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Role of resveratrol metabolites in adipose function

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ROLE OF RESVERATROL METABOLITES IN ADIPOSE FUNCTION

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To my husband, Matthew, who makes me a better person.

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LIST OF ABBREVIATIONS

3G	Resveratrol-3-O-glucuronide
3S	Resveratrol-3-O-sulfate
4G	Resveratrol-4′-O-glucuronide
AA	Amino acid
ABHD5	α/β hydrolase domain containing protein 5
AC	Adenylyl cyclase
ACC1	Acetyl-CoA carboxylase 1
ACL	ATP-citrate lyase
AMPK	5'-AMP-activated protein kinase
ATGL	Adipose triglyceride lipase
BAT	Brown adipose tissue
BMI	Body mass index
C/EBPα	CCAAT/enhancer binding protein alpha
C/EBPβ	CCAAT/enhancer binding protein beta
C/EBPδ	CCAAT/enhancer binding protein delta
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary DNA
CGI-58	Comparative gene identification-58
CHOP	C/EBP Homologous Protein
COLO25	Human colorectal carcinoma cell line
CPT1	Carnitine palmitoyltransferase I
CWR22Rv1	Prostate cancer cell line
DAG	Diacylglycerol

DEX	Dexamethasone
DGAT	Diglyceride acyltransferase
DMEM	Dulbecco's modified eagle's medium
DMI	Dexamethasone-methylisobuthylxanthine-insulin
DMSO	Dimethyl sulfoxide
DNL	De novo lipogenesis
Du145	Human prostate cancer cell line
ER	Endoplasmic reticulum
FABP	Fatty acid binding protein
FAO	Fatty acid oxidation
FAS	Fatty acid synthase
FBS	Fetal bovine serum
FCS	Fetal calf serum
FFA	Free fatty acids
FSK	Forskolin
GAPT	Glycerol-3-phosphate acyltransferase
HepG2	Human liver carcinoma cell line
HFD	High fat diet
HPLC	High performance liquid chromatography
HP-β-CyD	Hydroxypropyl-β-cyclodextrin
HSL	Hormone sensitive lipase
HT-29	Human colon adenocarcinoma cell line
HUVEC	Human umbilical vein endothelial cell line
IGF-I	Insulin-like growth factor-1
IL-6	Interleukin 6
IP	Intraperitoneal
ISP	isoproterenol
LD	Lipid droplet
LNcaP	Human prostate adenocarcinoma cell line
MAG	Monoacylglycerol

MAPK	Mitogen-activated protein kinase
MCAD	Medium chain acyl-CoA dehydrogenase
MCE	Mitotic clonal expansion
MCF-7	Human breast adenocarcinoma cell line
MGL	Monoglyceride lipase
MIX	Methylisobuthylxanthine
mRNA	Messenger RNA
MTT	3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide
MUFA	Monounsaturated fatty acids
NEFA	Non-esterified fatty acids
NRF1/2	Nuclear respiratory factors 1/2
ORO	Oil red O
PA	Phosphatidic acid
PBS	Phosphate buffered saline
PC-3	Human prostate cancer cell line
PGC-1α	PPARγ-cocactivator-1α
pHSL	Phosphorylation of HSL
PIC	Piceatannol (3, 5, 3', 4'-*trans*-tetrahydroxystilbene)
PKA	Protein Kinase A
PP-1	Protein phosphatase 1
PPARγ	Peroxisome proliferator activated receptor gamma
Prdm16	PRD1-BF-RIZ1 homologues domain-containing protein-16
Pref-1	Preadipocyte factor-1
RES	Resveratrol
RT-PCR	Reverse transcription polymerase chain reaction
RXR	Retinoid X receptor
SCD1	Stearoyl-CoA desaturase 1
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Ser	Serine
SREBP1c	Sterol regulatory element binding transcription factor 1c

SRS	Stimulated Raman scattering
TAG	Triacylglycerol
Tbx1	T-box 1
Tfam	Mitochondrial transcription factor A
Tmem26	Transmembrane protein 26
TMR	*Trans*-3, 4', 5-trimethoxy resveratrol
TNF-α	Tumor necrosis factor alpha
TPEF	Two-photon excitation fluorescence
TTBS	Tween-tris buffered saline
UCP1	Uncoupling protein 1
UV-VIS	Ultraviolet visualization
VEGF	Vascular endothelial growth factor
WAT	White adipose tissue

ABSTRACT

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The rise in obesity rate has drastically increased over the last few decades and has quickly grown into a worldwide epidemic. Large increases in visceral adipose tissue accumulation increase risk for metabolic disorders including insulin resistance, type 2 diabetes, and cardiovascular diseases. Currently, increased levels of circulating non-esterified fatty acids (NEFA) have been suggested to be the mediator linking obesity and type 2 diabetes. Studies identifying ways to alleviate mobilization of NEFA in adipose tissue via dietary phytochemicals may useful as a therapeutic approach. Despite the established benefits of resveratrol in health and in the development of obesity, the role of resveratrol metabolites in lipid metabolism remains unknown.

The objectives of this study were to determine the role of resveratrol metabolites in adipose function with a focus on the effect in lipolysis and lipogenesis. Resveratrol metabolite, piceatannol, previously demonstrated its role as an antilipolytic agent through the suppression of lipolysis via protein degradation of adipose triglyceride lipase (ATGL) and comparative gene identification-58 (CGI-58). We hypothesized other resveratrol metabolites exhibit similar effects in modulating the lipolysis process. In our study, we examined several resveratrol metabolites and identified *trans*-3, 4', 5-trimethoxy resveratrol (TMR) having ability to suppress lipolysis in basal and stimulated conditions in mature murine adipocytes at non-cytotoxic levels. Upon further mechanistic studies, TMR at 50 μM reduces ATGL and CGI-58 protein expression with greatest efficacy in an acute, three hour treatment. mRNA expression analysis displayed that TMR may also play a role in transcriptionally regulating lipolytic genes. Additionally, we investigated

the role of TMR in lipogenesis in maturing preadipocytes. Although TMR did not display significant reductions in lipid accumulation during lipogenesis, gene expression profiling indicates it may induce transcriptional remodeling of adipocyte function. Of interest, TMR upregulates expression of genes involved in mitochondria function suggesting increased catabolic processes, thermogenesis, and potential enhanced capacity for energy expenditure during development. Collectively, our study provides evidence that TMR, a resveratrol metabolite, might have a therapeutic potential in attenuating adiposity and its associated metabolic disorders.

CHAPTER 1. REVIEW OF THE LITERATURE

1.1 Introduction

The rise in obesity rate has drastically increased over the last 30 years and has quickly grown in to worldwide epidemic (Rosen and Spiegelman 2006; Van Gaal et al. 2006). Obesity, a disease of excess body fat, has attracted more attention due to the influence it has on regulation of overall energy homeostasis and coordinated responses (Rosen and Spiegelman 2006). Individuals with large visceral (central) adipose tissue deposition have elevated risk for metabolic disorders, morbidity, and mortality (Van Gaal et al. 2006). The large energy imbalance seen in obesity can be attributed to a variety of different factors including environmental, genetic, and metabolic function of central tissues (Kim et al. 2013).

Currently, investigations identifying the role of phytochemicals on human health is being done, however there is less work examining the modulation obesity via action by phytochemicals (Kim et al. 2013). The role of natural bioactive components have been shown to have a wide range of action in signaling pathways and cellular functions (Kim et al. 2013). In this review of the literature, several molecular pathways involved in adipose function and lipid metabolism are outlined in regards to the development of obesity and its associated metabolic disorders. Additionally, dietary compounds, specifically resveratrol metabolites, are defined and examined as a potential therapeutic strategy in specific adipocyte functions related to the development of obesity and its associated diseases. Taken together, this review offers a central background of information for further understanding concerning the selected research goals and objectives of our study.

1.2 Obesity

Obesity is defined as an excess accumulation of body fat to the ration of lean body mass. Obesity is determined with a numerical value of body mass index (BMI) of 30 or greater (CDC 2012). BMI is calculated with a formula based on weight in kilograms divided the height of adult in meters (CDC 2011). Although obesity is commonly associated with extreme body weight, it is a heterogeneous group of conditions which have compounded by multiple causes and determined by genetics, environmental factors, energy intake and expenditure, and culture (Kopelman 2000). Until recently, obesity has only been considered a metabolic condition, and primarily isolated to developed western civilization. However within the 20th century, global increases in body weight have been on the rise in developing countries as well (Caballero 2007). Changes in global food systems have caused a complex issues resulting in overnutrition and the promotion of obesity meanwhile nearly one billion people are malnourished (Swinburn et al.). The World Health Organization estimated that in 2008, 1.4 billion adults were overweight and 500 million obese (WHO 2013). Over the last two decades, the rapid rise in obesity rate in the United States has drastically increased to an astonishing level of more than one-third the population , 35.7% , with 17% of adolescents and children, who are obese (Ogden et al. 2014). The development of obesity and increased adiposity is coupled with physiological changes in function and in the distribution of adipose tissue. With contribution by obesogenic factors, obesity expansion is caused by increased intake of energy-dense foods, reduced physical activity, with increases in sedentary lifestyles. The imbalance between energy intake and energy expenditure over time results in positive energy balance with increased energy stores, as adipose mass, and weight gain (Bleich et al. 2011).

The American Medical Association recently recognized obesity as a disease which now requires medical interventions and is altering the way the medical community treats the complex issue and other chronic illness related to obesity (Breymaier 2013). Obesity is associated with many other metabolic disorders including cardiovascular disease, type 2 diabetes, inflammation, and certain cancers (Despres and Lemieux 2006). Also widely seen with excess fat accumulation is a composite of metabolic syndrome; a

combination of health issues that is largely seen with visceral obesity which is not exclusive to hyperlipidemia, insulin resistance, high blood pressure, and hypertension (Despres and Lemieux 2006). Moreover, obesity has also been reported as the underlying cause of sleep apnoea and sleep-related strokes which can lead to pulmonary hypertension, heart and respiratory failure (Kopelman 2000). Not only does the obesity rate cause a threat to global health, there is causation of severe financial implications. A conservative estimate for US national healthcare expenditures was estimated at $3.5 trillion in 2012, and that number is projected to increase to $5 trillion by 2020 (CMS 2010; 2014) with $150 billion attributed solely to obesity (Finkelstein et al. 2009). Defining strategies to alleviate not only the medical affliction of the obesity epidemic, but also the financial spending magnitude are imperative. It has been reported that modest weight loss can help to reduce risk factors for obesity associated diseases (Ben-Menachem 2007; NIH 2005). Nonetheless, of the 51% of Americans who desire to lose weight, less than half are actively working to shed pounds (Brown 2013). Therefore, identifying therapeutic approaches is critical to help prevent or treat obesity and its associated metabolic disorders.

1.3 Adipose biology

Adipocytes play a critical role in whole body energy homeostasis and metabolism. Adipose tissue stores fatty acids (FA) in the efficient form of triglycerides via esterification to glycerol, and in times of excess energy expenditure or an energy shortage, it releases them into circulation (Galic et al. 2010). There are two types of adipose tissue: white and brown adipose tissue (BAT), each containing specific physiological roles (Mohamed-Ali et al. 1998). White adipose tissue (WAT) is the primary adipose tissue depot in humans and is largely characterized by unilocular adipocytes with single lipid inclusion used for storage and mobilization (Ahima and Flier 2000). Excess energy is stored as triacylglycerides in adipose tissue and can increase in tissue volume through increases in size of an adipocyte lipid droplet (LD), hypertrophy, and in number of adipocytes, hyperplasia, or both (Spiegelman and Flier 1996). In adults, most expansion of adipocytes occurs from increased LD size in adipocytes due to tight regulation of the

number of fat cells, holding constant throughout adulthood in lean and obese individuals (Spalding et al. 2008). Although, changes in body weight are primarily due to increase adipocyte volume, there is a 'critical fat cell size,' seen in obesity, which activates hyperplasia, and induces the conversion of preadipocytes to adipocytes. Therefore, after losing body weight, it can be particularly hard to maintain a lean weight due to the constant number of adipocyte cells (Arner and Spalding 2010).

Adipose tissue is a complex, active, and essential metabolic endocrine organ comprised of two-thirds adipocytes, and one-third connective tissue, nerve tissue, stromovascular cells, and immune cells. The traditional idea of adipose tissue solely used for energy storage is no longer relevant, as it has been widely studied for its metabolism and endocrine functions (Kershaw and Flier 2004). Adipose tissue is responsible for secreting endocrine, paracrine, and autocrine signals which include a range of proteins termed adipokines and lipoprotein regulators; such as leptin, tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), adiponectin, resistin, lipoprotein lipase, cholesterol ester transfer protein, and apoliporotein E (Mohamed-Ali et al. 1998). Adipokines participate in a variety of metabolic action via physiological processes which include inflammation and immune health (Lago et al. 2007). Overall, excess accumulation of adipose tissue poses a public health threat as whole body health and metabolism is affected and negatively effecting longevity and reduceing life expectancy (Olshansky et al. 2005). Moreover, identifying therapeutic strategies to specific adipose tissue targets may have large potential in curbing obesity and its associated syndromes.

1.4 Adipogenesis

Adipocytes are derived from multipotent mesenchymal stem cells which allow them to have a two-step process in becoming mature adipocytes (Rosen and MacDougald 2006). This process begins with commitment to the adipocyte lineage followed by induction of terminal differentiation (Otto and Lane 2005). The first step of determination results in a preadipocyte that has lost its ability to differentiate into other cell lines. Widely used and accepted as a model for adipocyte biology include 3T3-L1 cells, originally clonally isolated from Swiss 3T3 mouse embryos (Green and Kehinde 1975;

Green and Meuth 1974; Gregoire et al. 1998). Preadipocytes are morphologically similar to that of fibroblasts and expansion of preadipocytes via hyperplasia occurs until confluence, when appropriate induction of differentiation causes the cell to acquire characteristics of mature adipocytes (Rosen and MacDougald 2006). In order for the differentiation process to occur, there are several steps that are completed, including development and growth arrest, allowance of mitogenic and adipogenic signals, and biochemical transformation, shown in Figure 1.1. Induction of differentiation in 3T3-L1 cell culture requires a cocktail of hormonal stimulation that is given to cells comprising of dexamethasone (DEX), methylisobuthylxanthine (MIX), and insulin. Mitotic clonal expansion (MCE) occurs unique to non-primary preadipocytes which permits one or two more rounds of DNA replication. Terminal differentiation is accompanied by a transcriptional cascade which creates a distinctive spherical shape and accumulates LDs (Gregoire 2001; Gregoire et al. 1998; Otto and Lane 2005).

1.4.1 Preadipocyte development, growth arrest, and differentiation

In order to differentiate, preadipose cell lines which have proliferated and display a fibroblastic morphology are required to reach a level of confluence (Gregoire et al. 1998; Hausman et al. 1980; Reichert and Eick 1999). The physical contact of cells results in growth arrest at which point preadipocyte cells withdraw from the cell cycle to undergo adipose conversion. It has been noted that cell to cell contact is not a sole requirement for this conversion into adipocytes, as it has been observed that cells plated at low density still maintain and go through differentiation (Gregoire 2001; Gregoire et al. 1998). Pre-confluent proliferation and growth arrest are necessary for preadipocytes to experience the appropriate morphological and distinct gene expression before their transformation to adipocytes.

Subsequent to growth arrest, preadipocytes must receive signals to further their development through differentiation which allow them to make specific biochemical alterations. CCAAT/enhancer binding protein α (C/EBPα) and peroxisome proliferator-activated receptor γ (PPARγ) have been identified as two genes which transactivate specific adipocyte genes (Gregoire et al. 1998). Nonetheless, normal preadipocytes

maintain high levels of preadipocyte factor-1 (pref-1), C/EBP homologous protein (CHOP-10), and GATA transcription factors until they are down-regulated upon differentiation (Mei et al. 2002; Tang and Lane 2000; Tong et al. 2000). Pref-1 is a transmembrane protein inhibitor of adipogenesis (Mei et al. 2002), and is highly abundant in the stromal-vascular fraction of preadipocytes yet absent in mature adipocytes (Smas et al. 1999). Growth arrested preadipocytes express CHOP-10 which is responsible for C/EBPβ herterodimerization binding and plays an impeding role on C/EBPβ transactivation, resulting loss of C/EBPβ activity and initiation of adipogenesis (Ron and Habener 1992; Tang and Lane 2000). GATA-2 and GATA-3 transcription factors bind specifically to DNA sequences, and are found in high levels in WAT and solely expressed in preadipocytes (Tong et al. 2000). Inherent expression of both represses differentiation in adipocytes, however with induction of differentiation these factors are down-regulated (Tong et al. 2005).

In order for these transcription factors to be expressed, cells are stimulated to differentiate with a standard adipogenic mixture supplemented to fetal bovine serum (FBS) containing medium including the glucocorticoid DEX, cyclic adenosine monophosphate (cAMP)-phosphodiesterase inhibitor MIX, and a high dose of insulin (Macdougald and Lane 1995; Rosen and Spiegelman 2000; Sadowski et al. 1992). It is important to note that different cell models require diverse cocktails for the induction of differentiation due to the nature of the reagents used (Gregoire 2001). In 3T3-L1 cell linage culture, this standard mix is used to induce the cells to begin their transformation into adipocytes. As mentioned, DEX is a synthetic glucocorticoid responsible for the induction of C/EBPδ expression and production (Hajduch et al. 1995; Wu et al. 1996), meanwhile blunting the expression of Pref-1 (Smas et al. 1999). MIX heightens differentiation by increasing cAMP levels and through the downstream actions of the cAMP pathway (Pantoja et al. 2008) by enhancing levels of C/EBPβ (Cao et al. 1991). Insulin is required for efficient differentiation of adipocytes because it not only increases the amount of lipid accumulation, but also it reduces apoptotic activity through insulin-like growth factor-I (IGF-I) (Kiess and Gallaher 1998). Insulin action in differentiation is via cross-activation of IGF-I receptors inducing downstream signaling, stimulating many

adipogenic effects (Rosen and Spiegelman 2000). Taken together, MIX, DEX, and insulin create favorable conditions for 3T3-L1 cells to undergo morphological and metabolic changes during differentiation to allow for cytoplasmic lipid accumulation (Zhou et al. 1992).

1.4.2 Mitotic clonal expansion

Once differentiation has occurred in growth arrested cells at G_0/G_1 cycle, they are able to return to the cell cycle for one or two more rounds of DNA replication and cell doubling; a progression known as MCE (Tang et al. 2003). MCE occurs approximately 24 hours after differentiation has been induced and is thought to be a necessary process to allow for the unwinding of DNA, subsequently allowing access for transcription factors to begin appropriate regulatory responses for the development of a mature adipocyte phenotype (Ntambi and Kim 2000). This DNA replication during mitosis also alters the promoter control elements helping to activate regulatory genes which initiate differentiation (Cornelius et al. 1994). However, whether MCE is truly required for differentiation has been deemed controversial; yet studies have identified evidence showing it is a prerequisite and that certain proteins acting as checkpoints for mitosis are important in the regulation of adipogenesis (Otto and Lane 2005; Rosen and MacDougald 2006). Insulin was able to induce MCE solely, similarly to when 3T3-L1 cells are induced into the differentiation process through an adipogenic cocktail (Qiu et al. 2001). Certain growth proteins are essential during clonal expansion and include retinoblastoma proteins such as pRB, p107, p130 each which bind to E2F/DP resulting in an inactivation specific transcriptional growth factors (Gregoire et al. 1998). When there is inhibition of MCE in 3T3-L1 cells, differentiation of preadipocytes has been shown to be blocked (Nam et al. 2008). To note, cell division in primary adipocytes stemming from human adipose tissue is not a requirement when proceeding through differentiation. It is suggested that these specific type of cells have previously experienced the necessary cell divisions *in vivo*, and are already in the late stage of adipocyte development (Entenmann and Hauner 1996).

1.4.3 Adipogenic transcriptional cascade

Necessary communication of extracellular signals by differentiating adipocytes to the nucleus give rise to the introduction of a transcriptional cascade (Gregoire et al. 1998). The coordination of gene-expression events including the expression of PPARγ and C/EBPs, specifically C/EBPα, are transactived and are responsible for adipocyte differentiation (Rosen and MacDougald 2006). These factors trigger terminal differentiation and commitment of cells to the adipocyte lineage (Dani 1999). These changes compound a result of morphological changes and developed fat droplets.

1.4.3.1 PPAR family

PPARγ is a member of PPARs which are ligand-dependent transcription factors that bind the ligand of receptors leading to activation of a target gene (Kersten et al. 2000). PPARs behave similar to hormone receptors in which they bind the promoter region of a specific gene and form a heterodimer with retinoid X receptor (RXR), which results in activation responses in transcription when bound to the ligand hormone (Kersten et al. 2000). PPARγ has been observed in different tissues including white adipose, brown adipose, liver, kidney, heart, and skeletal muscle (Kersten et al. 2000). PPARγ exists as three different isoforms including PPARγ1, PPARγ2, and PPARγ3, each are transcribed from the same gene only with different splicing and promoters (Otto and Lane 2005). PPARγ2 is the adipocyte specific isoform and is of reference in this context. PPARγ is a critical, "master" regulator which functions in many adipose specific genes and its early binding in the differentiation process is required for the development of mature adipocytes (Spiegelman and Flier 1996; Tontonoz et al. 1994). PPARγ is involved in transcription of genes in FA uptake, storage, and specifically adipocyte P2 (aP2), lipoprotein lipase, acyl-coenzyme A synthase and others (Otto and Lane 2005). It has also been demonstrated that expression of PPARγ in low levels in mice has compromised ability to go through adipogenesis (Wu et al. 1999b), as well as having no formation of WAT and little amounts of BAT (Mohamed-Ali et al. 1998). Animals that contain natural PPARγ mutations have impaired adipose functionality, accompanied by lipodystrophy (Mohamed-Ali et al. 1998). In knockout mice models of PPARγ, there was reduced

adipose tissue mass and mouse embryonic fibroblasts resulted in impaired adipogenesis. Other PPARγ knockout mice studies have shown animals developed insulin resistance, however they maintained normal levels of adipose mass and morphology (Rosen and MacDougald 2006). Ectopic expression of PPARγ demonstrates its efficient conversion of fibroblasts to adipocytes when treated with PPARγ activators of ligands, and is a result of its regulatory transcriptional activity (Spiegelman and Flier 1996; Tontonoz et al. 1994). In 3T3-L1 cell culture model, its activity has been aptly demonstrated and shown to increase with insulin (Hu et al. 1995). Additionally, overexpression of the adipokine, adiponectin, also resulted in an increase in PPARγ transcription targets (Attie and Scherer 2009).

1.4.3.2 C/EBP family

The transcription factor family of C/EBPs contains a basic luecine zipper domain, allowing activation via homo-and hetero-dimerization (Gregoire et al. 1998). This dimerization is a criterion for DNA-binding (Otto and Lane 2005). For the regulation of adipogenesis, several different C/EBPs are included: C/EBPβ, C/EBPδ, and C/EBPα (Otto and Lane 2005). Upon hormonal stimulated differentiation *in vitro*, C/EBPβ and C/EBPδ are swiftly increased due to activation by DEX and MIX, respectively (Yeh et al. 1995). They are early regulators in the program of preadipocyte differentiation demonstrated by their ability to accelerate adipogenesis with protein overexpression, specifically in 3T3-L1 cells (Otto and Lane 2005). C/EBPβ, though rapidly expressed after differentiation stimulators, does not possess the DNA binding ability until its phosphorylation occurs and it loses its association with CHOP-10, thus gaining DNA binding activity (Tang et al. 2004). Then, C/EBPβ is translocated to the nucleus where it can participate in DNA binding after a 12 to 16 hour lag phase, simultaneously with the S-phase of MCE (Tang and Lane 1999). Subsequent to DNA binding, C/EBPβ interacts with C/EBP regulating elements within their promoter regions of C/EBPα and PPARγ, to initiate the transcription activation (Zhang et al. 2004). The transcriptional activators increase the expression of C/EBPα and PPARγ, which initiates the progression of many adipogenic genes and terminal differentiation action (Fajas et al. 1999). It is also been

observed that both C/EBPβ and C/EBPδ mediate the expression levels of C/EBPα during day two of differentiation to approximately day five after differentiation induction (Ntambi and Kim 2000). To note, embryonic fibroblasts which did not contain both C/EBPβ, or C/EBPδ were impaired greatly in their development and maturation as adipocytes (Tanaka et al. 1997). Furthermore, a dominant-negative C/EBP compound, termed A-C/EBP, can actively block C/EBPβ DNA binding preventing the translocation of C/EBPβ to the nucleus and progression of MCE and adipogenesis (Zhang et al. 2004).

C/EBPα is imperative in the development of adipose tissue due to its ability to induce adipocyte genes directly (Rosen and MacDougald 2006). C/EBPα gene promoters contain C/EBP regulatory elements, and thereby can act as a positive feedback loop that, once expressed, accounts for the maintenance of both C/EBPα and PPARγ expression (Tang et al. 2004). C/EBPα has been described as one of the central regulators for energy metabolism, due to its modulation of adipocyte genes and encoding proteins specific to adipose tissue (Darlington et al. 1995). In a study done, C/EBPα knockout mice were unable to adequately store body fat. C/EBPα is necessary for normal energy functionality in liver, and in white and brown adipose tissues (Wang et al. 1995). In 3T3-L1 preadipocytes, C/EBPα overexpression resulted in efficient ability to terminally differentiate into mature adipocytes; demonstrating its important role in modulating differentiation (Freytag et al. 1994). Not only is C/EBPα necessary for differentiation, it has been reported as sufficient to activate growth arrested cells without use of hormonal stimulation (Lin and Lane 1994). C/EBPα has been reported to have anti-mitotic activity, thus early expression of this gene would inhibit preadipocytes from entering MCE, a necessary requirement for 3T3-L1 differentiation (Otto and Lane 2005). Importantly, PPARγ and C/EBPα can interact and cooperate together resulting in strong adipogenic responses to maintain terminal differentiation (Spiegelman 1998).

1.4.4 Terminal differentiation

Terminal differentiation is a state in which adipocytes have largely increased in de novo lipogenesis and are sensitive to insulin (Gregoire et al. 1998). Cells are no longer participating in the cell cycle and lose their capacity to proliferate; although after

differentiation, preadipocytes may be able to de-differentiate and re-enter mitosis (Cornelius et al. 1994). Nonetheless, once cells have surpassed the G_D point, they have become fully committed to terminal differentiation (Otto and Lane 2005). Observable changes take place with increased lipogenesis and LD formation. Mature adipocytes have lost their preadipocyte fibroblastic characteristics and appear spherical with the acquisition of biochemical and morphological traits of adipocytes (Macdougald and Lane 1995). Cells have also increased in protein levels related to lipid metabolism and enhanced expression of adipose tissue specific products, such as aP2 and FA binding protein (FABP). Furthermore, lipogenic enzymes and adipokines are highly expressed including leptin, adiponectin, and resistin (Gregoire et al. 1998; Otto and Lane 2005).

1.5 Lipogenesis

Triacylglycerides (TAG) are made up of a glycerol backbone esterified with three FA chains. TAG synthesis activity in WAT is an imperative factor for determination of adipose tissue mass and the release of non-esterified FA (NEFA). As seen in fasting rats, approximately 57% of newly hydrolyzed FA were recirculated for TAG synthesis (Wang et al. 2008). Although FA mainly come from the diet, 1-2% are made in adipose tissue itself (Wang et al. 2008). The carbon movement from glucose to FA is highly regulated by lipogenesis and involves a series of enzymatic reactions (Ameer et al. 2014). During FA synthesis, the first step includes a series of conversion reactions of citrate to acetyl-CoA by ATP-citrate lyase (ACL) (Ameer et al. 2014). Carbons coming from acetyl-CoA are added to a growing chain of FA. The key rate limiting step is ATP dependent on acetyl-CoA carboxylation to malonyl-CoA carboxylase 1 by acetyl-CoA carboxylase 1 (ACC1) (Gathercole et al. 2013). Malonyl-CoA is then transformed by multiple steps into a 16 carbon FA, palmitate, by fatty acid synthase (FAS), the major enzyme accounting for FA biogenesis (Zaidi et al. 2013). Further reactions lead to the production of complicated FA originating from palmitate, resulting in de novo lipogenesis (DNL) products of palmitate, and small amounts of stearate and shorter FA (Ameer et al. 2014).

Once FA have been generated by DNL, they are converted into substrates for TAG formation by becoming saturated and transformed into monounsaturated FA

(MUFAs) by stearoyl-CoA desaturase 1 (SCD1). SCD1 has preference to convert saturated stearate and palmitate to their MUFAs oleate and palmitoleate, respectively. SCD1 is a rate limiting reaction and its desaturase activity has been proposed to reduce FA oxidation and stimulate DNL (Hulver et al. 2005). Biogenesis of TAG is a series of esterification of FA moieties that are consecutively added to a glycerol backbone (Farese and Walther 2009). The acylation of a glycerol-3-phosphate and the first FA occurs by glycerol-3-phophate acyltransferase (GPAT) and the second by diacylglycerol acyltransferase (DGAT) yielding phosphatidic acid (PA), which is then dephosphorylated to produce diacylglycerol (DAG) (Gathercole et al. 2013; Kuerschner et al. 2008). DAG, by a third acylation, then becomes TAG, but could be used as a prerequisite for phospholipids. DGAT2, which is highly associated with adipocytes, is localized to the endoplasmic reticulum (ER) and is responsible for the catalyzed reaction into TAG (Kuerschner et al. 2008). Of the enzymes which are involved in TAG synthesis, they are commonly found in the ER as well as the mitochondria, or in mitochondrial-associated membranes and have different isoforms. Newly generated TAG have been suggested to be at large to go into an associated lipid bilayer and locating to cytosolic LD, or to cells which secrete TAG (Yen et al. 2008).

The biogenesis of LDs are defined as the initial accumulation of neutral lipids tightly connected at the ER (Digel et al. 2010). LDs are linked to a wide variety of functions which include lipid storage, energy and membrane formation, and protein degradation. LD synthesis occurs de novo and are generated in the ER (Walther and Farese 2012). LDs are primarily comprised of an organic phase of TAG and sterol esters in the core, yet other neutral lipids are present such as free cholesterol and retinol ester (Thiele and Spandl 2008). However the majority of the volume in adipocytes is TAG. The initial step is followed a gathering of proteins and increased lipid synthesis to create a globular LD. LDs have been observed in contact with the ER when formed and the specific membrane associated proteins moved between the two complexes (Walther and Farese 2012). In periods of excess energy, TAG need to be stored in LD and allowance for growth in LD requires local coordinated TAG synthesis or a translocation from the ER. This involves large amounts of TAG being located to its core (Farese and Walther

2009). LD droplet formation include ER budding which allows LD to grow from the ER bilayer and then bud off (Walther and Farese 2012). The main thought is that TAG biosynthesis is done between the leaflets of the ER membrane, prior to budding off and forming a LD. It was demonstrated that TAG is synthesized near the surface of the LD, accordingly indicated LDs grow by influx. Thus LDs can grow in size with droplet to droplet fusion and via increasing TAG levels (Kuerschner et al. 2008).

In adipose tissue, all the major lipogenic enzymes are present, however only low levels have been observed compared to rodents (Gathercole et al. 2013). The low levels of ACL, which produces cytosolic acetyl-CoA for FA synthesis, is suggested to be reason DNL in adipose tissue accounts for a small percentage of lipid generation (Gathercole et al. 2013; Wang et al. 2008). Nonetheless, DNL *in vitro* is regulated partly by hormones and the dysregulation of the lipogenic pathway can cause disruption to overall lipid metabolism and homeostasis (Ameer et al. 2014).

1.6 Lipolysis

LDs are a critical and active organelle found in the intracellular cytoplasm of many cells. Previously, adipose tissue and LDs were considered only inert storage locations for excess fat (Ahmadian et al. 2009). However in recent years, LD biology has been an increasing interest due to studies identifying proteins and mechanistic pathways specific to LDs and represent a progressive area in cell biology (Farese and Walther 2009; Walther and Farese 2012). Within the core of the LD, TAG, which are highly hydrophobic, reduced and concentrated, are used for storing energy, occupying much of the space in the cytoplasm (Farese and Walther 2009). Surrounding the LD is a phospholipid monolayer covered with proteins functioning to stabilize and regulate (Brasaemle 2007).

The mobilization of the stored energy stems from lipolysis; the hydrolysis of TAG into NEFA and a single glycerol backbone (Zechner et al. 2012) as shown in Figure 1.2. Lipolysis is an absolute prerequisite for cellular uptake and of release of FA to and from the bloodstream, first observed by Whitehead in 1909 (Whitehead 1909). This catabolic process allows for NEFA to be released during fasting, in times of metabolic need, as

well as when TAGs are in a surplus amount (Zechner et al. 2012). Nonetheless, the process of lipolysis is an intricate phenomenon which orchestrates the use of cellular lipases, plasma membrane transporters, FA binding proteins, and LD associated proteins (Langin 2006a). This mobilization of NEFA from adipose tissue via lipolysis, can be released to enter the bloodstream and taken up by organs or used for β-oxidation and further for ATP formation. NEFA may also be transported to the liver in order to be used as substrates for ketogenesis, and glycerol for gluconeogenesis (Ahmadian et al. 2010).

1.6.1 Lipolytic cascade

Lipolysis occurs in periods of increased energy demand or expenditure, and is activated by hormones (Zimmermann et al. 2004). The concentration of NEFA is highly regulated between a balance of triglyceride hydrolysis and NEFA esterification (Langin 2006b). Hydrolysis points occur at the primary and secondary ester bonds between the FA and the glycerol backbone (Zimmermann et al. 2004). Adipocyte lipolysis occurs in a meticulous order of events and in a regulated fashion, with each step utilizing a different enzyme; sequentially adipose triglyceride lipase (ATGL), hormone sensitive lipase (HSL), and monoglyceride lipase (MGL) (Ahmadian et al. 2009; Walther and Farese 2012; Zechner et al. 2012). ATGL performing the initial cleavage of the first FA is the rate limiting step in which it transforms the TAG to form a diacylglyceride (DAG) and a NEFA. The second lipase, HSL, is a multifunctioning enzyme in that it has the capacity to hydrolyze TAG, DAG, and monoacylglyeride (MAG); its cleavage of FA from the acylesters contain a breadth (Lass et al. 2011). In fact, HSL was formerly thought to be the rate limiting lipase in TAG hydrolysis (Duncan et al. 2007), however, Haemmerle demonstrated through HSL knockout mice a shift in composition of fat: rather than accumulating TAG, KO mice increased in DAG levels, observably showing that HSL is primarily responsible for DAG hydrolysis (Haemmerle et al. 2002). Lastly, MGL removes the final FA, freeing the last NEFA and glycerol (Duncan et al. 2007; Lass et al. 2011). This simple explanation for describing the lipolysis process is tightly regulated by a highly complex system of signals and mechanisms.

Increased levels of NEFA and glycerol in serum to be used as oxidative substrates are products of stimulated lipolysis (Duncan et al. 2007). Catecholamines are the major regulators in fasting induced lipolysis and are considered the starting point in the metabolic pathway that modulates TAG hydrolysis and NEFA release (Duncan et al. 2007). Catecholamines are able to impact lipolysis once they bind to different adrenoceptor subtypes, such as $\beta_{1\text{-}3}$ and $\alpha_{1\text{-}2}$, each linked to a G-protein (Jocken and Blaak 2008). Norepinephrine, a catecholamine, binds to β-adrenergic receptors, on the adipocyte plasma membrane, which is coupled to a G-protein. Of the three β-adrenoreceptors, in humans the first and second are highly active, whereas in rodents β_3 is most active (Barbe et al. 1996; Jocken and Blaak 2008). The G-protein subunit, α_s, then sends a signal to adenylyl cyclase (AC) which then activates the production of cAMP (Duncan et al. 2007). The increase in cAMP intracellular levels leads to the activation of a cAMP dependent protein kinase (PKA) (Carmen and Víctor 2006). Once cAMP binds PKA, this results in the controlling subunits to disassociate with the catalytically active stretch, thus increasing activity of the complex (Kim et al. 2005). This activation of PKA results in the phosphorylation of HSL and perilipin, a LD surface protein, and to further the catalysis of TAG to release NEFA and glycerol (Carmen and Víctor 2006) (Jocken and Blaak 2008). In addition, in the fasted state high levels of glucocorticoids also increase ATGL transcription and generate a signaling cascade (Villena et al. 2004). There are other signaling pathways which also induce lipolytic reactions in 3T3-L1 cells including protein kinase C and its stimulation by phorbol ester PMA in a dependent mitogen-activated protein kinase (MAPKs) and independent MAPK pathway (Carmen and Víctor 2006). Catecholamines can also bind to α_2-adrenoceptors that are coupled to inhibitory G-proteins (Gi). These Gis will inactive AC, thus reducing cAMP levels and muting PKA activity (Jocken and Blaak 2008).

During a fed state, insulin acts as an antilipolytic agent via a regulatory pathway which involves both cAMP dependent and independent pathways (Duncan et al. 2007). In adipocytes, the cAMP dependent cascade involves insulin binding to its receptor which transmits a signal resulting in activation of phosphodiesterase 3B by phosphorylation, in turn degrading cAMP levels (Ahmadian et al. 2010). This reduction of cAMP levels

relieves PKA from activation and blunting the phosphorylation mediated activity of HSL and perilipin (Duncan et al. 2007). The cAMP independent modulating pathway by insulin is through the stimulating phosphorylation of protein phosphatase-1(PP-1) causing an alteration in its regulatory subunit. Activating phosphorylation of PP-1 results in the dephosphorylation and inactivation of HSL, reducing the lipolytic rate (Duncan et al. 2007; Razani et al. 1999). Insulin also has action in regulating the expression of ATGL; insulin has been shown in 3T3-L1 cells to down-regulate ATGL possibly through FoxO1 (Ahmadian et al. 2010; Kershaw et al. 2006).

The physiological relevance of the controlling pathways of lipid metabolism is critical due to the volume of fat that is predominately found in adipose tissue (Jocken and Blaak 2008). Dysregulation of lipolysis and lipid metabolism can lead to severe physiological consequences. Specifically in a high fat diet-induced obesogenic state, there is increased basal lipolysis resulting in increased levels of circulating NEFA (Gaidhu et al. 2010). Defective regulation of lipolysis leads to excessive NEFA accumulation in visceral adipose tissue, and is highly correlated to insulin resistance which can result in impairment of the this hormone to act as an antilipolytic agent (Gaidhu et al. 2010). Overall, alternations in the regulation of this catabolic process can induce physiological impacts that can lead to defective mechanisms and lead to metabolic disorders.

1.6.2 ATGL

In 2004, three different groups identified a novel lipolytic enzyme which was responsible for TAG hydrolysis, and each gave the lipase a different name including ATGL (Zimmermann et al. 2004), desnutrin (Villena et al. 2004), and phospholipase A2ζ (Jenkins et al. 2004). In the scope of this review, ATGL will be the name utilized. ATGL is a 54 kDa protein that has an N-terminal domain similar to patatin, a plant acyl hydrolase, which encompasses its catalytic activity that cleaves at the primary ester bond at sn-1(3) position (Duncan et al. 2007; Lass et al. 2011; Walther and Farese 2012). ATGL is tightly associated with the LD in differentiated 3T3-L1 cells and maintains a hydrophobic region for binding to the LD (Duncan et al. 2007); loss of this domain

results in loss of intracellular activity because of its inability to locate and bind the LD (Zechner et al. 2012). ATGL is considered to be the major lipase in adipose tissue lipolysis with its high substrate specificity to TAG (Ahmadian et al. 2010), showing minimal activity toward other lipids including DAG, MAG, cholesterylesters or retinylesters (Lass et al. 2006; Lass et al. 2011). The localization of ATGL has been determined, with the majority of ATGL locating in the cytoplasm (approximately 50%), yet a portion of it is found to be tightly associated to the LD, around 10% (Zimmermann et al. 2004). This amount of ATGL localized with the LD is not affected by the stimulation of lipolysis, with Zimmermann observing the co-localization to the LD in 3T3-L1 cells (Zimmermann et al. 2004). In energy demand, increased levels of ATGL translocate from the cytosol to the surface of the LD upon activation (Bezaire et al. 2009). When ATGL is genetically altered *in vivo*, NEFA release by lipolysis was reduced greater than 75% in ATGL-deficient mice (Haemmerle et al. 2006; Haemmerle et al. 2002).

ATGL expression and enzymatic activity is tightly regulated. ATGL requires an activator protein which markedly increases its activity known as comparative gene identification -58 (CGI-58) or α/β hydrolase domain containing protein 5 (ABHD5) (Zimmermann et al. 2009) (Lass et al. 2011). Through interaction of ATGL and CGI-58, maximum stimulation is seen at equal concentrations of both proteins (Lass et al. 2006). Studies identifying how the activation occurs are consistent, ATGL's patatin domain inside the αβα sandwich fold is responsible for its activity where CGI-58 interaction also occurs. The C-terminal end of the ATGL includes its regulatory function and location of its LD interaction (Lass et al. 2011). The activation of ATGL by CGI-58 is necessary for lipolytic action and acts independently of HSL (Bezaire et al. 2009). Another key regulator for ATGL involves its interaction with perilipins; proteins which are highly associated with LD surface, in which many are phosphorylated by AMP-dependent PKA upon lipolytic stimulation (Clifford et al. 1998). Studies identifying the complex system of how ATGL is activated as the initial step in lipolysis suggest CGI-58 and perilipins engage the enzyme and mediate ATGLs LD targeting and hydrolysis of TAG (Walther and Farese 2012). Hydrolytic activity of ATGL in fat cell lipolysis, in both the basal and

hormone stimulated conditions, to proceed is necessary and maintains as the first step in the sequential regulation of lipolysis (Lafontan and Langin 2009).

Studies observed ATGL ablation in 3T3-L1 cells showed that with compromised lipolysis, cells were not able to compensate, even with the presence of other lipases (Duncan et al. 2007). ATGL-null mice have extreme TAG accumulation in the heart which resulted in premature heart failure (Brasaemle 2007), as well as reduced lipolysis which lead to fat deposition in many tissues, including fat, liver, muscle, kidney, spleen, and lung tissues (Lass et al. 2011). This has also been demonstrated with patients that contain *ATGL* gene mutations. Similar to ATGL null mice, there is massive TAG accumulation located in multiple tissues as well as the development of cardio myopathy, which is highly lethal (Lass et al. 2011). ATGL mediated TAG catabolism is an essential in all cells and when deficient, there is inability to maintain normal energy metabolism during fasting or energy expenditure (Lass et al. 2011). However, in studies analyzing the effect of high fat diet included obesity, there is disrupted signaling in the lipolytic cascade inducing upregulation of ATGL and its co-activator CGI-58. This results in increased basal lipolysis but blunted catecholamine-induced lipolysis yielding a dysfunctional mechanism, increased circulating NEFA, and contributing to obesity induced metabolic disease state (Gaidhu et al. 2010).

1.6.3 HSL

HSL was first discovered in the 1960s in which its isolation and characterization in adipose tissue demonstrated its innate ability to hydrolyze both DAG and TAG (Zechner et al. 2012). However, this 84 kDa cytoplasmic enzyme has a much greater affinity for DAG with relatively an eleven fold higher hydrolase activity toward DAG than TAG and preference for FA in the sn-1 or sn-3 position (Duncan et al. 2007; Lafontan and Langin 2009; Raclot 1997). Much of the recent knowledge of HSL stems from a study done with HSL-null mice, which demonstrated no changes in TAG accumulation in adipose tissues and nonadipose tissue, yet the level of DAG was enhanced (Haemmerle et al. 2002; Zechner et al. 2012). This led to the accepted thought that HSL is the second rate-limiting step in lipolysis and ATGL is responsible for initial

TAG hydrolysis. HSL also retains specificity to other lipid ester bonds such as cholesteryl esters, retinyl esters, and short-chain carbonic acid esters (Duncan et al. 2007; Zechner et al. 2012). The largest expression concentrations of *HSL* mRNA and protein include white and brown adipose tissues, although there is some low levels seen in other organs (Zechner et al. 2012). To date, there are three regions of HSL which make up its functional capacities including the N-terminal domain, C-terminal domain, and a third region presenting as the controlling component (Lafontan and Langin 2009). The N-terminal region has been suggested as the modulator of lipid binding, dimerization of the enzyme, and interface with FA binding protein 4 (FABP4) (Lass et al. 2011). The action of HSL is increased by the presence of aP2 and FABP4, both which interact and aid in the movement to the surface of LD (Wang et al. 2008). The C-terminal domain contains a commonly seen enzymatic α/β hydrolase fold. The regulatory region is found in the catalytic domain where HSL's five phosphorylation sites are located (Lass et al. 2011; Yeaman 1990).

HSL is regulated by hormones, similar to ATGL, and thus results in the second step in lipolysis. Through β-adrenergic stimulation, HSL is the target of PKA phosphorylation and other kinases which regulate its enzymatic activity (Lass et al. 2011) (Zechner et al. 2012). HSL phosphorylation at 5 serine residue sites by PKA, AMPK and MAPK are a critical regulatory steps for hormone-induced lipolysis and increases HSL lipolytic activity (Zimmermann et al. 2009) (Walther and Farese 2012). Through overall modulation of cAMP levels, catecholamines and insulin levels play a role in the regulation of HSL action (Lafontan and Langin 2009). This phosphorylation also allows for the recruitment of HSL to the surface of the LD and increased HSL activity 100 fold (Schweiger et al. 2006). The regulation of HSL induces a two-step process of activation: PKA phosphorylates HSL at several serine sites (Ser81, Ser222, and Ser276) resulting in translocation from the cytosol to the LD and binds HSL to a perilipin adipose differentiation-related protein, and secondary phosphorylation of serine-660 (Ser660) is responsible for the imperative LD interfacial activation and hydrolysis (Walther and Farese 2012; Wang et al. 2009). The activation of the signaling kinase pathway which stimulates lipolysis via phosphorylating HSL at Ser660 has been demonstrated *in vitro*

(Greenberg et al. 2001). The lack of HSL greatly affects overall adipocyte function and the hydrolysis of DAG and release of NEFA (Zimmermann et al. 2009).

1.6.4 MGL

The final step in lipolysis is catalyzed by MGL, the rate-limiting point for cleaving the final FA from the glycerol backbone. MGL's breakdown of MAG is resultant after the intracellular TAG hydrolysis by ATGL and DAG by HSL, and occurs in the cytoplasm of the cell (Zechner et al. 2012). This 33 kDa lipase was found in 1975 and purified from rat adipose tissue with its role clarified as the monoacylglyeride enzyme (Tornqvist and Belfrage 1976). MGL is responsible for cleaving the 1(3)- or 2-ester bonds of MAG, with specificity to only MAG, and no observed activity against TAG, DAG, or cholesteryl esters (Duncan et al. 2007). Similar to the other lipolytic enzymes, MGL has amphiphilic character and also contains a α/β hydrolase fold (Karlsson et al. 1997). Structurally, MGL's catalytic triad have been proposed as Ser122, in a GXSXG motif, Ap239, and HIS 269; mutation at any of these sites results in complete ablation of its lipase ad esterase activity (Duncan et al. 2007; Lafontan and Langin 2009; Lass et al. 2011). MGL is highly expressed in adipose tissue, but has been seen in other tissues (such as kidney and testis), and has demonstrated its ability to degrade MAG has been studied intensively via mutant mouse models (Chanda et al. 2010; Lass et al. 2011; Zechner et al. 2012). Without MGL activity in adipose tissue the lipolysis process is compromised and increases in levels of MAG are observed (Zechner et al. 2012). Recently, a new role for MGL in endocannabinoid signaling has been suggested, yet more investigations to fully understand its role in regulating appetite, pain sensations, and mood control need to be addressed (Lass et al. 2011). Currently, the literature reveals that the activity of MGL is not dependent upon hormonal stimulation, but necessary for complete degradation of TAG *in vitro* (Zimmermann et al. 2009). MGL activity has not been observed as rate limiting due to the abundance of the enzyme. Nonetheless, it plays a pivotal role as a modulator of 2-acylglyerol level release of the final NEFA, and is the last, but necessary step for the lipolysis process (Chanda et al. 2010; Zimmermann et al. 2009).

1.6.5 Perilipin

Perilipins are proteins which are tightly associated with adipocyte LD at the surface (Clifford et al. 1998). There are three protein isoforms stemming from the translation of alternatively spliced mRNA. These include perilipin A, which the largest with 517 amino acids (AA) and found in high numbers on the surface of the LD in adipocytes; perilipin B, that shares 405 AA with perilipin A , and perilipin C (Brasaemle 2007). Perilipin C is the shortest isoform of the three and is expressed solely in steroidogenic tissue cells (Brasaemle 2007).

For lipolysis to occur, soluble lipases need to access a hydrophobic LD and begin action on the TAG substrate, and perilipin proteins are a prerequisite for their action and contact with the LD (Duncan et al. 2007). During lipolysis perilipins are highly phosphorylated and stimulated by PKA, alongside HSL. Perilipins which lack phosphorylation by PKA, act as a barrier to lipid lipases and protect against hydrolysis (Clifford et al. 1998). The primary LD associated protein that is present in mature adipocytes is perilipin A(1), both in differentiated 3T3-L1 cells and murine primary cells (Brasaemle et al. 2000) (Shen et al. 2009). Perilipin A is a member of the PAT family and regulates WAT breakdown by controlling ATGL and HSL via mediating activity (Lass et al. 2011). It is highly expressed in adipocytes and interacts with the LD, specifically at the three C-terminal hydrophobic stretch (these are able to penetrate the LD) (Walther and Farese 2012). At the surface of the LD, perilipin A contains an amphipathic area, which has been suggested to add to lipid binding (Walther and Farese 2012). In unstimulated conditions, ATGL and HSL are located at the surface of the LD and are seen up to 50%; perilipin A and B which coat the LD are the only functional barrier that inhibit their action (Brasaemle et al. 2000). Perilipin A, the greatest phosphorylated protein behind PKA, plays an imperative role in the modulation of both basal and stimulated lipolysis. This is demonstrated by increased basal lipolysis and perilipin null mice loss of PKA-stimulated lipolysis (Martinez-Botas et al. 2000; Shen et al. 2009). Simultaneous to HSL phosphorylation, PKA also phosphorylates perilipin A on six different serine residues (Shen et al. 2009; Wang et al. 2009; Zechner et al. 2012). Increased TAG storage and inhibition of lipolysis has been observed with increased

expression of perilipin A. Discovered in 3T3-L1 cells, preadipocytes subjected to perilipin A transfection showed large reductions in lipolytic rates and even larger increases in TAG storage (Brasaemle et al. 2000; Duncan et al. 2007). Perilipin proteins located on the LD are critical for PKA stimulation of HSL translocation, and has been shown to be necessary for maximal lipolysis (Miyoshi et al. 2006). The phosphorylation of perilipin by PKA may also enable interaction with HSL on the LD, thus increasing enzymatic activity (Duncan et al. 2007; Miyoshi et al. 2006). Perilipin, during fasting conditions, also allows for the recruitment of ATGL and CGI-58 to the LD via dissociation of CGI-58 from perilipin A and activation of ATGL (Ho et al. 2011) (Lass et al. 2011; Zechner et al. 2012). Although these proteins are not critical for LD establishment, they are necessary in regulating lipid metabolism (Walther and Farese 2012). Overall perilipin A plays an important dual role in regulating lipolysis, first in preventing basal lipolysis and second upon PKA stimulation, increasing the process (Langin 2006a; Shen et al. 2009).

1.7 NEFA

Under normal conditions, NEFA have the ability to act as a fuel source alternative to glucose, occurring most often during times of fasting or increased energy expenditure (Unger 1995). In a fed state, insulin acts as an antilipolytic agent by suppressing NEFA levels and allowing glucose to be metabolized (Unger 1995). In obesity and type 2 diabetes, plasma NEFA are elevated in these conditions due to their lack of suppression by feeding as a result of insensitivity to insulin by adipocytes or increase in adipocyte mass (Kahn et al. 2006; Unger 1995). Excess NEFA toxicity, along with reduced normal oxidative metabolism frequently results in ectopic fat accumulation in non-adipose tissues leading to pancreatic β-cell dysfunction, cardiomyopathy and hepatic steatosis; termed lipotoxicity (Weinberg 2006). Excess lipid accumulation in other tissues other than adipose tissue can result in severe metabolic complications, including insulin resistance, diabetes, coronary heart disease, and even heart failure (Listenberger et al. 2003). Evidence has shown that increased circulating NEFA are contributors to this deposition to non-adipose tissues causing lipotoxicity and subsequent disorders

(Listenberger et al. 2003) as shown in Figure 1.3. Multiple pathways can be altered as a result of both acute and chronic effects of excess NEFA concentrations (Weinberg 2006). NEFA are important for normal pancreatic β-cell function and adaptive response to insulin. However, β-cell dysfunction leads to weakened glucose tolerance and fasting glucose, and can lead to insulin resistance and type 2 diabetes (Kahn et al. 2006). The loss of β-cell function is progressed by increased NEFA plasma levels, with chronic exposure of NEFA resulting in impaired glucose stimulated insulin secretion, insulin biogenesis and induction of apoptotic pathways in pancreatic β-cells (Sarafidis and Bakris 2007). Thus, NEFA at amplified levels are a primary factor mediating the link between obesity and type 2 diabetes due to its causation of insulin resistance and β-cell dysfunction (Kahn et al. 2006).

NEFA plasma concentrations are increased in an obesogenic condition and are highly indicative of the dysregulation of lipolysis (Lafontan and Langin 2009). Two components of lipolysis which could contribute to obesity include reduced plasma delivery from adipose tissue during energy expenditure or fasting, or impaired suppression of NEFA by insulin. The latter leads to the therapeutic potential of identifying antilipolytic agents due to the fact excessive lipolysis results in high circulating levels of NEFA and development of dyslipidemia and metabolic syndrome (Langin 2006a). Insulin mediated glucose uptake in cells as well glucose tolerance has been shown to improve upon acute action with an antilipolytic agents which helps to reduce NEFA levels (Kahn et al. 2006). It has been shown that cellular TAG accumulation is not toxic and in fact, accumulation in TAG pools of surplus NEFA, rather than locating to other pathways (in non-adipose tissues) that lead to cytotoxicity, act as a buffer against lipotoxicity (Listenberger et al. 2003). Thus, identifying antilipolytic agents which prevent lipolysis can be used as a therapeutic approach to avoid amplified NEFA levels all together.

1.8 Lipolysis as a therapeutic target for metabolic disease

With obesity, a state of sufficient increase in adipose tissue mass causing adverse health effects, dramatically increased, so has the amount metabolic disorders (Spiegelman

and Flier 2001). Metabolic syndrome is described as an array of contributing factors which negatively impact human health which includes insulin resistance, visceral (central) obesity, hypertension, a proinflammatory state, and increased circulating TAG and NEFA concentrations (Gertow et al. 2006). Obesity is associated with many metabolic syndromes, but specifically obesity results in an increase in basal levels of lipolysis, which can lead to insulin resistance (Duncan et al. 2007). Although basal lipolysis can be cut in half upon weight loss, often the desire to lose is greater than action taken (Brown 2013; Reynisdottir et al. 1995). Insulin insensitivity in adipocytes is a contributing factor to increased basal lipolysis as well as β-adrenergic receptors to stimulated lipolysis are often compromised in an obese individual (Duncan et al. 2007). Increased levels of lipolysis results in enhanced levels of circulating NEFA which are highly correlated to harmful whole body metabolic effects as discussed above.

There is therapeutic potential in regulating and manipulating lipolysis as a means to reduce levels of NEFA. In an obesogenic condition, multiple disorders can arise as a result of adipocyte lipolysis including ketogenesis, ketolysis, mitochondrial disease, and glycogen storage diseases (Wang et al. 2008). It was observed that both obese and non-obese relatives of obese patients alike share in atypical lipolytic processes (Wang et al. 2008). Lipolysis suppression could be very beneficial with inborn errors of fatty acid oxidation (FAO) and ketone metabolism (Wang et al. 2008). Altering lipolysis has large potential in re-balancing NEFA flux and regulating fat mass as a way to reduce the risk of obesity-induced complications in metabolic syndrome.

Currently, identification of mechanisms and potential targets to develop anti-obesity drugs are being done, many which relate to metabolic syndrome (Grundy 2006). Specifically in adipose tissue, there are metabolic pathways which could be significant in targeting as obesity contributors to metabolic syndrome conditions. However, drug development is in preliminary stages and remains to be safe and effective, especially if chronic use is needed with minimal side effects (Grundy 2006). Specifically, identifying compounds which inhibit lipolysis in an effort to lower serum levels of NEFA is a strategy to manage obesity associated diseases, like type 2 diabetes (Ahmadian et al. 2010). Increased NEFA being released at higher rates than oxidative capacity can also

cause ectopic TAG storage and lipodystrophy; and chronic levels of NEFA in circulating plasma can have a lipotoxic effects (Ahmadian et al. 2010; Lass et al. 2006). Taken together a vicious cycle ensues with increased mobilization of NEFA from adipose tissue, typically suppressed by insulin, yet adipocytes that become insulin resistant, consequently lipolysis is further increased (Karpe et al. 2011). Therefore identifying was to alleviate lipolysis and NEFA release will in turn produce approaches to treat obesity-induced metabolic syndromes.

1.8.1 Lipolysis and cancer cachexia

Lipolysis may also be involved in cancer-associated cachexia (Zechner et al. 2012). Cancer cachexia is defined as an intricate metabolic syndrome commonly associated with illness and characterized by persistent erosion of body mass due to a malignant growth. It has also been termed as an auto-cannibalism or wasting disease (Evans et al. 2008). Also commonly associated with cancer cachexia is anorexia, inflammation, insulin resistance, anemia, and hypogonadism (Evans et al. 2008). This wasting disease is primary due to the involuntary loss of skeletal muscle and adipose tissue, which are the majority and best studied targets of cachexia (Beutler and Cerami 1988). Typically, there are increased circulating factors that can induce lipolysis which include TNF-α, IL-6 and zinc-α-glycoprotein 1 (Zechner et al. 2012). TNF-α was isolated as a mediator of cachexia via its stimulation of adipocyte breakdown (Tracey et al. 1988). Cancer patients with cachexia have been observed to have higher activity of lipolytic enzymes, NEFA and glycerol levels, suggesting that lipolysis signaling is involved in the severe loss of adipose tissue and muscle. Although the fundamental mechanisms remain unknown, inhibition of lipolysis may play a role in helping to prevent or attenuate cachexia in people with cancer or other diseases (Evans et al. 2008; Zechner et al. 2012).

1.9 Dietary intervention of obesity

As the obesity epidemic continues to progress, there is large opportunity for functional ingredients that may be beneficial for weight control to be used in food product development (Kovacs and Mela 2006). Therefore, to establish a dietary

intervention strategy to combat obesity, a variety of factors need to be taken into consideration for practical feasibility. In addition, to mechanistic and clinical efficacy evidence, a proposed food ingredient should satisfy other criteria including safety, stability in processing and shelf life, raw ingredient quality and reliable sourcing, regulatory usage levels and dosing schedule, cost, and adequate and appealing food vehicle (Kovacs and Mela 2006). Bioactive components from food are attractive due to their extensive history of safe consumption as well as their health benefits, including weight control. The following focuses on dietary sources of functional components that help to prevent weight gain, aid in weight control, or reduce risk factors associated with excessive adipose mass.

1.9.1 Role of phytochemicals in adipose function

The potential for natural products to combat obesity is large due to the dissatisfaction of current anti-obesity drugs on the market (Yun 2010). Natural products that could be used to treat obesity include crude extracts or plant sourced isolated compounds. There have been many studies identifying and demonstrating the benefit of different dietary components in regards to their impact on energy balance (Astrup et al. 2010). Specifically, research has aimed to improve our understanding how bioactive food component help to inhibit energy storage, reduce lipid uptake or formation, stimulate fat mobilization, or increase levels of fat oxidation via different mechanisms (Kovacs and Mela 2006).

Adipocytes play a large role in overall homeostasis and energy balance by storing TAG and releasing NEFA based on energy demand as previously described. Studies have looked to identify anti-obesity biomaterials which inhibit adipocyte proliferation or differentiation (Yun 2010). However, contemporary research have suggested that inhibiting adipogenesis can be unhealthy and may lead to pathogenesis of type 2 diabetes or other metabolic diseases (Lefterova and Lazar 2009). Therefore pharmacological targeting of lipolysis has been investigated to help partially block the release of the already high levels of circulating NEFA in obesity and dyslipidemia. Many of the natural components which had been found to affect lipolysis do so by stimulation or activation of

the lipolytic pathway rather than reduce it. It was found that Nobiletin, a polymethoxylated flavone found in citrus fruits, dose-dependently stimulated lipolysis in 3T3-L1 adipocytes through activation of cAMP/PKA pathway (Saito et al. 2007). *Toona sinesis* leaf extracts have also been shown to enhance lipolysis in differentiated 3T3-L1 cells (Hsu et al. 2003). The aromatic compound of red raspberries, raspberry ketone, has the ability to increase norepinephrine–induced lipolysis in white adipocytes through enhanced translocation of HSL to the surface of the LD (Morimoto et al. 2005). The hot water soluble extract of *Salacia reticulata,* which is a Hippocrateaceae plant, demonstrated stimulation of lipolysis in rat epididylmal adipocytes (Yoshikawa et al. 2002). Oolong tea also has anti-obesity properties through its major bioactive component, caffeine, that acts on the lipolysis process via interactions with LD lipases (Han et al. 1999; Yun 2010). In fact, caffeine and other methylxanthines stemming from tea and coffee are well characterized and known to stimulate lipolysis through increases in cAMP levels (Duncan et al. 2007). Flavonoids genistein, diadzein, coumestrol, and zeralenone all show a dose-dependent effect in enhancing lipolysis in rat adipocytes, along with quercetin, luteolin, and fisetin, imposing dose- and time-dependent increases (Rayalam et al. 2008a). Resveratrol, a highly studied polyphenol found in grapes, has also been studied in regards to its stimulatory effect on lipolysis through activation of ATGL (Lasa et al. 2012b). Ethanol contains acute antilipolytic effects resulting in reduced levels of FFA in serum as well as in chronic conditions adipocytes show suppression of β-adrenergic receptor mediated lipolysis (Duncan et al. 2007; Kang et al. 2007). Of these dietary sources, most show an induction of lipolysis, and few food components have demonstrated an antilipolytic effect.

1.10 Resveratrol and resveratrol metabolites

Resveratrol (*trans*-3,5,4'-trihydroxystilbene) is a phytoalexin polyphenolic, naturally occurring compound that is produced in plants when there is a pathogenic attack or induced stress, not exclusive to fluctuations in climate, sunlight, and heavy metals (Athar et al. 2007; Fremont 2000). It is present in food such as berries, grapes, and peanuts and has many biological activities which include acting as an antioxidant, anti-

inflammatory, neuroprotective, anti-aging, anti-cancer, and cardioprotective (Baur and Sinclair 2006; Fremont 2000; Wang et al. 2004). The efficacy of oral administration of resveratrol depends on absorption, metabolism, and tissue distribution, yet rapid metabolism generally leads to the production of sulfates and glucuronides (Baur and Sinclair 2006; Hoshino et al. 2010). Due to the nature of the breakdown, it has been suggested that the physiological significance of having increased concentrations of resveratrol, used in *in vivo* studies, more likely should be given to resveratrol metabolites and their effects (Hoshino et al. 2010), structurally shown in Figure 1.4. Levels of resveratrol are generally exceeded by resveratrol metabolites due to phase II metabolism and longer plasma half-life of its metabolites; resveratrol has a short plasma half-life while the metabolites can maintain a plasma half-life of 9.2 hours (Athar et al. 2007; Patel et al. 2010). Due to its rapid breakdown and extensive metabolism, there is a 20 fold higher concentration of resveratrol conjugates which circulate, than resveratrol itself, equating to less than 1% (Andres-Lacueva et al. 2012). This is a major issue in maintaining resveratrol's title as the efficient functional compound, and raises the question of the biological activities and the effect of resveratrol metabolites themselves (Lasa et al. 2012a).

Research that has investigated resveratrol and its metabolites *in vivo* has detected glucuronide and sulfate conjugates, with only a small amount of resveratrol that was left unaltered (Hoshino et al. 2010; Urpi-Sarda et al. 2005). For example, it was shown that with oral ingestion of 25 mg of dietary relevant resveratrol, plasma concentration detected was in the nanomolar range, whilst its metabolites maintained levels in the micromolar range (Calamini et al. 2010; Walle et al. 2004). It has also been reported that generally speaking, glucuronides have been the leading metabolite in rodents, whereas sulfates are more prevalent in human plasma (Burkon and Somoza 2008; Emilia Juan et al. 2010; Lasa et al. 2012a). After glucuronidation and sulfation of resveratrol in both the gut and liver, resveratrol metabolites are taken up dependently by ABC transporters (Kenealey et al. 2011). The distribution of the resveratrol metabolites also depends on specific location, the concentration delivered, and the species (Andres-Lacueva et al. 2012). These facts suggest that if not most, some of the biological effects stimulated by

resveratrol should be attributed to its metabolites (Hoshino et al. 2010) and their activity (Wenzel and Somoza 2005). Furthermore, resveratrol metabolites have shown significant effects in biological activities (Lasa et al. 2012a).

Research identifying tissue distribution of resveratrol metabolites has primarily focused on profiling the liver, kidney, lungs, brain, and testis, however more recently, resveratrol metabolites were found located at intermediate levels in adipose tissue and even low levels in skeletal muscle (Andres-Lacueva et al. 2012). The majority investigations of resveratrol metabolites are researched in regards to their distribution and profile of their relative levels in tissues, with limited studies focusing their action. Resveratrol metabolites do exhibit levels of antioxidant activity, yet it is lower than the parent compound because their radical scavenging –OH groups may be blocked by sulphation, glucuronidation, or methylation (Halliwell 2007). In a study investigating the chemoprotective activity of sulfate metabolites of resveratrol, two were able to inhibit COX activity and NO production (Hoshino et al. 2010). It has also been demonstrated that resveratrol metabolite, resveratrol-4‘-O-sulfate inhibits COX-1 and COX-2 activity, which help to exert cardioprotective and anticancer effects. Other sulfate metabolites, have been suggested to promote longevity through inhibition of SIRT1 (Calamini et al. 2010). Resveratrol-3-O-sulfate was able to inhibit colon cancer cell proliferation having the potential to act as a chemotherapeutic drug to induce apoptosis (Aires et al. 2013). Interestingly, a mixture of metabolites, resveratrol-3-O-sulfate, resveratrol-3-O-D-glucuronide and resveratrol-4’-O-D-glucuronide, induced anti-proliferative activity greater than resveratrol (Aires et al. 2013). It has also been reported that the sulfate conjugates have anti-inflammation properties (Hoshino et al. 2010; Lasa et al. 2012a). Piceatannol (3, 3’, 4, 5’-*trans*-trihydroxy stilbene) another resveratrol metabolite, has also shown potential to have activity in activation of AMPK and GLUT4 translocation, suggesting prevention and improvement for diseases such as type 2 diabetes (Minakawa et al. 2012). Also it has been studied for its antioxidant, anti-cancer, and apoptotic effects (Piotrowska et al. 2012). Accordingly, resveratrol metabolites are involved in diverse biochemical processes that have the potential to exert positive health promoting effects; yet their action is dependent on the specific function investigated.

1.10.1 Resveratrol metabolites in lipid metabolism

Currently, there is minimal knowledge of the effect of resveratrol metabolites in adipose tissue and lipid metabolism. One study demonstrated several resveratrol metabolites had a significant reduction of TAG levels in maturing pre-adipocytes and mature adipocytes (Lasa et al. 2012a). Resveratrol-4'-O-glucuronide and resveratrol 3-O-sulfate both showed significant reductions in lipid species in maturing pre-adipocytes greater than resveratrol. Resveratrol-3-O-glucuronide and resveratrol-4'-O-glucuronide both showed delipidating effects in mature adipocytes. Taken together, resveratrol metabolites, target both maturing pre-adipocytes as well as mature adipocytes and may been involved in anti-obesity effects (Lasa et al. 2012a)

In studies identifying the tissue distribution and levels of resveratrol metabolites, have noted that not all the metabolites have been identified (Cottart et al. 2010). There is large inter-individual variability in metabolism and the fate of up to 30% of resveratrol dosage may not be recovered in urine or feces. Due to the various distribution of resveratrol and its metabolites, there may be other metabolites which have been bound to cellular membranes of lipophilic fractions and tissue and gone undetected (Cottart et al. 2010). Therefore other resveratrol metabolites, such as *trans*-3, 4', 5-trimethoxy resveratrol could also have potential and bioactive functionality. Identification of resveratrol metabolites may be method to help resolve the issues of resveratrol's poor bioavailability and rapid metabolism, and be a an alternative source for increasing health (Minakawa et al. 2012).

1.11 *Trans*-3, 4', 5-trimethoxy resveratrol

Stilbenes are widely available in foods such as mulberries, grapes, red wine, and peanuts (Shi et al. 2012). Food and herbs which contain beneficial phytochemicals have potential at curbing the increase of metabolic disorders and diseases. Although studies have supported the use of resveratrol in prevention of diseases, stilbenes related to resveratrol with the same backbone but differing substituents may contain greater potency (Dias et al. 2013). In an attempt to understand the role of resveratrol analogs that possess biological effects, new attention has been turned to *trans*-3, 4', 5-

trimethoxystilbene or *trans*-3, 4', 5-trimethoxy resveratrol (TMR). It is naturally found in the *Pterobolium hexapetallum* plant (Aggarwal et al. 2004) and has also be isolated from *Virola cuspidata* (Blair et al. 1969). The methoxy groups attached to TMR enhances the transportation to cells as well as metabolic stability; it is partially protected from further metabolism by enzymatic glucuronidation or sulfation. Also, TMR has greater lipophilicity and is highly likely to be distributed into fat tissues, and generally found in larger amounts in major organs than plasma (Dias et al. 2013). In a pharmacokinetic study, TMR maintains enhanced properties in clearance, plasma exposure, as well as absolute bioavailability (Lin et al. 2009).

1.11.1 Anti-cancer activity

Dietary factors that have the ability to modify progression and carcinogenesis are of large interest in epidemiological studies. Several studies have identified TMR as a potential anti-cancer agent, acting *in vitro* and *in vivo,* for its chemoprotective properties in various cancer types which include colon, leukemia, breast, and prostate. TMR has been shown to be different that other stilbenes in regards to its mechanism of cytotoxicity on neoplastic cells; suggesting its efficacy in different cancers (Simoni et al. 2006). TMR observed in HL60 cells, was highly active due to its structure-function activity relationship. The methoxy derivatives from resveratrol showed significantly better activity against HL60 leukemic cells (Simoni et al. 2006). Also noted, was that in many studies, stilbenes act as phase-specific drugs, blocking cells in a specific phases of the cell cycle. For example, resveratrol and piceatannol induced a block in the S phase, suggesting that they act as phase-specific cytotoxic agents. However, TMR demonstrated no modification in cell cycle distribution (G_0-G_1, S, or G_2-M) compared to the control. Overall, TMR maintained cytotoxic and apoptotic-induced activity and an ability to reduce neoplastic cells in all phases of the cell cycle (Simoni et al. 2006). TMR also was more potent than resveratrol against different human cancer cells lines including HT-29 (human colon adenocarcinoma), PC-3 (human prostate cancer), and COLO25 (human colorectal carcinoma) (Pan et al. 2008). The presence and location of the methoxy groups can be attributed for the biological activity, seen within hours of treatment. The induction

of apoptosis with TMR treatment is credited to its strong inhibition of cell viability, causing potent apoptosis and DNA laddering in COLO25 cells (Pan et al. 2008). TMR is thought to trigger apoptosis via p53 dependent proteins and effecting mitochondrial function. *In vivo*, TMR significantly reduced tumor growth in SCID mice with no cytotoxic effects in the mice when dosed at 50 mg/kg (Pan et al. 2008). Taken together, TMR may have potential in inhibiting cancer proliferation in colorectal carcinomas.

Similarly, in human prostate cells, TMR showed limited DNA damage, which could trigger reduced stimulation of p53 and subsequently p21 (Hsieh et al. 2011a). TMR maintained little effect on the cell cycle transition in CWR22Rv1 cells, but did decrease G_1 and expanded G_2M in PC-3 cells, different from other resveratrol metabolites. This suggests that TMR is active against different stages in prostate cancer cells and may use distinct mechanisms (Hsieh et al. 2011a). TMR studies in cell proliferation assays in three types of cells, LNcaP, Du145, and PC3M, demonstrated higher potency in growth inhibition than resveratrol with highest potency in LNCaP cells (Dias et al. 2013). Also, TMR dose-dependently inhibited colony formation in LNCaP-Luc cells *in vivo* with large reductions in size and number of colonies (Dias et al. 2013). Finally, to elucidate the anti-cancer effects *in vivo* using xenografts with prostate cancer cells, TMR pretreatment in mice helped to attenuate tumor development and progression. It was proposed that the phytochemicals created a microenvironment in which tumor initiation and development was reduced. Reported data shows TMR had enhanced anti-proliferative, anti-clonogenic, and anti-inflammatory properties, also higher amounts of TMR located in serum and target tumor tissue compared with resveratrol (Dias et al. 2013). With *in vitro* studies with breast cancer cell lines, TMR was able to induce apoptosis. And in biochemical analysis it is suggested that upon binding TMR to integrin αvβ3, a MAPK signaling pathway is stimulated leading to p53 activation and apoptosis which is different than routes seen with resveratrol and piceatannol (Hsieh et al. 2011b).

1.11.2 Antiangiogenic activity

Uncontrolled endothelial cell proliferation is commonly seen in tumor neovascularization and angioproliferative diseases. Two models to affect

neovascularization include identification of antiangiogenic compounds which prevent new blood vessel development, and disruption by vascular-targeting agents within neovasculature (Belleri et al. 2005). TMR has demonstrated efficacy in both areas and is identified as a microtubule-destabilizing agent, causing rapid, microtubule depolymerization in endothelial cells. Results indicated that TMR has antiangiogenic and vascular-targeting capacity and inhibits tumor proliferation (Belleri et al. 2005).

TMR evaluated for its cytotoxicity in human cancer lines was studied in human endothelial cells and zebra fish blood-vessel formation *in vivo* (Alex et al. 2010). TMR demonstrated its high potency as an antiangiogenic and vascular disrupting agent, greater than resveratrol *in vitro* and *in vivo*. TMR showed different efficacy in three cancer cells lines (HepG2 (human hepatoma), and human breast adenocarcinoma (MCF-7 and MDA-MB-231)) *in vitro*, compared to resveratrol. And in HUVECs (human umbilical vein endothelial cells) *in vitro* and zebra fish embryo *in vivo,* TMR's antiangiogenic activity was more potent and expressed greater specific cytotoxic effects on endothelial cells. Mechanistically, further insight showed TMR may act through down regulation VEGFR2 expression in the VEGF/VEGR pathway and cause cell-arrest at G_2/M phase (Alex et al. 2010).

1.11.3 Other bioactive properties

Allergies and allergic reactions stem from hypersensitivity to a person's immune system which induces a defense system to harmless substances (Matsuda et al. 2004). TMR was able to significantly inhibit the release of β-hexosaminidase by ionomycin in RBL-2H3 cells, which correlates to anti-allergenic activity (Matsuda et al. 2004). In murine hippocampal HT22 cells, TMR failed to protect the cells from glutamine-induced cytotoxicity and was unable to increase HO-1 expression and SIRT1 activation. HO-1 has been reported as one of the main mediator of antioxidant activity and cytoprotection (Kim et al. 2012). Therefore increased expression HO-1 is partially responsible for cytoprotective actions in HT22 cells. The data suggests that the methoxy groups of TMR interfere with its ability to resist oxidative stress (Kim et al. 2012).

Overall, TMR presents as a biologically active resveratrol metabolite and analog in variety of cellular functions and in different cells types. However, its specific role in lipid metabolism is still to be investigated. Understanding TMR's effects in other systems reveals its activity and suggests it may have potential in a wide range of areas for beneficial health effects.

1.12 *In vitro* model

In vitro systems used to study adipocyte function have been used for the last 30 years and helped to identify molecular mechanisms and distinct cellular events (Gregoire et al. 1998). Cell lines which are morphologically similar to primary preadipocytes in a fibroblast like shape, and are able to transform into round, lipid accumulating mature fat cells have been established. Preadipose cell lines have already committed to adipocyte lineage and are able to represent different stages of adipocyte differentiation (Cornelius et al. 1994; Gregoire et al. 1998). One of most frequently used and studied cell lines includes 3T3-L1 cells. They are beneficial in studying molecular events in adipocyte biology because they are well characterized, a reliable model, and can be passaged indefinitely allowing for a consistent source for investigation (Ntambi and Kim 2000). Additionally, since these cells are derived from cloning their homogeneity helps to yield conclusive results to different treatments. 3T3-L1 cells were initially selected as a consistent model for preadipocyte differentiation because of their ability to accumulate TAG (Cornelius et al. 1994). When injected into mice, 3T3-L1 preadipocytes not only differentiated and formed fat pads, they were histologically indistinguishable from the normal adipose tissue (Cornelius et al. 1994; Ntambi and Kim 2000). In cell culture protocols, 3T3-L1 rapidly and in sync can proliferate and differentiate with very similar characteristics of adipocytes from animal tissue; formation of LD are also very similar to those seen *in vivo* adipose mass (Ntambi and Kim 2000). These cells are also very coordinated to the pathways of de novo FA and TAG biogenesis demonstrated through expression of every enzyme involved (Macdougald and Lane 1995).

Nonetheless, there are some disadvantages to using cell lines in studying adipocyte biology. Being that these cells are aneupolid, they may not always accurately

resemble preadipocytes found *in vivo* along with the fact that they are out of the context of a normal extracellular matrix (Ntambi and Kim 2000; Rosen et al. 2000). And while these types of cell lines have similar characteristics, across the board there may be differences between the established lines. Presumably, these differences are due to the stage of preadipocyte differentiation that the cloning occurred (Macdougald and Lane 1995). Preadipocytes in these cell lines represent early, intermediate, or late stage development, therefore the response to different hormones or treatments may be varied (Macdougald and Lane 1995; Ntambi and Kim 2000). Most all cell lines differentiate into WAT exclusively, and do not represent any changes to BAT. Finally, preadipose cells lines may not be derived from the same fat depot, which is an important consideration because there are known differences in metabolic behavior of mature adipocytes between visceral and subcutaneous fat pads (Rosen et al. 2000). However, almost all work in investigating adipogenesis and other adipocyte functions have used clonal cells lines, stemming from the stromal vascular fraction. Overall, established preadipose cells lines used *in vitro* represent a useful tool in preliminary understanding of *in vivo* systems.

1.13 Conclusions and research objectives

Metabolic syndrome is associated with a variety of health risk factors including abdominal obesity, blood lipid disorders, inflammation, insulin insensitivity, type 2 diabetes, and elevated risk of cardiovascular disease (Despres and Lemieux 2006). In an obesogenic condition, dysfunctional adipose tissue is unable to appropriately store excess energy and increased basal lipolysis occurs; leading to increased levels of circulating NEFA which can have severe metabolic consequences (Duncan et al. 2007). A surplus of NEFA results in uptake in non-adipose tissue and ectopic fat deposition which can induce other conditions including dyslipidemia, hyperglycemia, inflammation, and insulin resistance (Despres and Lemieux 2006). Studies identifying novel ways to alleviate adipose lipolysis and NEFA release may provide approaches to treat obesity-induced type 2 diabetes and other metabolic syndromes. Identification of dietary components as a strategy to combat obesity and its associated disorders may be via phytochemicals. Currently, there is a knowledge gap in investigation the regulation of adipose tissue

lipolysis by dietary components. To date, there is no clear evidence of adipose lipolysis modulation by dietary components, expect one report recognizing resveratrol action on the stimulation of lipolysis (Lasa et al. 2012b). Our lab has previously demonstrated that piceatannol, a resveratrol metabolite, has antilipolytic activity *in vitro* and *in vivo* (Kwon, unpublished). However, little is known about resveratrol metabolites, specifically, TMR, in lipid metabolism and its affect in specific adipose functions. Thus, the work in this thesis has been completed with the following objective, aims, and hypothesis.

Our overall working hypothesis is that resveratrol metabolites affect lipid metabolism by modulating the lipolysis process.

Objective: Identify and determine the effect of different resveratrol metabolites and their role in adipose function

Aim 1: Determine the effect of resveratrol metabolites on lipolysis in adipocytes *in vitro*

Aim 2: Determine the effect of resveratrol metabolites on lipogenesis in adipocytes *in vitro*

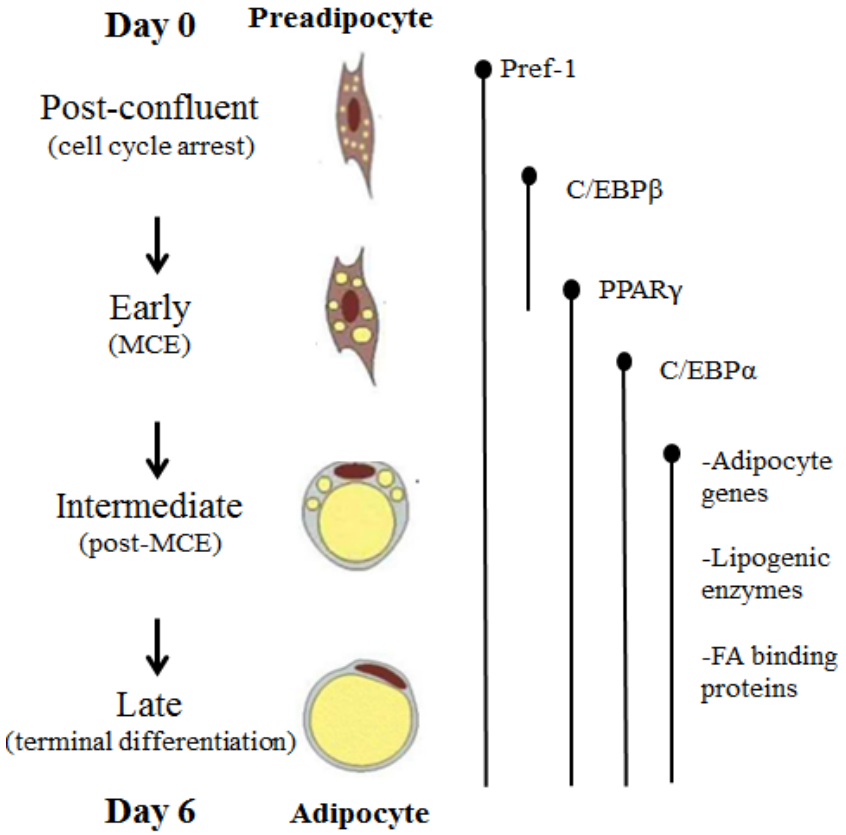

Figure 1.1 Cascade of 3T3-L1 preadipocyte differentiation and adipogenic program *in vitro*. With the appropriate environment and gene expression induction, preadipocytes undergo mitotic clonal expansion which is then followed by terminal differentiation. The conversion is a result of up- and down-regulation of specific transcription factors and genes. The process of differentiation takes place over the course of 6 days, beginning with a fibroblast-like preadipocyte until full maturation of a round lipid-containing adipocyte. Adapted from Gregoire and Ali (Ali et al. 2013; Gregoire et al. 1998).

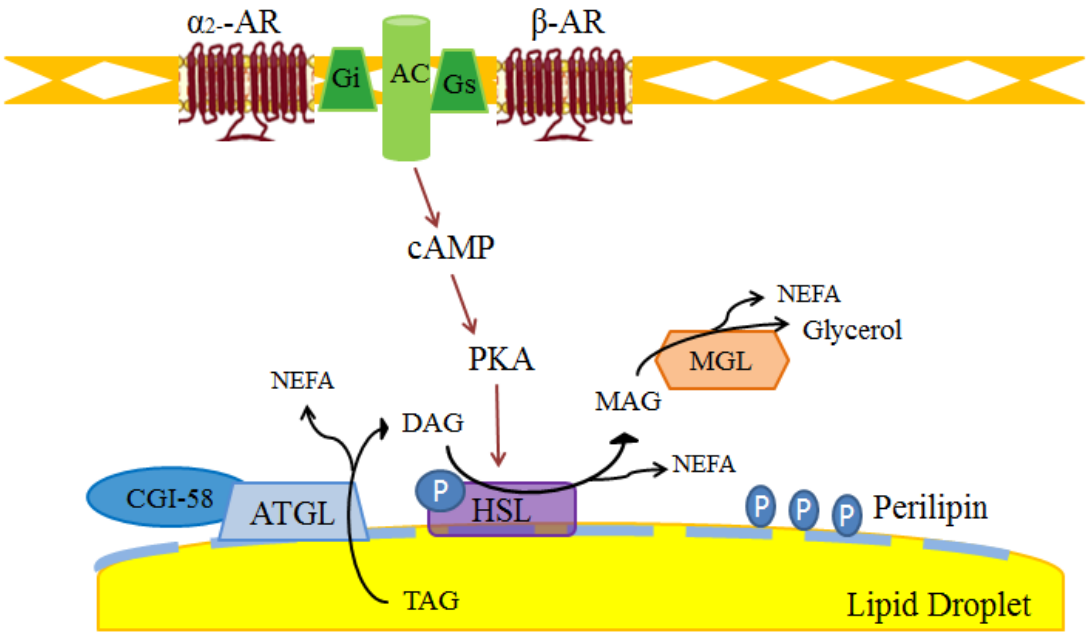

Figure 1.2 Lipolytic cascade in adipocytes. ATGL binds to CGI-58 and facilitates lipolysis via hydrolysis of TAG to DAG and NEFA. With signal transduction from catecholamines via β-adrenergic receptors (β-AR) and G protein-mediated signaling (Gs), activates adenylyl cyclase(AC), resulting in increased levels of cAMP, activating PKA, which phosphorylates HSL and perilipin A. Conformational changes occur in perilipin A, and pHSL translocates to the surface of the LD and hydrolyzes DAG to MAG and NEFA. Cytosolic MAG hydrolyzes MAG into NEFA and a glycerol backbone. NEFA and glycerol are released into circulation. Adapted from Lafontan and Brasaemle (Brasaemle 2007; Lafontan and Langin 2009).

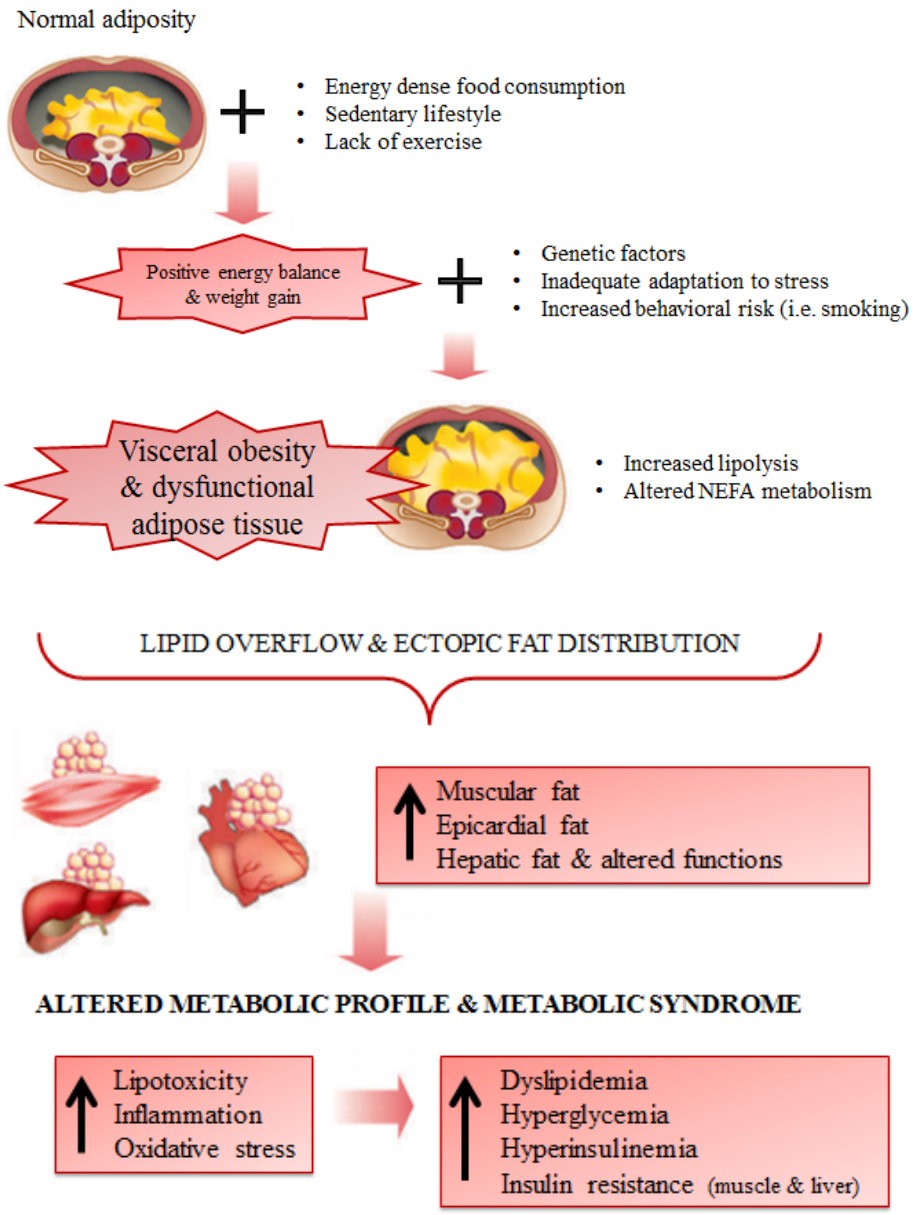

Figure 1.3 Metabolic syndromes with ectopic fat distribution. Excess visceral fat accumulation is associated with insulin resistance and also a marker of dysfunctional adipose tissue due to inability for the body to appropriately store surplus energy. In metabolic dysfunction, with high levels of circulating NEFA, fat deposition is stored in undesirable locations including the liver, heart and muscle. Metabolic syndrome ensures with this condition as well as other metabolic consequences including visceral obesity, insulin resistance, dyslipidemia, and inflammation, which could result in increased risk of other obesity associated disorders. Adapted from Despres (Despres and Lemieux 2006)
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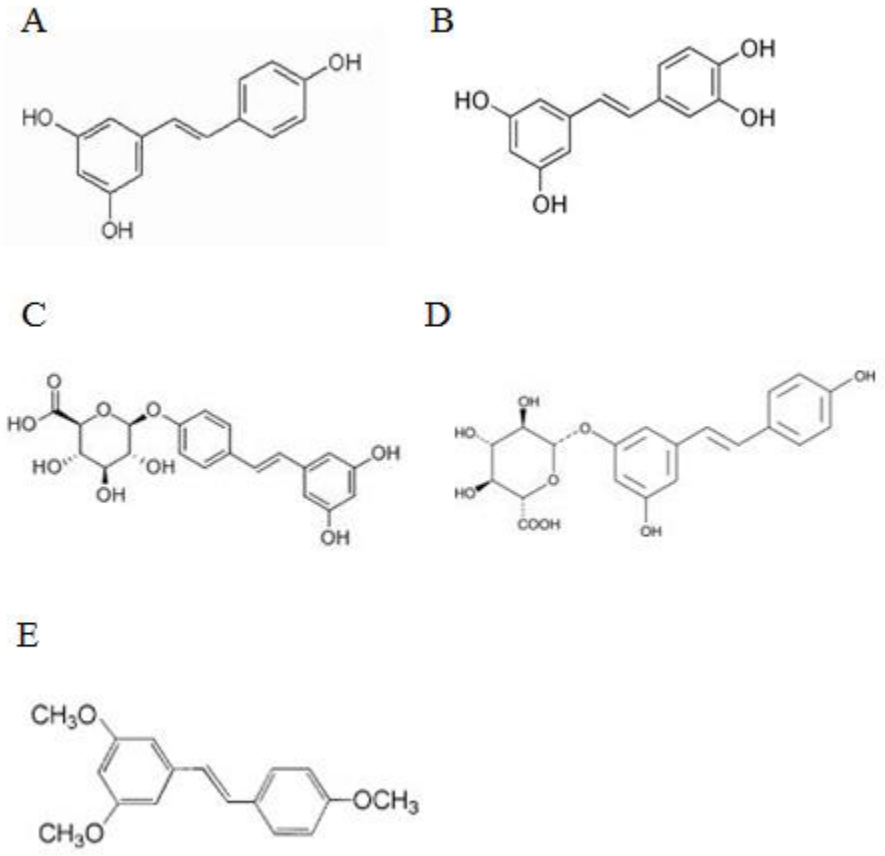


Figure 1.4 Structure of resveratrol and resveratrol metabolites. (A) Resveratrol, (B) Piceatannol, (C) Resveratrol-4′-O-glucuronide, (D) Resveratrol-3-O-glucuronide, (E) T*rans*-3,4',5-trimethoxy resveratrol.

CHAPTER 2. MATERIALS AND METHODS

2.1 Introduction

To test our overall goal and achieve our specific aims we used a variety of techniques to elucidate the effects of resveratrol metabolites. We utilized an *in vitro* model by means of 3T3-L1 cells as an established cell line. The cells were passaged as preadipocytes and differentiated into mature adipocytes and incubated in the presence of different resveratrol metabolite treatments to understand their role in lipolysis. Upon further investigation, preadipocyte cells were differentiated in the presence or absence of *trans*-3, 4', 5-trimethoxyresveratrol (TMR) to gain understanding its role in lipogenesis. We utilized both qualitative and quantitative techniques to investigate our findings. Additionally, biochemical and molecular tools were used to gain knowledge into specific mechanisms of action. The following accounts for the materials and methods used during our research processes.

2.2 Materials and methods

2.2.1 Materials and reagents

Trans-3, 4', 5-trismethoxy resveratrol (TMR) was provided by Cayman Chemical (Ann Arbor, MI). *Trans*-resveratrol-3-O-β-D-glucuronide (3G) and *trans*-resveratrol-4'-O-β-D-glucuronide (4G) were obtained from Toronto Research Chemicals (North York, ON, Canada). Piceatannol (PIC) was purchased from Alexis Biochemicals (Lausen, Switzerland). Dulbecco's Modified Eagle's Medium (DMEM) was purchased from Thermo Scientific (Waltham, MA). Penicillin-streptomycin, sodium pyruvate, and 0.25% trypsin EDTA were purchased from VWR (Randor, PA). Dexamethasone (DEX),

3-isobutyl-1-methylxanthine (IBMX), insulin, free glycerol reagent and RNase Away were purchased from Sigma-Aldrich (St. Louis, MO). Fetal calf serum (FCS) and fetal bovine serum (FBS) were obtained from PAA (Darthmouth, MA). TRIzol® reagent as well as SuperScriptII was bought from Invitrogen. Protein assay kit was from Bio-Rad laboratories (Hercules, CA). 3-(4.5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Alfa Aesar (Ward Hill, MA). Antibodies against ATGL, β-actin, and secondary mouse antibodies were from Santa Cruz Biotechnology (Santa Cruz, MA). Phospho-HSL (ser660) and secondary rabbit antibodies were obtained from Cell Signaling Biotechnology (Beverly, MA). CGI-58 was used from BioVision (Milpitas, CA). Acetonitrile, methanol, water (Mallinckrodt-Baker, Phillipsburg NJ), and formic acid (Sigma-Aldrich St. Louis, MO) used in sample preparation and analysis were certified HPLC-MS and ACS grade.

2.2.2 3T3-L1 cell culture

3T3-L1 preadipocytes were sourced from American Type Culture Collection and were cultured in 10% (v/v) FCS-DMEM in a humidified environment with 5% CO_2 at 37^{o}C. Cells were designated known passages and were not cultured past passage nine. Preadipocytes had new FCS-DMEM changes every two days until growth had reached approximately 80% confluence, at which point cells were split and expanded into 100mm, 60mm, 6-, 12-, or 24-well plates for further proliferation until differentiation. Preadipocytes were kept in a sub-confluent state until induced to differentiate after two days of post-confluence (entitled Day 0), with standard adipogenic cocktail (DMI) comprising of 1 µg/ml insulin, 5 µg/ml DEX, and 0.5 mM IBMX in 10% FBS-DMEM for two days. After two days of incubation, 10% FBS-DMEM medium including only insulin was added to the maturing adipocytes and at day four to solely 10% FBS-DMEM, and was changed every two days until adipocytes are fully differentiated to mature cells (day 8-10) when lipid droplets are clearly visible. Cells which had differentiated with greater than 90% into mature adipocytes were used for experiments. All media consisted of 100 U/ml penicillin, 100 µg/ml streptomycin and 0.11 g/L sodium pyruvate.

2.2.3 Treatment condition

Purchased metabolites (TMR, 3G, 4G, and PIC) were each dissolved in dimethyl sulfoxide (DMSO) as recommended by the supplier. Stock solutions were diluted to 50 or 100 mM and aliquots were generated to keep freeze-thaw cycles to a minimum, with a maximum thaw number of three times. For treatment of cells, stock solutions were diluted into DMEM media to a final working concentration and applied to cells. Preadipocytes were induced to differentiate with the adipogenic cocktail and in the presence or absence of a specific treatment and continued to receive treatment every two days until day 6. Mature adipocytes at day 8 to day 10 after differentiation were treated in serum free medium for a set period of time with the designated treatment conditional to the experimental design.

2.2.4 Oil Red O (ORO) staining

ORO staining was done on day six to visualize and quantify intercellular lipid droplet accumulation (Koopman et al. 2001). 3T3-L1 cells were subjected to oil red O lipid soluble dye as described with protocol adjustments (Ramirezzacarias et al. 1992). Briefly, medium was removed from cells in each well and washed twice with phosphate buffered saline (PBS). Cells were then subjected to 3.7% formaldehyde for 0.5-1 hour at room temperature or overnight at 4^{o}C to fix the cells. Plates were then washed with double distilled water twice and a filtered working solution of ORO was applied to fixed cells and incubated at room temperature for greater than 30 minutes. Cells were then rinsed with distilled water by submersion and dried. Scanned images were taken of ORO-stained cells. Quantification of ORO-stained lipid droplets was done with isopropyl alcohol extraction and absorbance measured spectrophotometrically at 490 nm with a microplate reader (Bio-Rad). The quantification was calculated based on the ORO stain as a percent of the control.

2.2.5 Cell viability assay

A 3-(4.5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction was utilized in order to determine the effect of different concentrations of *trans*-3, 4', 5-

trismethoxy resveratrol (TMR) on cell viability. This assay allows for the quantification of active mitochondrial function based on their ability to reduce the yellow MTT solution in the presence of dehydrogenase enzymes into a purple formazan product (Gerlier and Thomasset 1986). Both proliferating preadipocytes and mature adipocytes were incubated with different concentrations of TMR for 24 hours in serum free DMEM. The cells were then incubated with the MTT assay solution (0.5 mg/ml) for one hour at 37^{o}C in 5% $CO_{2.}$ Upon development of the violet precipitate, the contents were then dissolved in DMSO and quantified by absorbance measured spectrophotometrically with a microplate reader (Bio Rad) at a wavelength of 595 nm. The amount of formazan, calculated as a percent of control, is representative of final cell viability.

2.2.6 Lipolysis assay

The measure of free glycerol in media samples is representative to the amount of lipolysis that occurs in samples based on hydrolysis of triglycerides into free fatty acids and a glycerol backbone. This method was used to determine the level of lipolysis in both basal and stimulated conditions of fully differentiated cells. The free glycerol reagent measures free glycerol by using the same coupled enzymes reactions and produces a quinoneimine dye, observed as a purple hue. Serum free DMEM with 10 µM isoproterenol and 0.5 mM IBMX (stimulated) or without (basal) added was added to mature adipocytes in the presence or absence of the different resveratrol metabolite treatments at different concentrations. After incubation periods, aliquots of the medium were collected and analyzed for free glycerol. Samples of the media were gently mixed with the free glycerol reagent and incubated at 37^{o}C for 5 minutes according to supplier protocol. Quantification of the product was measured spectrophotometrically at minimum absorbance of 540 nm using a microplate reader (Bio Rad). The increase in absorbance is directly proportional to the free glycerol concentration in the sample. Glycerol release measurement was normalized by protein concentration.

2.2.7 Immunoblot assay

Western blot analysis was employed to measure the amount of protein expressed by cells treated with different experimental groups as described (Burnette 1981) with modifications. The cells were cultured and treated as specified in each experiment and collected by scrapping in phosphate buffer saline (PBS). Samples were immediately centrifuged to remove excess PBS. Cell pellets were then transferred to lysis buffer comprising of Tris-HCl (100mM (pH 8.0)), NaCl (100mM), 0.5% Triton X-100, protease inhibitor blend, sodium fluoride (10mM) and sodium orthovanadate (1mM). The concentration of protein was quantified with the Bradford method (Bio-Rad Laboratories, Hercules, CA). 100 µg of protein sample were separated with a 10% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) and transmitted to a nitrocellulose membrane. A protein ladder, Precision Plus Protein Standards Kaleidoscope (Bio-Rad) was employed to identify protein size (kDa). Membranes were blocked in 5% non-fat dry milk in 1% Tween-Tris Buffered Saline (TTBS) for two hours at room temperature. Immunoblotting was completed with respective primary antibodies at 4^{o}C overnight, followed by washing in 1% TTBS (3 times for 5 minutes). Signals were seen with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) which were incubated for two hours at room temperature, followed by washing (3 times for 6 minutes) and enhanced with Pierce enhanced chemiluminescence plus Western Blotting reagents with autoradiography. Film was scanned and protein band intensity was quantified with NIH ImageJ software (ver 1.45S) normalized to band intensity of β-actin. Membranes were stripped with a mild stripping buffer (0.2 M glycine, 0.1% SDS, 1% Tween20, pH 2.2 1 L dd water) at room temperature, and re-blocked.

2.2.8 Total RNA isolation and real time reverse transcription polymerase chain reaction (RT-PCR)

TRIzol® (Invitrogen, Carlsbad, CA) was used to extract total RNA from 3T3-L1 cells as detailed in manufacturer's protocol. Mature adipocyte cells were treated for duration of time depending on experimental design or during differentiation at various concentrations of TMR. Media was aspirated and cells were washed with PBS. TRIzol ®

was added to the cells, harvested, and stored at -80^{o}C until subjected to RNA isolation. The integrity of the isolated RNA was determined with NanoDrop 2000c (ThermoScientific) using the 260nm/280nm ration**.** Samples that maintained a ratio greater than 1.8 were used. 1µg/µl of isolated RNA was subjected to the reverse transcriptase reaction utilizing SuperScriptII system, and cDNA was synthesized. Newly synthesized cDNA was used for real time reverse transcription PCR reactions using Applied Biosystems Step One real time PCR thermocycler. The cDNA was diluted and amplified using a SYBR premixed Taq reaction mixture with PCR primers. Reactions were performed in triplicate for each pair of 100 n/ml PCR primers with values normalized by housekeeping gene, β-actin, expression. The forward and reverse primer sequences used in the study are shown in Table 2.1. Analysis of RT-PCR data was performed using $\Delta\Delta C_T$ scheme and calculated as relative changes in gene expression.

Table 2.1. Primer sequences corresponding to genes used in quantitative RT-PCR (F) represents nucleotide sequence for the forward primer and (R) represents nucleotide sequence for the reverse primer.

Gene	(F) 5'- 3' sequence	(R) 5'- 3' sequence
PPARγ	CCC AAT GGT TGC TGA TTA CAA T	CTA CTT TGA TCG CAC TTT GGT ATT CT
FAS	GCC ACC CAC CGT CAG AAG	TGT CAC ATC AGC CAC TTG AGT GT
ACC	GAA TCT CCT GGT GAC AAT GCT TAT T	GGT CTT GCT GAG TTG GGT TAG CT
SCD1	GGT GAT GTT CCA GAG GAG GTA CTA C	AGC GTG GGC AGG ATG AAG
MGAT1	CTG GTT CTG TTT CCC GTT GT	TGG GTC AAG GCC ATC TTA AC
DGAT2	GCC GTG TGG CGC TAC TTC	GTG GTC AGC AGG TTG TGT GTC T
SREBP1c	GGC ACT GAA GCA AAG CTG AAT	GGC ACT GAA GCA AAG CTG AAT
Resistin	TGC CAG TGT GCA AGG ATA GAC T	CGC TCA CTT CCC CGA CAT
Leptin	CAC ACA CGC AGT CGG TAT CC	AGC CCA GGA ATG AAG TCC AA
Adiponectin	GAT GCA GGT CTT CTT GGT CCT AA	GGC CCT TCA GCT CCT GTC A
Adipsin	GCT ATC CCA GAA TGC CTC GTT	TTC CAC TTC TTT GTC CTC GTA TTG
UCP1	GCC AAA GTC CGC CTT CAG AT	TGA TTT GCC TCT GAA TGC CC
PGC-1α	CCC AGG CAG TAG ATC CTC TTC AA	CCT TTC GTG CTC ATA GGC TTC ATA
Tfam	TCC TGA GGA AAA GCA GGC ATA T	CAT TTC ATT GTC GTA ACG AAT CCT A
NRF1	GCC GTC GGA GCA CTT ACT G	GGC CAT GAT TTC TGG AAG CA-
NRF2	TCC CAGGTT GCC CAC ATT	TGC CAA AAG CTG CAT ACA GTC T
MCAD	CAA CAC TCG AAA GCG GCT CA	ACTTGCGGGCAGTTGCTTG
CPT1	GCT GCT TCC CCT CAC AAG TTC C	GCT TTG GCT GCC TGT GTC AGT ATG C
Tbx1	GGC AGG CAG ACG AAT GTT C	TTG TCA TCT ACG GGC ACA AAG
Tmem26	ACC CTG TCA TCC CAC AGA G	TGT TTG GTG GAG TCC TAA GGT
Prdm16	CAG CAC GGT GAA GCC ATT C	GCG TGC ATC CGC TTG TG
ATGL	GAG ACC AAG TGG AAC ATC	GTA GAT GTG AGT GGC GTT
CGI-58	GGT TAA GTC TAG TGC AGC	AAG CTG TCT CAC CAC
HSL	TGT GGC ACA GAC CTC TAA AT	GGCATATCCGCTCTC
Perilipin	TGC TGG ATG GAG ACC TC	ACC GGC TCC ATG CTC CA
β-actin	AGA TGA CCC AGA TCA TGT TTG AGA	CAC AGC CTG GAT GGC TAC GT

2.2.9 Stimulated Raman scattering (SRS) and two-photon excitation fluorescence (TPEF) imaging

Stimulated Raman scattering (SRS) imaging was performed on a femto-second laser (InSight DeepSee, Spectra-Physics, Mountain View, CA) system, as described previously (Wang et al. 2013; Zhang et al. 2011). Mature adipocytes were treated for three hours with 50µM TMR and subjected to both SRS and TPEF imaging. Briefly, with the stokes beam fixed at 1040 nm, the pump beam is tuned to 800 nm to match the CH2 stretch vibration at 2850 cm^{-1}. A water immersion objective lens (60X, UPlanSApo, Olympus, Tokyo, Japan) with numerical aperture of 1.2 was used to focus the light on the sample. The average power used is 30 mW for the pump beam and 75 mW for the stokes beam. For Two-Photon Excited Fluorescence (TPEF) imaging of intracellular TMR compound, the pump beam at 800 nm was used as the excitation source. Forward signal was detected by a PMT (H7422-40, Hamamatsu) after a 410/40 nm bandpass filter. The imaging acquisition time is 10 µs per pixel. Images were analyzed using Image J software.

2.2.10 High performance liquid chromatography (HPLC) with ultraviolet (UV) detection

In order to determine the effect of TMR stability in media an HPLC system with UV-VIS detector was used as previously described (Lin and Ho 2009; Lin et al. 2010) with modifications. Briefly, TMR at 50 µM added to serum free medium incubated at 37^0C were collected at 0, 3, 6, 9 and 24 hours and stored at -80 ^{0}C. Samples with 2% formic acid in acetonitrile were vortexed and centrifuged at 14,000 x g for 5 minutes to precipitate any proteins in the sample. Finally the supernatants were filtered through a 0.45 µm PTFE filter, and injected 10µL on a Waters 2695 Separation Module system equipped and a Waters 2996 Photodiode Array Detector. Separation was achieved with a RPC18column (XBridge BEH Shield RP18 2.5 µm, 2.1 x 100mm) through delivery of a mobile phase of 95% acetonitrile-water (75:25, v/v) containing 5% and 0.1% formic acid respectively at a flow rate of 0.25 ml/min with a run time of 10 minutes. TMR was detected at a wavelength of 300 nm and quantified using calibration curves constructed from serially diluted stock solutions of TMR prepared in the range of 500, 250, 75, and 25 µM.

2.2.11 Statistical analysis

Data are displayed as means ± SEM. Statistical analysis was performed using SAS 9.3 software. One-way analysis of variance was used to determine significance of treatment interactions and effect. Statistical significant comparisons between each group with the control were analyzed by Dunnette's multiple comparison. Each experiment was performed in duplicate or triplicate, with representative images. P values lower than 0.05 were regarded as statistically significant.

CHAPTER 3. RESULTS AND DISCUSSION

3.1 Introduction

The prevalence and growth of obesity has drastically increased and expanded into a global health issue (Kim et al. 2013). Currently two thirds of the population in the United States are overweight, with a third being considered obese (Ogden et al. 2014). Consequently, obesity is highly associated with other metabolic diseases including insulin resistance and type 2 diabetes, cardiovascular diseases, and certain cancers (Despres and Lemieux 2006). Adipose tissue plays a critical role in whole body energy homeostasis and lipid metabolism. Adipocyte development is a result of hypertrophy, an increase in cell size, as well as hyperplasia, an increase in cell number (Kim et al. 2013). Hypertrophy occurs in mature adipocytes and this process is critical for lipid storage and secretion of hormones which maintain overall energy balance. Hyperplasia is responsible for generation of new adipocytes primarily seen in adolescence and adult adipocyte turnover (Kim et al. 2013). Both processes are targets for the prevention and or treatment of obesity in therapeutic strategies.

Obesity is associated with metabolic diseases that include type 2 diabetes. Currently, NEFA at amplified levels have suggested as a mediator linking the two conditions (Despres and Lemieux 2006). Lipolysis is the process in adipose tissue responsible for the mobilization of these NEFA into circulation and it commonly seen at an increased level in obese patients, which can be precursor to other detrimental metabolic syndromes (Duncan et al. 2007). Therefore, studies investigating strategies to attenuate lipolysis and subsequent NEFA release may be an approach to treating obesity-induced type 2 diabetes. Currently, there is limited knowledge in regards to examining the role of dietary components and their regulation of adipose lipolysis. Our lab has

previously shown a resveratrol metabolite, piceatannol, has antilipolytic activity (Kwon, unpublished). Thus, we hypothesized that other resveratrol metabolites may also have an effect in in modulating lipid metabolism and play a role in reducing adipose lipolysis. Furthermore, the objective of this study is to identify and determine the effect of different resveratrol metabolites and their role in adipose function. Using cellular and biochemical techniques, we examined the role of the resveratrol metabolite, TMR, in lipid metabolism, with a focus on lipolysis and lipogenesis.

3.2 Adipose function: Lipolysis

WAT are fat stores and major energy reserves in mammals. With food intake, NEFA are esterified to a glycerol backbone and stored as neutral lipids in the form of TAGs and deposited in LD (Lass et al. 2011). During fasting or increased energy expenditure, TAGs are mobilized and FA are sequentially cleaved by lipases, released, and circulated to peripheral tissues to undergo β-oxidation and generate ATP (Lass et al. 2011). This mobilization and hydrolysis of FA from the glycerol backbone is known as lipolysis (Zechner et al. 2012). Adipocyte lipolysis occurs sequentially and is a highly regulated process in that each step is carried out by a different lipolytic enzymes (Walther and Farese 2012). The first FA cleaved from TAG is done by ATGL, the second is removed by HSL, and the third by MGL resulting in three NEFA and a glycerol backbone (Ahmadian et al. 2009).

NEFA plasma concentrations are increased in obesogenic conditions and are a common cause for the dysregulation of lipolysis (Lafontan and Langin 2009). Currently, there is evidence that shows impaired NEFA metabolism is a contributing factor for insulin resistance in visceral obesity (Despres and Lemieux 2006). Increased circulating NEFA pose a serious issue in that with excess fat accumulation and dysfunctional metabolic state, ectopic fat disposition occurs where excess energy is stored in undesirable locations including the heart, liver, and muscle (Despres and Lemieux 2006). Additionally, NEFA at increased levels are thought to be a mediator linking obesity and type 2 diabetes through β-cell dysfunction, and as previously mentioned, insulin resistance (Kahn et al. 2006). Therefore, it has been proposed that identifying

antilipolytic agents to partially inhibit lipolysis may help to attenuate the level of circulating NEFA, and the development of dyslipidemia and metabolic syndromes (Langin 2006a).

3.2.1 Impact of resveratrol metabolites on lipolysis in 3T3-L1 adipocytes

As reviewed in the literature, resveratrol metabolites have varying function and effects in different types of cells and their role is dependent on the function being analyzed. To better understand the effects of different resveratrol metabolites, specifically in lipolysis, several metabolites were tested on mature 3T3-L1 cells which had greater than 90% lipid accumulation. 3T3-L1 cells were treated with resveratrol metabolites including piceatannol (3, 5, 3', 4'-*trans*-tetrahydroxystilbene) (PIC), *trans*-3, 4', 5-trimethoxy resveratrol (TMR), resveratrol-3-O-glucuronide (3G), resveratrol-4′-O-glucuronide (4G) compared to the control vehicle, DMSO. Mature adipocytes were incubated with serum free medium and treated in both the basal and stimulated conditions. Incubating mature adipocytes in these two different conditions are necessary to mimic two different physiological conditions. The basal condition imitates a resting metabolic state in a neutrally temperate environment, or in other words, the energy that is expended by humans at rest, in a post-absorptive state sufficient for only functioning organs. A stimulated condition mimics a fasting condition such as after an acute exercise state where lipolysis is induced. In this cellular model, 3T3-L1 cells were treated in both conditions and stimulated by addition of isoproterenol (ISP) or forskolin (FSK). FSK works to stimulate and increase accumulation of cAMP in intact cellular systems (Seamon et al. 1981), and ISP is a β-adrenergic agonist that stimulates β-adrenergic receptors and increases levels of cAMP and AMPK to promote the lipolytic pathway (Fasshauer et al. 2002a; Minokoshi et al. 2002). Quantification of glycerol release, normalized by protein, allowed us to see the effect of metabolite action on lipolysis. Each metabolite was screened at a concentration of 50 μM; this is an intermediate range used in treatment of phytochemicals (Minakawa et al. 2012) and a level that has been previously used in our lab. Additionally, 50 μM is a frequently used with *in vitro* studies, including those examining the effects of resveratrol (RES) on 3T3-L1 adipocytes (Park et

al. 2008; Rayalam et al. 2008b). From an acute 1.5 hour treatment, PIC, TMR, and 3G show a significant reduction in lipolysis in the basal condition compared to the control (Figure 3.1 A). The significant inhibition of PIC on lipolysis has been demonstrated in our lab both *in vitro* and *in vivo* (Kwon, unpublished) and is confirmed in these data. Our new finding of the partial, significant inhibition of lipolysis by TMR and 3G in an acute, basal treatment has not been reported. The extent at which TMR reduced the level of lipolysis is similar to the efficacy seen with PIC. 4G demonstrated a slight reduction in the level of glycerol release, however, it was not reduced to a significantly different level as compared to the control. In a stimulated, acute treatment (Figure 3.1 B) we observed significant reduction in lipolysis by PIC as well as TMR, with similar inhibition level. 4G also significantly reduced the level of glycerol release in a lipolysis-induced environment in contrast to a basal condition. Interestingly, we note that 3G was unable to significantly reduce lipolysis, in this condition with an acute treatment, although we did observe a slight reduction. Overall, we observed, as hypothesized, that other resveratrol metabolites were able to partially inhibit lipolysis. To our knowledge, this is the first report of the effect of different resveratrol metabolites and their effect on lipolysis with short treatment time. Collectively, these results demonstrate the rapid action that resveratrol metabolites have on lipolysis in mature adipocytes *in vitro.*

The next question addressed was the impact of these resveratrol metabolites in an extended, chronic treatment in basal and stimulated conditions. A 24 hour treatment of the resveratrol metabolites at 50 μM was added to mature 3T3-L1 cells with greater than 90% lipid accumulation. Compared to the control, all the metabolites examined, PIC, TMR, 3G, and 4G demonstrated lipolysis-lowering ability in a chronic, basal environment (Figure 3.2 A). PIC and TMR significantly reduced lipolysis at similar levels, consistent with an acute treatment. 3G and 4G significantly diminished the glycerol release the most. This suggests that efficacy of 3G and 4G is enhanced with a longer treatment time. In the literature, there is one report identifying RES enhancing the level of lipolysis through increase in free fatty acid (FFA) release in mature 3T3-L1 adipocytes (Lasa et al. 2012b). Observed by Lasa, RES significantly increased FFA in both chronic (24 hours) basal and stimulated conditions, however, glycerol levels

remained unchanged. Consistent with this report, RES treated for 24 hours in the basal condition revealed no significant difference as compared to the control. However, we did not observe the same large induction detected by Lasa (Lasa et al. 2012b). There is greater variability observed with the chronic stimulated condition (Figure 3.2 B). Of the treatments, PIC and TMR alone were able to reduce lipolysis significantly. We observed similar levels between the control, 3G, and 4G. Interestingly, we observed a significant reduction in lipolysis by RES, contrasting with reported data. However, it must be noted that the concentration levels used in our experiment was half the dosage of that reported induction by RES. This may be a contributor to the discrepancy between the chronic, stimulated condition treatment of RES as well as the different lipolytic-stimulant utilized.

The distinct reduction in glycerol release correlating to the partial inhibition of lipolysis by resveratrol metabolites is not unreasonable. Treatment with TMR demonstrated significant reductions in both acute and chronic conditions in both basal and stimulated conditions. This resveratrol analogue gave rise to great interest as it was able to cause an effect in levels similar to PIC. To our knowledge, TMR has not been studied in lipid metabolism, although it has been shown to have anti-cancer activities in a variety of other *in vitro* cell systems (Dias et al. 2013; Hsieh et al. 2011b; Pan et al. 2008). The structure of TMR, containing three methoxy groups, lends itself to be more lipophilic and making it more favorable to be studied in a cellular adipocyte system. Although there has been no report of resveratrol metabolites in lipolysis, 3G and 4G have been previously studied for their delipidating effect in both maturing and mature 3T3-L1 adipocytes (Lasa et al. 2012a). It was reported that 3G was able to significantly reduce the TAG content in mature adipocytes upon a 24 hour treatment at both 10 and 25 μM. In comparison to a control, 3G was described as having slightly increased mRNA expression of *ATGL* and *HSL* levels, however this increase was not significant (Lasa et al. 2012a). This previous report is consistent with our results in the chronic, stimulated condition, where 3G shows very little action in reducing lipolysis. 4G was observed in the same study to have action in reducing TAGs significantly both in maturing and mature adipocytes at 25 μM. 4G also faintly increased *ATGL* mRNA expression and significantly increased *HSL* (Lasa et al. 2012a). Like 3G, these reported findings for 4G

are similar to our observation in Figure 3.2 A; where there is a significant reduction in lipolysis. RES was additionally examined in this experiment to demonstrate the structure-function differences that occur with slight molecular modifications. Although RES has been reported to significantly increase serum FFA in 3T3-L1 cells (Lasa et al. 2012b), it only slightly increased FFA release in SGBS cells in the basal condition, while glycerol levels remained unchanged in both cell lines. This is comparable to our results. Notably, SBGS are human adipocytes, and the effect in these cells are of higher resemblance to an actual human effect (Lasa et al. 2012b). Also, previously reported in our lab with 3T3-L1 cells, lipolysis was unaffected by RES, showing no difference from control cells in glycerol release or FFA (Kwon, unpublished). RES has been show as most active at reducing TAG levels (Lasa et al. 2012a), while its metabolites maintain action in other functions; indicating that resveratrol metabolites maintain relevancy in reducing lipolysis.

In obesity, there is increased level of basal lipolysis, as previously described, and in this system, that condition may be most similar to a chronic, stimulated condition in our *in vitro* model. Therefore in looking at these results (Figure 3.2 B), we see that PIC and TMR have the greatest potency in reducing the level of glycerol release. 3G and 4G did not elicit the same effectiveness and there is variability seen between basal and stimulated conditions. Additionally, 3G and 4G have already been studied in lipid metabolism with some emphasis of their role in the lipolytic pathway. For these reasons, we pursed the role of TMR in adipose function and studied deeper into identifying the mechanistic pathways in which it partially inhibits lipolysis *in vitro.*

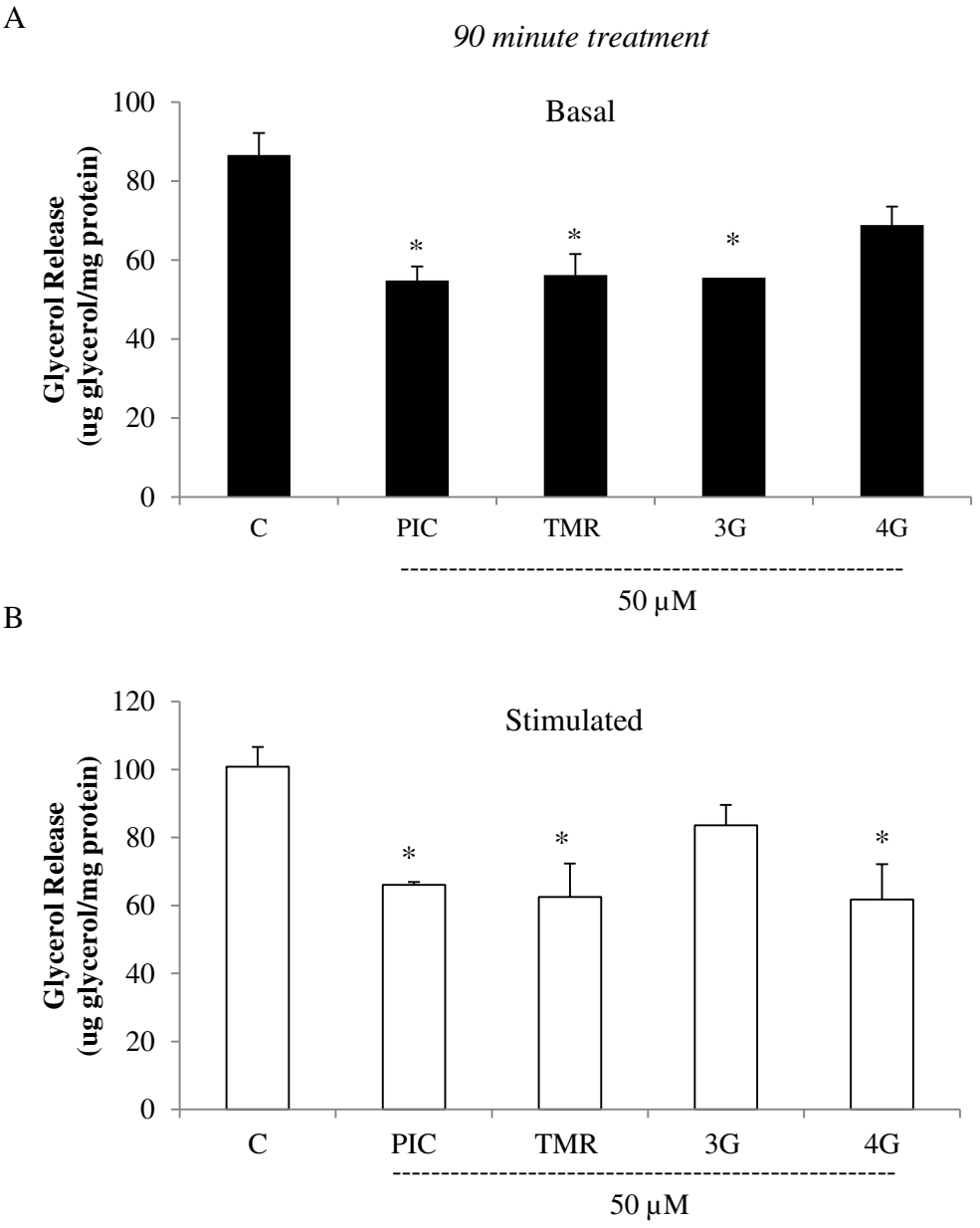


Figure 3.1 Effect of resveratrol metabolites on acute release of glycerol on mature adipocytes. Glycerol release in 3T3-L1 mature adipocytes treated for 1.5 hours at day 10 after differentiation in the presence of the vehicle (control [C]), dimethyl sulfoxide (DMSO), or at 50 µM with piceatannol (PIC), *trans*-3, 4', 5-trimethoxyresveratrol (TMR), resveratrol-3-O-glucuronide (3G), resveratrol-4′-O-glucuronide (4G) in DMSO. Comparisons between each treatment with control; subjected to basal (A) and stimulated (B) conditions. Data are represented as means ± SEM, n=3. *, P <0.05.

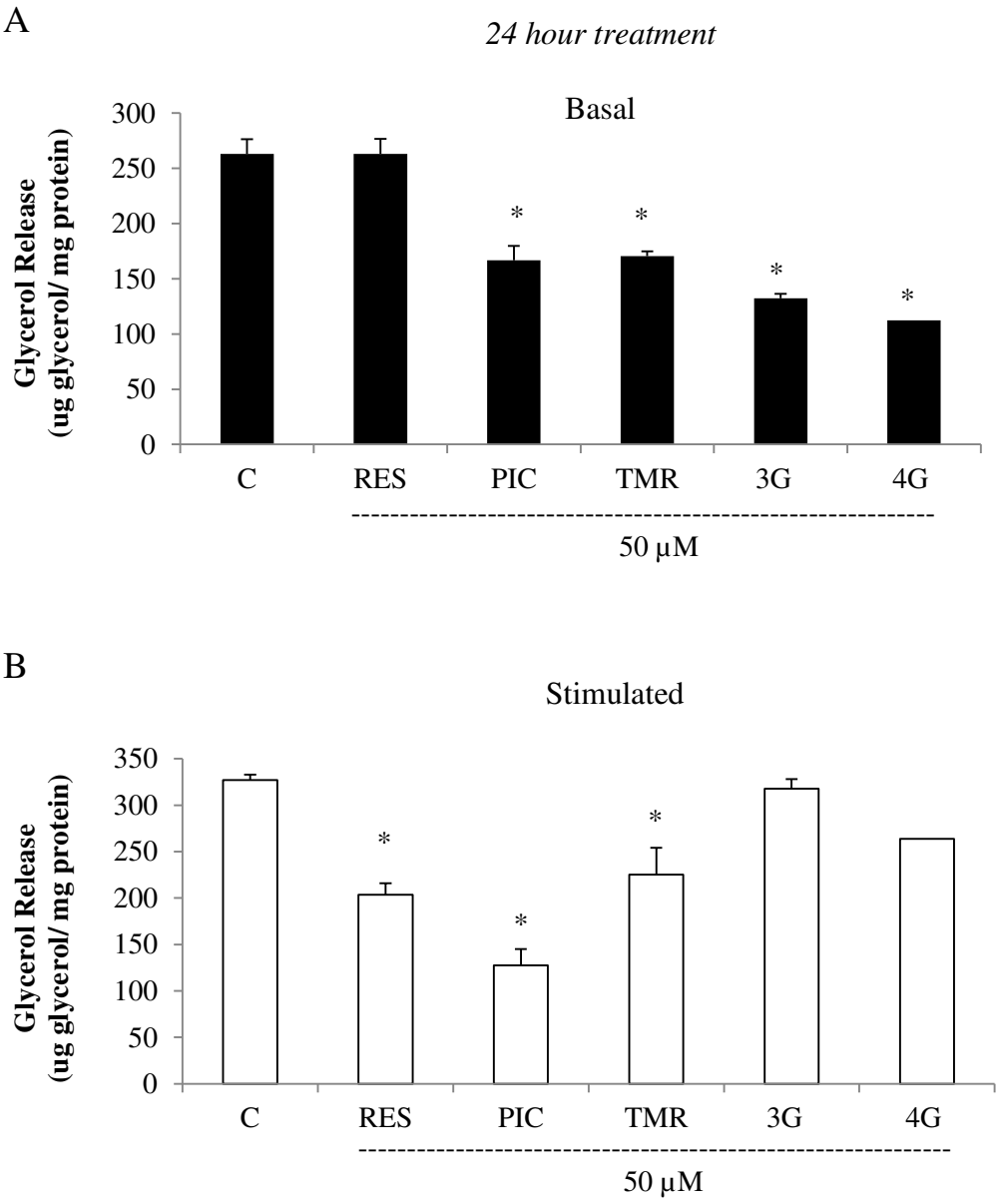


Figure 3.2 Effect of resveratrol metabolites on chronic release of glycerol on mature adipocytes. Glycerol release in 3T3-L1 mature adipocytes treated for 24 hours at day 10 after differentiation in the presence of the vehicle (control [C]), dimethyl sulfoxide (DMSO), or at 50 µM with resveratrol (RES), piceatannol (PIC), *trans*-3, 4', 5-trimethoxyresveratrol (TMR), resveratrol-3-O-glucuronide (3G), resveratrol-4′-O-glucuronide (4G) in DMSO. Comparisons between each treatment with control; subjected to basal (A) and stimulated (B) conditions. Data are presented as means ± SEM, n=3. *, P <0.05.

3.2.2 Impact of *trans*-3, 4', 5-trimethoxyresveratrol (TMR) on cell viability

TMR has been shown to possess the capacity to inhibit cell viability as well as stimulate apoptosis in a variety of cell systems including colon, prostate, and breast tumors *in vitro* and *in vivo* (Dias et al. 2013; Hsieh et al. 2011a; Pan et al. 2008; Simoni et al. 2006). Therefore, we wanted to ensure the partial inhibition of lipolysis by TMR was not due to action inhibiting cell viability, but through other antilipolytic action. Thus, the MTT assay was used to determine the effect of TMR in both proliferating 3T3-L1 preadipocytes along with mature adipocytes. Cells were treated with varying concentrations of TMR (0, 5, 10, 25, and 50 μM) for 24 hours. These conditions represent the maximum treatment concentration and time that was used in our study. Proliferating preadipocytes showed no significant reductions in cell viability (Figure 3.3). In fact with lower concentrations of TMR at 5 and 10 μM, we note slightly enhanced viability. At higher concentrations of TMR, 25 and 50 μM, we did observe some slight inhibition on viability, however these effects were not greater than 25% nor significant. Mature adipocytes were also examined. Two day, post-confluent cells were stimulated to differentiate with an adipogenic cocktail of DMI and grown to mature adipocytes with lipid accumulation. At day 10 after differentiation, cells were incubated with TMR and consistent with preadipocytes, TMR had very little effect on cell viability (Figure 3.4). At its highest concentration, TMR maintained the same level of cell viability as the control. Taken together, our results demonstrate that TMR does not contain cytotoxic effects in 3T3-L1 preadipocytes or mature adipocytes, and this data suggests that the reduction of lipolysis observed is caused by an alternative pathway.

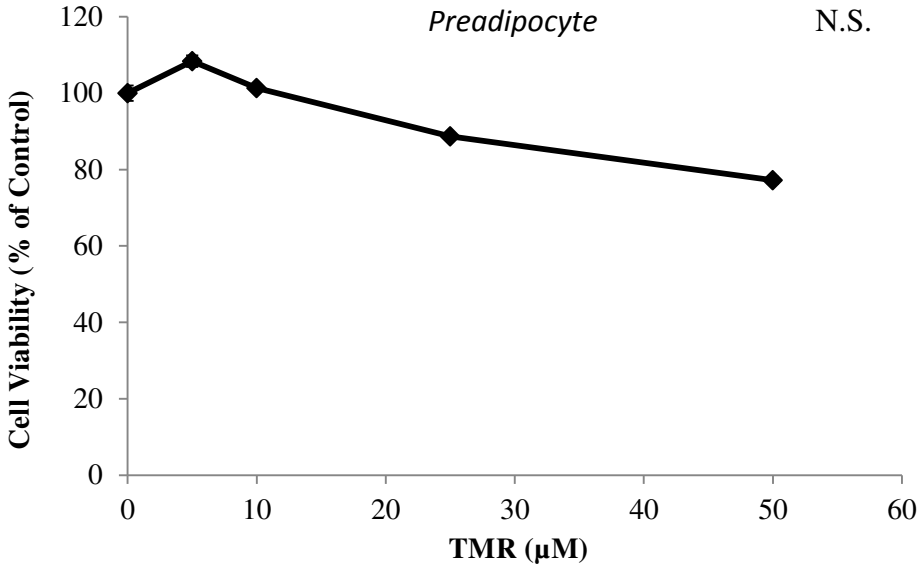


Figure 3.3 Effect of *trans*-3, 4', 5-trimethoxyresveratrol (TMR) on 3T3-L1 proliferating cell viability. Post-confluent 3T3-L1 preadipocytes were incubated with various concentrations of TMR for 24 hours and the viability of these cells was assessed by MTT assay. Data are presented as means ± SEM, n=3. Not significant denoted as N.S.

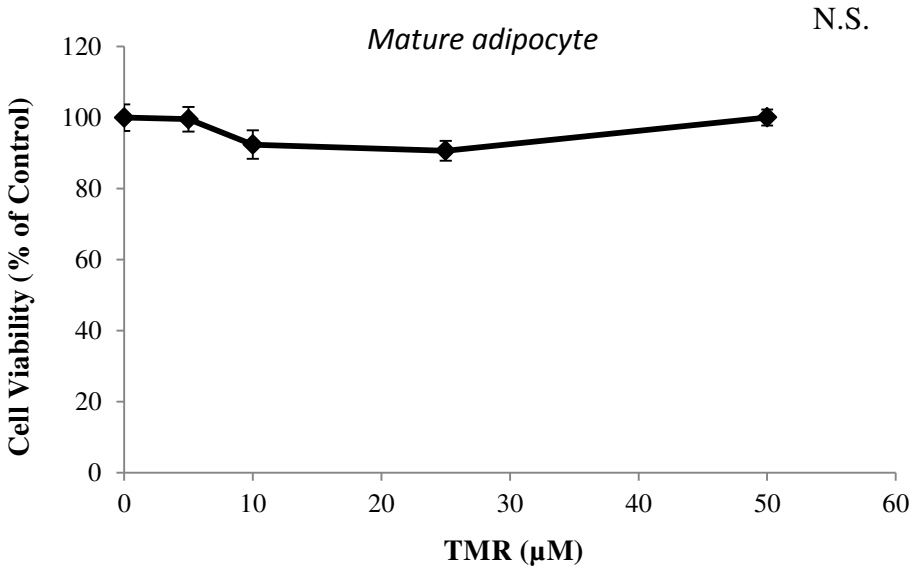


Figure 3.4 Effect of *trans*-3, 4', 5-trimethoxyresveratrol (TMR) on mature 3T3-L1 adipocyte cell viability. Mature 3T3-L1 adipocytes at day 8 after differentiation were incubated with various concentrations of TMR for 24 hours and the viability of these cells was assessed by MTT assay. Data are presented as means ± SEM, n=4. Not significant denoted as N.S.

3.2.3 TMR stability and intracellular localization

In order attribute the effects observed to TMR, a stability assay was performed to understand its degradation pattern in DMEM media. This assay is necessary and often an overlooked factor, with some phenolic compounds undergoing reactions with the cell culture medium (Halliwell 2008; Long et al. 2010). Modifications in stability, chemical structure, and interactions with other biomolecules may affect molecular targets (Delmas et al. 2011). RES incubated in DMEM at 37^{o}C results in some instability overtime (24 hours); however amounts of hydrogen peroxide, indicative of oxidation, were not significant (Long et al. 2010). This demonstrates the benefit of TMR having a degree of protection against extensive metabolism or oxidation due to its methoxylation (Lin and Ho 2009). Currently, there is little information on the stability of resveratrol metabolites in reference to their biological effects (Delmas et al. 2011). Resveratrol metabolite, PIC, has been shown to be present more intact than that of RES when studied in rats (Setoguchi et al. 2014). The pharmacokinetic profile of TMR was evaluated in rat plasma and found to have greater plasma exposure, longer half-life and lower clearance compared to RES, however this model was with TMR fully dissolved in hydroxypropyl-β-cyclodextrin (HP-β-CyD) (Lin and Ho 2009). In our *in vitro* model, the stability of TMR was analyzed to ensure its efficacy. TMR was added to medium, incubated at 37^{o}C, and collected at different time points (Figure 3.5). The half-life of TMR, as a percentage of the control, was determined to approximately 4 hours. Currently there are no published data on the stability of TMR in DMEM media, the closest comparison is relatively similar at approximately 5.8 hours seen in rat plasma dissolved in HP-β-CyD (Lin and Ho 2009). Between 6 and 9 hours, TMR concentration was undermined, which suggests a degree of degradation. However, the majority of the experiments completed in this study were well within the range of 6 hours, with most common treatment times of 1.5 and 3 hours.

In determination of stability, we also wanted to identify where in the cell TMR localizes. Mature 3T3-L1 cells were treated with TMR and incubated for 3 hours at 37^{o}C. Images of cells and accumulated lipid were captured by SRS imaging, while the fluorescence of TMR was photographed with TPEF (Figure 3.6). The fluorescence signal

of TMR is strong, and the green color (TMR) indicates its high intensity. These images allow us to see that TMR co-localizes very strongly with the LD. The merged picture of TMR and the LD demonstrate its lipophilicity and where its action may occur. Interestingly, images taken previously by our lab show RES and in the intracellular space of the cytosol (unpublished). Noting this, it distinctly sets apart TMR, in location and its action in the cell may be due to its more lipophilic structure. TMR has been described as being distributed in higher levels in major organs than in plasma and is highly likely to be found fat tissues (Dias et al. 2013).

Collectively, although the stability of TMR in media is found in low levels after 6 hours, it is important to note that TMR is highly located with the LD within 3 hours of treatment. This suggests that when given to the cells, much of TMR is taken up rapidly and can have action in cellular processes, such as lipolysis, within a short period of time *in vitro.*

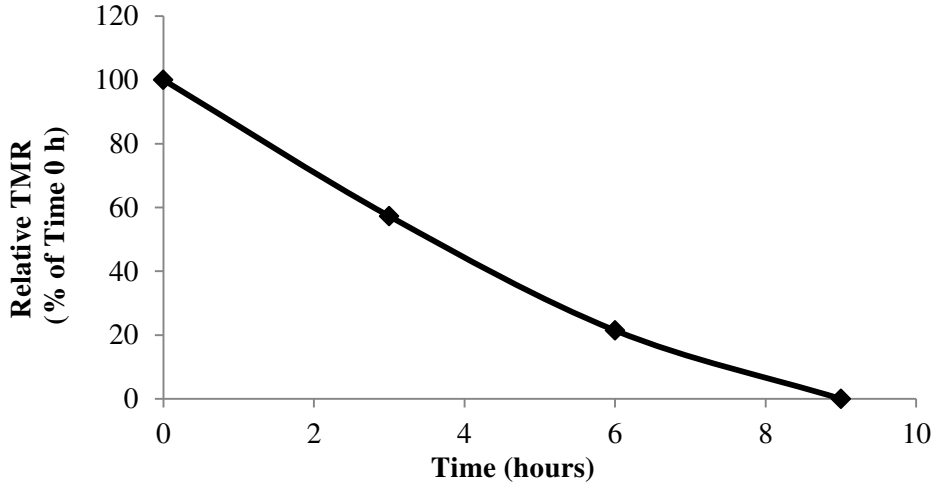


Figure 3.5 Stability of *trans*-3, 4', 5-trimethoxyresveratrol (TMR) in DMEM media. TMR was added to serum free media at 50 µM and incubated at 37 ^{0}C. Aliquots were collected at various incubation time points. Samples were quantified by fluorescence at 300 nm. Data are presented as relative TMR present as a percent at time 0 hour.

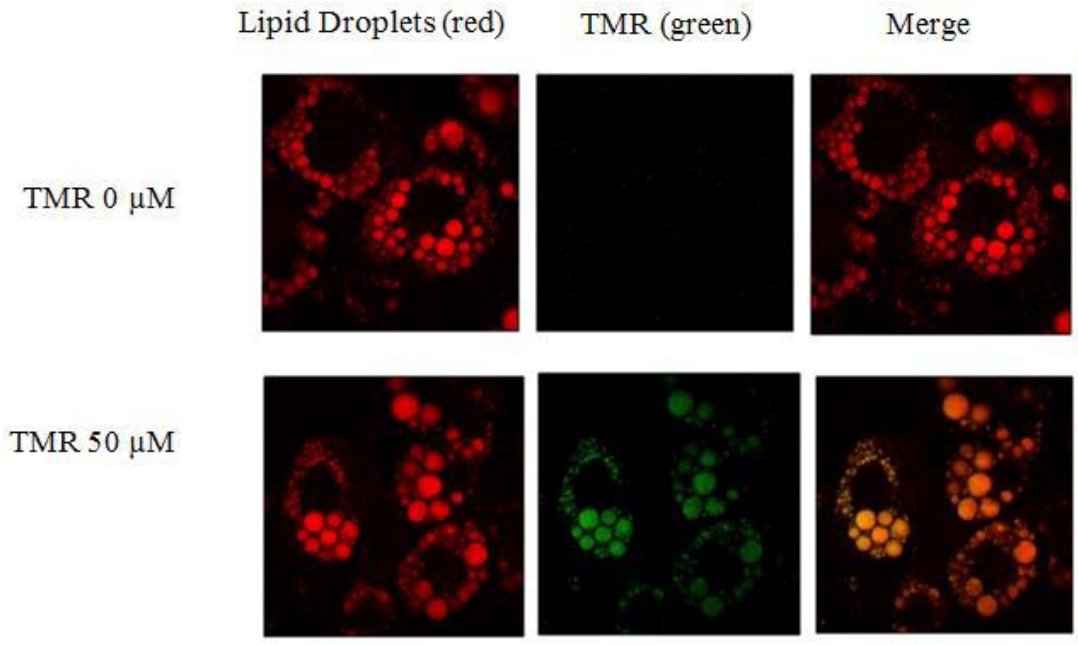


Figure 3.6 *Trans*-3, 4', 5-trimethoxyresveratrol (TMR) intracellular localization in adipocyte. 3T3-L1 mature adipocytes treated for 3 hours at day 11 after differentiation in serum free DMEM media in the presence or absence of TMR (0 and 50 µM). Mature adipocytes were subjected to SRS imaging to visualize lipid droplet accumulation (red color) and TPEF imaging to observe intracellular TMR (green color). Image dimensions 100µm x 100µm. Representative data shown; multiple images demonstrated similar results.

3.2.4 Dose-dependent effect of TMR on lipolysis in mature adipocytes

TMR has been studied as an anti-cancer agent; however, its role in lipid metabolism has not yet been investigated. Here we elucidate its impact on 3T3-L1 mature adipocyte lipolysis and its underlying molecular mechanism. In order to achieve this, fully differentiated cells were treated at day 10 in the presence or absence of TMR at increasing concentrations. Based on MTT assay, each concentration up to 50 µM is non-toxic and maintains no adverse effects on cell viability. Treatment of cells was done acutely at 1.5 hours. This is based on previous results which indicated TMR had a significant effect with a low treatment time. TMR was administered to cells at 0, 5, 10, 25, and 50 µM in both the basal and stimulated condition (Figure 3.7 A and B). In both conditions, TMR demonstrates significant, partial inhibition of lipolysis observed with reduced levels of glycerol released. This reduction in lipolysis is seen at each concentration of TMR. The efficacy of TMR and other resveratrol metabolites have been reported to show effects even at low concentrations (Hsieh et al. 2011a; Lasa et al. 2012a). Identifying a low concentration threshold with adequate efficacy is critical for making animal and human physiological comparisons for dosage. Furthermore, identifying this dose-dependent response in glycerol release indicates another model system in which the resveratrol analogue, TMR, has action.

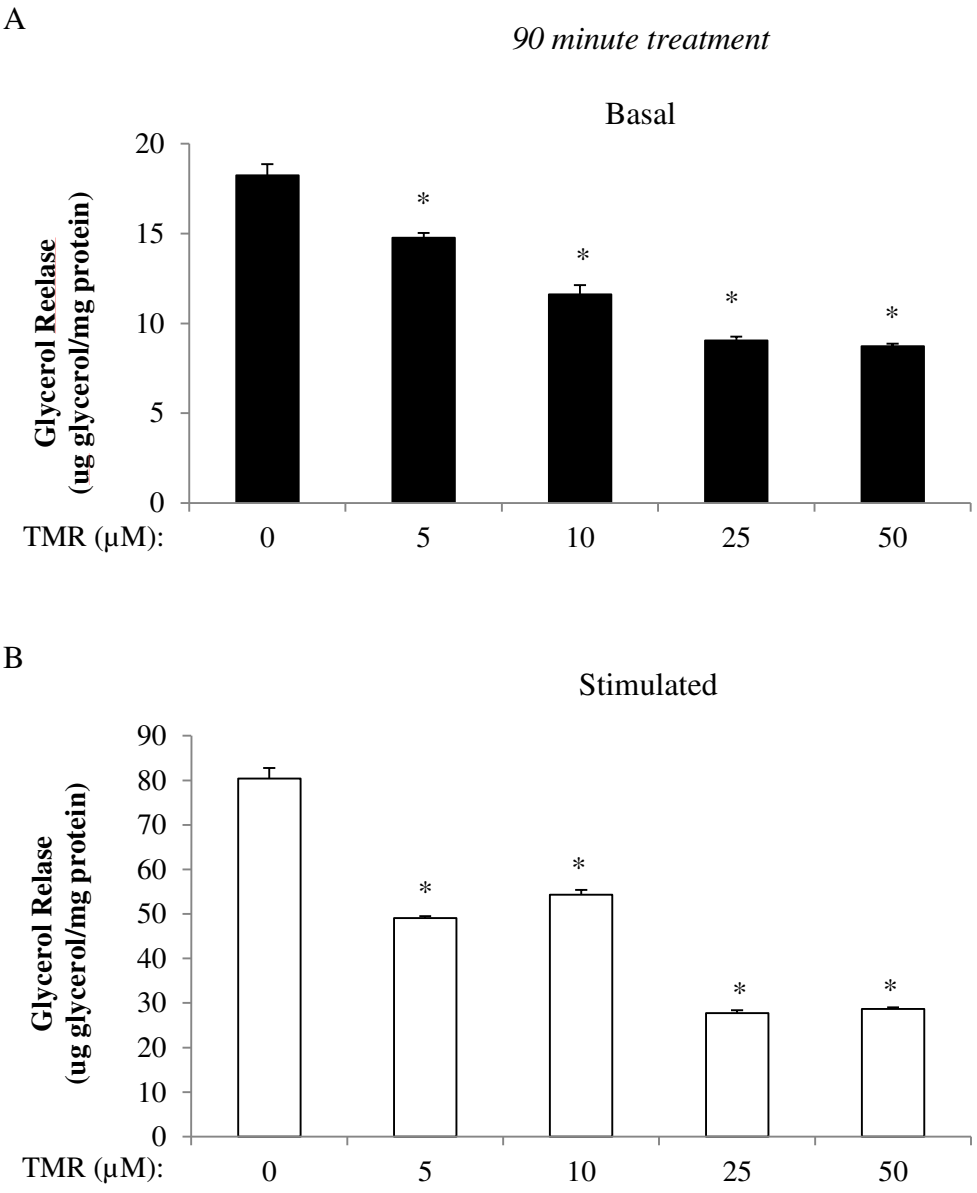


Figure 3.7 Dose-dependent effect of *trans*-3, 4', 5-trimethoxyresveratrol (TMR) on release of glycerol on mature adipocytes. Glycerol release in 3T3-L1 mature adipocytes treated for 1.5 hours at day 10 after differentiation in the presence or absence of TMR (0, 5, 10, 25, and 50 µM). Comparisons between each treatment with control; subjected to basal (A) and stimulated (B) conditions. Data are represented as means ± SEM, n=4. *, P <0.001.

3.2.5 Time-dependent impact of TMR on lipolysis in mature adipocytes

In order to understand the difference between an acute and chronic treatment, we turned our attention to the aspect of time and the role it plays in affecting lipolysis inhibition by TMR. Most treatments of RES and its metabolites on 3T3-L1 cells were done a longer treatment times; a 24 hour treatment in mature adipocytes or treatment was given every 48 hours in maturing adipocytes (Lasa et al. 2012a; Lasa et al. 2012b). In one report by Lasa, were cells treated for a shorter, 12 hour time period with RES. As mentioned in the literature review, the lipolysis process, though a simplistic idea, it is a highly regulated, complex system (Zechner et al. 2012). The cascade of events which occur by proteins and different transcription factors may be altered in their action due to the amount time that the cell is exposed to an antilipolytic agent. We proposed that perhaps there is an optimal time that TMR is able to modulate the lipolysis process.

TMR demonstrated an antilipolytic effect at 25 μM in glycerol release. We treated mature 3T3-L1 cells in the presence or absence of TMR (25 μM) for 3, 6, 21 and 24 hours (Figure 3.8). We observed that TMR has a significant reduction in the glycerol release at an acute treatment time of 3 hours, and we also noted a significant difference at 21 hours. Additionally, we also incubated mature adipocytes with 50 μM for 1.5 and 6 hours, in both basal and stimulated conditions (Figure 3.9 A and B). Similarly, we note that the shorter, acute treatment of TMR is most effective in partially blunting glycerol release. These results indicate that TMR acts rapidly in influencing major components in the lipolytic cascade.

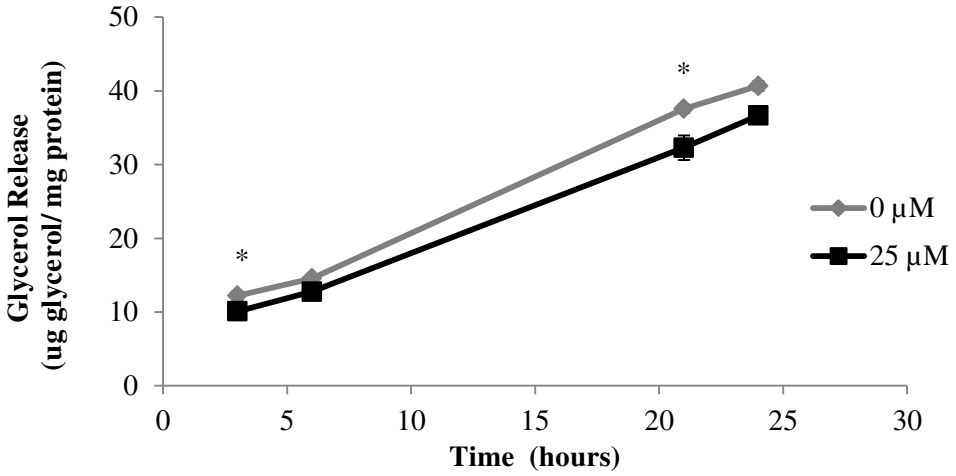


Figure 3.8 Time-dependent effect of *trans*-3, 4', 5-trimethoxyresveratrol (TMR) on release of glycerol on mature adipocytes with 25 µM. Glycerol release in 3T3-L1 mature adipocytes treated at different time points (1.5, 6, 21, and 24 hours) at day 10 after differentiation in the presence or absence of TMR (25 µM). Comparisons between each treatment with control; subjected to a basal conditions. Data are represented as means ± SEM, n=3. *, P <0.05.

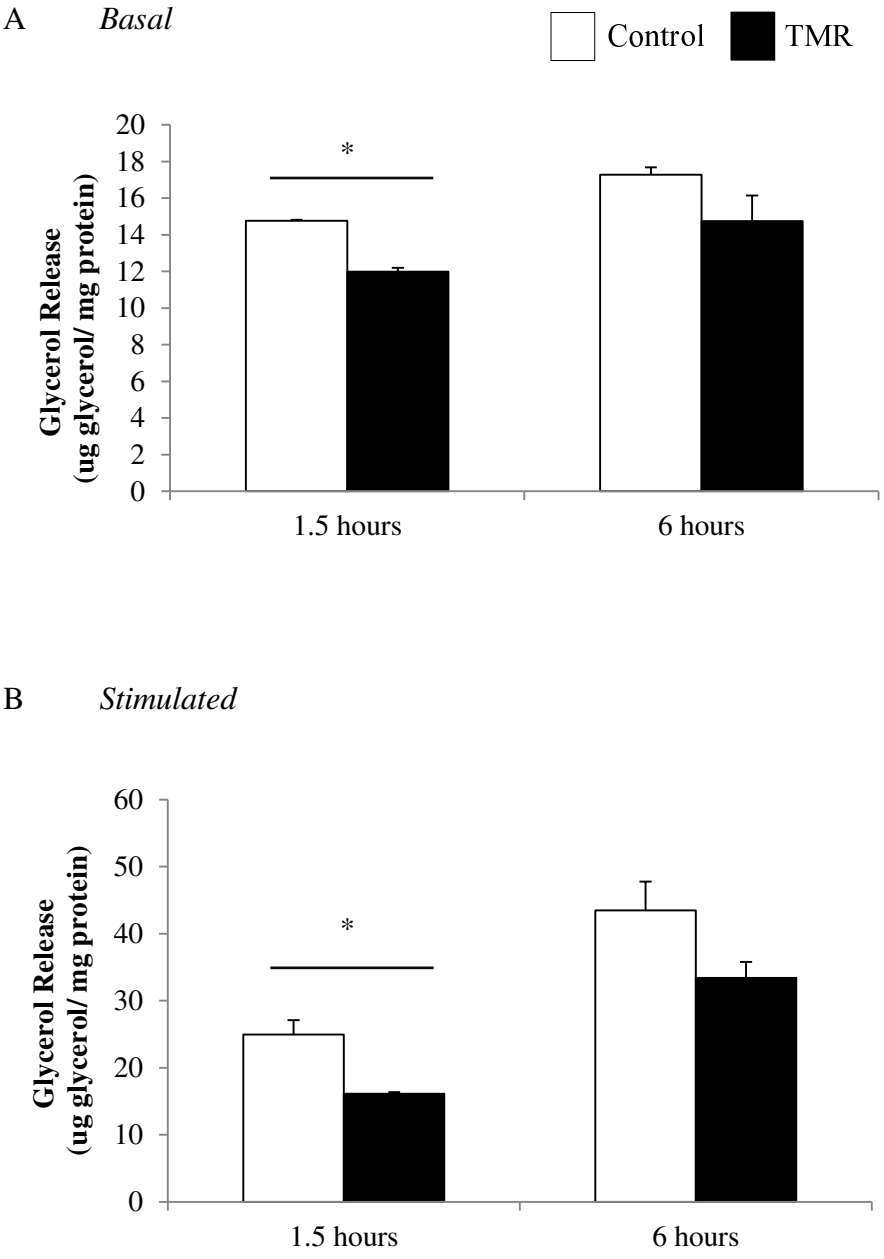


Figure 3.9 Time-dependent effect of *trans*-3, 4', 5-trimethoxyresveratrol (TMR) on release of glycerol on mature adipocytes with 50 µM. Glycerol release in 3T3-L1 mature adipocytes treated at different time points (1.5 and 6 hours) at day 10 after differentiation in the presence or absence of TMR (50 µM). Comparisons between each treatment with control; subjected to basal (A) and stimulated (B) conditions. Data are represented as means ± SEM, n=3. *, P <0.05.

3.2.6 Mechanistic effect of TMR on lipolysis in mature adipocytes

The next question we inquired involved the determination by what molecular mechanism does TMR elicit its partial inhibition of lipolysis. Lipolysis involves three different enzymes which work to hydrolyze LD TAGs into three FA and a glycerol backbone (Duncan et al. 2007; Zechner et al. 2012). This catabolic process and mobilization of NEFA into the bloodstream occurs during times of energy expenditure or fasting (Langin 2006a). As previously described, the first enzyme which acts primary on TAG is ATGL with activation by CGI-58, followed HSL which has high affinity to remove the second FA on DAG, and finally MGL acts to remove the final FA from the glycerol backbone (Ahmadian et al. 2010; Walther and Farese 2012). Formerly studied in our lab was the action of PIC on lipolysis. It has been shown that PIC works to partially inhibit lipolysis through protein degradation of ATGL and co-activator, CGI-58 (Figure 3.10), primarily through activation of autophagy pathway targeting ATGL and CGI-58 (Kwon, unpublished). Therefore, based on our results of PIC and TMR both inhibiting lipolysis at similar levels, the molecular mechanisms at which they suppress lipolysis may also be similar due to resemblance in their structure.

Immunoblot analysis of lipolytic enzymes was done with mature 3T3-L1 adipocytes that were treated in the presence or absence of TMR at varying concentrations (0, 5, 10, 25, and 50 μM) and PIC at 50 μM was used as a positive control. Western blot analysis was performed both in basal and stimulated conditions at an acute treatment time of 1.5 hours (Figure 3.11). In the basal condition, there is a dose-dependent response that mimics the effect seen in glycerol release. ATGL is observably reduced as compared to the control with different concentrations of TMR. Not surprisingly, we see that TMR is has greatest potency at its highest concentration of 50 μM. However, the level of ATGL reduction is not as substantial as PIC. Based on the quantification of band intensity (Figure 3.12 A), TMR is able to reduce ATGL protein expression by 40%, whereas PIC reduces it greater than 80%. TMR is also shows action in reducing CGI-58 protein expression. CGI-58 is necessary for activation and maximum stimulation of ATGL as well as regulation of the hydrolytic protein (Lass et al. 2006). TMR activity in the reducing CGI-58 is comparable to that of PIC, in which we see just over 50% reduced by

TMR and approximately 80% reduced by PIC. The second major lipolytic enzyme is HSL. Phosphorylation of HSL is the major regulator for activation of the enzyme as well as for the translocation to the surface of the LD (Schweiger et al. 2006). HSL phosphorylation occurs at multiple sites, but phosphorylation at serine-660 is responsible for interfacial activation and hydrolytic action of DAG (Walther and Farese 2012). Phosphorylation of HSL (pHSL) was also analyzed. TMR action of pHSL was surprisingly different than PIC in the basal condition. We observed slight reduction in the level of pHSL by TMR, whereas we see a large induction of pHSL of PIC. Overall, the level of reduction in lipolysis by glycerol release of TMR and PIC is very similar, yet the reduction level of ATGL and CGI-58 is greater in PIC than TMR. This discrepancy may be due to TMRs slight action inhibiting HSL activation in an acute basal treatment.

In the acute stimulated condition, we observe a similar, more pronounced trend in the action of TMR on lipolytic enzymes. ATGL protein expression is greatly reduced by TMR, similar to PIC (Figure 3.11 B) at approximately 80% (Figure 3.12 B). TMR only reduced CGI-58 by 20% as compared to the control, whereas we still observe a large reduction by PIC. The pHSL in this condition by TMR is slightly reduced maintaining a similar trend as seen in the basal condition. Again, this contrasts with action by PIC, which increases pHSL. This data suggests that TMR action on lipolysis in an acute condition is predominantly through reduction of protein expression of ATGL. However, it is worth noting, that TMR does have small action in reducing both CGI-58 and pHSL. The ability of TMR to interact and affect, at least in part, these major lipolytic enzymes may be attributed to its capacity to locate with the LD. The lipolysis process is occurs at the surface of the LD, accordingly, the catabolic enzymes are equipped with a hydrophobic region which allows binding (Duncan et al. 2007). Advantageously, the structure of TMR lends itself to be more lipophilic, and perhaps have greater versatility surrounding the LD.

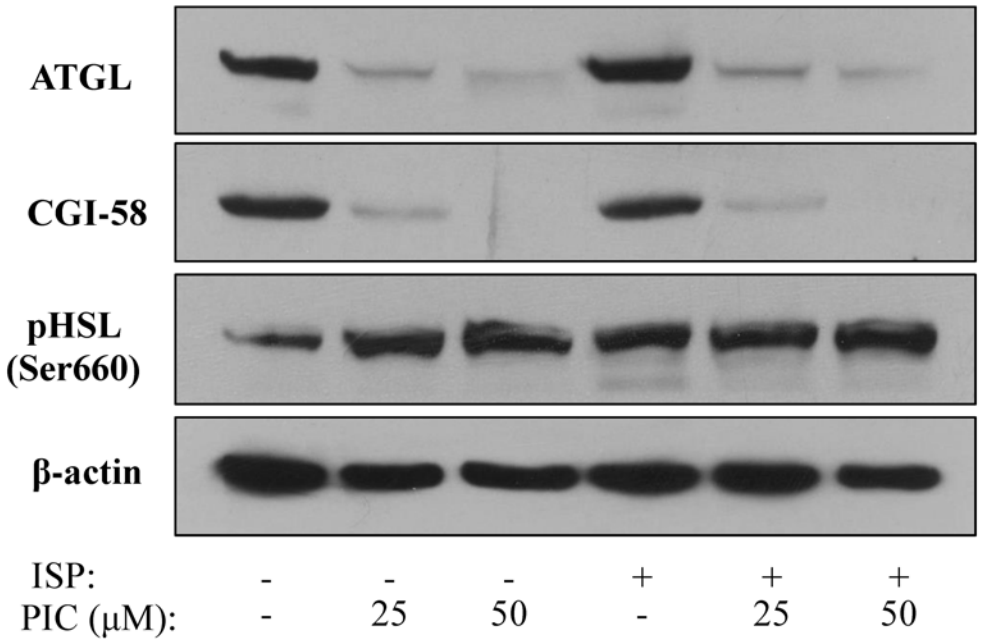
To further confirm that greatest effect of TMR action is seen with an acute treatment. Immunoblot analysis was performed on mature murine 3T3-L1 adipocytes which were treated for 3 hours in the presence or absence of TMR at 0, 25, and 50 μM concentrations (Figure 3.13). We observed large reductions in the levels of ATGL and

CGI-58 protein expression (Figure 3.14). These results show the reduction of lipolytic enzymes by TMR dose-dependently. The reductions in protein expression are similar to the levels observed by PIC with a 1.5 hour treatment. TMR, at 50 μM was able to effectively reduce ATGL expression by approximately 80% and CGI-58 by 70%. The level of pHSL was also consistent with our previous results, indicting slight action in blocking its phosphorylation. These data suggest the largest reduction of lipolytic enzymes observed by TMR at 3 hours. For comparison, TMR was treated for 6 hours on mature adipocytes and immunoblot analysis was done to observe its effects on enzyme expression (Figure 3.15). Observably, we note a reduction in ATGL and CGI-58 levels. Yet when analyzing the quantification of the band intensity, we see that the detected effect is not as large compared to a 3 hour treatment (Figure 3.16). TMR demonstrates reduction of ATGL protein expression by 40% and reduces CGI-58 protein level by 25%. Conversely, we see an upregulation of pHSL as compared to the control. Importantly, the data suggests that the most effective treatment of time of TMR is acute, at 3 hours, and time does play a role on the level of lipolytic inhibition.

In an attempt to further our understanding of the effect of TMR on lipolytic protein expression, we examined TMR in a basal, chronic condition. 3T3-L1 mature adipocytes were treated with different concentrations of TMR (0, 5, 10, 25 and 50 μM) for 24 hours and protein expression was analyzed with immunoblotting (Figure 3.17). Our protein expression levels of cells treated with TMR as compared to the control are reduced, however not to the same degree observed in an acute treatment of 3 hours. Here we observe an approximately 20% reduction in ATGL protein expression by TMR only at its highest concentration of 50 μM (Figure 3.18). PIC still maintains chronic efficacy in its degradative action of ATGL. CGI-58 protein expression had a similar trend as ATGL; TMR induced a slight reduction while PIC sustained its degradation (data not shown). pHSL after a 24 hour treatment was highly increased with TMR (25 and 50 μM) and PIC treatment with a 3-fold increase. This data suggests that there may be a compensatory effect that occurs in a chronic treatment of TMR. And although, glycerol release is still partially inhibited with a 24 hour treatment by TMR, within the cell only slight reductions in ATGL and CGI-58 are seen with pHSL being upregulated to possibly

compensate for the extended time and need for an energy substrate. Taken together, our results demonstrate TMR acts in a similar manner as PIC and the molecular mechanism at which TMR partially inhibits lipolysis is primarily with reduction of ATGL, with slight inhibition action on both CGI-58 and pHSL. This effect of TMR is seen at a greater potency in an acute treatment.

It has been reported *in vivo* high fat diet (HFD)-induced obesity disrupts the controlling pathways and causes changes in lipolytic protein expression and signaling (Gaidhu et al. 2010). In an obesogenic condition, it was noted that several key lipolytic elements are altered; this includes upregulation of ATGL and CGI-58 and down regulation of HSL and perilipin. This culminates in an increased level of basal lipolysis with alterations in the molecular regulation of FA metabolism and lipolysis, leading to increased metabolic dysfunctions (Gaidhu et al. 2010). Defects in the metabolic regulatory system ultimately results in severe consequences, as reviewed earlier, including increased levels of circulating NEFA and insulin resistance leading to ectopic fat distribution (Despres and Lemieux 2006). Here we describe an *in vitro* system with dietary compounds effects can help to attenuate some of the dysfunctional action in lipolysis. Our results demonstrate an altered cellular environment that would increase the health of a HFD-obesogenic condition. We observe reduction in glycerol release and definitive down regulation of ATGL and CGI-58, and slight reductions in the pHSL by TMR. This action is occurs acutely and is specific to the surface of LD, where TMR locates. The data suggests that TMR helps to modulate the first, rate-limiting step of lipolysis through action in reducing key lipase protein expression.



Kwon, unpublished

Figure 3.10 Treatment of piceatannol in 3T3-L1 mature adipocytes causes degradation of lipolytic enzymes. 3T3-L1 mature adipocytes were incubated in the presence or absence with piceatannol (PIC) at various concentrations (0, 25, 50 μM) for 1.5 hours in the basal and stimulated conditions. Cells in stimulated condition were incubated with isoproterenol (ISP). The cells were collected and protein samples were prepared. Protein levels of ATGL, CGI-58, and phosphorylated HSL (Ser660) in these cells were detected by immunoblot assay using their specific antibodies and β-actin as a loading control. Results from previous student, Kwon, unpublished.

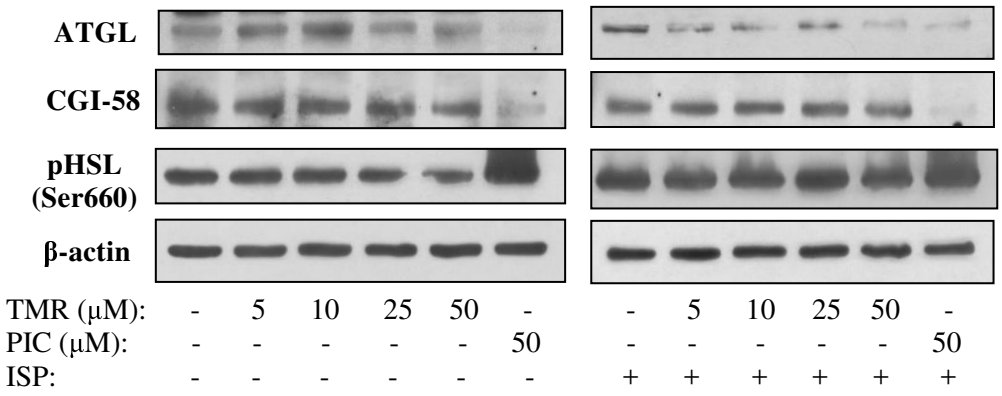
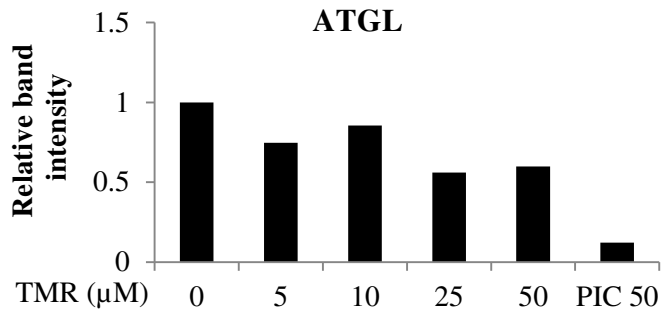
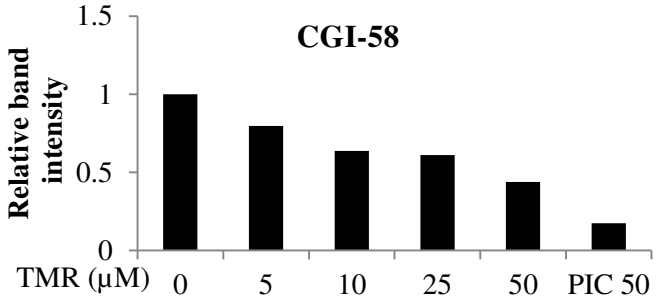
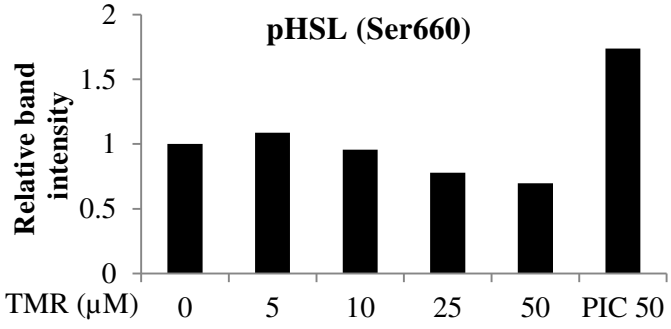


Figure 3.11 Dose-dependent acute effect of *trans*-3, 4', 5-trimethoxyresveratrol (TMR) on lipolytic enzymes in 3T3-L1 mature adipocytes. 3T3-L1 mature adipocytes were incubated in the presence or absence of TMR at various concentrations (0, 5, 10, 25, 50 μM) or piceatannol (PIC) at 50 μM for 1.5 hours in the basal and stimulated conditions. Cells in stimulated condition were incubated with isoproterenol (ISP). The cells were collected and protein samples were prepared. Protein levels of ATGL, CGI-58, and phosphorylated HSL (Ser660) in these cells were detected by immunoblot assay using their specific antibodies and β-actin as a loading control. The experiment was repeated at least twice with similar results.

A *Basal*


ATGL
Relative band intensity
1.5
1
0.5
0
TMR (μM)
0
5
10
25
50
PIC 50


CGI-58
Relative band intensity
1.5
1
0.5
0
TMR (μM)
0
5
10
25
50
PIC 50


pHSL (Ser660)
Relative band intensity
2
1.5
1
0.5
0
TMR (μM)
0
5
10
25
50
PIC 50

B *Stimulated*

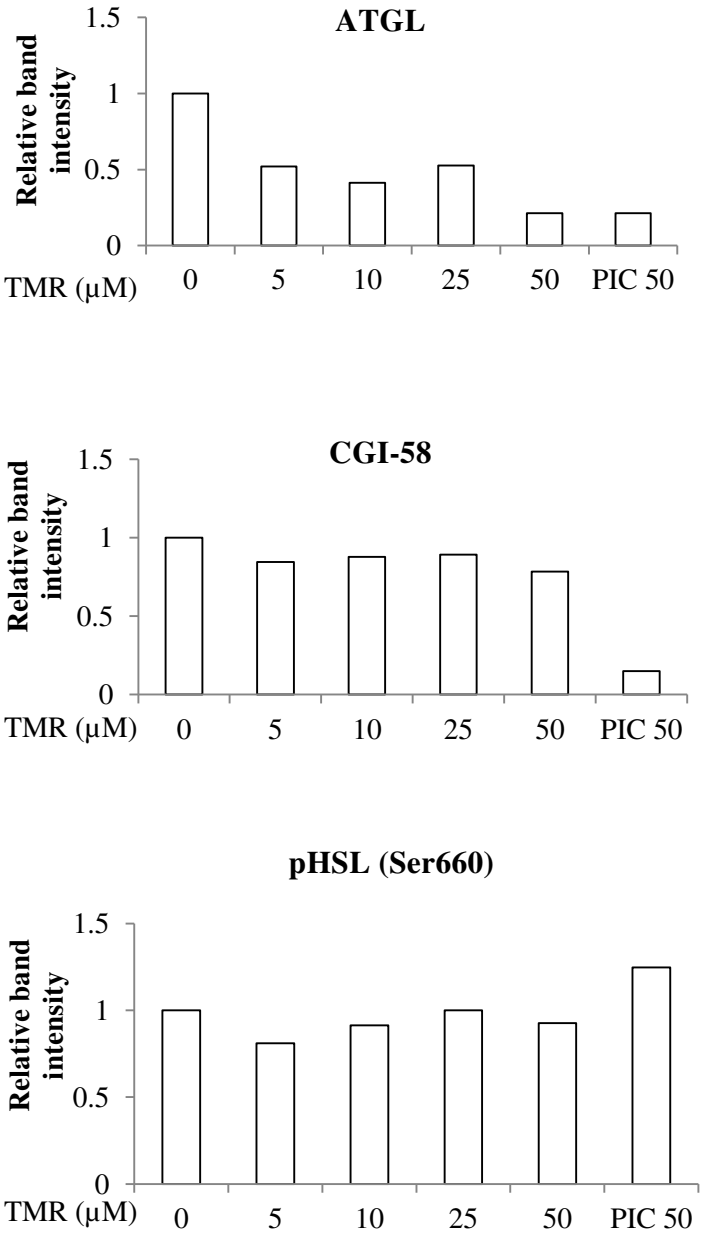


Figure 3.12 Quantification of immunoblot [shown in Figure 3.11] Film was scanned and protein band intensity was quantified with NIH ImageJ software (ver 1.45S), normalized to band intensity of β-actin. Cells were subjected to basal (A) and stimulated (B) conditions.

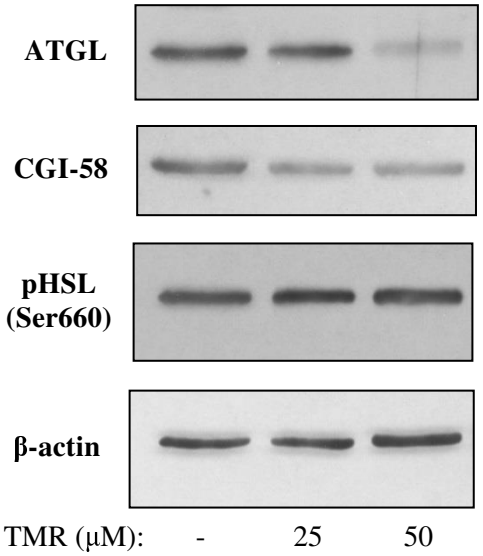


Figure 3.13 Three hour effect of *trans*-3, 4', 5-trimethoxyresveratrol (TMR) on lipolytic enzymes in 3T3-L1 mature adipocytes. 3T3-L1 mature adipocytes were incubated in the presence or absence of TMR at various concentrations (0, 25, 50 μM) for 3 hours in a basal condition. The cells were collected and protein samples were prepared. Protein levels of ATGL, CGI-58, and phosphorylated HSL (Ser660) in these cells were detected by immunoblot assay using their specific antibodies and β-actin as a loading control. The experiment was repeated at least twice with similar results.

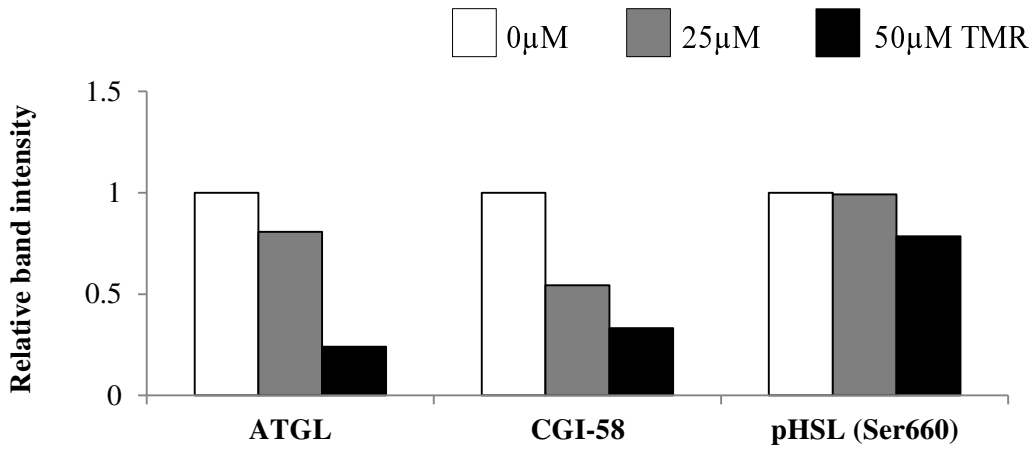


Figure 3.14 Quantification of immunoblot [shown in Figure 3.13] Film was scanned and protein band intensity was quantified with NIH ImageJ software (ver 1.45S), normalized to band intensity of β-actin. Cells were subjected to basal conditions.

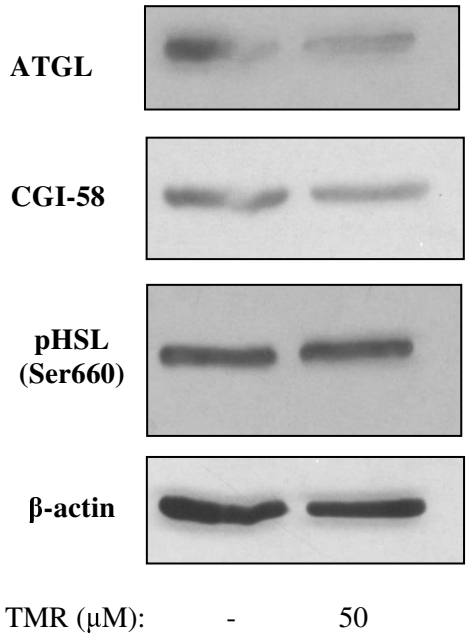


Figure 3.15 Six hour effect of *trans*-3, 4', 5-trimethoxyresveratrol (TMR) on lipolytic enzymes in 3T3-L1 mature adipocytes. 3T3-L1 mature adipocytes were incubated in the presence or absence of TMR at various concentrations (0, 50 μM) for 6 hours in a basal condition. The cells were collected and protein samples were prepared. Protein levels of ATGL, CGI-58, and phosphorylated HSL (Ser660) in these cells were detected by immunoblot assay using their specific antibodies and β-actin as a loading control.

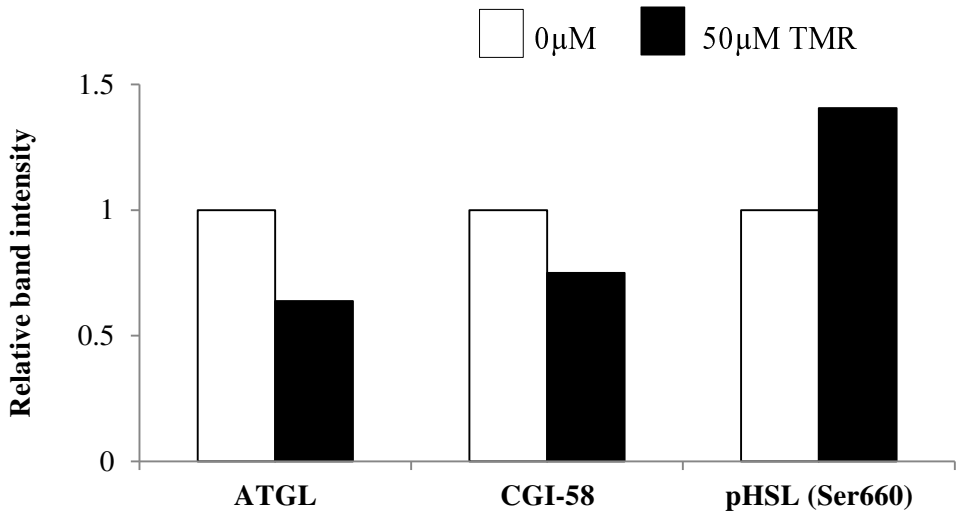


Figure 3.16 Quantification of immunoblot [shown in Figure 3.15] Film was scanned and protein band intensity was quantified with NIH ImageJ software (ver 1.45S), normalized to band intensity of β-actin. Cells were subjected to basal conditions.

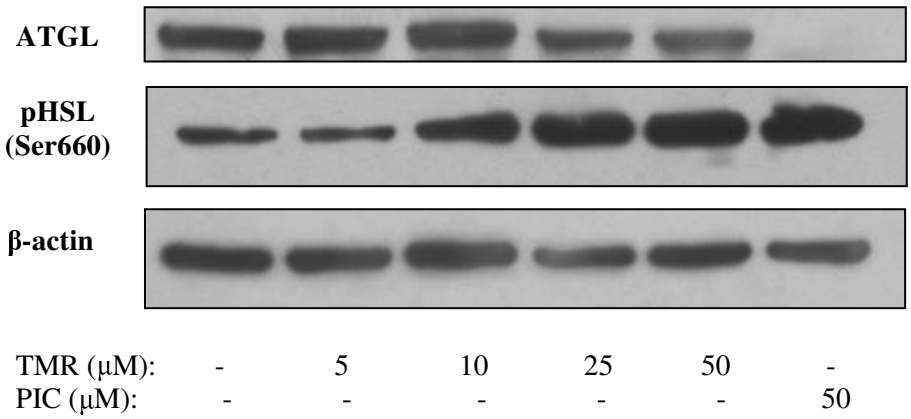


Figure 3.17 Dose-dependent chronic effect of *trans*-3, 4', 5-trimethoxyresveratrol (TMR) on lipolytic enzymes in 3T3-L1 mature adipocytes. 3T3-L1 mature adipocytes were incubated in the presence or absence of TMR at various concentrations (0, 5, 10, 25, 50 μM) or piceatannol (PIC) at 50 μM for 24 hours in a basal condition. The cells were collected and protein samples were prepared. Protein levels of ATGL and phosphorylated HSL (Ser660) in these cells were detected by immunoblot assay using their specific antibodies and β-actin as a loading control. The experiment was repeated at least twice with similar results.

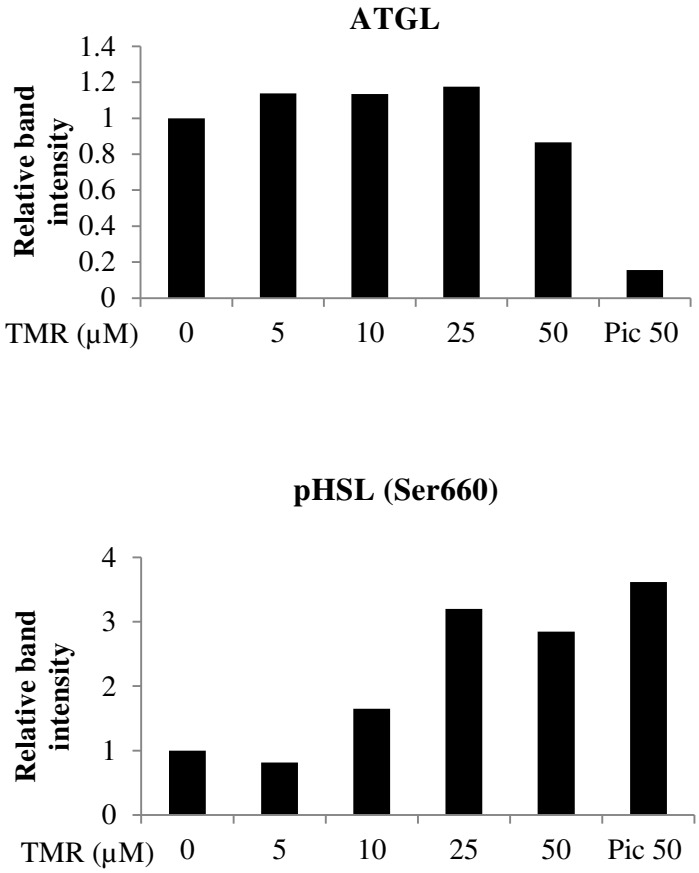


Figure 3.18 Quantification of immunoblot [shown in Figure 3.17] Film was scanned and protein band intensity was quantified with NIH ImageJ software (ver 1.45S), normalized to band intensity of β-actin. Cells were subjected to basal conditions.

3.2.7 TMR partially inhibits lipolysis in mature adipocytes via protein reduction

In order to observe and confirm the overall effect that TMR exhibits on mature adipocytes. We treated 3T3-L1 cells in the presence or absence of TMR (0, 25, 50 µM) for 3 hours in both a basal and stimulated condition (Figure 3.19). Here we validate results consistent with our previous data. TMR is able to modulate lipolysis in both conditions via reductions in protein expression of ATGL and CGI-58. To note, TMR is highly potent in the basal condition with significant reductions of ATGL at 70% and 60 % of CGI-58 (Figure 3.20 A). TMR also showed a slight inhibition of pHSL, consistent with previous 3 hour treatments. In the stimulated condition, we observe that TMR is able to reduce ATGL and CGI-58, yet the levels of reduction are not as pronounced as in the basal state (Figure 3.20 B). We detect 25% and 35% reduction in protein expression levels of ATGL and CGI-58, respectively. Also, pHSL was slightly increased compared to the control. This may suggest some increased signaling or alternative route the cell is trying to control with HSL, because although HSL mainly acts on DAG, it does maintain affinity for hydrolyzing TAG (Duncan et al. 2007). Nonetheless, these results provide confirmatory evidence on TMR partial inhibitory action of lipolysis through reduction of lipolytic enzymes and its modulating specificity in lipid metabolism.

Our study proposes significant, partial inhibition of mature adipocyte lipolysis by TMR requires a concentration up to 50 µM *in vitro*. Our data suggests that the physiological condition of mature adipocytes may be more sensitive to acute exposure to circulating levels of TMR, which exerts a beneficial function in a metabolic dysfunctional environment. The rate of NEFA release of adipose tissue is strongly associated with insulin resistance and type 2 diabetes (Lass et al. 2006). Therefore identifying a potential dietary compound, such as TMR, as an antilipolytic agent, which aids in modulating this process may be a beneficial therapeutic strategy. Given the importance of the lipolysis process in overall energy homeostasis, it imperative to mention that with treatment of TMR, we do not observe full inhibition of glycerol release and protein expression. It has been reported that patients with complete deficiencies in either ATGL or CGI-58 lead to defective lipolytic catabolism, resulting in accumulation of neutral lipid storage and an obese phenotype (Lass et al. 2006). Moreover, the partial

inhibition we see with TMR is more applicable as strategy to alleviate some, but not all, adipose lipolysis.

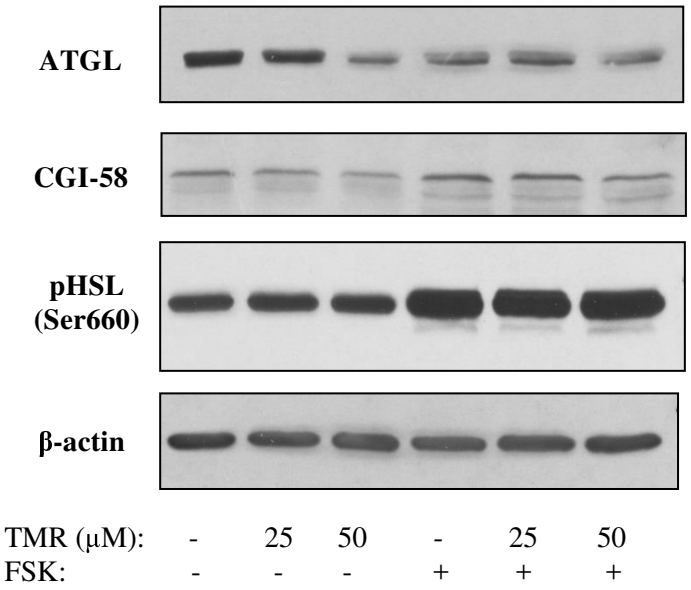


Figure 3.19 Acute effect of *trans*-3, 4', 5-trimethoxyresveratrol (TMR) on lipolytic enzymes in 3T3-L1 mature adipocytes. 3T3-L1 mature adipocytes were incubated in the presence or absence of TMR at various concentrations (0, 25, 50 μM) for 3 hours in the basal and stimulated conditions. Cells in stimulated condition were incubated with forskolin (FSK). The cells were collected and protein samples were prepared. Protein levels of ATGL, CGI-58, and phosphorylated HSL (Ser660) in these cells were detected by immunoblot assay using their specific antibodies and β-actin as a loading control. The experiment was repeated at least twice with similar results.

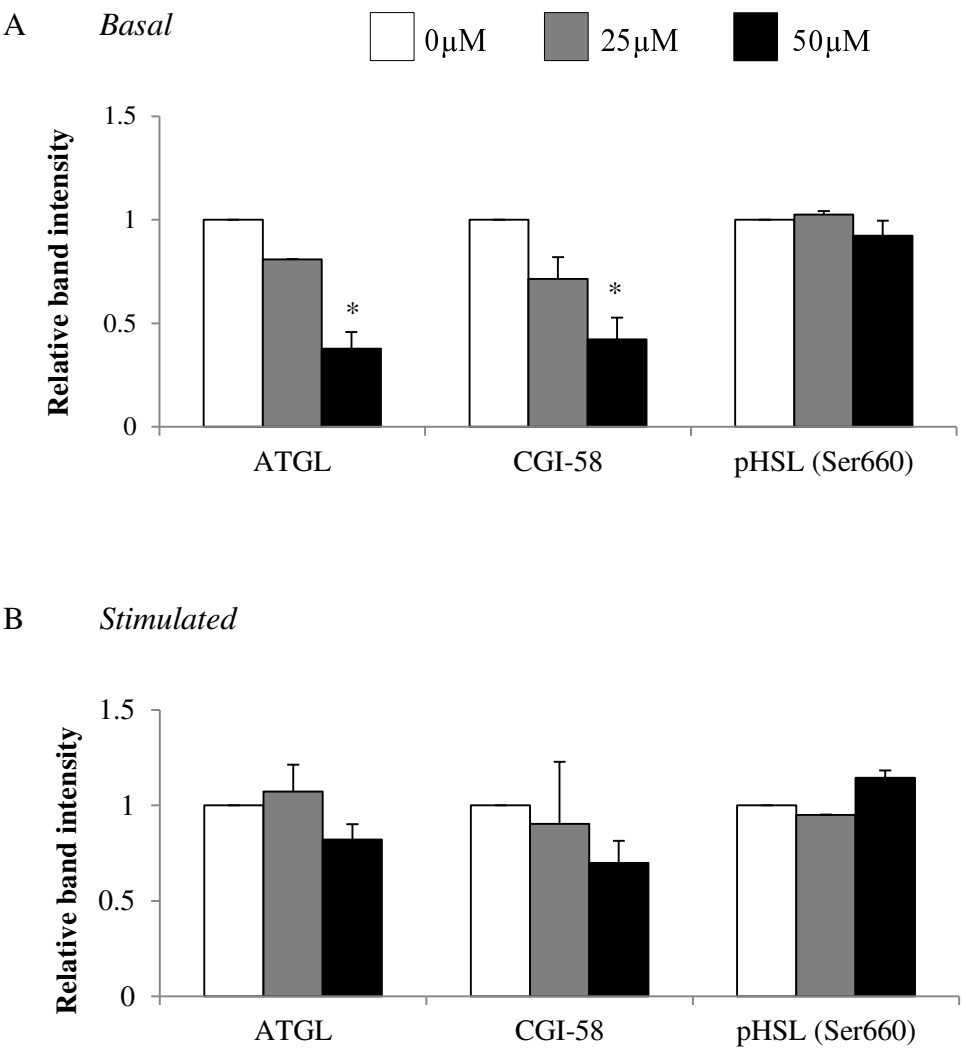


Figure 3.20 Quantification of immunoblot [shown in Figure 3.19] Film was scanned and protein band intensity was quantified with NIH ImageJ software (ver 1.45S), normalized to band intensity of β-actin. Cells were subjected to basal (A) and stimulated (B) conditions. Data are presented as means ± SEM, n=2-4. *, P <0.05.

3.2.8 Impact of TMR on lipolytic gene expression

There is large importance in understanding how specific lipolytic enzyme manifestation is effected by dietary compounds, such as TMR. However, other regulatory mechanisms take place in modulating the transcription and translation of specific lipases. Therefore, in order to understand if TMR acts at these levels, relative mRNA expression of lipolytic lipases was also analyzed (Figure 3.21). Here we observed as significant reduction in the relative expression of *ATGL*, as well as with the lipid associated protein, *perilipin*. We note that a treatment with TMR shows a trend of reduction in both *CGI-58* and *HSL* expression levels. The down regulation of these genes is not surprising as it has been previously reported that RES, shown to target ATGL, displayed upregulation in both protein and relative mRNA expression. It was also noted, however, that RES does not have any noticeable effect on *HSL* (Lasa et al. 2012b). Also reported, the resveratrol metabolite, resveratrol-3-O-sulfate (3S), displayed a reducing trend in *HSL* mRNA expression levels (Lasa et al. 2012a). Collectively, our protein analysis in combination with our mRNA analysis of lipolytic enzymes demonstrates a profound effect of TMR and its modulation of lipolysis. Our study observed that TMR reduced glycerol release to similar potency as PIC. And although we show that TMR also plays a role in the reduction of protein expression of ATGL and CGI-58, it was not seen to the same degree as PIC. Our relative mRNA expression of lipolytic enzyme illustrates another piece of the puzzle, suggesting TMR has some transcriptional activity regarding these enzymes.

In summary, our study including molecular imaging and biochemical analysis provides evidence that TMR is significant in partially inhibiting mature adipocyte lipolysis through the reduction of lipolytic enzymes, possibly via mediation of protein degradation, and potentially having activity which acutely affects enzymatic transcription. Our results also indicate that the structure of TMR allows its co-localization with the hydrophobic LD and may attribute to its high activity in lipid metabolism. Collectively, this study elicits new findings that TMR modulates lipolytic activity in murine adipocytes *in vitro* and can support further investigations of therapeutic approaches for metabolic disorders induced by obesity and metabolic syndrome.

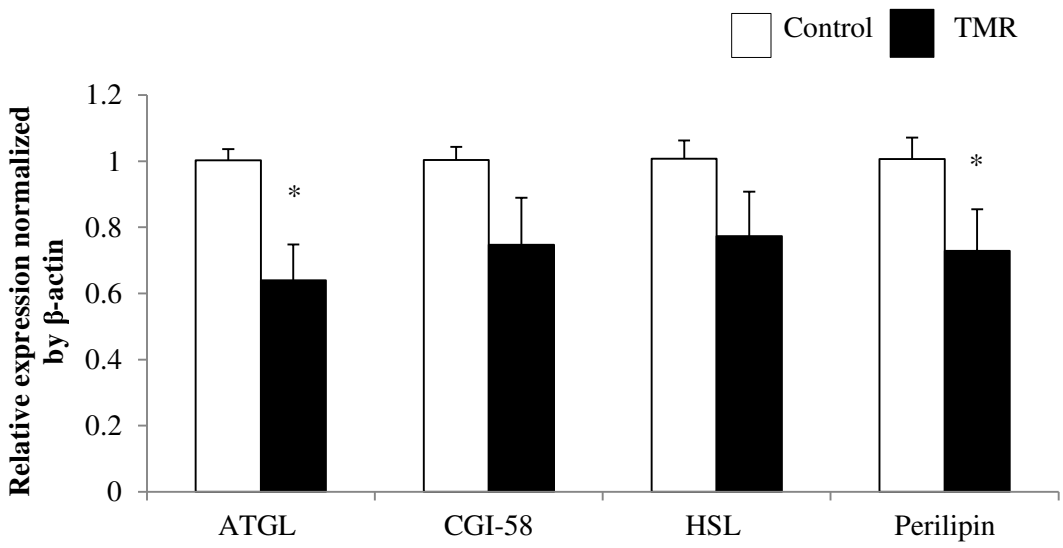


Figure 3.21 Effect of *trans*-3, 4', 5-trimethoxyresveratrol (TMR) on lipolytic transcription factor expression. 3T3-L1 mature adipocytes were incubated in the presence or absence of TMR (50 μM) for 3 hours in a basal condition. The cells were collected on day 10 and samples were prepared and subjected to quantitative gene expression analysis of ATGL, CGI-58, HSL, and perilipin by real-time (RT)-PCR. The signals were normalized by β-actin as an internal control. The values are displayed as fold change compared to the control; value arbitrarily set to 1. Data are presented as relative fold induction means ± SEM, n=3. *, $P < 0.05$.

3.3 Adipose function: Lipogenesis

As described in the review of the literature, during the terminal phase of adipogenesis, genes are encoded which result in the generation of FA and TAG (Kim et al. 2013). The process of carbon movement and series of enzymatic reactions yielding the production of FA is known as de novo lipogenesis (Ameer et al. 2014). The formation of TAG is also conducted by enzymes which involves a series of esterification of FA moieties to a glycerol backbone (Farese and Walther 2009). The molecular level of gene expression of these specific enzymes as well as adipokines secreted are tightly regulated and increased during TAG formation (Kim et al. 2013).

Dietary and molecular control of lipogenesis may be a potential target to prevent adipose mass increase and the development of obesity and its associated diseases. Yet, complete inhibition of lipid accumulation in adipocytes could lead to severe negative consequences including hypertrophy and or increased storage of TAGs in non-adipose tissues (Kim et al. 2013). Approaches involving increasing overall energy expenditure through increased activity of thermogenesis and fatty acid oxidation (FAO) may be an alternative route to positively affect obesity. The molecular mechanisms involved in the regulation of adipocyte differentiation and lipid accumulation can be widely altered by addition of bioactive dietary compounds. Furthermore, utilizing phytochemicals as a means to modulate obesity-preventing cellular metabolic pathways may be used as a therapeutic strategy (Jeong et al. 2012).

3.3.1 Impact of TMR on adipocyte differentiation and lipid accumulation

TMR has been shown in other cellular system to have efficacy, acting as an anti-cancer agent and can induce apoptosis (Dias et al. 2013; Pan et al. 2008). However, as reviewed in the literature, the action of phytochemicals is dependent on the function being analyzed. Currently, resveratrol metabolites have not been extensively studied in lipid metabolism; however, there is one report which details their role in adipogenesis and lipid accumulation. Lasa reports that treatment of 4G and 3S on maturing 3T3-L1 cells from day 0 to day 8, results in a significant reduction of relative TAG content (Lasa et al. 2012a). PIC has also been shown to inhibit adipogenesis through modulation of

MCE as well as through insulin receptor-dependent insulin signaling during the early stage of differentiation (Kwon et al. 2012). Based on our results, TMR has specific action in adipose function, specifically lipolysis, but its role in obesity development has not yet been elucidated. Also, because other resveratrol metabolites have demonstrated an effect on lipid accumulation, we hypothesize that TMR may also play a role in lipogenesis. In order to understand the function of TMR on lipid accumulation, we first examined the effect of TMR on the differentiation process in 3T3-L1 preadipocytes. Two day post-confluent preadipocytes were treated in the presence or absence of TMR (0 and 50 μM) and an adipogenic cocktail (DMI). Differentiating cells were treated with TMR every two days and were collected on day 6 after differentiation and lipid accumulation was quantified with ORO staining (Figure 3.22 A). Observably, there does not seem a dramatic reduction in lipid accumulation by TMR. Upon further inspection, we note that the sizes of the LDs in cells treated with TMR are slightly reduced. However, quantification of ORO stained intracellular LD was of no significance (Figure 3.22 B). Upon further confirmation of the effect TMR has on lipid accumulation, this experiment was repeated, yielding a large sample size (n=24). The repetition of this experiment was slightly contrasting with previous results we observed, in which we did see a reduction in LD formation (data not shown). Collectively, the lipid accumulation between the control and TMR are similar, however to fully understand TMR's effects during cellular development we investigated intracellular gene expression.

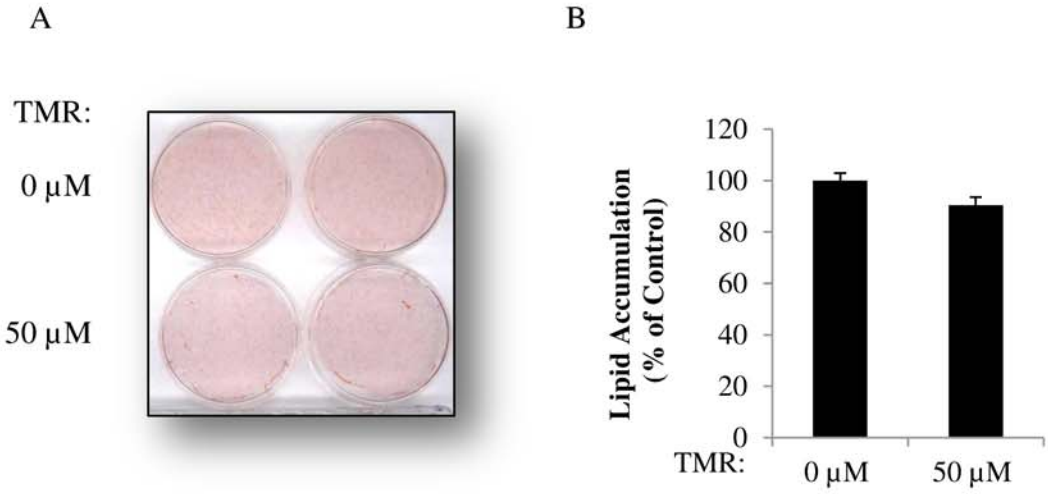


Figure 3.22 Effect of *trans*-3, 4', 5-trimethoxyresveratrol (TMR) on adipocyte differentiation and lipid accumulation. 3T3-L1 preadipocytes were subjected to adipocyte differentiation and an adipogenic cocktail. Cells were incubated in the presence of dimethyl sulfoxide (DMSO) or TMR (50 µM) in DMSO for 6 days. The cells were treated for 6 days with TMR and cells subjected to (A) Oil Red O (ORO) staining and (B) quantitative analysis of ORO stained intracellular lipids. Data presented as means ±SEM, n=24. *, P <0.05.

3.3.2 Impact of TMR on maturing adipocyte gene expression

As mentioned in the literature review, adipogenesis is a highly regulated program in which the cascade of transcription factors are stimulated and activated leading to terminal differentiation and ultimately developed LD (Cornelius et al. 1994). There is high coordination of gene expression events that occur and enhance the activity of de novo lipogenic enzymes and increase lipid accumulation (Rosen and MacDougald 2006). It is important to note, that although PIC and 3S have been reported to have significant action in reducing adipogenic gene expression markers of *C/EBPα* and *PPARγ*, other resveratrol metabolites, 4G and 3G demonstrated no significant difference from the control, and actually trended toward increasing these markers (Kwon et al. 2012; Lasa et al. 2012a).

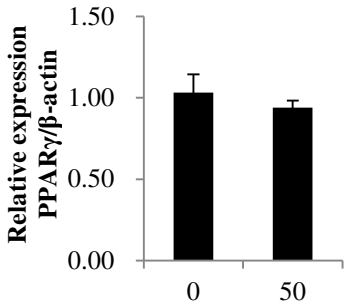
In order to investigate the trend of slightly reduced lipid accumulation in maturing 3T3-L1 adipocytes by TMR, we analyzed gene expression to understand if modulation occurred by TMR treatment. We wanted to determine if TMR was altering different gene expression markers during differentiation using quantitative real time RT-PCR. 3T3-L1 preadipocytes were stimulated to differentiate in the presence or absence of TMR at varying concentrations (0, 10, 25, and 50 μM) and treated every two days until collected on day six after differentiation. The gene expression profiles of several different functional adipose markers are discussed below.

3.3.2.1 <u>De novo lipogenesis</u>

As previously discussed in the literature review, the cascade of de novo lipogenesis involves series of reactions performed by enzymes which construct FA and generate TAG (Farese and Walther 2009). PPARγ has been coined the master coordinator of adipocyte differentiation for its binding to fat specific enhancers. TMR at 50 μM showed no significant difference in mRNA expression compared to the control (Figure 3.23 A). This data is consistent with reported data of RES, 3G, and 4G in which there was no significant change in *PPARγ* levels of maturing adipocytes (Lasa et al. 2012a). *FAS*, the major enzyme accounting for FA biogenesis, is dose dependently, and significantly upregulated by TMR (Figure 3.23 B). *ACC* is also upregulated significantly

by the highest concentration of TMR (50 μM) indicating increased production of malonyl-CoA to be used as substrate for FAS (Figure 2.23 C). We observed upregulation of *MGAT1* and *DGAT2*, but there is no significant change in *SCD-1* or *SREBP1c* (Figure 2.23 D-G). It is interesting to observe upregulation in genes that are involved in the production of FA and TAG synthesis, due to our previous collected data that demonstrates no significant induction in LD accumulation. This expression of lipogenic genes poses a thought-provoking question: how does TMR upregulate de novo lipogenesis genes and yet the level of lipid accumulation is unchanged and trends toward a slight reduction? To begin to answer this question, we looked at the previous report with resveratrol metabolites and their effect on *ACC* and *FAS*. Interestingly, 3T3-L1 mature adipocytes were subjected gene expression analysis of *ACC* and *FAS* after a 24 hour treatment of 10 μM of RES, 4G, 3G and 3S (Lasa et al. 2012a). *ACC* levels trended increasingly, although not significant, by RES, 3G, and 4G. The level of *FAS* expression was significantly reduced by 3G, however RES demonstrated an almost 1.5 fold increase as compared to the control (not significant). Nonetheless, the action seen by RES is comparable to that observed by TMR. In mature adipocytes, RES significantly reduced TAG content yet had a trending increase in gene expression levels of *FAS* (Lasa et al. 2012a). Although the authors do not comment on this occurrence, they did show increased levels of *ATGL* expression, which could indicate that RES has a higher capacity for energy expenditure through catabolic processes. In order to see if this is the case in our study and to fully understand this phenomenon in regards to TMR and its effect on maturing adipocytes, we further analyzed other gene expression markers.

A


Relative expression PPARγ/β-actin
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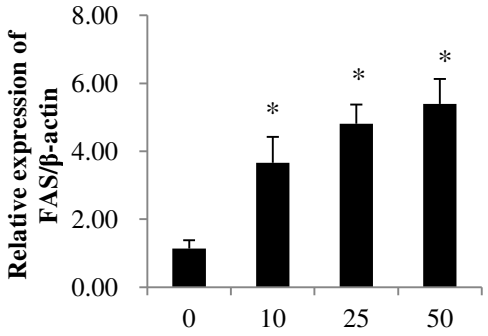
1.00
0.50
0.00
0
50

B


Relative expression of FAS/β-actin
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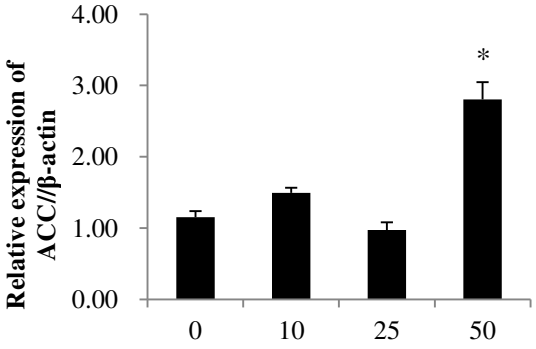
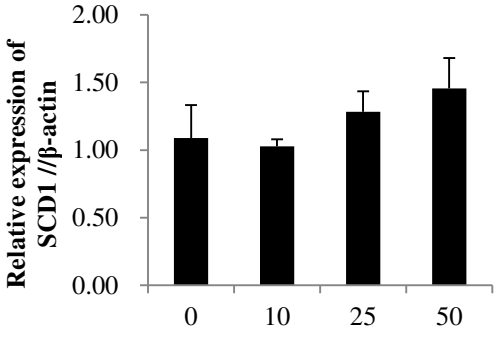
6.00
4.00
2.00
0.00
0
10
25
50
*
*
*

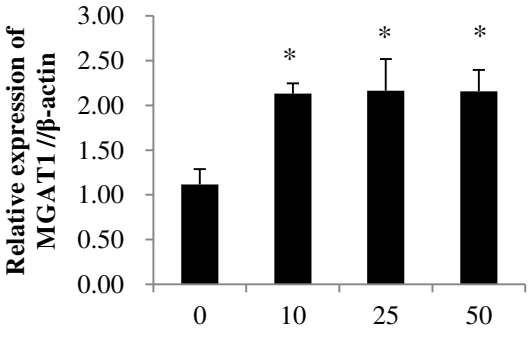
C


Relative expression of ACC//β-actin
4.00
3.00
2.00
1.00
0.00
0
10
25
50
*

D


Relative expression of SCD1 //β-actin
2.00
1.50
1.00
0.50
0.00
0
10
25
50

E


Relative expression of MGAT1 //β-actin
3.00
2.50
2.00
1.50
1.00
0.50
0.00
0
10
25
50
*
*
*

F G

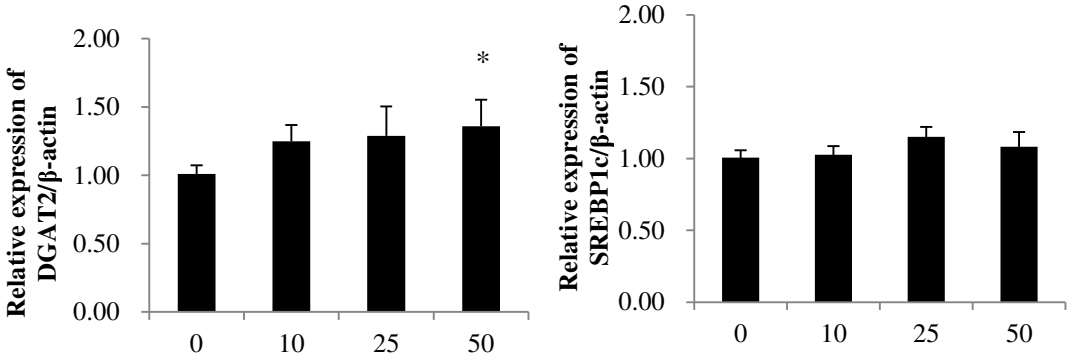


Figure 3.23 Dose-dependent effect of *trans*-3, 4', 5-trimethoxyresveratrol (TMR) on de novo lipogenic marker gene expression. 3T3-L1 preadipocytes were subjected to adipocyte differentiation and an adipogenic cocktail (DMI). Cells were incubated in the presence of dimethyl sulfoxide (DMSO) or various concentrations of TMR (0, 10, 25, and 50 μM) in DMSO for 6 days. The cellular total RNA was extracted and subjected to RT-PCR. The mRNA levels of (A) PPARγ (0and 50 μM), (B) FAS, (C) ACC, (D) SCD1, (E) MGAT1, (F) DGAT2, and (G) SREBP1c were quantified and normalized with the house keeping gene, β-actin. The values are displayed as fold change compared to the control; value arbitrarily set to 1. Data are presented as relative fold induction means ± SEM, n=3. *, P <0.05.

3.3.2.2 Adipokines

With TMR demonstrating a role in the marked increases in the lipogenic transcription program, we wanted to further investigate its effect on maturing adipocyte adipokine expression. Because adipose tissue is an endocrine organ, it has the capacity to secrete adipokines affecting metabolic pathways and energy homeostasis (Kershaw and Flier 2004). Therefore, we looked at four different adipokine gene expression profiles in cells treated with TMR to elucidate its potential involvement. *Resistin, adiponectin*, and *adipsin* were significantly upregulated, while the *leptin* was significantly reduced (Figure 3.24 A-D). First of all, *resistin* expression is induced during adipocyte differentiation and its levels are increased in diet-induced obesity and suppressed by insulin in 3T3-L1 adipocytes (Haugen et al. 2001) (Steppan et al. 2001). TMR demonstrated an increase in *resistin*. Its physiological function is to help regulate glucose tolerance and act as an adaptive response (Shojima et al. 2002). Comparatively, although in a different system, Mercader observed a 20 hour treatment of RES in mature adipocytes reduced *resistin* expression (Mercader et al. 2011). Leptin plays an important role in regulating food intake, energy expenditure, and physiologically when leptin plasma levels are increased, insulin secretion is inhibited (Cases et al. 2001). A reduced leptin concentration signals initiation for a starvation response (Ahima et al. 1996). Our results indicate a slight, significant reduction in the gene expression of *leptin* by TMR. This is similar to other published data with RES, which in maturing preadipocytes significantly reduces *leptin* expression (Eseberri et al. 2013) as well as rat adipocytes had reduced leptin secretion with RES treatment (Szkudelska et al. 2009). It was also noted that reduced *leptin* levels may be in part due to increased cAMP in fat cells, which could result in a parallel rise in lipolysis (Szkudelska et al. 2009). Our data suggests that the reduction in *leptin* levels by TMR may induce increased energy expenditure in the cells. Adiponectin is largely present in healthy human plasma and significantly lowered in obese subjects (Arita et al. 1999). It is an important modulator in lipid metabolism and is known to increase insulin sensitivity as well as increase FAO (Fasshauer et al. 2002b). TMR dose-dependently increases *adiponectin* expression significantly. This induction is consistent with the effect of other resveratrol metabolites in maturing adipocytes including 3G, 4G, and 3S

(Eseberri et al. 2013). Finally, adipsin is found in high levels in adipose tissue and its expression has been shown to be greatly reduced in obese mice, while increased levels are associated with a catabolic state in normal rodents (Flier et al. 1987; White et al. 1992). TMR effectively increases the expression of *adipsin* in 3T3-L1 maturing adipocytes which is consistent with an increased energy expenditure state. Collectively, the effect of TMR on adipokine gene expression is representative of cells which have altered lipid metabolism in that they have a higher propensity to expend energy. Taken together, this upregulation of adipokine gene expression, specifically *adiponectin* and *adipsin*, and down regulation of *leptin* possibly indicate an enhanced catabolic state which may result in reduced lipid accumulation.

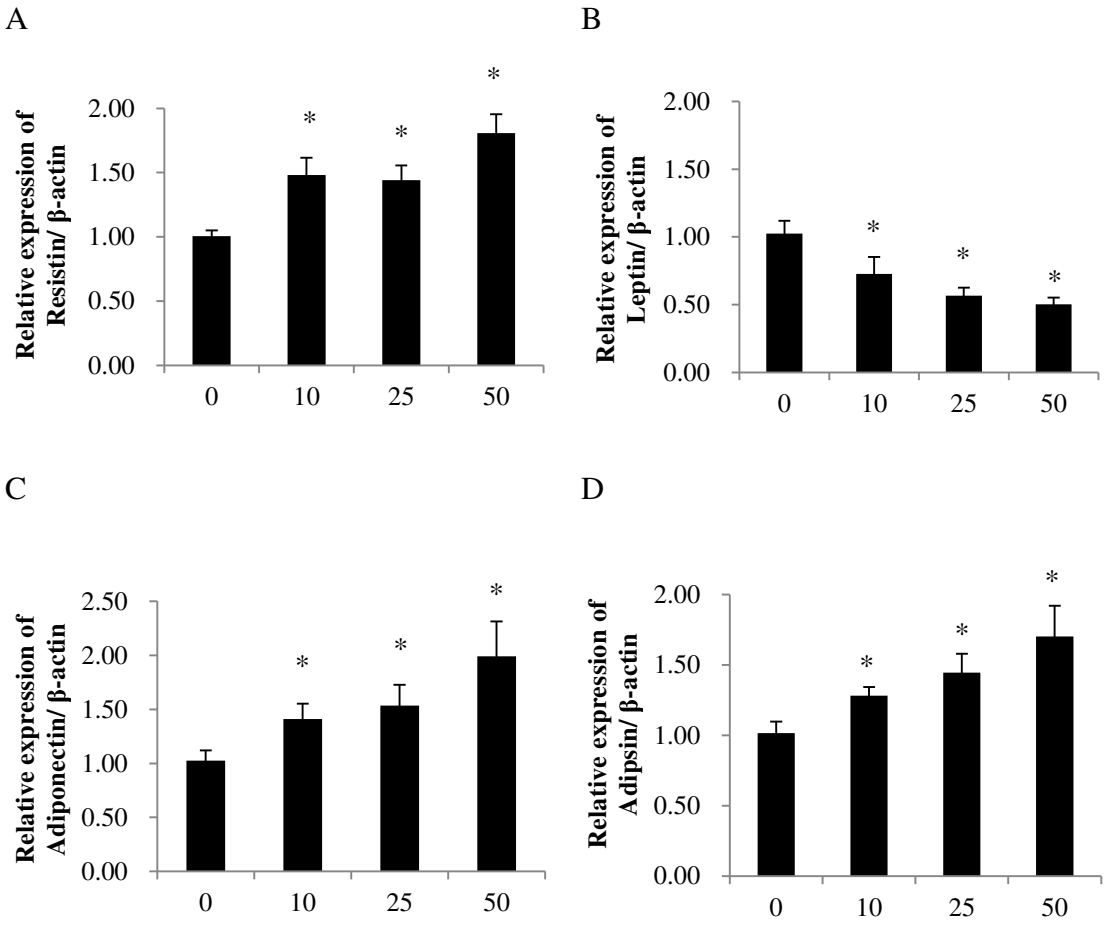


Figure 3.24 Dose-dependent effect of *trans*-3, 4', 5-trimethoxyresveratrol (TMR) on adipokine gene expression. 3T3-L1 preadipocytes were subjected to adipocyte differentiation and an adipogenic cocktail. Cells were incubated in the presence of dimethyl sulfoxide (DMSO) or various concentrations of TMR (0, 10, 25, and 50 μM) in DMSO for 6 days. The cellular total RNA was extracted and subjected to RT-PCR. The mRNA levels of (A) Resistin, (B) Leptin, (C) Adiponectin, and (D) Adipsin were quantified and normalized with the house keeping gene, β-actin. Data are presented as relative fold induction means ± SEM, n=3. *, $P<0.05$.

3.3.2.3 Lipolysis

To further investigate the overall effect of TMR on maturing adipocytes, lipolytic gene expression was analyzed to determine if catabolic processes were induced. *ATGL*, a major, rate-limiting step in the lipolysis was dose-dependently and significantly increased (Figure 3.25 A). *Perilipin*, a LD associated protein was also significantly increased when treated with 50 μM TMR (Figure 3.25 B). This transcriptional regulation by TMR demonstrates its ability to increased catabolic processes. This data represents that the action observed is highly dependent on the function being analyzed. TMR acts to increase energy expenditure in maturing adipocytes. Comparably, there is no report of other resveratrol metabolites concerning lipolytic gene expression in development stage of lipogenesis. Taken together, this data suggests that TMR modulates lipolytic transcription in maturing adipocytes which may be contributing factor of a trend toward a lessened lipid accumulation and size during development. This also shows TMR's ability to affect many major adipose functions in the progression of the cell growth.

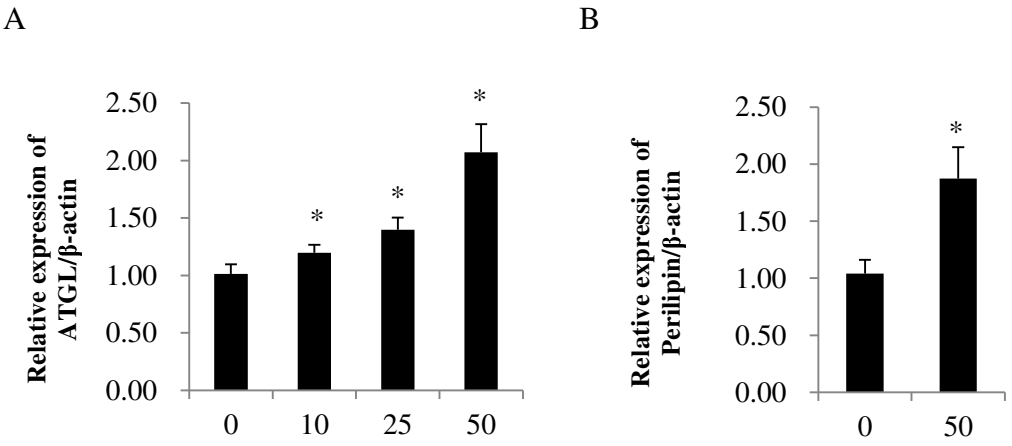


Figure 3.25 Dose-dependent effect of *trans*-3, 4', 5-trimethoxyresveratrol (TMR) on lipogenic gene expression. 3T3-L1 preadipocytes were subjected to adipocyte differentiation and an adipogenic cocktail. Cells were incubated in the presence of dimethyl sulfoxide (DMSO) or various concentrations of TMR (0, 10, 25 and 50 μM) in DMSO for 6 days. The cellular total RNA was extracted and subjected to RT-PCR. The mRNA levels of (A) ATGL and (B) Perilipin (0 and 50 μM) were quantified and normalized with the house keeping gene, β-actin. Data are presented as relative fold induction means ± SEM, n=3. *, $P<0.05$.

3.3.2.4 Mitochondrial biogenesis

TMR showed an ability to increase catabolic process transcriptional activity; therefore we wanted to explore its effect in mitochondrial biogenesis. We identified three genes which are highly involved in the biogenesis of the mitochondria including *mitochondrial transcription factor A* (*Tfam)* and *nuclear respiratory factors 1 (NRF1)* and *2 (NRF2)* (Figure 3.26 A-C). As energy demands of the cell can change through the process of differentiation, mitochondrial content is variable and can increase based on different physiological conditions, including adaptive thermogenesis (Goffart and Wiesner 2003). Although 95% of the genes required for mitochondrial biogenesis are encoded inside the nucleus, regulation of these genes are controlled by transcriptional mechanisms (Goffart and Wiesner 2003). *Tfam* activation is dependent on *NRF1* and *NRF2*, and is an important transcriptional activator stimulating specific mitochondrial transcription initiation as well as mitochondrial DNA maintenance (Escriva et al. 1999). Tfam protein controls mitochondrial DNA copy number and has been shown to be vital for embryonic development and biogenesis (Escriva et al. 1999). TMR showed a slight, significant increase in *Tfam*, supporting mitochondrial biogenesis, as well as demonstrated significant increases in both *NRF1* and *NRF2*. The NRFs have been reported to activate transcription of many genes involved in respiratory chain functions as well as regulating Tfam, translocating and activating the mitochondria and its replication and transcription (Wu et al. 1999a). NRF1 is important for mitochondrial haem synthesis and protein import into the mitochondria. It plays an important role in the coordination of nuclear and mitochondrial gene expression acting as a transcriptional activator for Tfam and in MRP endonuclease, which in required for mitochondrial DNA transcription (Goffart and Wiesner 2003). NRF1 also is critically involved in mitochondrial integrity and functioning (Goffart and Wiesner 2003). NRF2 binds to specific target sequences in the promoter region of a large number of nuclear encoded mitochondrial genes (Goffart and Wiesner 2003), and is responsible for the activity and expression of mitochondrial subunits (Virbasius and Scarpulla 1994). Our data suggest that TMR may be involved in altering and enhancing the coordinated expression of the genes that regulate

mitochondrial biogenesis pathway and may effective in increasing oxidative metabolism in maturing adipocytes *in vitro*.

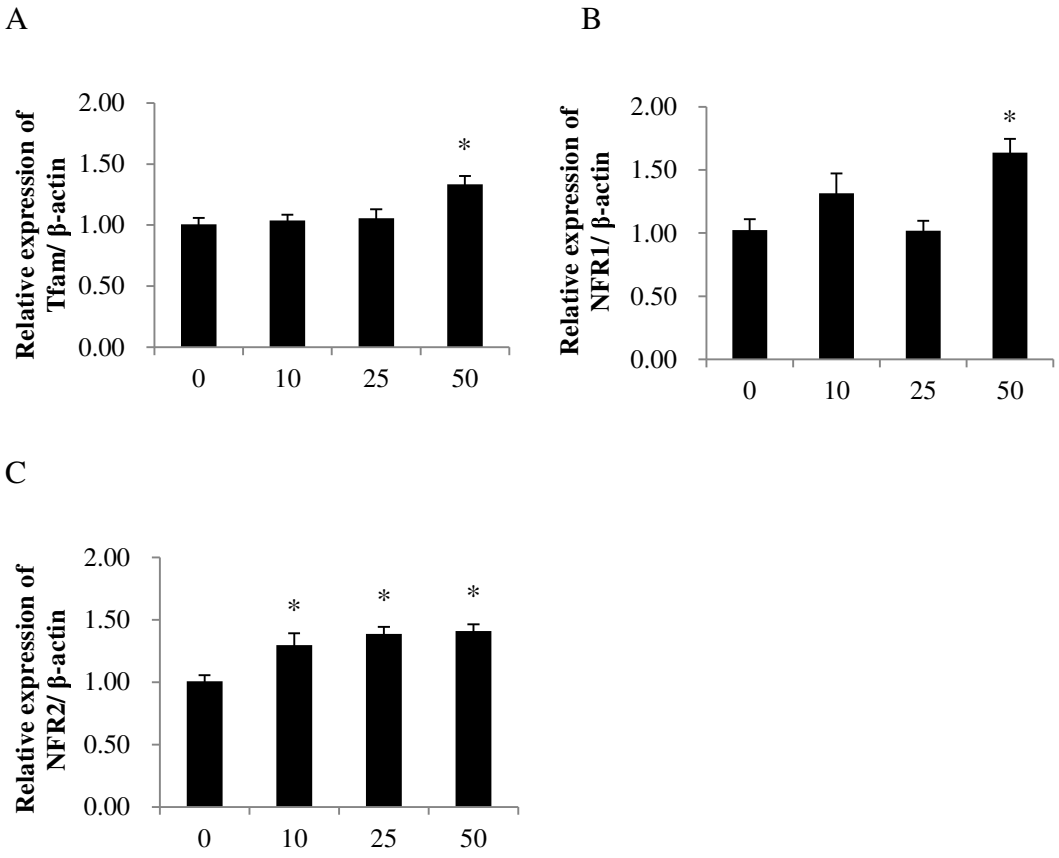


Figure 3.26 Dose-dependent effect of *trans*-3, 4', 5-trimethoxyresveratrol (TMR) on mitochondrial biogenesis marker gene expression. 3T3-L1 preadipocytes were subjected to adipocyte differentiation and an adipogenic cocktail. Cells were incubated in the presence of dimethyl sulfoxide (DMSO) or various concentrations of TMR (0, 10, 25, and 50 μM) in DMSO for 6 days. The cellular total RNA was extracted and subjected to RT-PCR. The mRNA levels of (A) Tfam, (B) NRF1, and (C) NRF2 were quantified and normalized with the house keeping gene, β-actin. Data are presented as relative fold induction means ± SEM, n=3. *, $P<0.05$.

3.3.2.5 Fatty acid oxidation

Genetic alteration in adipocyte metabolism has global implications in overall energy homeostasis; and increases in FAO in WAT has been suggested as a way to reduce adiposity (Rosen and Spiegelman 2006). Since TMR showed inductions in mitochondrial biogenesis gene expression, we proposed that it may also demonstrate upregulation of genes involved in FAO. Our findings show that TMR significantly increased *MCAD* expression, yet at its highest concentration, showed no change compared to the control in *CPT1* (Figure 3.27 A and B). Increases in *CPT1* and *MCAD* expression exert important effects in β-oxidation due to the nature that these two proteins act as the controlling, limiting steps in the process (Tiraby et al. 2003). Importantly, *uncoupling protein 1 (UCP1)* upregulation and its activity in uncoupling cells to undergo FAO without kinetic limitation by respiratory control (Tiraby et al. 2003). TMR was able to increase the expression of *MCAD*, a rate limiting enzyme involved in mitochondrial FAO (Mottillo et al. 2012), which is consistent with our observation of trending toward reductions in TAG content and TMRs proposed effect to allow the cell to maintain a higher capacity to burn energy. Yet, we did not see a similar induction with *CPT1*, another rate limiting enzyme highly involved in FAO by mitochondria. It is worth noting that an analogous phenomenon was also observed when RES was treated in mature adipocytes (Mercader et al. 2011). Overall a reduction in TAG content was observed with RES, yet of the two FAO genes examined, *RIP 140* and *CPT1-L*, there was a significant increase in only one gene expression profile, while the other showed no difference from control. Nonetheless, our data suggests TMR directly promotes transcriptional remodeling in maturing adipocytes and trends toward increasing oxidative metabolism. Regulation of gene expression of *PPARγ-cocactivator-1α (PGC-1α)* and *UCP1* have potential to increase the capacity of FAO (Tiraby et al. 2003). TMR may have activity in increasing the expression and activation of *PGC-1α* in adipocytes, which then could contribute to *UCP1* expression induction and increase FAO, resulting in reduced fat mass.

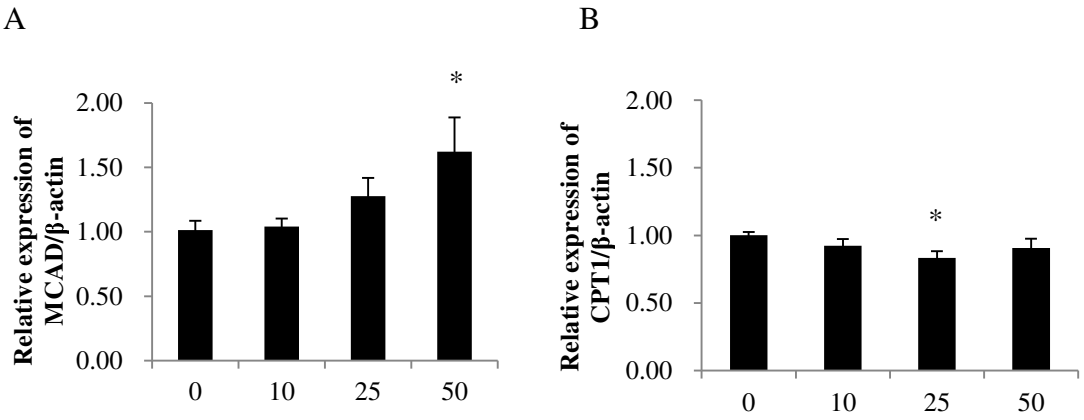


Figure 3.27 Dose-dependent effect of *trans*-3, 4', 5-trimethoxyresveratrol (TMR) on fatty acid oxidation (FAO) marker gene expression. 3T3-L1 preadipocytes were subjected to adipocyte differentiation and an adipogenic cocktail. Cells were incubated in the presence of dimethyl sulfoxide (DMSO) or various concentrations of TMR (0, 10, 25, and 50 μM) in DMSO for 6 days. The cellular total RNA was extracted and subjected to RT-PCR. The mRNA levels of (A) MCAD and (B) CPT1 were quantified and normalized with the house keeping gene, β-actin. Data are presented as relative fold induction means ± SEM, n=3. *, $P<0.05$.

3.3.2.6 Thermogenesis

The impact of TMR gives evidence that is complicatedly involved in altering the normal functions of the cell. To gain more insight as to what is happening transcriptionally, we analyzed thermogenic marker gene expression in these cells. TMR significantly increases both *UCP-1* and *PGC-1α* (Figure 3.28 A and B). UCP1 is highly abundant in the mitochondria of BAT and when activated has the ability to short circuit the electrochemical gradient driving ATP synthesis, and consequently stimulates respiratory chain action (Harms and Seale 2013). UCP1 biosynthesis is highly regulated at the transcription level, and increases cAMP, a primary trigger of UCP1 (Ricquier 2011). It has been known that WAT can contain cells which express increased levels of *UCP* and have the ability to take on multi-locular characteristics and pathways which promote increased cAMP levels (Wu et al. 2012). Our data shows that treatment of TMR increases *UCP1* expression, suggesting that it may play a role in increasing the thermogenic capacity of the cell. In coordination with this increase in *UCP1*, we also observed an increase in *PGC-1α*. *PGC-1α* is seen at high levels in BAT and can also be induced upon cold exposure or stimulated β-adrenergic pathways (Rosen and Spiegelman 2006). In WAT, PGC-1α causing a switch "on" to many key factors of brown fat cells, including mitochondrial biogenesis and UCP1 action (Rosen and Spiegelman 2006). PGC-1α is currently recognized as a "master regulator" of both oxidative metabolism and mitochondrial biogenesis in many cells types. It is a central transcription effector inducing adrenergic action and thermogenesis in adipocytes (Harms and Seale 2013). Presently, there has been indirect evidence that WAT adipocytes can acquire brown adipocyte characteristics during conditions of cold exposure or catecholamine excess (Rosen and MacDougald 2006). Taken together, with increases in both *UCP1* and *PGC-1α* transcription by TMR, this suggests that it may play an important role in increasing the cells capacity for energy expenditure. Our data showing the upregulation of mitochondrial biogenesis genes is consistent with our observation in the upregulation of *PGC-1α* by TMR. It has been reported that *PGC-1α* stimulates a large induction in *NRF1* and *NRF2* expression (Wu et al. 1999a). TMR may affect the thermogenic program causing induction of thermogenic genes, possible through the activation of β-adrenergic

signaling pathway which leads to upregulation of *PGC-1α* and other thermogenic expression in the maturing adipocytes. This higher capacity to utilize energy may be the cause our observation in lipid accumulation. It is of interest to note that in mature 3T3-L1 cells, RES has been shown to increase *PGC-1α* gene expression (Lasa et al. 2012a). Resveratrol metabolites, 3G and 4G also increased *PGC-1α* expression, however not in significant levels. Collectively, action of thermogenic gene expression may be a potential target to this class of phytochemicals.

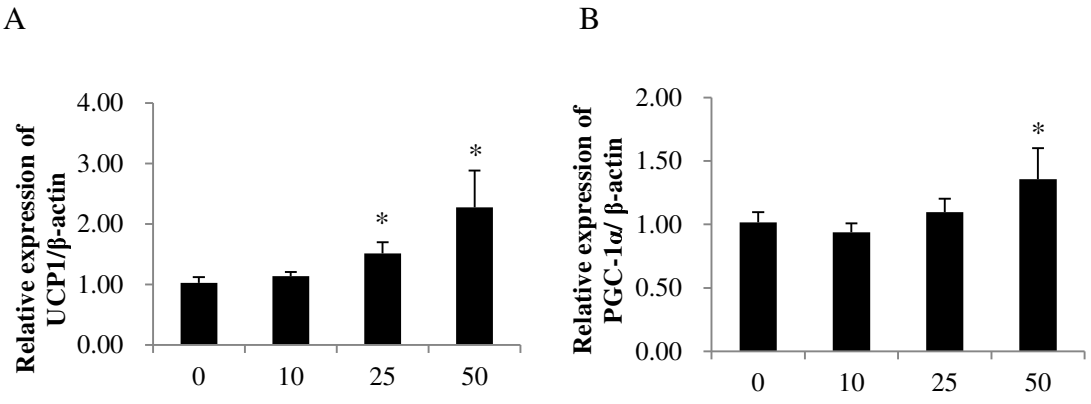


Figure 3.28 Dose-dependent effect of *trans*-3, 4', 5-trimethoxyresveratrol (TMR) on thermogenic marker gene expression. 3T3-L1 preadipocytes were subjected to adipocyte differentiation and an adipogenic cocktail. Cells were incubated in the presence of dimethyl sulfoxide (DMSO) or various concentrations of TMR (0, 10, 25, and 50 μM) in DMSO for 6 days. The cellular total RNA was extracted and subjected to RT-PCR. The mRNA levels of (A) UCP1 and (B) PGC-1α were quantified and normalized with the house keeping gene, β-actin. Data are presented as relative fold induction means ± SEM, n=3. *, $P<0.05$.

3.3.2.7 Beige fat markers

The development of beige fat, is described as WAT having multi-locular LD morphology, high mitochondrial content, and increased expression of specific brown fat genes, including *UCP1* and *PGC-1α* (Harms and Seale 2013). Beige fat cells also have increased ability for thermogenesis. Gene expression of beige-selective gens can help to differentiate beige fat cells, from those of BAT and WAT (Wu et al. 2012). Among these beige fat markers include transcription factors T-box 1 (Tbx1), transmembrane protein 26 (Tmem26), and PRD1-BF-RIZ1 homologues domain-containing protein-16 (Prdm16) (Rosen and Spiegelman 2014). In order to observe any effect by TMR on the induction of these specific beige fat markers, we examined their gene expression. Overall we not that TMR has a variable effect (Figure 3.29 A-C). There is a significant induction of *Tbx1* expression by TMR, yet the opposite effect on *Tmem26*, showing a significant reduction. TMR shows no significant difference in the expression *Prdm16*, however it trends toward increasing. T-box genes are essential for the developmental process and has been identified as an enriched beige fat marker (Chapman et al. 1996; Harms and Seale 2013). With increases in *Tbx1*, this suggests that TMR may have the ability to induce alterations in the cell to increase genes with a phenotype more closely related to beige cells. *Tmem26* is another enriched beige fat marker which is commonly expressed higher in inguinal fat compared to brown fat (Wu et al. 2012). *Tbx1* and *Tmem26* are both beige markers which have also been suggested to be present in classical brown tissues. Interestingly, TMR showed a reduction in *Tmem26*, contrasting from a large increase in *Tbx1*. Currently, the determinant of white and brown adipocyte gene programs are not completely understood, although analysis of beige fat cells have shown a distinction between the two (Villanueva et al. 2013; Wu et al. 2012). Therefore, we tested a third gene that is a key transcription factor for both brown and beige fat. *Prdm16* ectopic expression is known to convert white fat precursors into thermogenic adipocytes which contain UCP1(Harms and Seale 2013). It has been identified as a co-regulator in differentiation of brown and beige cells, with increased expression leading to beige adipocyte development and suppression of metabolic disease (Harms et al. 2014). To note, based on the metabolic rate of BAT in mice, is was calculated that 40-50 grams of BAT could account for 20% of daily energy

expenditure (Rosen and Spiegelman 2014). Although our data shows no significant alteration in *Prdm16* gene expression, it trends toward increasing with TMR; coupled with increased expression of *UCP1* may suggest an altered adipocyte metabolism with distinctive changes in physiological functions. Taken together, treatment of TMR may induce transcriptional cell remodeling in the metabolic efficiency of energy expenditure through a higher capacity to burn fat (Figure 3.30). Although more analysis is needed, there may be therapeutic potential with TMR due to its involvement of induction of thermogenic genes.

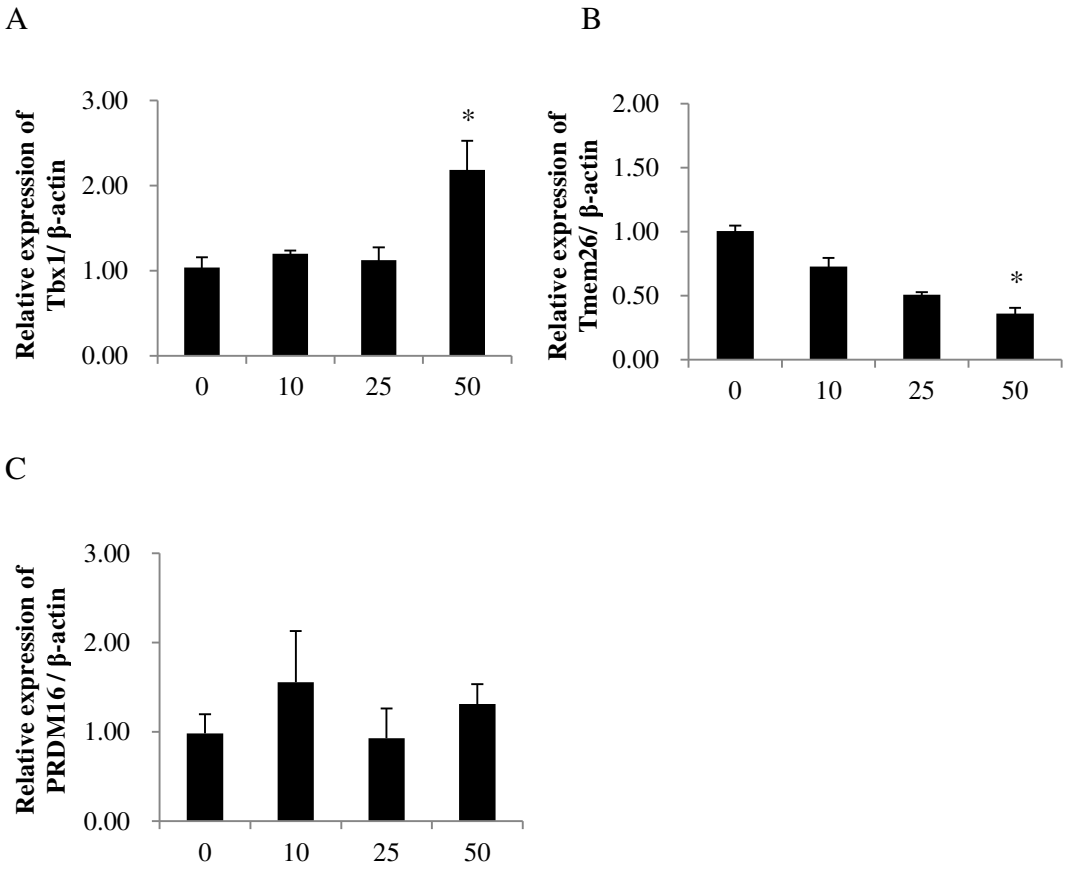


Figure 3.29 Dose-dependent effect of *trans*-3, 4', 5-trimethoxyresveratrol (TMR) on beige fat marker gene expression. 3T3-L1 preadipocytes were subjected to adipocyte differentiation and an adipogenic cocktail. Cells were incubated in the presence of dimethyl sulfoxide (DMSO) or various concentrations of TMR (0, 10, 25, and 50 μM) in DMSO for 6 days. The cellular total RNA was extracted and subjected to RT-PCR. The mRNA levels of (A) Tbx1, (B) Tmem26 and (C) Prdm16 were quantified and normalized with the house keeping gene, β-actin. Data are presented as relative fold induction means ± SEM, n=3. *, $P<0.05$.

3.4 Summary and conclusions

The major objectives of this study were to identify the effect of different resveratrol metabolites in adipose function *in vitro*. Due to the fact that RES is quickly metabolized in the body, biological activities of its metabolites may be more physiologically relevant to study (Lasa et al. 2012a). We singled out TMR as a resveratrol metabolite and analogue having specific action in lipid metabolism, specifically for its action in adipocyte lipolysis and lipogenesis. Our results demonstrate the first identification of TMR acting as an antilipolytic agent in mature adipocytes as well as having action in the development of maturing adipocytes. In our study, we were able to establish TMRs efficacy with significant, partial inhibition of glycerol release in mature adipocytes, with action greater than other resveratrol metabolites examined. TMR was shown to have non-cytotoxic effects over a range of concentrations extending to safe usage at 50 μM displayed by cell viability assays in both pre- and mature adipocytes. Also taken into consideration was the stability of TMR in the medium of treated cells. Additionally, we determined TMRs rapid uptake into the cell and co-localization with the LD, attributing this to TMRs structure and methoxylation, providing a degree of protection and enhancing transportation to the cell (Dias et al. 2013). Further studies are needed to fully understand the scope of the location of TMR in the cell, but our imaging data provides preliminary evidence.

TMR was able to significantly reduce glycerol release in mature adipocytes in both an acute and chronic exposure. Previous research in lab demonstrated a resveratrol metabolite, PIC, was able to significantly degrade a primary lipolytic enzyme, ATGL, and its co-activator CGI-58 mechanistically through autophagy (intracellular degradation of components via lysosome) (Kwon, unpublished). Due to the structural similarity of TMR and PIC, we hypothesized that TMR action in lipolysis is also similar. TMR demonstrated reduction in protein expression of both ATGL and CGI-58 with acute treatment, and to a less extent with a chronic treatment. TMR also demonstrated action in slightly blocking the activation of HSL through a slight reduction in phosphorylation. We discovered TMRs action in the lipolysis process was greatest seen with an acute treatment time with greatest potency with a three hour treatment in basal and stimulated

conditions. TMR alters lipolytic gene expression of mature adipocytes which may also be a contributor to the reduction in glycerol release and lipolysis. Overall, our data suggests that treatment of TMR has the ability to acutely, partially inhibit lipolysis in mature adipocytes via possible transcriptional regulation, potential mediation of protein degradation or a combination of both. Importantly, treatment of TMR may be a therapeutic approach to reduce the level of circulating NEFA in an obesogenic condition and help to attenuate the induction of obesity induced insulin resistance and type 2 diabetes. Additionally, the identification of a dietary antilipolytic compound may also help to attenuate the effects of cancer cachexia.

In an effort to further examine the role of TMR in adipose function, we also studied its effect in the development of maturing adipocytes and lipogenesis. Our study suggests a role of TMR in lipogenesis and cellular events in early adipocyte development. We observed a slight trend toward reduction in LD formation. This data suggests that TMR influences the lipogenic pathway and may primarily working through transcriptional regulation during differentiation and development of maturing adipocytes. Interestingly, TMR increased lipogenic gene expression, although no differences were seen compared to the control in lipid accumulation. Further analysis of cellular gene expression showed transcriptional increases in catabolic processes and mitochondrial biogenesis. TMR significantly upregulates the expression of thermogenic genes, *UCP1* and *PCG-1α*, suggesting an increased capacity for energy expenditure and thermogenesis. Taken together, the TMR may play a significant role in transcriptional regulation during adipocyte development and may be responsible for redirecting cellular processes toward activating energy expenditure in maturing adipocytes. The trend towards reduced lipid accumulation and increased lipogenic gene expression can possibly be connected through TMRs ability to maintain balance within cellular pathways between energy storage and utilization.

This work in identifying the effect of TMR in maturing preadipocytes may be therapeutically important because this type of cell plays a role in obesity development. Accordingly, during child development and adolescence, obesity mainly stems from induction of hyperplasia, which is suggestive to the differentiation of preadipocytes into

mature adipocytes (Eseberri et al. 2013). Furthermore, understanding mechanisms to prevent or reduce obesity development may be a strategy to combat this complicated disease. Conversely, in adulthood, obesity is primarily of as result of hypertrophy of mature adipocytes. Therefore deeper investigations into the effects on mature adipocyte by phytochemicals are needed for the prevention of obesity and associated metabolic diseases. A schematic diagram of our proposed mechanism is shown in Figure 3.31 and 3.32.

Our study is of significance because it illuminates the role and potential that stilbenes can have in overall energy homeostasis. This study suggests the possible role of TMR remodeling in adipose tissue to more metabolically active cells. It also provides evidence utilizing dietary compounds in modulating adipose lipolysis. Our data represents novel suggestions and potential for phytochemicals and their possible applications in adipocyte biology. In turn, finding ways to promote the health and welling being, especially in an obesogenic diseased state, may lessen the burden of this preventable epidemic.

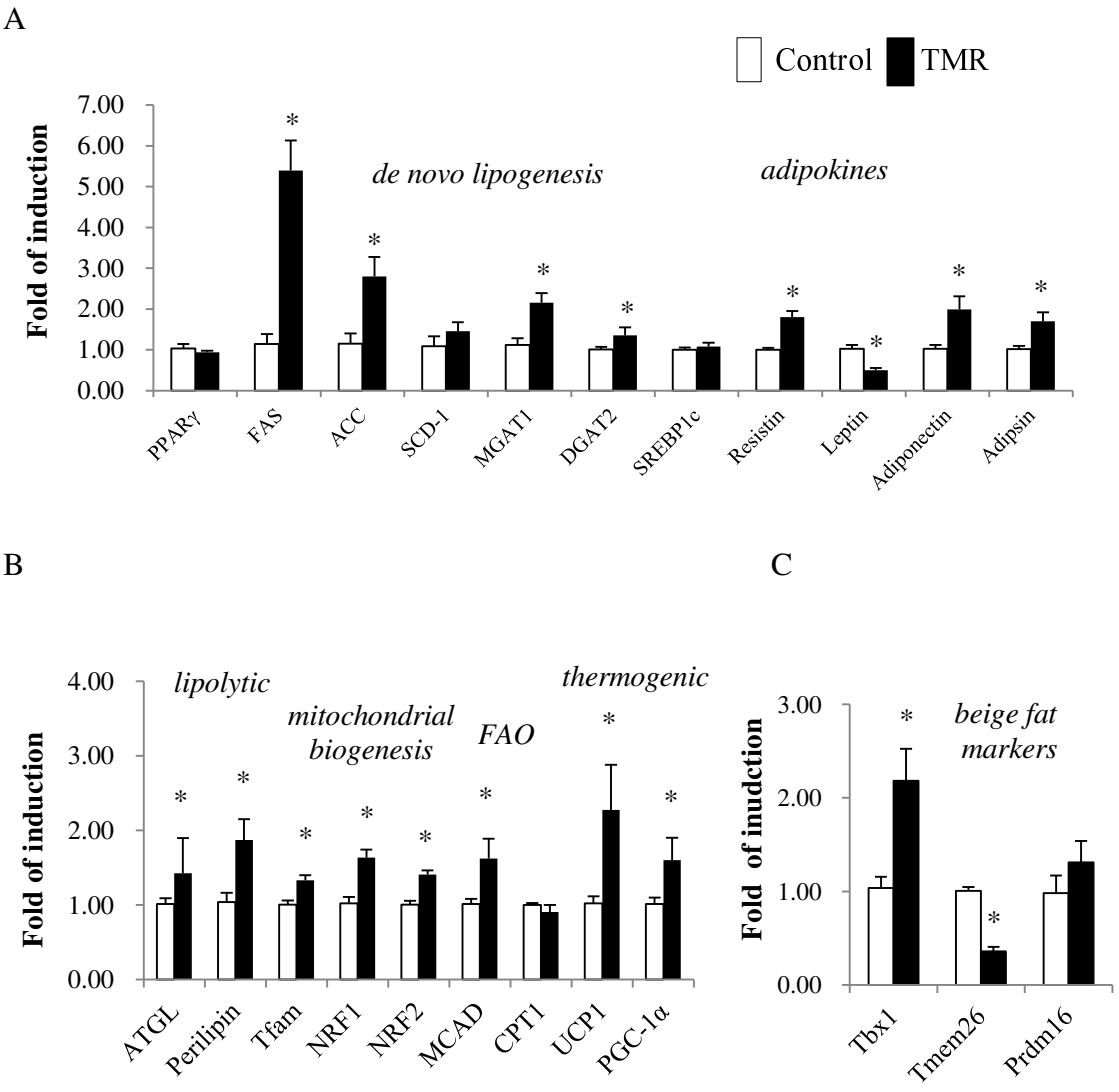


Figure 3.30 Summary of *trans*-3, 4', 5-trimethoxyresveratrol (TMR) on functional adipocyte gene expression. 3T3-L1 preadipocytes were subjected to adipocyte differentiation and an adipogenic cocktail. Cells were incubated in the presence of dimethyl sulfoxide (DMSO) or TMR (0 and 50 μM) in DMSO for 6 days. The cellular total RNA was extracted and subjected to RT-PCR. The mRNA levels of (A) de novo lipogenesis and adipokine marker genes, (B) lipogenic, mitochondrial biogenesis, fatty acid oxidation, and thermogenic genes, and (C) beige fat marker genes were quantified and normalized with the house keeping gene, β-actin. Data are presented as relative fold induction means ± SEM, n=3. *, $P<0.05$.

Mature adipocyte

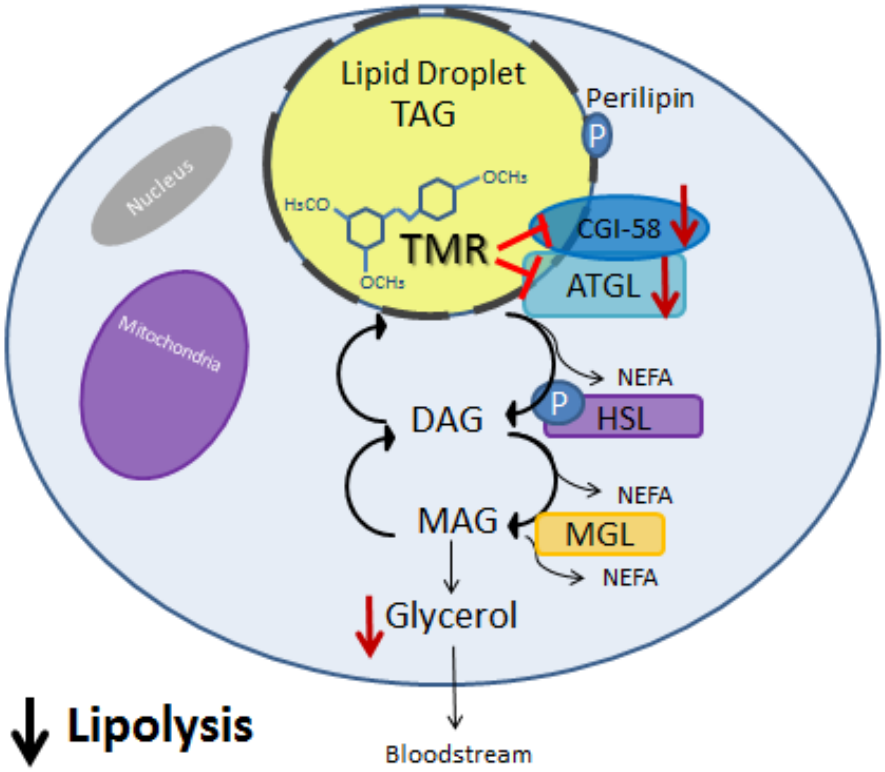


Figure 3.31 TMR action in mature adipocyte *in vitro*. Acute treatment of TMR in mature adipocyte results in reduction of protein expression and overall reduction in glycerol release. TMR action in the cell suggests modulation of lipolytic enzymes and the lipolysis process.

Maturing adipocyte

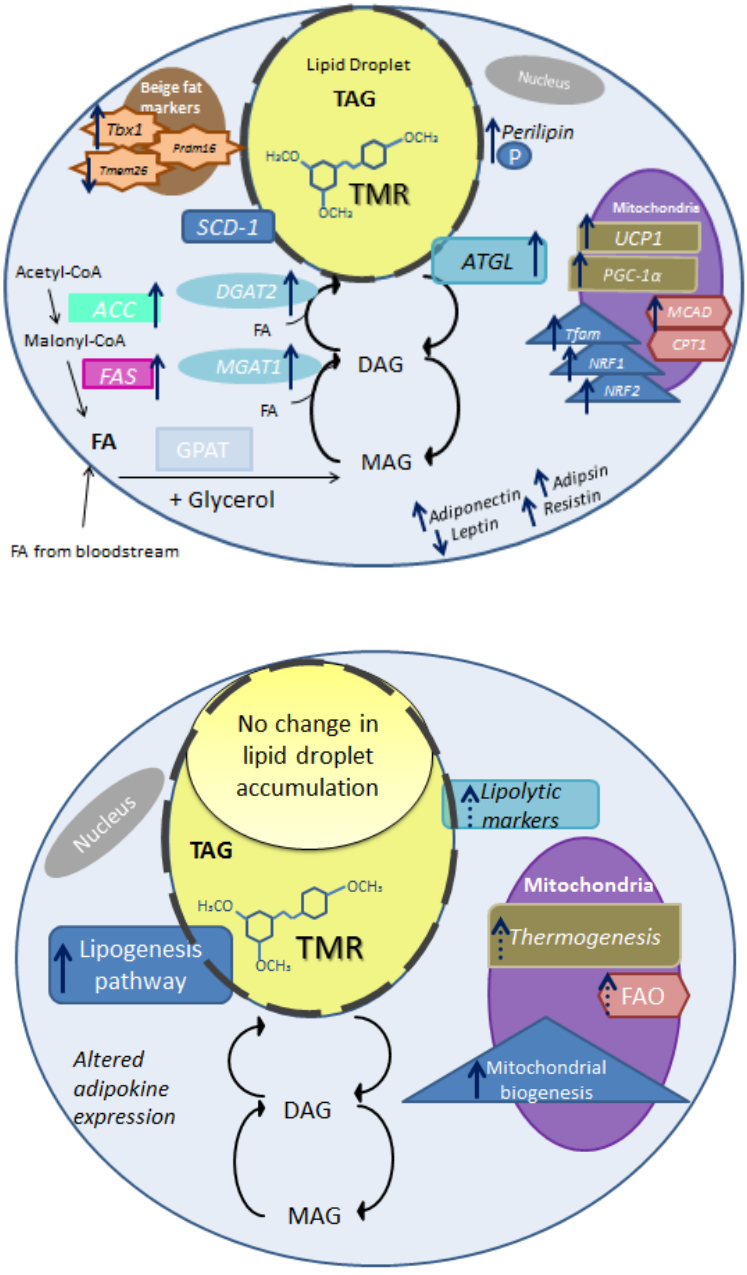


Figure 3.32 TMR action in development of maturing adipocyte *in vitro*. Changes induced by treatment of TMR in maturing adipocyte during lipogenesis. mRNA expression profiles are altered by TMR during development, suggestive of transcriptional remodeling and potential balance between energy utilization and storage by cell.

CHAPTER 4. FUTURE WORK

4.1 Limitations of study

Chapter 3 outlines our results and demonstrates the significant, partial inhibition of lipolysis by an acute treatment of TMR. It also shows the effect of TMR in the development of maturing adipocytes and the transcriptional remodeling of the adipocyte toward increased energy expenditure. However, there are some limitations to the study that are important to consider:

Firstly, the data collected from our experiments were entirely based on *in vitro* results. The 3T3-L1 preadipocyte cell line is widely established, however, due to the fact that the results in this study are trying to mimic actual physiological conditions of human obesity, the role of prevention, development, and associated metabolic disorders; understanding the effect of dietary compounds *in vivo* is critical. Additionally, our experiments were conducted in a tightly controlled atmosphere with one type of cell. To be able to have more substantial evidence, a more complex and intricate model such as an animal *in vivo* system, is needed to translate the effects to humans.

Our study also had technical limitations. Our data in regards to the inhibition of lipolysis was done primarily through the level of glycerol release. To further confirm our findings, a free fatty acid assay and measurement of TAG, DAG, and MAGs levels should also be completed which would account for specific steps in lipolysis that were effected. Our study utilized protein expression and analysis to determine the relative level of activity in the cell, whereas biochemical enzyme assays to measure the actual activity of the enzymes would allow greater specificity in determining the modulation in the cell. Furthermore, these types of assays done in primary murine and human adipocytes would also strengthen our data, as well as demonstrate reproducibility and reliability.

The lipogenesis piece of our study is restricted to only mRNA gene expression analysis and technically limited. Additional confirmatory data with replication and reproducibility of the effects we observed will help to strengthen our gene expression analysis results. Furthermore, biochemical measurement of the correlating protein levels and enzymatic activity of the genes we observed changes in is critical to the proposed effect induced transcriptionally by TMR. Other technical assays including mitochondrial biogenesis and ATP production techniques would also help connect the data observed in the gene profiling and aid to substantiate TMRs effect.

Mechanistic pathways are a large part in identifying the beneficial effect of phytochemicals. In regards to TMRs effect in lipogenesis, this is a limitation of our study. Our data lacks knowledge on specific pathways and how those pathways may be directly altered by TMR. One technique in identifying changes in lipogenesis is the utilization of radioisotope labeling to track changes in the biosynthesis of TAGs. In addition, SRS imaging with labeled glucose is another method that allows the visualization of the source and construction of the LD.

Finally, the largest drawback of the study is the fact that the data is presented on findings based *in vitro* and should be tested in an *in vivo* system. TMR showed efficacy at 50 μM, however this is higher than a physiological condition. Current literature is limited, however one studied noted using 4 mg/kg with an intraperitoneal (IP) injection and 10 mg/kg via oral gavage, however this concentration was fully dissolved in a HP-β-CyD (Lin and Ho 2009; Lin et al. 2010). Previous research in our lab for PIC utilized 10 mg/kg/day in an animal model. For animal treatment with TMR, a concentration range up to approximately 10-15 mg/kg would be sufficient maximum dose. The bioavailability of TMR needs to be considered when choosing a method of treatment. According to the study by Lin, they analyzed the pharmacokinetic profile of TMR when given via oral gavage and found that it has a short absorption time and prolonged elimination time, with a calculated bioavailability F value of 54.9% (Lin and Ho 2009). Taking this into consideration, giving the animals an IP injection would allow us to see the direct effect of TMR and it would prevent any modifications to the compound. Oral gavage of TMR dissolved in a vehicle would give a better understanding of the physiological relevance

once ingested. To note, it has been suggested that food may enhance the bioavailability of TMR, by stimulated bile secretions (Lin and Ho 2009). In an experimental model that is identifying the health benefit of a compound in regards to prevention of obesity, it would be most applicable to administer TMR in the diet. Because the nature of this study would be a high fat diet-induced obesity model, the increased fat content may help to increase the bioavailability of the compound via normal bile secretions. However, stability of TMR in the food matrix would need to be examined to ensure its functionality is still intact. Depending on the adipose function being analyzed, different animal models should be employed. Specific parameters are further discussed in detail below.

The following outlines experiments and future work that should be done to better understand the role of TMR in affecting overall energy homeostasis.

4.2 Future Work: TMR in lipolysis

Additional research is needed to fully understand mechanistically how TMR acts in the cell specific to the lipolytic pathway. Future work needs to be completed both *in vitro* and *in vivo* studies to characterize TMR in overall modulation of lipolysis.

Further characterizations of the lipolytic enzymes involved in the lipolysis process are needed to gain insight into the overall action of TMR. We mainly examined ATGL, the first step in lipolysis, however HSL and MGL are still players in the overall progression of lipolysis and their roles maintain specificity. HSL can hydrolyze both TAG and DAG, yet it has a higher affinity for DAG (Duncan et al. 2007). We measured the phosphorylation of HSL at serine-660; responsible for the LD interfacial activation and hydrolysis of DAG (Walther and Farese 2012). However measuring the total level of HSL would also be beneficial in gaining insight into TMR's breadth of regulation and potential degradation or induction. Similarly, identifying the total protein expression level of MGL and perilipin would complete the picture of TMRs modulating role in the entire, sequential process of lipolysis. Additionally, other technical measurements need to be explored include biochemical enzyme assays order to confirm the action of TMR. Biochemical analysis of enzymatic activity of enzymes involved in lipolysis would give a clear evidence to the level TMR modulates this process.

Our results lack the knowledge and specificity of how possible enzymatic degradation occurs: through autophagy or proteasome proteolysis. Our lab has previously demonstrated that degradation of ATGL by PIC is not proteasome-dependent, but dependent on autophagy and occurs through an autophagy pathway, via the use of different inhibitors. Macroautophagy is a lysosomal degradative pathway present in cells and is responsible for degrading cytosolic organelles and protein aggregates which are too large for proteasome degradation. Autophagy is a highly regulated process in which whole organelles and regions of cytoplasm are sequestered inside an enveloping double-membrane structure termed, autophagosome, which translocates to lysosomes for fusion and content degradation (Lee et al. 2010; Singh et al. 2009). Proteolysis involves proteases which hydrolyze peptide bonds and then free amino acids are released (Glickman and Ciechanover 2002). This occurs under different physiological circumstances and allows the cell to accommodate to changes and adapt to its environment (Ciechanover 1994; Glickman and Ciechanover 2002). TMRs apparent reduction of ATGL and CGI-58 is done through an unknown mechanism, and identifying how TMR works would further our knowledge of its modulation, but also increase understanding in the markers and regulation of the machinery involved. We also observed transcriptional changes by TMR with an acute treatment. This suggests rapid turnover in the cell, however this would need to be confirmed through additional biochemical analysis experiments.

The most critical future experiment is to utilize our results and correlate them into an *in vivo* system. It would be advantageous to conduct primary cell culture before entering into an *in vivo* study. This would be a critical step to get a more accurate picture of how cells would respond. Testing the efficacy of TMR in both murine and human primary cells would be supportive data before taking on an animal model. An animal model testing TMRs effect in lipolysis would consist of approximately 16 week old male C57B/6J mice that are obese and continued to be fed a high fat diet (60% of calories from fat) (HFD) for 1 week prior to treatment. Due to the fact we saw that TMR is most effective with an acute treatment, with an *in vivo* model, this correlates to about 2-4 weeks of treatment. Two groups (n=8) should be fed as followed: HFD and HFD + 10

mg/kg/day TMR. In this treatment mice should have access to water and food *ad libitum*. Body weight and food intake should be recorded about every 3rd day, as well as blood samples to measure basal glycerol release, FFA, TAG, and glucose levels. Physiological and biochemical parameters after sacrifice should be done upon collection of all tissues, including but not limited to, histology, gene profiling, and immunoblot analysis.

4.3 Future Work: TMR in lipogenesis

We were able to observe that TMR increased catabolic and thermogenic gene expression, however, our study was limited to only gene profiling *in vitro*. Further biochemical and proteomic analysis is needed and additional focus on signaling pathways involved in observed upregulation.

It would be beneficial to collect confirmatory data as well as test other genes involved in lipogenesis, catabolic processes, and include gene expression that is specific to beige fat markers. As previously described, beige cells in murine WAT are defined by their morphology, high mitochondrial content, and expression a core set of brown fat genes (Harms and Seale 2013). Our data lacks biochemical analysis of the effects obtained with gene expression. Therefore, analyzing in depth a wider range of genes and correlating proteomic analysis and utilizing enzymatic activity assays would elucidate TMRs overall effect during the development of the cell, and would also indicate any metabolic switch. Additionally, techniques involving mitochondrial biogenesis, respiration, and ATP production need to be investigated to confirm our gene expression analysis.

Further investigation in the location and effect of TMR during the development stage of maturing adipocytes is needed. Deuterium-labeled glucose added into the medium during the early stages of differentiation and photographed with SRS imaging would allow us to visualize the progression of lipid droplet formation and the source of energy utilized. This assay would give us a better understanding of how TMR is influencing cell during growth and de novo lipogenesis. Additionally, radioisotope labeling of FA is another technique that would allow detection of direct pathway changes. Increased characterization of TMR is needed by these types of methods to better

understand the pathways it's affecting and possibly modulating, which is critical to knowledge of its therapeutic potential.

Analyzing the effect of TMR *in vivo* is imperative. Investigating the remodeling role of TMR *in vivo* would be more applicable for interpretation of physiological conditions and human comparison. Examining the role of TMR treatment during the development of young mice in a HFD-obesity-induced model, would allow us to critically analyze its effect. 3 week old male C57B/6J mice with three groups (n=8) fed as HFD, HFD + low dose (3 mg/kg/day TMR), and HFD + high dose (15 mg/kg/day TMR). During the experimental period, mice should have access to water and food *ad libitum.* Body weight and food intake will be measured every 3 days with all groups followed for approximately 4-6 weeks, around when potential weight diverges are seen for at least 1 week. If significant change in body weight does not occur, dosages may need to be adjusted. Fecal samples will be collected for 1 week and respiratory exchange ratio will be analyzed. To determine a potential energy expenditure changes and increased health of adipose tissue of TMR, specific biochemical parameters associated with obesity development should be measured including serum indicators, gene profiles, and protein expression levels in different tissues to show the critical impact on adipogenesis and lipogenesis. Other tests should be completed not exclusive to glucose tolerance test, serum levels of triglyceride, glucose, insulin, glycerol, and FFA. Additionally, adipose tissue, liver, kidney, intestines and other major tissues should be collected, weighed with histology, PCR, and immunoblot analysis measured along with other biochemical analysis, and measurements specific to mitochondrial biogenesis and thermogenesis. Mitochondrial uncoupling has been associated as means of weight-loss therapy and increasing the activity of either brown fat, beige fat, or both has large potential for the treatment of obesity and metabolic disease by dietary compounds (Harms and Seale 2013). This model would be representative of a therapeutic treatment during adolescent development and growth, in which we could identify any alteration in overall energy balance and changes in homeostasis.

4.4 Conclusion

Collectively, our study elucidates the beneficial activities of dietary compounds, such as TMR, in the context of adipose function. Our data represents foundational work that can be expanded through future studies, including further investigations not mentioned and those mentioned above. In the future, additional *in vivo* models will help to gain new insights into the potential, therapeutic application of TMR and other resveratrol metabolites in adipose biology. The over-arching big picture is that this type of research can help people who suffer from a variety of metabolic disorders. Identifying ways to improve overall health and well-being can improve quality of life and increase longevity. The use of phytochemicals as a means to treat conditions has been around for centuries, as stated by Hippocrates, "Let food by thy medicine and medicine by thy food." This idea of food components as a means to improve health is fundamental and needs further investigation in discovering the specific pathways and their impact on energy balance. A focus on lipid metabolism is extremely relevant to the current health status America and application of functional food components may be key. Overall, there is vast potential for investigation of dietary components to help reduce the incidence of obesity and attenuate its associated metabolic disorders.

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