inhibitory factors repressing adipogenesis and modulating insulin sensitivity will remain of great interest.

AMPK

AMPK, or AMP-activated protein kinase, is a well-known energy sensor that is crucial to energy metabolism and regulation. Although research on AMPK is well established, its role in adipose function is still relatively enigmatic. Various studies have investigated how AMPK may promote adipocyte browning and illuminate its effect on energy homeostasis. Exposure to increased AMPK levels during adipogenesis has been shown to inhibit differentiation of WAT, and instead, seems to induce transcription of browning factors to produce BAT or

beige adipocytes in 3T3-L1 cells by reducing expression of PRDM16[87, 88]. AMPK has been found to be indispensable to fatty acid metabolism, thermogenesis, and development of adipose tissue. Understanding the effect of AMPK on adipogenesis will prove to be essential to the future research explored in the following manuscript.

3. Therapeutic Approaches

Standard Methods

Fewer topics have engendered as much controversy as the health concerns associated with excess fat mass. Obesity was once a novel concern. Now, obesity rates have extended across nations and populations all around the world. A series of chronic conditions have been associated with the development of obesity and its influence over metabolism. These conditions have been linked to a host of other disorders that include respiratory issues and infertility. In summation, as a large-scale analysis of over 1.4 million adults showed, obesity was directly linked with an overall increase in mortality[89].

The importance of addressing this growing epidemic with methods to reduce instances of obesity need not be argued. Instead, efforts have concentrated on determining and evaluating therapies for weight reduction. Considering available methods, little more can be said for the effectiveness of increased physical activity and monitored dietary intake. Dietary therapies come in an innumerable number of packages, but focus primarily on decreasing dietary

intake to create an energy deficit. Many strategies often employ the reduction of saturated fats and carbohydrates[90]. This method requires patience and consistency, as weight reduction often involves time. Obese individuals should consider contacting nutritional specialists or seeking counsel to properly follow dietary restrictions unique to their needs[91]. In order to reduce weight, an energy deficit of 500/kcal per day is often prescribed, although certain cases may require more extreme strategies[92].

Physical activity, often in conjunction with changes in dietary intake, has been shown to not only reduce weight, but improve other health parameters associated with obesity[93]. Various forms of physical exercise are available, including strength training, circuit training, endurance training, and high intensity interval training[94]. Although each method may be found to have different levels of energy expenditure, a mixed approach may provide the best results in combining weight reduction and improving overall health. It is suggested that a weight reduction of 2 kg within 6-12 months is deemed appropriate[95]. Though behavior and lifestyle modifications continue to be the most effective long-term treatments for obesity and weight reduction, dire cases have escalated the necessity for more immediate pharmaceutical therapies. Several medications are available through over-the-counter and prescription methods. More recognized medications include Orlistat, a lipase inhibitor that works to prevent the degradation of dietary triglycerides and increases their excretion[96]. Medications such as this include uncomfortable adverse effects and may decrease absorption

of fat-soluble vitamins. Phentermine is a prescription drug that decreases desire for food, but does not influence metabolism. Similar pharmaceutical options also work to inhibit serotonin, norepinephrine, and dopamine reuptake and influence dietary behaviors, though long term use is not advised[97].

Innovative Nutrient Strategies

For many, the adverse effects of pharmaceuticals perpetuate a sense of unease about their safe use. Instead, a growing body of evidence suggests that a new era of nutraceuticals may provide health specialists new means of treating disease through the use of phytochemicals. Phytochemicals are biologically active compounds found in plants that play a role in plant growth or defense against pathogens, competitors, or predators[98]. In clinical studies, phytochemicals have shown potential to attenuate oxidative stress and inflammation[99].

Traditional use of phytochemicals can be traced back to ancient Chinese and Indian medicine where a variety of herbs and fruits were used to treat ailments such as infertility, headaches, skin detoxification, and improve eyesight. Ancient medicines also proffered therapeutic agents such as diuretics, cough expectorants, and treatments for inflammatory disorders[100]. A variety of compounds were used to treat disorders such as diabetes and cardiovascular disease and touted hepatoprotective attributes. Current research has collected theories from these practices to consider their health value in clinical trials; however, results have been mixed[101-103]. One of the primary limitations to current

literature is the elevated number of possible compounds investigated and the poor means of assessing effective dosage. As an example, a PubMed search of the term “phytochemical” alone yields over 25,000 publications with a plethora of polyphenols making narrowing options for analysis difficult to determine. Other topics of concern include whether researchers should isolate the most bioactive compound or consider whole nutrient sources. Decisions among these factors may continue to be one of the most challenging obstacles for investigators yet.

4. Zyflamend

Introduction

Zyflamend is a phytonutrient supplement that includes a variety of different herbal extracts[104]. Containing the extracts from ginger, rosemary, turmeric, Chinese goldthread, holy basil, Hu Zhang, barberry, oregano, green tea, and Chinese skullcap, Zyflamend was originally developed based on their anti-inflammatory properties. A review of associated literature has reinforced this claim as several compounds found in Zyflamend seem to inhibit the pro-inflammatory pathways of MAPK’s and transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB)[105, 106]. Studies investigating the beneficial potential of phytochemicals tend to focus on large doses of individual bioactive ingredients. Alternatively, the therapeutic results of Zyflamend interventions have been based on human-dose equivalency. Moreover, certain

studies of Zyflamend have proposed that its robust effects may in part be due to the synergistic capacity between ingredients[104].

Currently, research efforts have asserted Zyflamend holds new relevancy as a potent anti-cancer therapy[107, 108]. Although the mechanisms involved are still being reviewed, these studies point to a potential influence over genes regulating cell cycle and apoptosis[107]. NF-kB is a protein complex that is involved in cytokine production and regulates cell survival through apoptosis inhibition[109]. Previous studies have tied some of the anti-cancer effects of Zyflamend to the regulation of NF-kB signaling pathway[110]. Recently, new studies have begun to investigate how these anti-cancer pathways may interact in non-cancer cells. There remains limited evidence on the relevancy of Zyflamend in normal cells as its main impact with publications has been through cancer research; yet studies have unmasked NF-kB as the primary pathway responsible for inflammation in excess adipose tissue[111]. Further examination of the anti-inflammatory and anticancer pathways effected by Zyflamend treatment suggest there may be other connections to metabolic dysregulation. Little research has been published on this topic, leaving many properties unexplored.

History

The first reference of Zyflamend in scientific literature came with Bemis et al.'s publication on its nonselective cyclooxygenase-2 (COX-2) inhibition in prostate cancer[112]. This research sparked a procession of studies determining Zyflamend supplementation could induce apoptosis in prostate cancer cells.

Several authors, several studies, several cancers, and several discoveries later and Zyflamend research continues to yield therapeutic potential for a variety of conditions.

A Review of the Literature

A review of the scientific literature on Zyflamend emphasizes a focus on its therapeutic properties in cancer. Even so, general mechanistic studies have revealed various effects on anti-inflammatory pathways. Several studies have suggested that Zyflamend may attenuate inflammation through multiple signaling pathways[107, 113, 114]. Some studies concluded Zyflamend may suppress COX 1/2 activity, although many studies emphasized inhibition of NF-kB as the major pathway of interest, specifically through its primary transcription factor Re1A[107, 113, 115]. Suppression of NF-kB resulted in reduction of TNF-alpha and other pro-inflammatory mediators and improvement of pro-apoptotic events.

Other studies on Zyflamend's effect on metabolomics expressed its ability to modify cellular energetics. Zyflamend has been shown to activate AMPK in vivo[116]. The mechanisms of AMPK activation suggest an ability to regulate specific pathways promoting lipolysis and promoting adipose tissue reduction through tumor suppressor liver kinase B-1 (LKB1)[104]. These preliminary studies suggest potential in utilizing Zyflamend in metabolic research. However, understanding the true mechanism involved may provide researchers with the

insight necessary to determine how effective Zyflamend may be as a potential therapy.

Components

The ingredients of Zyflamend include ginger, rosemary, turmeric, Chinese goldthread, holy basil, Hu Zhang, barberry, oregano, green tea, and Chinese skullcap. Many of these herbs are touted for their various anti-diabetic, anti-cancer, and anti-oxidant benefits. Of the ingredients, several have been shown to exhibit anti-inflammatory properties as well. Health specialists have deemed obesity a chronic low-grade inflammatory disease[13]. Research has suggested that results of phytochemical supplementation may contribute to decreased inflammation attributed to metabolic diseases; however, the translatability to clinical trials has much to be desired. This review explores the shift towards holistic therapeutic approaches to find increased effectiveness when treating metabolic disease. Furthermore, this field is shifting from novel basic science research towards real clinical application as the development of phytochemical therapies continues to show promise.

5. Research Objectives

Specific Aims

Obesity is a continued global health concern that directly correlates with significant health consequences. Prevalence of obesity has been associated with a multitude of metabolic disorders like type 2 diabetes mellitus, dyslipidemia, cardiovascular disease, hypertension, as well as other physiological disorders and injuries like liver dysfunction, cancer, and respiratory disorders. These comorbidities are exacerbated by the pro-inflammatory nature of the disease, thus, determining potential new therapeutic approaches to decrease adipose tissue is vital for more effectively treating obesity.

Found in a variety of fruits, vegetables, and spices, phytochemicals are naturally occurring compounds commonly consumed. These therapeutic phytochemicals also use a variety of pathways ranging from increasing the efficacy of certain enzymes' antioxidant activity, posttranslational modification of key signaling molecules, and regulation of gene expression[4]. Zyflamend, an herbal supplement has been used standardly as a medicinal anti-inflammatory agent. Previous studies have tied the anti-cancer effects of Zyflamend to the regulation of the NF-kB signaling pathway and phosphorylation of AMPK suggesting that Zyflamend may influence metabolic factors that could provide therapeutic treatment to combat obesity. However, certain questions remain unanswered: (1) what is the effect of Zyflamend on non-cancer cells, such as

white adipocytes, and (2) what molecular mechanisms mediate the effectiveness of Zyflamend and its influence over adipogenesis?

The central hypothesis of this study is that Zyflamend supplementation attenuates adipogenesis; however, its mechanisms will be determined.

Specific Aim 1: **To determine the effects of Zyflamend in vitro on adipocyte differentiation using molecular techniques.** We will use the well-established model that employs 3T3-L1 cells. Through innovative methods, we expect to explore the interactions that occur with a translatable human dose of Zyflamend treatment.

CHAPTER TWO

THE CROSS-TALK BETWEEN PKA AND JNK MEDIATES THE ANTI-ADIPOGENIC POTENTIAL OF ZYFLAMEND, A UNIQUE HERBAL BLEND

ABSTRACT

Zyflamend, a polyphenol herbal supplement, includes numerous different extracts. This botanical complex has been used as an anti-inflammatory due to its impact on the NF-kB signaling pathway. However, recent research has exhibited its potential as an anti-cancer agent by stimulating AMPK and shifting the metabolic dynamic in cancer cells. Currently, the effects of Zyflamend in non-cancer cells has yet to be elucidated. This study sought to explore the interactions of Zyflamend treatment on non-cancer cells such as 3T3-L1 adipocytes. The results of this study showed that Zyflamend significantly attenuated adipogenesis. As evidenced in previous research, Zyflamend was found to increase phosphorylation of AMPK and downregulate markers of lipogenesis, decreasing glucose uptake and upregulating lipolytic enzymes. It was determined this pathway occurred by upregulation of PKA-regulated lipolysis through pHSLS660. In addition, Zyflamend drastically increased activity of MAPK activity, specifically JNK. Through investigation of these effects, partial rescue of adipogenesis was observed through treatments with PKA inhibitor H89 and AMPK inhibitor BML275; however JNK inhibitor SP600125 more significantly rescued the effects of Zyflamend on differentiation. These results suggest Zyflamend's effect on attenuating adipogenesis may be modulated by upregulation of JNK.

SIGNIFICANCE

Zyflamend has been shown to downregulate NF-kB and upregulate AMPK activity in cancer cells, resulting in a shift in metabolism that results in cancer cell death. AMPK activity is also an essential part of metabolism and can impact the differentiation of non-cancer cells. Currently, methods to promote activation of AMPK have been used in metabolic research to influence the differentiation of adipocytes, primary energy storage cells. By addressing the mechanisms associated with adipocyte differentiation, adipogenesis has become a target for a variety of therapeutic approaches in metabolic research. Zyflamend's ability to shift energy metabolism and promote energy expenditure may yield therapeutic potential as a cell differentiation modulator.

1. Introduction

Obesity is a global health concern that directly correlates with significant health consequences. Obesity is caused by excessive energy consumption and can lead to an increased mortality rate through a variety of comorbidities making therapeutic and preventative approaches to these conditions of top interest to health professionals[118]. Type 2 diabetes mellitus, dyslipidemia, cardiovascular disease, hypertension, as well as other physiological disorders, like liver dysfunction, musculoskeletal and respiratory disorders, and cancer, all have shown a close correlation with BMI and prevalence of increased body weight[119]. These comorbidities are exacerbated by the pro-inflammatory nature of the disease related to a dynamic immune response in adipocytes[120]. Customary ways to decrease the accumulation of adipose tissue are effective through long-term efforts; however, finding inventive methods to attenuate adipose tissue may be instrumental to improving health prognosis for extreme cases.

Directed efforts to understand the intricate changes in adipocyte morphology may hold the key to treating adipocyte dysfunction associated with obesity[121]. Various transcription factors have been identified that promote the differentiation of white adipocytes from adipocyte precursors. Peroxisome proliferator-activated receptor gamma (PPAR-γ) initiates lipid uptake and drives differentiation of adipocytes. In addition, sterol regulatory element-binding transcription factor 1 (SREBF1) promotes glucose metabolism and supports fatty acid and lipid production within the cell[122, 123]. These factors, and others

associated with energy storage and metabolism, promote the maturation necessary for fully differentiated adipocytes. Once mature, these cells act as energy storage sites and modulate hormone signaling to a variety of tissues. Targeting adipogenesis has been on the forefront of obesity research, making downregulation of transcription factors that drive this process a natural mark for study[124].

In clinical studies, certain phytochemicals have been found to induce a catabolic state by activating energy sensor adenosine monophosphate-activated protein kinase (AMPK) promoting fatty acid oxidation and even adipocyte "browning" [125,126]. Many phytochemicals have been shown to express anti-inflammatory, antioxidative, anticancer, and anti-obesity affects[127]. Thorough investigation into a multitude of these compounds have yielded promising results to counter a variety of ailments, including pomegranate flavonoids, sulforaphane, turmeric, and quercetin. Each therapeutic phytochemical can vary in its mechanistic pathway or target ranging from increasing the efficiency of certain enzymes' antioxidant activity, activating or preventing PPAR-γ and the inflammatory NF-kB pathway, to upregulating gene expressions associated with autophagy[128].

As mentioned, research investigating the effects of phytochemicals on adipose tissue development often illustrate changes in metabolism through AMPK. AMPK is a key regulator in cellular metabolism, known to heighten energy production and attenuate energy storage processes[129]. AMPK's role in

energy homeostasis is well evidenced through its stimulation of lipolytic mechanisms and its ability to improve insulin sensitivity[130]. Additionally, cyclic AMP-dependent protein kinase A (PKA) is a multifunctional protein that can initiate lipolytic events by phosphorylating hormone-sensitive lipases (HSL)[130]. Phosphorylated HSL translocates from the cytosol to stored triglycerides in the reservoir lipid droplet in adipocytes and exhibits an increase in hydrolytic activity[131]. Both PKA and AMPK have been shown to stimulate these hydrolytic events by HSL through different serine residues[132]. Upregulation of this pathway can inhibit normal differentiation processes associated with adipose tissue maturation. Several studies have confirmed fatty acid oxidation and mobilization can effectively halt adipogenesis when AMPK and HSL activity is promoted[133].

Zyflamend has shown efficacy in regulating inflammation and metabolism in cancer cells; perhaps in part due to the synergy expressed by its ingredients[133-137]. Although these mechanisms are not well understood, studies point to potential influence over genes regulating the cell cycle, autophagy, and apoptosis[138]. Furthermore, they have identified AMPK as responsible in shifting the metabolic dynamic in cancer cells, promoting apoptosis. As AMPK activity can support changes in metabolism through upregulation lipolysis, we wished to investigate how Zyflamend may activate this pathway in non-cancer cells, such as in adipocytes[139].

In this study, we examined the anti-adipogenic potential of Zyflamend and the underlying mechanisms involved. The results showed that Zyflamend

significantly decreased adipocyte differentiation *in vitro* by upregulating lipolysis activity and suppressing the activity of differentiation transcription factors PPAR-y and lipogenic enzymes fatty acid synthase and perilipin A. Additionally, upregulation of lipolytic mechanisms were noted. Furthermore, these processes were shown to be mediated by upregulation of JNK and increased activity of PKA-mediated HSL activity.

2. Materials and Methods

Chemicals and Reagents. Media, sera and trypsin for cell culture were purchased from Gibco (Thermo Fisher Scientific, Waltham, MA). Zyflamend was purchased from New Chapter Inc. (Brattleboro, VT). Primary antibodies and secondary antibodies were acquired from varying sources (Table 1). Chemical reagents such as dithiothreitol (DTT), percoll, digitonin, phenylmethylsulfonyl fluoride (PMSF), protease inhibitors cocktail, sodium deoxycholate, Triton X-100, ethylene glycol-bis-(2-aminoethyl)-N,N,N',N'-tetraacetic acid (EGTA), sodium fluoride (NaF), Sodium phenylbutyrate (4-PBA), Hoechst 33258, propidium iodide, autophagy inhibitor (DBeQ), JNK inhibitor (SP600125), Thapsigargin, AICAR, TUDCA, HS38, H89, SB203580, STK1, Fasudil, and KT572 were acquired from Millipore-Sigma (Burlington, MA). Finally, AMPK inhibitor (BML275) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA)

Cell Culture. Brown precursor cells were isolated from newborn wild type mouse by collagenase digestion as described previously, from brown fat pads[140]. 3T3-L1 MBX (ATCC ® CRL-3242) cells were maintained in DMEM containing 25 mM glucose, 10% FBS, 50 U/ml penicillin and 50 µg/ml streptomycin. To induce cell differentiation, white, brown and 3T3-MBX pre-adipocytes were grown to confluence in culture medium containing 10% FBS. Confluent cells were then switched to differentiation media containing 10% FBS, 20 nM insulin and 1 nM triiodothyronine [T3] for 48 hours. Adipocyte differentiation was induced by treating cells for 48 h in differentiation medium further supplemented with 5 µM dexamethasone, 0.5 mM isobutylmethylxanthine, and 0.125 mM indomethacin (induction media). After induction, cells were returned to differentiation medium and exhibited a fully differentiated phenotype with accumulation of fat droplets.

Zyflamend Treatment. Zyflamend was dissolved in DMSO at a concentration of 800mg/ml for the stock solution. Cells were treated with Zyflamend at the indicated concentration and for the indicated duration. Treatment was stopped by two washes with ice-cold phosphate buffer saline (PBS). Plates were then flash frozen in liquid nitrogen and stored at -80ºC until further analyses.

Cytotoxicity Assay. Cytotoxicity assays were performed using sulforhodamine B (Millipore-Sigma) as previously described[141]. Briefly, 3T3-MBX pre-adipocytes were treated with Zyflamend at 37°C in an atmosphere of 10% CO2 for the

indicated time. After the incubation, the 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 washed with water and dried, and the stain was dissolved in 0.2 mL 10 mM Tris (pH 9). Quantification of sulforhodamine B was carried out using the Synergy™ HTX Multi-Mode microplate reader (BioTek Instruments, Inc. Winooski, VT) at a wavelength of 540 nm. The relative plating efficiency of each cell line was determined by dividing the absorbance observed for a given treatment by the absorbance detected in the absence of treatment and expressed as a fold change.

Quantification of Lipid Accumulation and Oil Red O Staining. On Day 12 of differentiation, cells were fixed with 10% PBS buffered formalin for at least 12 hour 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).

Western Blotting Analysis. Cells were lysed in radio-immunoprecipitation assay (RIPARIPA: 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) as we previously described[142]. Lysates were clarified by centrifugation at 15,000 g for 10 min, and

protein concentrations were determined using a bicinchoninic acid assay kit (Thermo Scientific Pierce BCA Protein Assay). Proteins (10-30 µg) were resolved by SDS-PAGE and transferred to PVDF membranes. Immunoblotting of lysates was performed with primary antibodies (Table 1), and after incubation with secondary antibodies, proteins were visualized using Luminata™ Western Chemiluminescent HRP Substrate (Millipore). Pixel intensities of immunoreactive bands were quantified using FluorChem Q Imaging software (Alpha Innotech Corp, San Leandro, CA). Data for phosphorylated proteins are presented as phosphorylation normalized to protein expression while total protein expression was normalized to β-actin as loading control.

RNA Extraction, RT-PCR And Real-Time PCR. RNA was extracted from cells using TRIzol reagent (Invitrogen). The concentration of RNAs was determined using the NanoDrop® ND-1000 spectrophotometer (Thermo Scientific). Specific DNA products were generated with an RT-PCR system (high-capacity cDNA Synthesis Kit (Applied Biosystems). Primer sets are described in Table 2 and expression of different genes was assessed by quantitative real-time PCR using SsoAdvanced™ Universal SYBR® Green Supermix (BioRad) and the Bio-Rad CFX96™ system. Relative abundance of target gene mRNA was measured using the ΔΔCT method and normalized to Tata-box binding protein (Tbp) as previously described.

Annexin V Staining. Annexin V staining was performed as previously described with modification[143]. Briefly, 3T3-MBX pre-adipocytes were exposed to Zyflamend [for the indicated duration] then washed with PBS and resuspended in 0.5ml of binding buffer (10 mM HEPES/NaOH, pH 7.5, 140 mM NaCl, and 2.5 mM $CaCl_2$) containing 5 µl of fluorescein isothiocyanate (FITC)-conjugated Annexin V (BD Pharmingen, San Diego, CA) and 10 µl of propidium iodide (PI) (Millipore-Sigma, Burlington, MA) 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-FITC (530 nm) and PI (585 nm) were collected on FL1 and FL3 channels, respectively. Both viable (negative for Annexin V and PI staining) and apoptotic cells (positive for Annexin V, but negative for PI staining) were quantified using CellQuest software (BD Biosciences, San Jose, CA).

Cell Cycle Analysis. Cell cycle analysis was determined by examining the DNA content of cells stained with propidium iodide as previously described with modification[144]. Briefly, 3T3-MBX pre-adipocytes were exposed to Zyflamend [for the indicated duration] then washed with PBS and fixed in 70% ethanol for 12hours at 4°C. After, cells were washed twice with cold PBS and then incubated in RNase solution in PBS (100 U/ml) for 30 min at 37°C. Cells were then incubated in solution of propidium iodide solution (10 µg/ml in PBS) overnight at

4°C. Fluorescence was measured using the Guava® easyCyte flow cytometer and analyzed with ModFitLT™ cell cycle analysis program (Becton Dickinson).

2-Deoxyglucose Uptake Assay. 2-Deoxyglucose uptake was determined using the colorimetric glucose uptake assay kit (Biovison INC., Milpitas, CA). Briefly, differentiated 3T3-MBX adipocytes were starved in low glucose (1mM) and 0% FBS media then treated with insulin for 30 minutes 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 are expressed as means +/- standard error of the mean (SEM). All statistical analyses were performed with JMP program (SAS Institute, NC) using an unpaired heteroscedastic two-tail Student's t test. Differences were considered significant at $p<0.05$ and highly significant at $p<0.01$. Single symbol (such as *) corresponds to $p<0.05$, while double (such as **) corresponds to $p<0.01$.

3. Results

In this study, we sought to investigate the effects of Zyflamend on lipid accumulation and differentiation of white adipocytes. 3T3-MBX white pre-adipocytes were treated with increased concentrations of Zyflamend and

differentiation into fully mature white adipocytes was carried as described in the methods and outlined in **Fig. 1B-C**. On Day 12 of differentiation, cells were stained using the fat-specific dye Oil Red O to monitor lipid accumulation (**Fig. 1C-E**) To rule out off-target effects, control cells (0 ug/ml) were treated with the highest concentration of the vehicle DMSO (1.25 ul/ml). On day 12 of differentiation, these cells accumulated fat droplets and exhibited a fully differentiated phenotype with more than 85% of the cells containing fat droplets **(Fig. 1D).** In contrast, cells treated with Zyflamend resulted in a dose-dependent decrease in lipid accumulation and only a small percentage of cells (<10%) were able to accumulate fat. Notably, cells treated with 1 mg/ml of Zyflamend exhibited blunted differentiation on Day 12 and were comparable to the non-differentiated cells at Day 1 of differentiation.

To determine whether Zyflamend would alter adipogenesis of 3T3-MBX at this dose, we examined changes in lipid accumulation over a time course experiment over 12 days of differentiation. Consistent with the previous experiment, this dose significantly attenuated adipogenesis as judged by Oil red O staining and lipid accumulation **(Fig. 2A-B)**.

A growing body of literature indicates that Zyflamend exhibits anti-survival and pro-apoptotic effects on several cancer models[135, 145-146]. However, its effects on non-cancer cells have yet to be investigated. To address this, we examined the effects of Zyflamend on pre-adipocyte proliferation using the sulforhodamine B cytotoxicity assay. Zyflamend inhibited differentiation of 3T3-MBX pre-adipocytes

in the presence of Zyflamend (200ug/ml) compared to DMSO (0.25ul/ml) as a vehicle control as shown in (**Fig. 2C**),

We demonstrate that the expression of cyclins D1 and D2, which repress adipocyte differentiation, were significantly attenuated in Zyflamend treated cells in comparison to the control. The exception was D3, which did not show significant differences in response to Zyflamend.

Expanding on these findings, we examined alterations in cell cycle and cell death in response to Zyflamend treatment. To examine whether attenuation of adipogenesis is caused by changes in cell proliferation, we also examined cell death of 3T3-MBX pre-adipocytes. These cells were cultured in the presence of Zyflamend (200ug/ml) or DMSO (0.25ul/ml) as a vehicle control for up to 48 hours. Cells treated with Zyflamend exhibited blunted proliferation. The intensity of the SRB staining reflected significant variation in the cell proliferation rate from a 12-hour time point to 48 hours ($p<0.05$) between treated and non-treated cells.

Patterns of differentiation correlate with expression of adipogenic markers: To further investigate the effects of Zyflamend on white adipose differentiation, we determined the expression of adipogenic markers fatty acid synthase (Fas), pyruvate carboxylase (PCB), perilipin, peroxisome proliferator-activated receptor gamma (PPARγ), CCAAT-enhancer binding protein alpha (c/EBPα), adiponectin, and glucose transporter 4 (Glut 4). Consistent with previous report, FAS, PCB, c/EBP adiponectin, PPARγ exhibited a progressive increase in expression during differentiation in control cells treated with the vehicle DMSO

(**Fig. 3A**)[147]. Conversely, cells treated with Zyflamend at a dose of 200 ug/ml exhibited blunted expression of these proteins throughout differentiation. In addition, we examined changes in other proteins associated with the non-differentiated state of white adipocytes such as pre-adipocyte factor 1 (Pref1), α5 integrin, fibronectin, and suppressor of cytokine signaling 9 (Sox9). The expression pattern of Pref1, α5 integrin, fibronectin, and Sox9 were comparable during the differentiation of control cells and exhibited a blunted expression by day 12 of differentiation. Cells treated with Zyflamend, on the other hand, maintained high levels of these proteins throughout differentiation. Consistent with protein expression, pref1 RNA was higher in Zyflamend treated cells compared to controls (**Fig 3.B**). Next, the expression of several white fat specific genes was determined during differentiation of control and Zyflamend-treated cells. Consistent with the protein data, control cells exhibited a marked increase of mRNA in *fas*, *ap2*, *resistin*, *c/ebp1a*, and *adiponectin*. Zyflamend treatment abolished the expression of these genes. Additionally, changes in glucose uptake were also noted, with Zyflamend treatment modulating insulin response (**Fig. 4**). Taken together our data suggest that cells treated with Zyflamend exhibited attenuated differentiation, as indicated by lipid accumulation and changes in protein and mRNA expression.

Zyflamend induces lipolysis in fully differentiated 3T3-MBX adipocytes: To investigate the molecular basis for the attenuated adipogenesis and decreased lipogenesis in Zyflamend-treated cells, we assessed the levels of adipogenesis and lipolysis markers in these cells. Zyflamend induces the

phosphorylation and activation of AMPK in a dose dependent manner. The increased AMPK phosphorylation and its activation were associated with a significant increase in ACC phosphorylation at Ser9, inhibiting its activity (**Fig. 5A-B**). CPT1a expression was also reduced in Zyflamend treated-cells (**Fig. 5A-B**).

To examine whether the reduction in fatty acid synthesis was associated with increased lipolysis, we evaluated changes in energy storage markers. We conducted a dose response of Zyflamend and examined changes in protein expression of hormone-sensitive lipase (HSL) (**Fig. 5C**). As Zyflamend concentration increased, pHSL expression increased at serine sites 563, 565, and 660. PKA-mediated phosphorylation of HSL at S563 and S660 is essential for HSL translocation to lipid droplets and triacylglycerol hydrolysis[148-149]. During a time course experiment over 12 days, alterations in pHSL activity were noted between Zyflamend and DMSO. In the control, pHSL at S563 showed an increased and then proceeding decrease of activity, whereas Zyflamend treatment showed little phosphorylation until Day 12. Over the time course, phosphorylation of pHSL at S565, which is dependent on AMPK, was shown to decreased during normal differentiation activity with the DMSO control. Conversely, it remains at low activity during Zyflamend treatment. Phosphorylated HSL at S660 is shown to remain at low expression during the control treatment, but is significantly upregulated with Zyflamend treatment (**Fig. 5D**). Consistent with these findings, PKA activity was significantly higher during

differentiation of Zyflamend-treated cells compared to controls as judged by the phosphorylation of PKA substrates (**Fig. 5G**). Collectively, this data indicates that the Zyflamend-induced lipolysis in 3T3-MBX is PKA dependent by upregulation of pHSL by S660.

To determine if browning was a factor in changes of metabolic activity, we assessed the expression of the brown adipose marker UCP1. UCP1 protein was expressed in brown adipocytes (positive control) after 12 days of differentiation but not in Zyflamend-treated cells. Similarly, the expression of PRDM16 and BMP7 were highly expressed in brown adipocytes but not in white adipocytes treated with Zyflamend, confirming that Zyflamend treatment, while altering lipolysis and energy metabolism, does not induce browning of white 3T3-MBX adipocytes (**Fig. 5I**).

We examined activation of MAP kinases during the course of differentiation of 3T3-MBX cells. Zyflamend exhibited a marked increase in JNK phosphorylation, particularly on Day 6 (**Fig. 6A**). Consistent with the activation of JNK, Zyflamend also increased the phosphorylation and activation of the JNK canonical downstream effectors, namely c-Fos and c-Jun. To further prove this hypothesis, we treated differentiating 3T3-MBX cells with Zyflamend along with specific inhibitors of PKA, p38 and JNK. We also used BML275 as a specific inhibitor for AMPK. As shown in **Fig. 7**, inhibition of JNK or PKA with SP600125 and H89, respectively, reversed the effects of Zyflamend on differentiation as judged by lipid accumulation Oil Red O staining (**Fig. 7A-C**). Inhibition of p38

using SB203580 did not rescue the effects of Zyflamend on differentiation (data not shown). Inhibition of AMPK on the other hand, resulted in a moderate rescue of Zyflamend-induced attenuation of lipid accumulation in 3T3-MBX cells (**Fig. 7A-B**). Together these data suggest that the impaired differentiation by Zyflamend is mainly mediated through the activation of PKA and JNK signaling pathways.

The cross-talk between PKA and JNK mediates the anti-adipogenic potential of Zyflamend: Despite the large body of evidence implicating both PKA and JNK in stimulating lipolysis during adipocytes differentiation, little is known about the crosstalk between these two proteins in coordinating the hydrolysis of triglycerides and how this affects the overall differentiation process. To address this question, we used PKA and JNK inhibitors and examined changes in differentiation and triglyceride content (**Fig. 7A**). We also evaluated the effects of inhibiting both proteins on the overall differentiation process by examining changes in the expression of the differentiation markers; Fas, PCB, Perilipin, and Pref1 (**Fig 7B**). Our study indicates that inhibition of PKA markedly attenuated the phosphorylation and activation of JNK while rescuing the effects of Zyflamend on the differentiation markers. On the other hand, while inhibition of JNK restored the expression of the differentiation markers in Zyflamend-treated cells, Zyflamend-induced PKA activation appears to decline (**Fig. 7C-D**). These results suggest that JNK is downstream target the PKA signaling and that our study demonstrates that Zyflamend attenuates differentiation of 3T3-MBX

adipocyte through the activation of PKA, which in turns activates JNK. Finally, future studies are necessary to further determine the underlying mechanisms of JNK activation by PKA, such as examining the molecular mechanisms mediating the cross-talk between PKA and JNK.

4. Discussion

Zyflamend's proven ability to activate AMPK and downregulate the NF-kB inflammatory pathway has identified it as a potent moderator for cancer. However, this study's intent was to identify how Zyflamend treatment using an evidenced human dose equivalent may influence metabolism in non-cancer cells and determine its impact on cell metabolism.

This study is the first of its kind to explore a direct relationship in Zyflamend treatment and metabolic research. We sought to investigate the effect of Zyflamend on adipocyte differentiation and determine the potential mechanisms involved. We discovered that Zyflamend significantly attenuated adipogenesis in 3T3-MBX adipocytes by inhibiting lipid storage and upregulating lipolysis. We propose that these affects are due to Zyflamend's ability to increase PKA and JNK signaling pathways. Considering these effects, we determine Zyflamend may have strong, natural therapeutic potential to address the development of adipose tissue, novel to metabolic research.

The differentiation process of adipocytes relies heavily on cell signaling pathways that identify and respond to anabolic stimuli. As adipose tissue is a key regulator and storage site of energy intake, the development of adipose tissue

bears great responsibility in managing growth, energy accessibility, and other metabolic processes. The ability for pre-adipocytes to fully differentiate and mediate energy storage in the form of triglycerides is essential for the development of obesity. Previous cancer research has established the efficacy of the human dose equivalent 200 ug/ml as an effective modulator in cancer studies. As this study is novel in design, our initial focus was to verify its efficacy in normal cells. First and foremost, we determined that 200 ug/ml was sufficient to promote changes in adipocytes differentiation. We noted attenuation of differentiation of 3T3-MBX cells as judged by the abolished lipid accumulation, and decreased expression of differentiation markers. Due to changes in lipid metabolism, we also sought to verify Zyflamend's ability to activate AMPK and determine this effect's contribution to the shift in energy metabolism. Our observations support previous research, suggesting Zyflamend can effectively activate AMPK and decrease the activity of growth and survival pathways through AKT.

Certain ingredients of Zyflamend previously studied exhibit the potential to activate AMPK and mediate adipogenesis. To name a few ginger, turmeric, and green tea have been shown to disrupt the onset of obesity, noting their ability to inhibit adipose expansion and suppress differentiation via activation of AMPK through food derived components[150]. Similar studies have noted the impact of Turmeric on activation of lipolysis through adipose triglyceride lipase and hormone sensitive lipase[151]. This activity can stunt the process of differentiation by decreasing the cell's ability to develop the lipid droplet and suppress anabolic

mechanisms that promote growth. The ability for the ingredients of Zyflamend to mobilize stored triglycerides supports our findings without the necessity of doses such 500g/kg body weight as evidenced in previous literature[151]. As discussed, Zyflamend exhibits unique synergistic affects improving the efficacy of the ingredients' biological reactions at relatively low quantity of the components promoting the translatability of our study. Furthermore, Zyflamend increased the activity of HSL at S660, suggesting PKA may be mediating lipolysis activity.

We were able to determine the effects of Zyflamend increasing lipolytic mechanisms in white pre-adipocytes were not due to the upregulation and expression of a brown phenotype. We determined that Zyflamend inhibited UCP1 expression, suggesting that the metabolic shift was independent of browning mechanisms. Currently, investigations to increase rates of brown or beige adipocytes has led the forefront of obesity research. The thermogenic potential of this phenotype has supported many research efforts to increase natural energy expenditure. As this area of research continues to grow, we propose exploring other biological avenues of obesity interventions. Previous research investigating the metabolomic potential of Zyflamend has previously reported attenuation of adipose tissue in C57BL/6 mice treated with Zyflamend[204]. However, this study did not differentiate the prevalence of brown adipose tissue, suggesting our findings are supportive of the potential of Zyflamend treatment through an alternative strategy.

We sought to determine how AMPK, JNK, and PKA activation induced by Zyflamend influenced the process of differentiation. Using specific inhibitors of each, we observed that inhibition of these key regulators impacted the overall effects of Zyflamend on the differentiation process. However, inhibition of JNK through SP600125 was able to rescue the effects of Zyflamend most significantly and promote normal adipogenesis. This outcome suggests it is Zyflamend's ability to activate JNK that prevents maturation of 3T3-BMX pre-adipocytes.

This treatment attenuated adipogenesis and significantly decreased the uptake and storage of glucose and lipids. Some studies have published similar effects on adipogenesis through some of the components of Zyflamend. Previous research determined the effects of curcumin as suppressing differentiation by promoting apoptosis of 3T3-L1 adipocytes[151]. In our investigations, the compound Zyflamend was able to suppress these effects without significantly altering cell proliferation rates.

We observed increased activity of HSL at S660. As we described, HSL as S660 is activated not through AMPK, but through upstream activator PKA. This has yielded rare insight into the complex interactions occurring from Zyflamend treatment. Other studies have yet to observe this interaction between PKA activity. When exploring how PKA inhibitor H89 influenced the reaction of Zyflamend treatment during adipogenesis, we observed a stronger rescue reaction from inhibition of PKA in comparison to AMPK inhibitor BML275. This suggests that there may be more mechanisms at play than what has been previously published.

We aimed to determine the key regulator attenuating the adipogenesis we observed in 3T3-MBX pre-adipocytes treated with Zyflamend. We determined that JNK activity was particularly upregulated in these cells. Although the mechanisms that promote this activation have yet to be explored, our study evidenced JNK has having a potent lipolytic role. By exploring how JNK inhibitor SP600125 interacted with the effects of Zyflamend, we observed a rescue effect suggesting JNK activation is essential for Zyflamend's effect on abolishing lipid storage and differentiation. There is reference to the lipolytic action of JNK in various studies; although we have yet to determine what mechanisms may be driving this force, previous literature hypothesizes autophagy and ER stress.

This study provides novel insight into the potential of phytochemical interventions in metabolic disease. Despite this study being novel within the current literature, future studies are warranted to investigate the relationship between JNK activation and the differentiation process of adipocytes. The transition towards abandoning conventional pharmaceutical methods and embracing holistic and synergistic bioactive nutrient therapies presents a transformative paradigm to disease research. This study was designed to incorporate a shift in perspective utilizing nutrition basic science research to establish a counterargument to more traditional therapeutic approaches. Zyflamend has already established itself in previous cancer research to provide therapeutic potential when more traditional strategies are exhausted. In this study, we demonstrated the plausibility of future Zyflamend research toward addressing metabolic diseases. Further analysis of the

mechanisms at play are required before solidifying Zyflamend's role in obesity interventions, however the potential remains.

CHAPTER THREE

CONCLUSION AND FUTURE PERSPECTIVES

1. Conclusion

Recent studies have demonstrated that Zyflamend triggers a dose-dependent anti-lipogenic response in prostate cancer cells through the activation of the energy sensor AMP-activated protein kinase (AMPK)[131, 133, 138, 147]. The findings from this study suggest that Zyflamend-induced PKA activation may alter lipid metabolism in adipocytes and dysregulate adipocyte differentiation. Adipocyte differentiation is a complex process that requires integration of a multitude of stimuli including nutrients and hormones and is often marked by the increased expression of several genes involved in adipogenesis and lipid accumulation[27]. In this study, we sought to investigate the effects of Zyflamend on lipid accumulation and differentiation of white adipocytes. Several factors may explain the decreased differentiation of Zyflamend-treated cells such as increased cell death, elevated lipolysis, reduced lipogenesis, loss of contact inhibition, inhibition of clonal expansion, transdifferentiation into brown-like adipocytes, etc. Thus, we conducted a detailed analysis of the effects of Zyflamend on pre-adipocyte proliferation, cell cycle, cell death, and differentiation. Together with our findings on PKA, these results suggest that PKA and/or JNK signaling pathways are what potentially mediate the effects of Zyflamend on adipocytes differentiation.

2. Future Perspectives

As Zyflamend has been shown across the literature to be a modulator of inflammation and regulator of metabolism in cancer cells, future research should continue to investigate and explore the effects of Zyflamend treatments against non-cancer cells. This study explored the potential of Zyflamend to regulate normal metabolic function within adipocytes and provided evidence of its potential as a modulator of adipocyte differentiation. These methods will help to elucidate the mechanisms that regulate this process through upregulation of JNK and PKA-mediated lipolysis. However, the necessity to further determine the complex mechanisms at work may show its potential in metabolic studies.

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APPENDIX

Table S1: List of primers used to quantitate the mRNA levels of markers of differentiation

Gene	*Forward 5'->3'*	*Reverse 5'->3'*
18s	*GCAATTATTCCCCATGAACG*	*GGCCTCACTAAACCATCCAA*
Adiponectin	*TGTTCCTCTTAATCCTGCCCA*	*CCAACCTGCACAAGTTCCCTT*
Ap2	*GCTTTGCCACAAGGAAAGTG*	*CATAACACATTCCACCACCA*
Cebp1	*GGTCAACAGGAGAATCTCCCAG*	*CTCTGTTTTATGCTGTTATGGGTGA*
Fas	*AGAGATCCCGAGACGCTTCT*	*GCCTGGTAGGCATTCTGTAGT*
Pref1	*CCCAGGTGAGCTTCGAGTG*	*GGAGAGGGGTACTCTTGTTGAG*
Resistin	*CTGTCCAGTCTATCCTTGCACAC*	*CAGAAGGCACAGCAGTCTTGA*

Table S2: List of primary antibodies and conditions of use.

Antibodies	Source	Host	Dilution
ACC	Cell Signaling Technology	Rabbit	1:2,000
Adiponectin	Abcam	Rabbit	1: 1,000
AMPK	Cell Signaling Technology	Rabbit	1:5,000
BMP7	Santa Cruz Biotechnology	Mouse	1:1,000
C/EBP	Santa Cruz Biotechnology	Mouse	1:1,000
c-Fos	Santa Cruz Biotechnology	Mouse	1:1,000
c-Jun	Santa Cruz Biotechnology	Mouse	1:1,000
CPT1a	Abcam	Mouse	1: 1,000
Cyclin D1	Cell Signaling Technology	Rabbit	1:5,000
Cyclin D2	Cell Signaling Technology	Rabbit	1:2,500
Cyclin D3	Cell Signaling Technology	Rabbit	1:5,000
Fas	Santa Cruz Biotechnology	Mouse	1:5,000
Fibronectin	Santa Cruz Biotechnology	Mouse	1: 1,000
Glut4	Santa Cruz Biotechnology	Mouse	1:5,000
HSL	Cell Signaling Technology	Rabbit	1:1,000
JNK1/2	Santa Cruz Biotechnology	Mouse	1:1,000
MnSOD	Santa Cruz Biotechnology	Mouse	1:2,500
p38	Santa Cruz Biotechnology	Mouse	1:1,000
PCB	Santa Cruz Biotechnology	Mouse	1:5,000
Perilipin	Santa Cruz Biotechnology	Mouse	1:1,000

Table S2 Continued

Antibodies	Source	Host	Dilution
Phospho-ACC^{S9}	Cell Signaling Technology	Rabbit	1:1,000
Phospho-AMPK^{T172}	Cell Signaling Technology	Rabbit	1:2,500
Phospho-c-Jun^{S63}	Santa Cruz Biotechnology	Mouse	1:1,000
Phospho-HSL^{S563}	Cell Signaling Technology	Rabbit	1:1,000
Phospho-HSL^{S565}	Cell Signaling Technology	Rabbit	1:1,000
Phospho-HSL^{S660}	Cell Signaling Technology	Rabbit	1:1,000
Phospho-JNK1/2$^{T183/Y185}$	Santa Cruz Biotechnology	Mouse	1:1,000
Phospho-p38$^{T180/Y182}$	Santa Cruz Biotechnology	Mouse	1:1,000
Phospho-PKA Substrate (RRX$^{S*/T*}$)	Cell Signaling Technology	Rabbit	1:5,000
PPARγ	Cell Signaling Technology	Rabbit	1:1,000
PRDM16	Abcam	Mouse	1: 2,000
Pref 1	Cell Signaling Technology	Rabbit	1:1,000
Sox9	Santa Cruz Biotechnology	Mouse	1:1,000
UCP1	Santa Cruz Biotechnology	Mouse	1:5,000
α5 Integrin	Santa Cruz Biotechnology	Mouse	1: 1,000
β-Actin	Santa Cruz Biotechnology	Mouse	1:20,000

A

Extract	Latin Name	Amount mg/serving
Rosemary	Rosmarinus officinalis	150
Turmeric	Curcuma longa	110
Ginger	Zingiber officinale	100
Holy Basil	Ocimum sanctum	100
Organic Green Tea	Camellia sinensis	100
Hu Zhang	Polygonum cuspidatum	80
Chinese Goldthread	Coptis chinensis	40
Barberry	Berberis vulgaris	40
Organic Oregano	Origanum vulgare	40
Chinese Skullcap	Scutellaria baicalensis	20

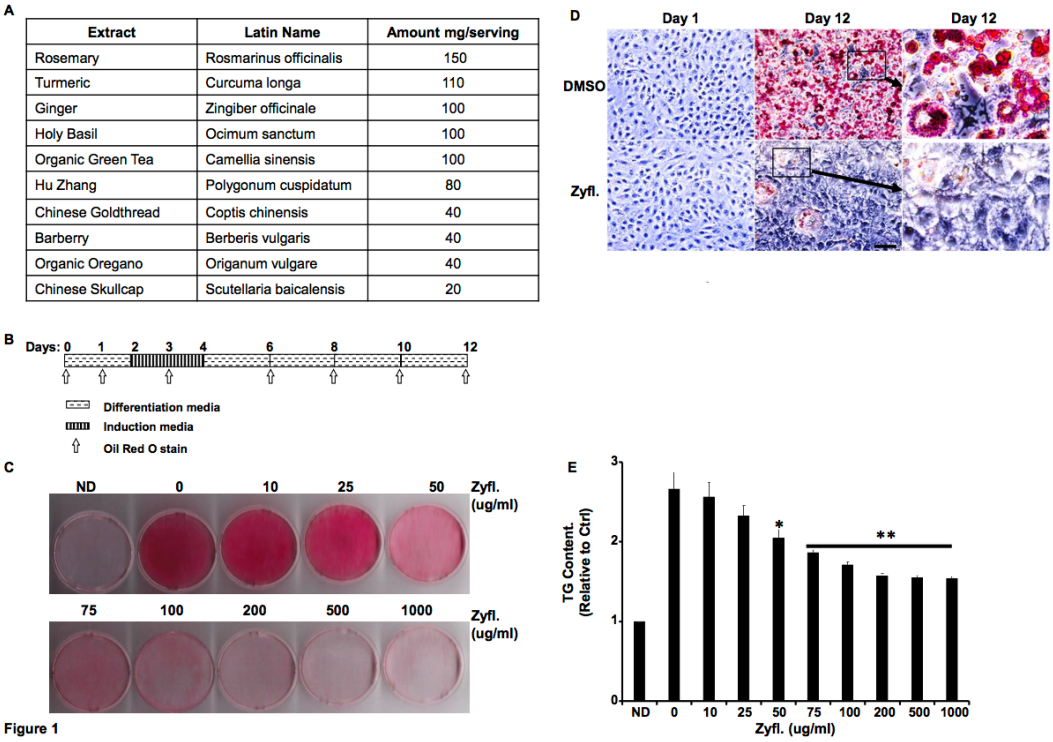


Figure 1

Figure 1. Dose Dependent Effect of Zyflamend on adipocytes differentiation. A) Composition of Zyflamend. **B)** A Schematic illustration of the differentiation procedure. C**)** 3T3-MBX were differentiated in the presence of increasing doses of Zyflamend for 12 days. Cells were fixed and stained with oil red O. **C)** Oil red O-stained and (**D**) phase contrast mages of non-differentiated (day 1) and differentiated adipocytes (day 12). Scale bar: 200 µm Images on the right panel are magnification of the boxed regions. **D)** Oil Red O stain was extracted and its absorbance (520 nm) was quantitated. Graph represents data from six independent experiments, and data are expressed as mean + SEM. *p<0.05, **p<0.01 indicate significant difference between non-treated and Zyflamend treated cells. **E.)** Dose Dependent Effect of Zyflamend on adipocytes Zyflamend treated cells and triglyceride content.

Figure 2. A physiologically relevant dose of Zyflamend inhibits adipocytes differentiation and glucose uptake. A) 3T3-MBX pre-adipocytes were treated with DMSO or 200 ug/ml of Zyflamend and differentiated as described in the Methods section. At different days of differentiation, cells were fixed and stained with oil Red O, and then triglyceride content was quantitated spectrophotometrically (520 nm) quantitated (**B**). Graph represents data from six independent experiments, and data are expressed as mean + SEM. **p*<0.05, ***p*<0.01 indicate significant difference between the indicated time and day one of differentiation. †*p*<0.05, ††*p*<0.01 indicate significant difference between Zyflamend and control (DMSO) treated cells. **C**) 3T3-MBX pre-adipocytes were treated with DMSO or 200 ug/ml of Zyflamend for the indicated time and cell survival was assessed using the sulfide-reducing bacteria (SRB) cytotoxicity assay as detailed in the Methods section. Bar graphs represent the intensity of SRB staining reflective of the cell number and presented as means + SEM. **p*<0.05, ***p*<0.01 indicate significant difference between cell proliferation rate at the indicated time point and 12hours. †*p*<0.05, ††*p*<0.01 indicate significant difference between non-treated and Zyflamend treated cells (50 ug/ml–500 ug/ml). **D**) Assessment of DNA content and cell cycle analysis in 3T3-MBX pre-adipocytes treated with DMSO or Zyflamend. Representative histogram distributions for each treatment are shown. **E**) Bar graphs represent the percentages of cells in each phase of the cell cycle which were estimated using the GuavaSuite Software package and are presented as means +/- SEM from

three independent experiments. *p<0.05, **p<0.01 indicate significant difference the indicated concentration and control cells treated with the vehicle DMSO. **F**) Immunoblots of cell cycle regulators cyclins D1, D2, and D3 in cells treated with 200 ug/ml of Zyflamend at various stages of differentiation. Representative Immunoblots from three independent experiments are shown. **G)** Bar graphs represent Cyclin D1, D2 and D3 normalized to β-Actin as a loading control and presented as means + SEM. *p<0.05, **p<0.01 indicate significant difference the indicated time point and day 1 of differentiation. †p<0.05, ††p<0.01 indicate significant difference between Zyflamend and control (DMSO treated) cells. **H-I)** 50% confluent cells were treated with increasing concentrations of Zyflamend (50 ug/ml–500 ug/ml), and then labelled with Annexin V-FITC and 7-AAD. Representative dot plots are shown. Annexin V positive and /7-AAD negative cells (lower right quadrants) represent early stages of apoptosis, whereas cells that are positive for both Annexin V and 7-AAD (upper right quadrants) are in late stage of apoptosis. Bar graphs represent live, early, and late apoptotic cells are presented as means +/- SEM of at least three independent experiments. *p<0.05, **p<0.01 indicate significant difference the indicated concentration and control cells treated with the vehicle DMSO.

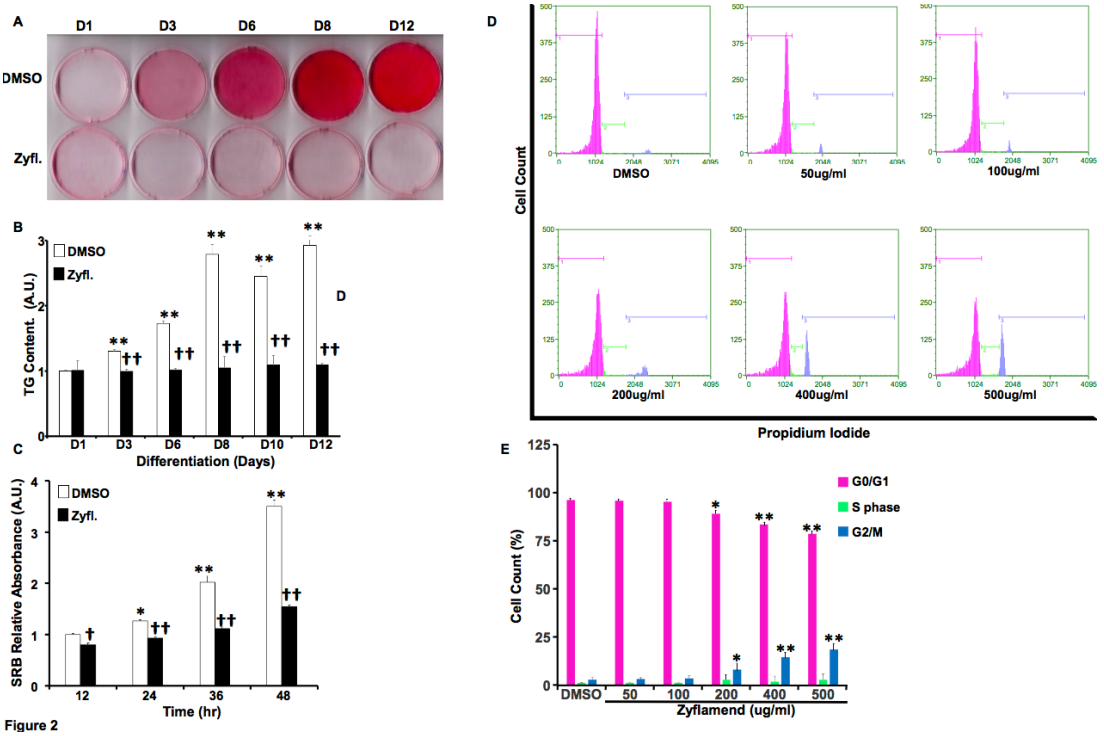


Figure 2

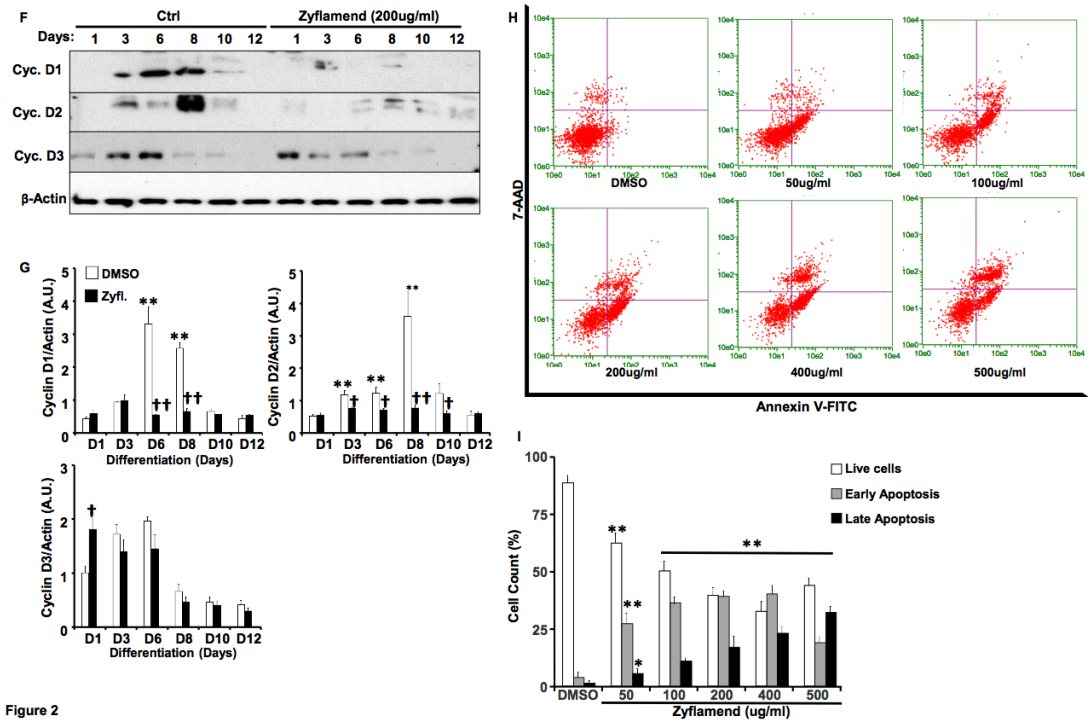
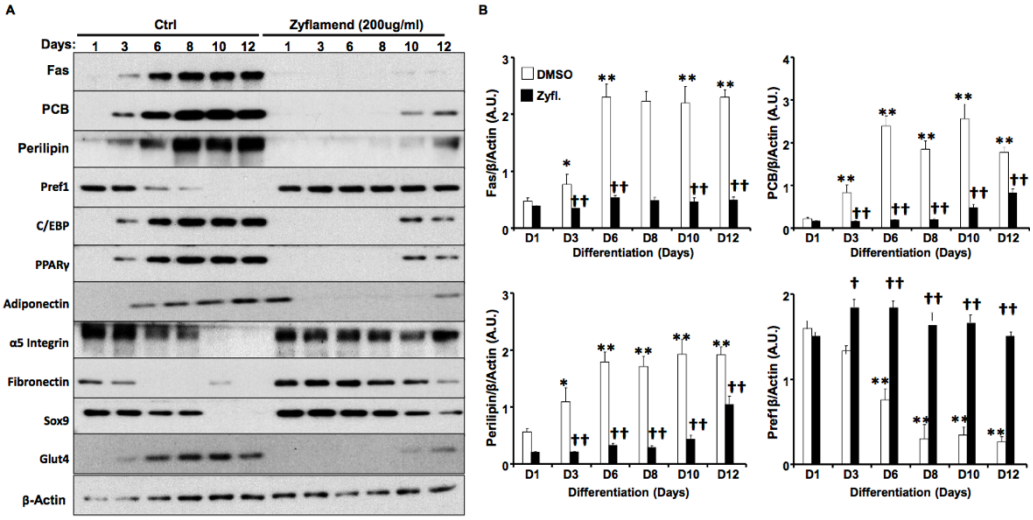


Figure 2

Figure 3. Zyflamend inhibits differentiation of 3T3-MBX pre-adipocytes. A) Immunoblots of adipogenic markers in 3T3-MBX cells treated or non-treated with Zyflamend (200ug/ml) at varying stages of differentiation. Lysates were blotted for β-actin to control for loading. Representative Immunoblots from three independent experiments are shown. **B**) Bar graphs represent the indicated protein normalized to β-Actin as means + SEM. *p<0.05, **p<0.01 indicate significant difference between indicated time points and day 1 for each cell type. †p<0.05, ††p<0.01 indicate significant difference between Zyflamend and control (DMSO) treated cells. **C**) Quantitative (q) RT-PCR of *Fas*, *Pref1*, *Resistin*, *Ap2*, *Adiponectin*, *Cebp1a*, *Pparg*, *Perilipin*, *Sreb1c*, *Fadb4*, *Visfatin*, and *Cidea* mRNA levels in control and Zyflamend (200 ug/ml) treated cells at various days of differentiation. Data are normalized to *18S ribosomal RNA* (*18S rRNA*). Results are representative of three independent experiments and data are expressed as mean +/- SEM. *p<0.05, **p<0.01 indicate significant difference between indicated time points and day 1 for each treatment. †p<0.05, ††p<0.01 indicate significant difference between Zyflamend and control (DMSO) treated cells.



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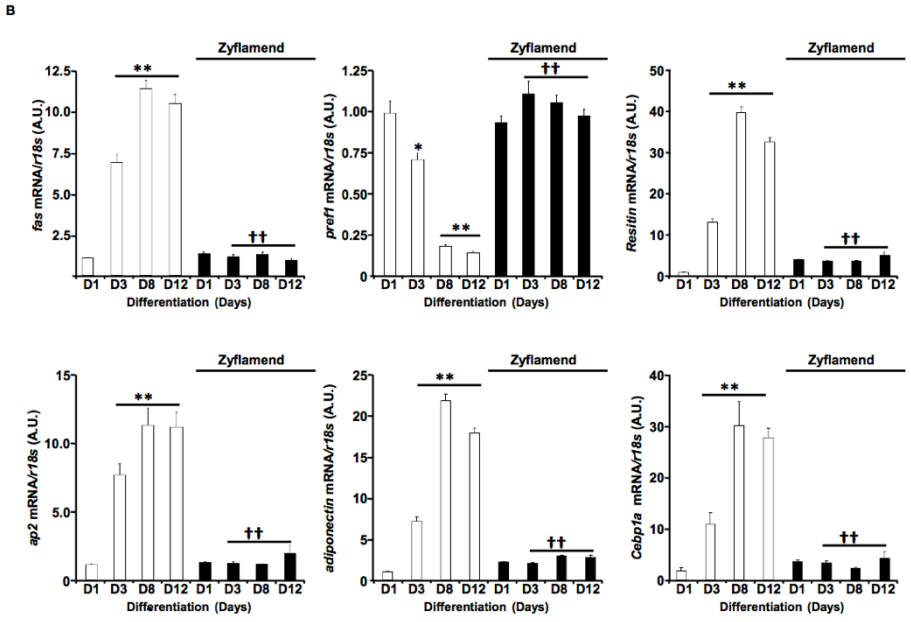

Figure 3

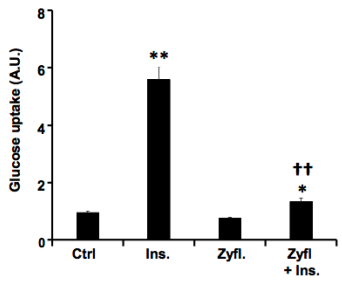

Figure 4

Figure 4. Zyflamend inhibits glucose uptake. 3T3-MBX pre-adipocytes were treated with DMSO or 200 ug/ml of Zyflamend and differentiated as described in the methods section. On day 12, cells were starved overnight in low glucose (1 mM) and 0% FBS media then treated with insulin for 30 minutes in the presence of 2-deoxyglucose. 2-doxyglucose uptake was quantified and presented as means +/- SEM of three independent experiments. Graph represents data from four independent experiments, and data are expressed as mean +/- SEM. *p<0.05, **p<0.01 indicate significant difference between the insulin treated and non-treated cells. †p<0.05, ††p<0.01 indicate significant difference between Zyflamend and control (DMSO) treated cells.

Figure 5. Zyflamend inhibits lipogenesis and induces lipolysis in fully differentiated 3T3-MBX adipocytes. Total lysates from fully differentiated 3T3-MBX adipocytes treated with the indicated concentrations of Zyflamend (0-400 ug/ml) for 24 hrs were immunoblotted for markers of lipogenesis (**A-B**) and lipolysis (**C-D**). Representative Immunoblots from three independent experiments are shown. **B-D**) Bar graphs represent $pAMPK^{T172}$/AMPK, Fas/β-Actin, $pACC^{S79}$/ACC, CPT1α/β-Actin, $pAKT^{S473}$/AKT, $pGSK^{S9}$/GSK, $pHSL^{S563}$/HSL, $pHSL^{S565}$/HSL, $pHSL^{S660}$/HSL, and HSL/ β-Actin as means + SEM. *p<0.05, **p<0.01 indicate significant difference between indicated concentration of Zyflamend and control (0) untreated cells. **E-F**) Immunoblots of $pHSL^{S563}$, $pHSL^{S565}$, $pHSL^{S660}$, and HSL in 3T3-MBX cells treated or non-treated with Zyflamend (200 ug/ml) at varying stages of differentiation. Representative Immunoblots from three independent experiments are shown. **F**) Bar graphs represent $pHSL^{S563}$/HSL, $pHSL^{S565}$/HSL, $pHSL^{S660}$/HSL, and HSL/ β-Actin as means + SEM. *p<0.05, **p<0.01 indicate significant difference between indicated time points and day 1 for each treatment. †p<0.05, ††p<0.01 indicate significant difference between Zyflamend and control (DMSO) treated cells. **G-H**) Immunoblots of phosphorylated PKA substrate in 3T3-MBX cells treated or non-treated with Zyflamend (200ug/ml) at varying stages of differentiation. Representative Immunoblots from three independent experiments are shown. **H**) Bar graphs represent phosphorylated PKA substrate /β-Actin as means + SEM. *p<0.05, **p<0.01 indicate significant difference between indicated time points

and day 1 for each treatment. †p<0.05, ††p<0.01 indicate significant difference between Zyflamend and control (DMSO) treated cells. **I**) Immunoblots of UCP1, prdm16, and BMP7 in 3T3-MBX cells treated or non-treated with Zyflamend (200ug/ml) at varying stages of differentiation. Primary inguinal fully differentiated brown adipocytes (BAT) were used as positive control.

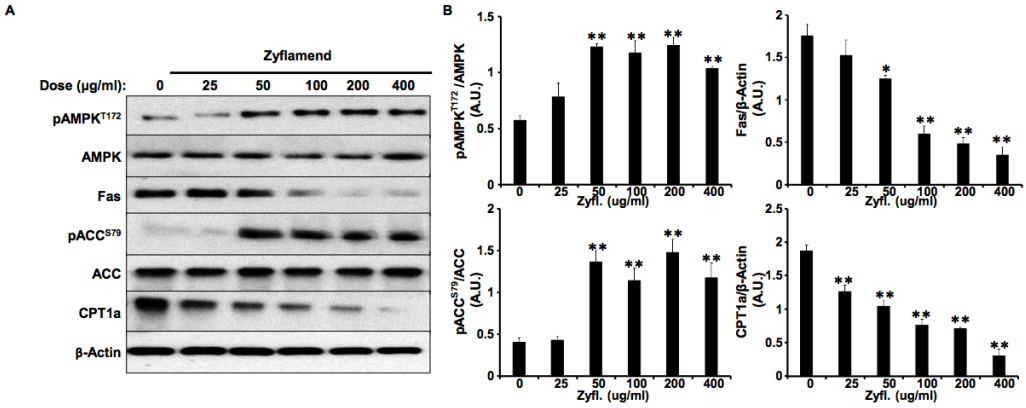

Figure 5

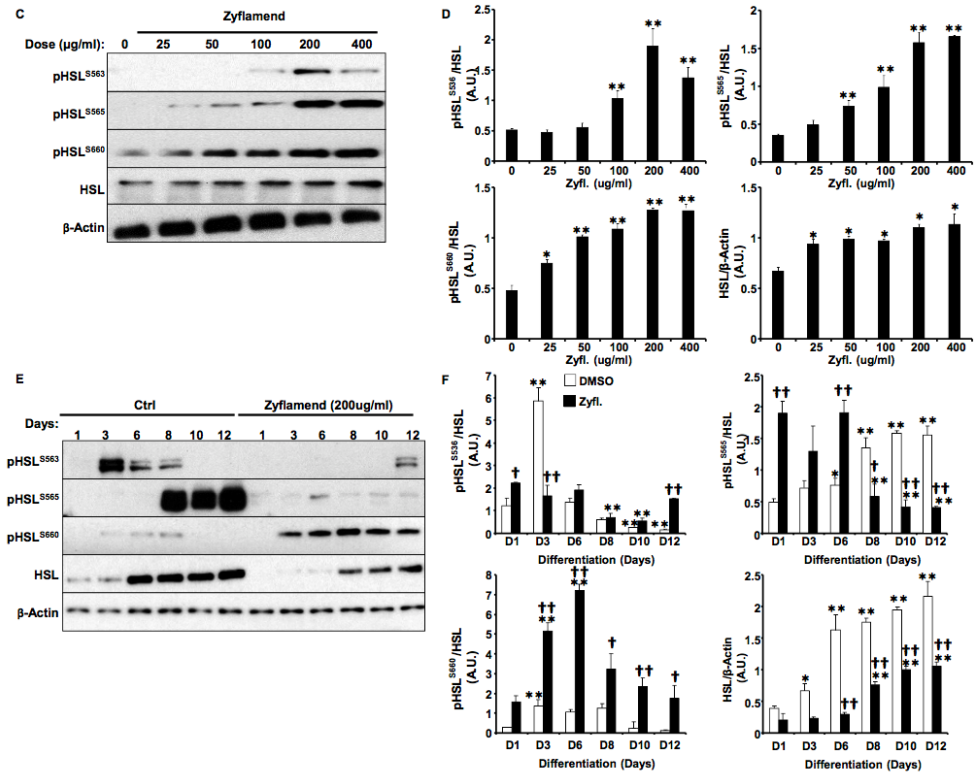

Figure 5

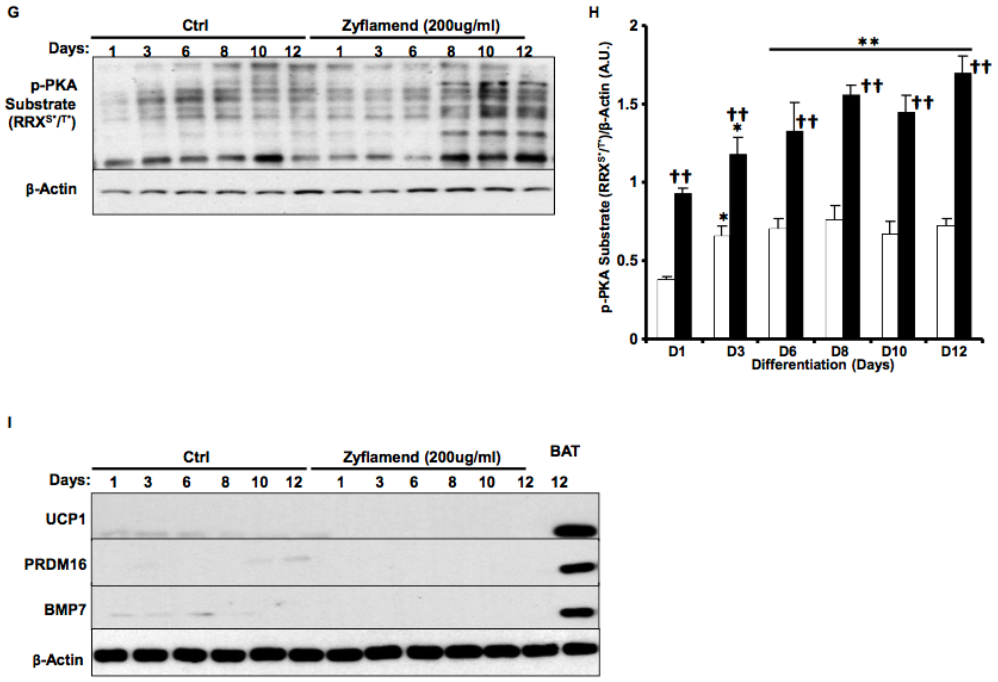

Figure 5

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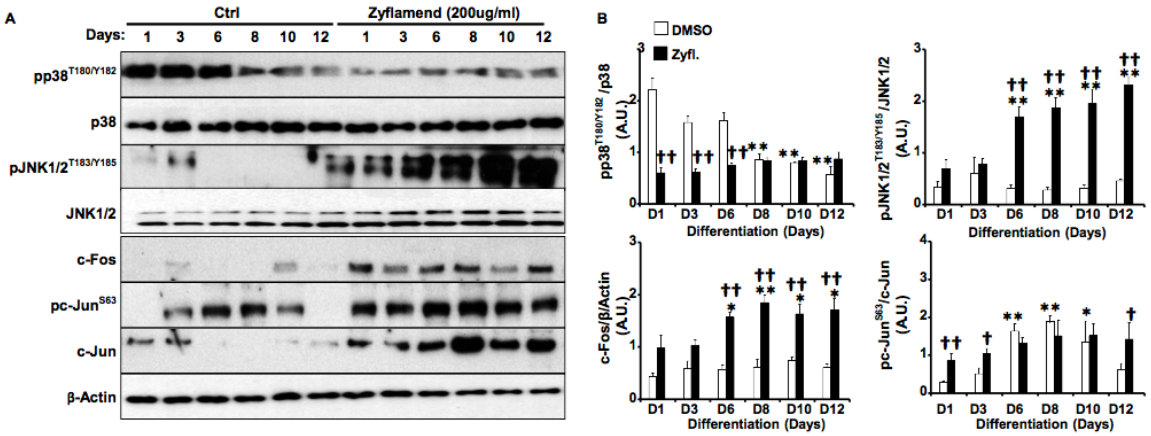

Figure 6

Figure 6. Zyflamend activates JNK and PKA during the differentiation of 3T3-MBX adipocytes. Total cell lysates from control and Zyflamend treated cells at various stages of differentiation were immunoblotted for (**A**) phosphorylated p38, JNK, c-Jun and their respective unphosphorylated proteins, c-Fos. Samples were also blotted for β-Actin as a loading control. Representative immunoblots from three independent experiments are shown. **B**) Bar graphs represent pp38$^{T180/Y182}$/p38, pJNK$^{T183/Y185}$/JNK, pc-Jun^{S63}/c-Jun, and c-Fos/β-Actin as means + SEM. $p<0.05$, $^{**}p<0.01$ indicate significant difference between indicated time points and day 1 for each treatment. †$p<0.05$, ††$p<0.01$ indicate significant difference between Zyflamend and control (DMSO) treated cells.

Figure 7. Inhibition of PKA and JNK abrogates the effects of Zyflamend on differentiation A) Oil red O-stained images of non-differentiated (day 1) and differentiated adipocytes (day 12) treated or non-treated with AMPK (BML-275), JNK (SP600125) or PKA (H89) inhibitors. **B**) Oil Red O stain was extracted and its absorbance (520 nm) was quantitated. Graph represents data from at least six independent experiments, and data are expressed as mean +/- SEM. **p*<0.05, ***p*<0.01 indicate significant difference between non-treated and Zyflamend treated cells. †*p*<0.05, ††*p*<0.01 indicate significant difference between cells treated with the indicated inhibitor together with Zyflamend and cells treated with Zyflamend only. **C**) Phase contrast images of non-differentiated (day 1/D1) and differentiated adipocytes (day 12/D12) treated or non-treated with SP600125 or H89. Images in the right panel are magnification of the boxed regions. Scale bar: 200 μm. **D-G**) Immunoblots of adipogenic markers in 3T3-MBX cells treated or non-treated with Zyflamend (200ug/ml) with or without PKA inhibitor (H89; **D**) or JNK inhibitor (SP600125; **F**). Lysates were blotted for β-actin to control for loading. Representative Immunoblots from three independent experiments are shown. **E-G**) Bar graphs represent the indicated protein normalized to β-Actin as means +/- SEM. **p*<0.05, ***p*<0.01 indicate significant difference between day 12 and day 1 of differentiation for each treatment. †*p*<0.05, ††*p*<0.01 indicate significant difference between inhibitor-treated and non-treated cells.

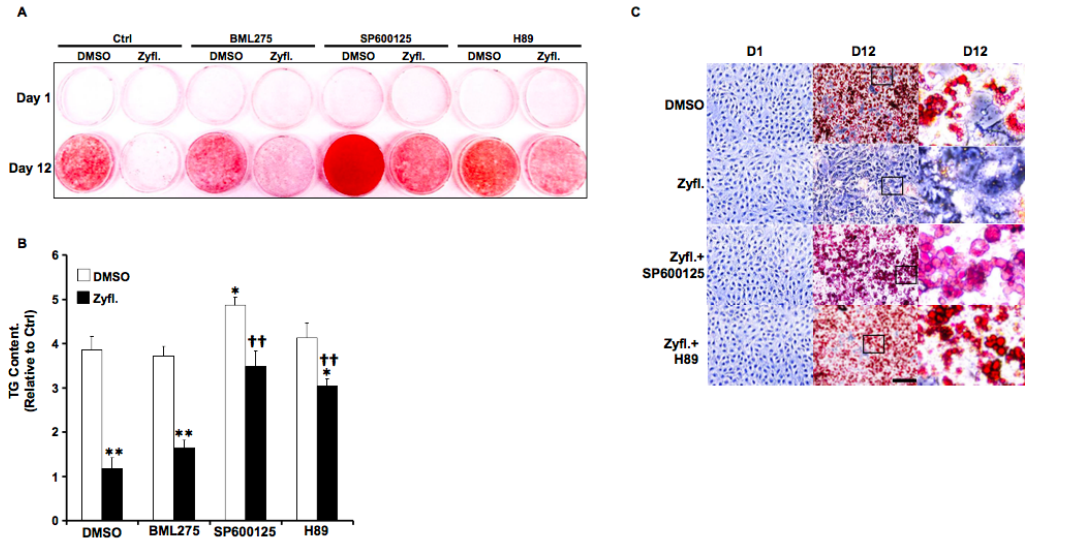

Figure 7

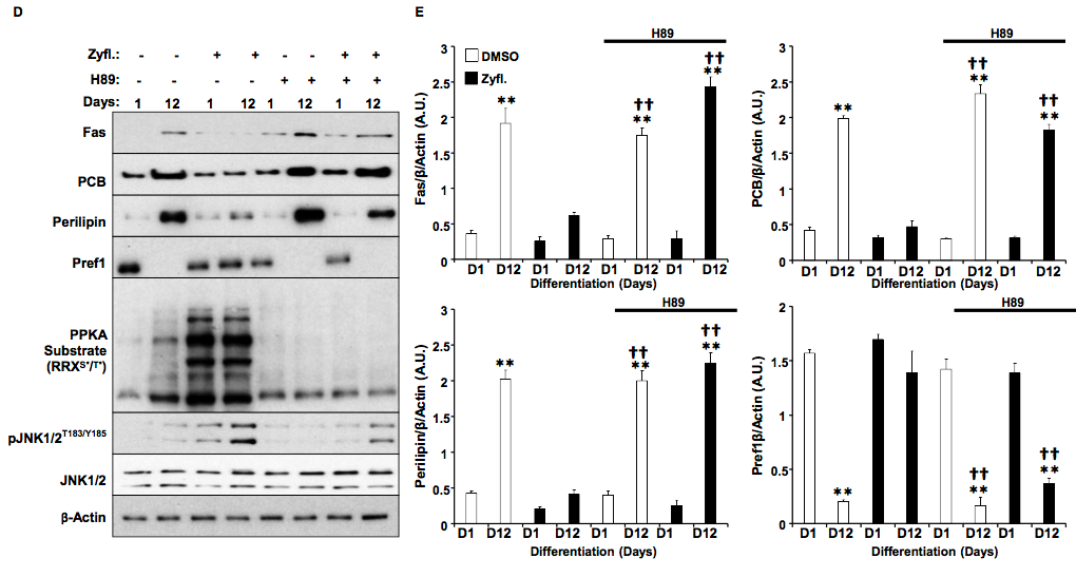

Figure 7

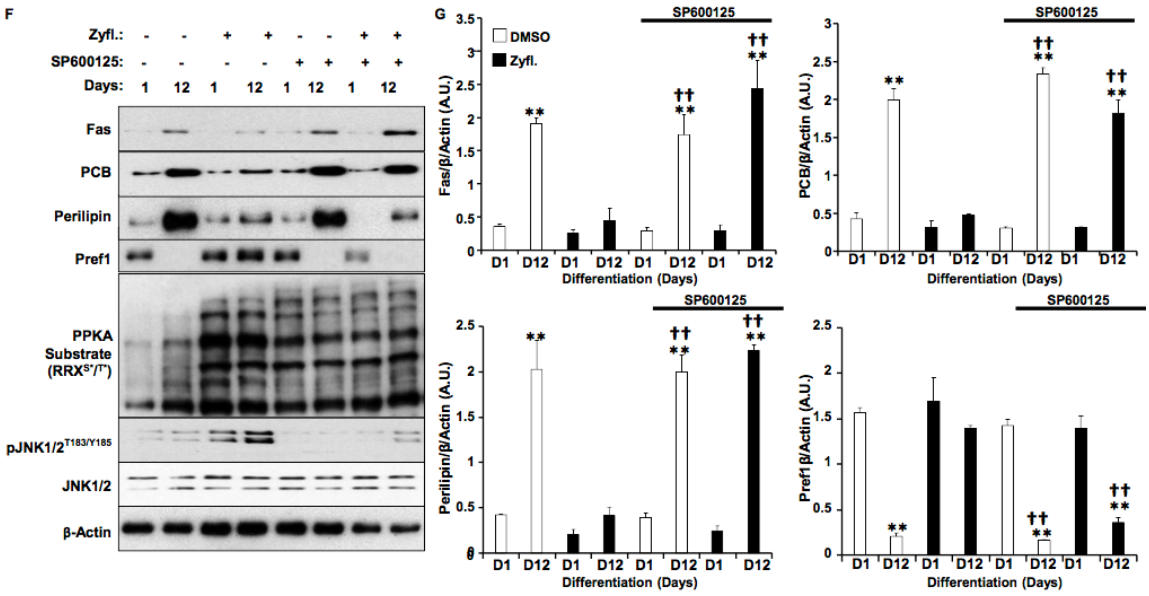

Figure 7

Figure 7 continued

VITA

Victoria Danielle Frankel was born in Melbourne, Florida and raised in Knoxville, TN to the parents of Dr. Jay I Frankel and Valerie L Frankel on December 11, 1989. She has two older sisters and a niece and nephew. Victoria graduated from Bearden High School in 2008 and attended University of Tennessee, Knoxville where she obtained her Bachelor of Health Science degree in Nutrition Dietetics.

In 2014 she started her own meal preparation company, The Wooden Spoon Kitchen, selling healthy, homemade meals and offering nutrition consulting and with a unique focus on athletics and personal fitness.

Victoria enjoys coffee, writing, and spending as much time as possible with her friends, family, and German Shepherd. Victoria is currently pursuing her Master of Science in Cellular and Molecular Nutrition at the University of Tennessee, Knoxville and hopes to continue her life pursuing truth, happiness, and scientific discovery.

"A table, a chair, a bowl of fruit and a violin; what else does a man need to be happy?"
-Albert Einstein

"A coward is incapable of exhibiting love; it is the prerogative of the brave."
-Mahatma Gandhi