



Effect of the Flavonoid Quercetin on Adipocytes

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**EFFECT OF THE FLAVONOID QUERCETIN ON
ADIPOCYTES**

A Thesis Presented

By:

JENNIFER C SWICK

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DEDICATION

To those not eating fruits and vegetables; your mother was right.

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ABSTRACT

EFFECT OF THE FLAVONOID QUERCETIN ON ADIPOCYTES

SEPTEMBER 2011

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Obesity is an urgent global public health concern as prevalence rates continue to increase, especially among children. Obesity is defined at the cellular level as an increase in adipocyte number (hyperplasia) and size (hypertrophy). Both lead to the dysfunction of adipose tissue, which has been identified as the link between obesity and chronic disease. Bioactive compounds, naturally occurring in fruits and vegetables, hold enormous potential in regulating adipocyte biology. Quercetin, the most commonly consumed dietary flavonoid, is a strong potential anti-obesity agent that has been implicated as an AMP-activated protein kinase (AMPK) activator and shown to ameliorate symptoms of metabolic syndrome *in vivo*. Here we investigated quercetin's effect on (1) adipogenesis, the process of increasing adipocyte number, and (2) metabolism of mature adipocytes. In 3T3-L1 preadipocytes, quercetin dose-dependently inhibited adipogenesis, as evidenced by decreased lipid accumulation and expression of adipogenic markers such as peroxisome proliferator-activated receptor (PPAR) γ , CCAAT/ enhancer binding protein (C/EBP) α , adipocyte fatty acid binding protein 2 (aP2), and acetyl-CoA carboxylase (ACC) on mRNA and protein levels. This inhibitory effect was limited to the early stages of adipogenesis (0-36 hours), and

quercetin treatment altered the normal expression pattern of cell cycle related genes Cyclin A and p27, indicating quercetin may inhibit adipogenesis through cell cycle events. We next investigated quercetin's ability to activate AMPK and the metabolic pathways related to AMPK activation: lipolysis and β -oxidation. Quercetin increased phosphorylation of AMPK and its downstream target ACC. Further, quercetin treatment (100 μ M) increased free fatty acid content in the media through an AMPK-dependent mechanism. Quercetin up-regulated mRNA expression of uncoupling proteins 3 (UCP3) and peroxisome proliferator-activated receptor-gamma co-activator 1 alpha (PGC-1 α), indicating that quercetin may induce mitochondrial oxidative pathways, also through an AMPK-dependent pathway. These findings suggest (1) quercetin inhibits adipogenesis through the regulation of early cell cycle events required for adipogenic differentiation, and (2) quercetin's activation of AMPK induces lipolytic and oxidative pathways. Taken together, quercetin could be further developed as an anti-obesity agent because of its potential to inhibit both hyperplasia and hypertrophy *in vitro*.

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CHAPTER 1

BACKGROUND

1.1 Overview

The increasing prevalence of obesity is a pressing concern for health officials worldwide because of its link to the development of type 2 diabetes, cardiovascular disease, and cancer. In the United States alone, with nearly 2/3 of adults and 17% of children overweight and obese, it is estimated that \$147 billion is spent annually on obesity-related health care [1]. In the obese state, adipose tissue expands to accommodate excess energy intake through an increase in the number (hyperplasia) and size (hypertrophy) of adipocytes. Hyperplasia leads to the irreversible expansion of adipose tissue in unwanted depots, specifically the visceral cavity [2]. Hypertrophy leads to the dysfunction of adipocytes, as they become insulin resistant and recruit macrophages to the tissue for their own destruction [3]. Together, these events lead to the development of metabolic syndrome. Therefore targeting adipocyte biology, both hyperplasia and hypertrophy, and identifying potential factors that regulate these processes are of great importance in the prevention and treatment of obesity.

In recent years, researchers have turned to the potential of dietary bioactive compounds to prevent this adipocyte dysfunction and therefore help combat the rising obesity epidemic and reduce health care costs [4, 5]. Quercetin, the most commonly consumed dietary flavonoid, has been shown to ameliorate symptoms of metabolic syndrome in *in vivo* animal studies [6-10], however the exact effect of quercetin on adipose tissue was not explored.

Research has shown that quercetin inhibits adipogenesis in both human and mice cell models [11-13], and induces lipolysis [14, 15] in mature mice adipocytes; however the underlying mechanisms are unknown. Interestingly, quercetin has recently been implicated as an AMP-activated protein kinase (AMPK) activator [13]. AMPK is the major metabolic regulator of the cell, and a major target for obesity-related conditions because of its ability to induce fatty acid oxidation [16]. However, quercetin's ability to alter the metabolic state of mature adipocytes has not been explored. Therefore, our focus was to investigate (1) mechanisms of quercetin's anti-adipogenic effect, and (2) metabolic effects of quercetin in mature adipocytes through AMPK activation. These findings will largely improve our understanding of quercetin's ability to ameliorate hyperplasia and hypertrophy, and thus inform its development as an anti-obesity agent.

1.2 The Extent of the Problem of Obesity

Obesity has become one of the most urgent concerns of health care officials over the past two decades, as dramatic increases in prevalence rates have been seen not only in the United States but worldwide. In the U.S. alone, with nearly 2/3 of adults and 17% of children overweight or obese, it is estimated that \$147 billion is spent annually on obesity-related health care [1]. The World Health Organization outlines the severity of the problem with the following: (1) worldwide obesity has more than doubled since 1980, (2) overweight and obesity are the 5th leading cause of death globally, (3) in 2008 more than 10% of the population was obese, (3) in 2010 nearly 43 million children

under the age of five were overweight, and (4) obesity accounts for the incidence of 44% of diabetes, 23% of ischemic attacks, and up to 41% of certain cancers [17]. This places an enormous burden on health care systems and impacts health care costs worldwide.

Obesity has been linked to all of the following: coronary heart disease, type 2 diabetes, cancer (specifically breast and colon), hypertension, dyslipidemia, stroke, liver and gallbladder disease, sleep apnea, osteoarthritis, and gynecological complications [18]. In an effort to classify the most common conditions related to obesity, the term “metabolic syndrome” has been used to include the development of hypertension, dyslipidemia, hyperglycemia, and insulin resistance. This term was coined because of the surprising combination of conditions seen repeatedly in overweight and obese patients. Currently 8.3% of the US population is affected by diabetes [19], 12% by cardiovascular disease [20], and 34% of adults are classified as having metabolic syndrome [21]. If no action is taken, the prevalence of metabolic syndrome will likely only increase in the population, as childhood obesity has tripled over the last 25 years, and continues to grow [22].

At the cellular level, adipose tissue expands its capacity to store lipid by increasing adipocyte size (hypertrophy) and number (hyperplasia). Hyperplasia leads to the expansion of adipose tissue in unwanted depots, specifically the visceral cavity, through the process of adipogenesis. Adipogenesis occurs in two stages: (1) the commitment of precursor mesenchymal stem cells to preadipocytes, and (2) the differentiation of preadipocytes to mature adipocytes [3]. Because adipocytes are

particularly resistant to apoptosis, it has been suggested that hyperplasia may be associated with less reversibility of metabolic consequences from obesity [23, 24].

Hypertrophy of adipocytes directly leads to their dysfunction. The accumulation of excess lipid leads to the malfunction of the cytosolic organelles resulting in insulin resistance. Eventually macrophages are recruited to the tissue, which creates systemic low-grade inflammation. This adipose tissue dysfunction has been identified as the major link between obesity and metabolic syndrome [3]. Therefore there are two ways to target the development of metabolic syndrome on a cellular level; (1) targeting adipogenesis, and (2) targeting adipocyte metabolism to prevent excess lipid accumulation and dysfunction. Developing prevention and treatment methods that make use of both strategies is vital to obesity-related research and would impact the financial burden facing health care systems.

1.3 The Role of Adipose Tissue in the Development of Obesity

1.3.1. Adipogenesis

The regulation of adipogenesis plays a critical part in the development of obesity. During childhood and adolescence, adipocyte number increases based on genetic and environmental factors. However, once adulthood is reached, adipocyte number remains relatively constant [2], even though the tissue is still capable of recruiting new adipocytes. Studies have shown hypertrophy precedes hyperplasia in adults during excess energy intake and hyperplasia is only stimulated under sustained energy imbalance, such as during the development of obesity [23, 25]. Further, weight

loss results in a reduction in adipocyte volume but not necessarily adipocyte number [2].

Adipocytes are extremely resistant to apoptosis; therefore adipose tissue that has been expanded by hyperplasia it will be maintained, making it harder for an individual to sustain weight loss and worsening the prognosis for treatment [26, 27]. Targeting hyperplasia is thus crucial for preventing the progression of childhood and adult obesity.

The development of new adipocytes through the adipogenic process, occurs in two phases: the proliferation of preadipocytes from stromal vascular cells, and the terminal differentiation of preadipocytes to lipid-laden mature adipocytes. This transition is tightly regulated by cell cycle events and the induction of adipogenic factors.

Nearly all the progress made in understanding the regulation of adipogenesis at the molecular level has come from *in vitro* studies. Researchers have found that behavior of cell line models for adipogenesis, whether from mouse or human, can vary significantly based on depot origin, *in vivo* imprinting, and pluripotency stage [28]. As previously discussed, *in vivo* adipogenesis proceeds in two stages; therefore, cell models may reflect one or the other depending on their source of origin. 3T3-L1 murine preadipocytes are pre-committed to terminally differentiate into adipocytes, in response to hormonal stimulation. This cell line has been the most widely used cell model for studying adipogenic program. The 3T3-L1 cell line was derived from disaggregated Swiss 3T3 mouse embryos [29] and consists of unipotent preadipocytes, that have been shown to require mitotic clonal expansion (MCE) for terminal differentiation [30, 31] although it is still debated [32]. The research done in this model system thus reflects what might occur *in vivo* to pre-committed preadipocytes. Other cells lines commonly

used are 10T1/2 mesenchymal stem cells, and various human vascular stem cells isolated from individual patients.

In the 3T3-L1 cell line model, post-confluent, growth arrested preadipocytes can be induced to differentiate by an adipogenic cocktail consisting of methylisobutyl-xanthine (MIX), dexamethasone (DEX), and insulin (MDI). DEX and MIX stimulate glucocorticoid receptor and cAMP signaling, respectively, causing the initial up-regulation of CCAAT/enhancer binding protein (C/EBP) δ and C/EBP β . Cells at this time enter into mitotic clonal expansion (MCE), a period of replication, for roughly 48 hours. 12-18 hours after MDI stimulation, C/EBP β is dually phosphorylated and translocated into the nucleus where it binds to the DNA to help elicit PPAR γ and CEBP α expression [33]. PPAR γ and C/EBP α are the major adipogenic transcription factors for terminal differentiation that bring about a cascade of changes in gene expression and increased lipid synthesis [34]. In addition to measuring PPAR γ and C/EBP α as indicators of adipogenesis, lipid accumulation and lipogenic genes, such as fatty acid synthase (FAS), acetyl-CoA carboxylase (ACC) and adipocyte fatty acid binding protein (aP2), are used to determine the degree of differentiation and lipogenesis.

In vivo studies have clearly shown that cell cycle regulation is necessary for control of hyperplasia. The loss of cyclin-dependent kinase inhibitors in p27 and p21 knockout mice resulted in increased proliferation and recruitment of preadipocytes [35]. Therefore, targeting the molecular interplay between cell cycle and terminal differentiation is a novel approach to inhibiting the progression of enhanced hyperplasia in childhood and adult obesity.

In vitro, MCE is induced by DEX and MIX stimulation from the media.

Growth-arrested cells, held in G0 phase, are stimulated to transition into G1 phase, typically within the first 12 hours, which prepares the cell for replication. In order for the cell to transition into S phase, when DNA replication occurs, it must first pass through a checkpoint to ensure the cell is in proper condition. This checkpoint between G1 to S phase requires the accumulation of Cyclin A within the cytosol. Cyclin A is part of the cyclin family, which regulates the cell cycle by binding and activating cyclin dependent kinases (CDKs). It is the accumulation of these complexes and individual proteins that allow a cell to pass through checkpoints. P27, a CDK inhibitor, binds cyclin and CDKs, and inhibits cell cycle progression. Therefore, if the cell is functioning properly, expression of p27 will be transiently down-regulated during checkpoint transitions, allowing cyclin-CDK complexes to accumulate. When the cell enters S phase, typically between 18-36 hours, it begins DNA replication. Between 36-72 hours, the cell enters G2 phase when it prepares for final mitosis, the cleaving of two daughter cells. It has been shown that, during MCE events, induction of PPAR γ increases the expression of CDK inhibitors p18 and p21, and thus shifts the cell toward cell cycle exit [36] which is seen typically 48-72 hours post MDI induction.

Reactive oxygen species (ROS) signaling is required for MCE and the addition of ROS enhances terminal differentiation in 3T3-L1 preadipocytes [37]. Hypertrophied adipocytes, incapable of managing excess ROS production, secrete excess ROS to surrounding tissue, stimulating precursor cells residing within the tissue to differentiate. This is likely the mechanism by which hyperplasia is accelerated in conditions of excess caloric intake, and thus explains why hypertrophy precedes hyperplasia in

adulthood. Therefore anti-oxidant treatment, capable of quenching excess ROS, may be one potential way of inhibiting hyperplasia *in vivo*.

1.3.2 Adipocyte Metabolism

In a state of excess energy intake, severe hypertrophy leads to adipocyte dysfunction. Adipocytes can expand upwards of 100 μm in diameter, nearly 10 times that of a typical mammalian cell. Visceral adipocytes greater than 75 μm in diameter have been linked to the development of metabolic syndrome [38]. Therefore, preventing or alleviating excess lipid storage is a strategic approach in the fight against obesity that involves understanding how adipocytes regulate the ebb and flow of lipid synthesis and breakdown.

The primary role of adipocytes is to store energy, in the form of triglyceride, for times of need, such as during exercise and fasting. Circulating free fatty acids are readily taken up and stored as triglyceride, while excess energy in the form of glucose is synthesized into triglyceride through the process of *de novo* lipogenesis. When needed, adipocytes release this stored lipid into the blood stream through the process of lipolysis, in which triglyceride is broken down into free fatty acids (FFA) and glycerol that are released into the bloodstream [39]. Another fate of stored lipid is β -oxidation, in which the adipocyte itself uses free fatty acids to generate energy. Investigating ways to manipulate lipolysis and β -oxidation is an approach to reduce the amount of lipid stored in hypertrophied adipocytes and thus alleviate the metabolic demand they face.

1.3.2.1 De Novo Lipogenesis

Glucose is the primary source of energy for most cells. Therefore, it is readily metabolized through glycolysis to produce pyruvate. Pyruvate is further converted into acetyl-CoA, which along with citrate is a substrate for aerobic respiration. If excess glucose is present in the cell, acetyl-CoA and citrate will accumulate in the cytoplasm. This buildup metabolically shifts the cell from an energy-burning to an energy-storing state. Citrate buildup allosterically enhances the function of Acetyl-CoA Carboxylase (ACC), which converts acetyl-CoA to malonyl CoA, the substrate for lipid synthesis. Subsequently, the buildup of malonyl CoA inhibits the function of carnitine palmitoyltransferase1 (CPT1), which transports lipids across the mitochondrial membrane for β -oxidation. Therefore, the buildup of malonyl CoA promotes lipogenesis while blunting oxidation [40]. This response is one of the major ways adipocytes switch between energy storage and energy expenditure.

1.3.2.2 Lipolysis

Adipocytes purge stored triglyceride through the process known as lipolysis. Triglyceride is stored in a lipid droplet surrounded by perilipin protein that serves as a protective coating. When lipolysis is activated by β -adrenergic signaling, the second messenger cAMP activates protein kinase A (PKA). PKA, in turn, phosphorylates perilipin and hormone sensitive lipase (HSL) [41]. Phosphorylated perilipin detaches from the lipid droplet giving phosphorylated HSL access to begin breaking down the stored lipid. Two other constitutively expressed lipases involved in lipolysis, acyltriglyceride lipase (ATGL) and monoacylglycerol lipase (MAGL), have been

recently discovered [39]. However, both of their activities are not as tightly regulated during lipolysis as that of HSL. ATGL is primarily responsible for releasing the first free fatty acid; HSL the second, and MAGL, the third [42]. The resulting glycerol and free fatty acids are transported out of the cell and into the bloodstream for other tissue to take up.

Lipolysis is of interest to anti-obesity researchers because of its ability to reduce lipid storage; however, the resulting increase in circulating FFA and thus potential harm to the circulatory system is a source of much concern. Therefore, another approach involves investigating how to block the release of lipolyzed FFA from and redirect FFA to β -oxidation within the cell.

1.3.2.3 β -oxidation

Lipid is the most energy dense storage unit the body has. When energy is needed, the body taps into this store through the process of β -oxidation, which yields significantly more ATPs than carbohydrate and protein metabolism. To carry out this process, the cell must transport FFA across the mitochondrial lipid bilayer. It does so through the tight regulation of carnitine palmitoyltransferase 1 (CPT1) [40], which is the enzyme responsible for trafficking FFA through the mitochondrial membrane. As mentioned previously, the build up of malonyl CoA, the substrate for *de novo* lipogenesis, allosterically inhibits CPT1. Therefore adipocytes can easily shift between lipid synthesis and breakdown.

Once in the mitochondria, fatty acid carbon chains are oxidized by niacin adenine dinucleotide (NAD⁺) and flavin adenine dinucleotide (FAD), and the

remaining acetyl-CoA is further degraded by the citric acid cycle. NADH and FADH₂ transport H atoms to the electron transport chain, where the build up of H atoms across a membrane supplies the energy for ATP synthesis. NAD and FAD are then recycled back for further oxidation [40].

A transcriptional regulator of β-oxidation is the coactivator peroxisome proliferator-activated receptor (PPAR)-γ coactivator (PGC)-1α. PGC-1α interacts with a broad range of transcription factors to regulate expression of genes related to metabolic processes in the cell, such as thermogenesis, mitochondrial biogenesis, and glucose/fatty acid metabolism. Specifically, PGC-1α is known to enhance transcription of Uncoupling Protein 1, which is the major thermogenic gene found in muscle cells and brown fat and a suggested therapeutic target for obesity treatment and prevention [43]. AMP- activated protein kinase (AMPK), the major metabolic regulator of the cell, is also known to stimulate β-oxidation and increase expression of PGC-1α to improve ATP/AMP ratios [44].

Of great interest to obesity research is the ability of mitochondria to uncouple β-oxidation from ATP synthesis, by the use of uncoupling proteins (UCP1, 2, and 3). These proteins dissipate the buildup of H atoms across the inner mitochondrial membrane, resulting in thermogenesis instead of ATP synthesis. It has been suggested that adipocytes carry out a basal level of uncoupled β-oxidation that protects the body from fatty acid leakage, and, in the hypertrophied state, mitochondrial dysfunction impairs the cell's ability to keep up with the increased flux of free fatty acids [45]. Therefore, finding factors that stimulate or enhance β-oxidation, and specifically

uncoupled β -oxidation, is a promising approach to preventing and alleviating adipocyte hypertrophy.

1.4 The Role of AMP- activated protein kinase (AMPK) in Adipocytes

1.4.1 Overview

When targeting obesity and the development of metabolic syndrome through hyperplasia and hypertrophy, it is important to consider a major metabolic regulator of the cell, AMP-activated protein kinase (AMPK). The function of this protein affects both differentiation and metabolic pathways in adipocytes.

AMPK is a well-known sensor of cellular ATP/AMP ratios and activation of AMPK shifts the cell toward an energy-producing state when ATP levels are too low. AMPK is comprised of 3 subunits (α , β , and γ) and is active in its phosphorylated state (α -subunit p-172) [46]. The γ - subunit binds AMP causing a conformational change in the protein that protects the α -subunit p-127 from cytosolic phosphatases [47].

Although AMPK activation is nearly 100-fold greater with AMP bound, AMP-independent pathways also lead to AMPK activation. These pathways include calmodulin-dependent kinase kinase (CaMKK), protein kinase A (PKA), and liver kinase B1 (LKB1) [47]. There are several downstream targets of pAMPK, but of interest in adipocyte research is the phosphorylation of acetyl coenzyme A carboxylase (ACC), a lipogenic enzyme that is inhibited by its phosphorylation.

1.4.2 Activators and Inhibitors

The use of AMPK activators and inhibitors has allowed researchers to begin to understand the role of AMPK in white adipose tissue (WAT) [48-54]. The well-known AMPK activator, 5-aminoimidazole-4-carboxamide-1- β -D-ribofuranoside (AICAR), mimics AMP in the cell, thus activating AMPK by inducing a perceived increased AMP/ATP ratio. The well-known inhibitor, Compound C (CC), enzyme-specifically inhibits phosphorylation of p127 on the α -subunit, without altering upstream kinases.

1.4.3. Role of AMPK in Adipogenesis

AMPK has been shown to play a critical part in adipogenesis, in particular the progression of mitotic clonal expansion (MCE), and is therefore a likely candidate for targeting hyperplasia. Habinowski et al. [49] showed that AICAR, when added at the initial time of differentiation in 3T3-L1 preadipocytes, inhibited differentiation as seen by decreased lipid accumulation and expression of adipogenic factors. They further demonstrated that AICAR blunted MCE, as measured by cell counting on Day 3. AICAR did not alter C/EBP β expression but did inhibit PPAR γ and C/EBP α , suggesting that AMPK activation may prevent C/EBP β priming for DNA binding. Interestingly, they also found that AICAR's effect was time-sensitive, having the strongest inhibitory effect when added on Day 0, a mild effect at Day 3, and no significant effect when added on Day 5, although this pattern has since been disputed [50].

Compound C has also been shown to inhibit adipogenesis through MCE. Gao et al. found that Compound C at high concentrations ($>10\mu\text{M}$) inhibited lipid accumulation

and expression of PPAR γ , C/EBP α , FAS, and aP2 [55]. This effect was limited to early time points as treatment after the first two days of differentiation had no impact on lipid accumulation or adipogenic factors. Further, Compound C treatment decreased cell number from Day 0 to Day 3 of differentiation. Nam et al. also found Compound C inhibited adipogenesis through MCE, as Compound C increased levels of p21, a cyclin kinase dependent inhibitor, levels [53].

Taken together, the general consensus in the literature is that either activation or inhibition of AMPK alters MCE events and inhibit adipogenesis. This effect is understandable, as the cell's energy needs must be tightly regulated during such a demanding transition. Therefore, identifying factors that target AMPK are of interest in preventing hyperplasia. Interestingly several researchers have shown that certain bioactive compounds exert their anti-adipogenic effect through AMPK activation [47, 56].

1.4.4 Role of AMPK in Mature Adipocytes

Much interest surrounds the role of AMPK in the metabolism of mature adipocytes. Specifically, activation of AMPK to induce lipolysis and increase mitochondrial oxidation of fatty acids would lead to a possible approach to alleviate hypertrophy.

In general, activated AMPK turns on catabolic pathways that produce ATP and turns off anabolic pathways that consume ATP. Likewise, it has been shown that AMPK increases β -oxidation in muscle and liver cells [57], but the effect on adipocytes remains unclear. In theory, AMPK activation would activate oxidative pathways while

inhibiting lipid synthesis. It is known that AMPK phosphorylates and deactivates the lipid synthesizing enzyme ACC, resulting in a cytosolic decrease in malonyl CoA. This decrease subsequently results in the availability of CPT1 to transfer fatty acids into the mitochondria, thus shifting the cell toward β -oxidation. Gaidhu et al. [58] found these effects in isolated epididymal rat adipocytes. Their findings showed that chronic AICAR exposure (15 hours) increased pACC, fatty acid oxidation, and PGC-1 α , CPT1, acetyl-CoA oxidase mRNA expression, while UCP1 and UCP2 were unaltered by AICAR treatment. The authors concluded that AMPK activation could remodel adipocytes toward energy dissipation.

Also of particular interest is AMPK's effect on lipolysis. Upon normal β -adrenergic stimulation of lipolysis, cAMP activates PKA, which in turn phosphorylates and activates HSL for lipolysis. Djouder et al. found that PKA concurrently inactivates AMPK in order to promote efficient lipolysis [59]. However other researchers have found that AMPK activation through cAMP was necessary for induced lipolysis, as lipolysis was no longer stimulated in the presence of mutant AMPK alpha subunits [60]. When AMPK was therapeutically activated by the use of AICAR in isolated rat adipocytes, AICAR inhibited HSL activation and glycerol release, but over the course of 15 hrs increased FFA release [52]. The authors here concluded that the length of AMPK activation determined the lipolytic response; acute treatment resulted in an inhibition of lipolysis, while chronic activation resulted in an increase of fatty acid release. This difference may explain some of the controversy observed among researchers. Because glycerol release did not also increase with time, the authors suggested that AMPK activation may prevent the complete hydrolysis of triglyceride

molecules through the inhibition of HSL phosphorylation.. If triglyceride hydrolysis is incomplete triglycerides are broken down to diacylglycerides by ATGL but not further, resulting in the release of some FFA and the shunting of diacylglycerides to other metabolic pathways in the cell. This provides a potential mechanism and indicates that AMPK may play a critical role in the cells ability to undergo lipolysis.

AMPK regulation provides a promising new avenue toward alleviating adipocyte hypertrophy as it has been implicated in β -oxidation and lipolysis. Recently, several bioactive compounds have been implicated as AMPK activators [16], which could provide a natural approach toward targeting hyperplasia and hypertrophy in the progression of obesity.

1.5 Potential Health Effects of Bioactive Compounds in the Development of Obesity

To prevent chronic disease, increasing fruit and vegetable consumption has been the longstanding advice. Consistently, fruit and vegetable intake is negatively correlated with the occurrence of obesity and metabolic syndrome [61, 62], and further, increasing fruit and vegetable consumption may decreases risk for heart disease and diabetes [63, 64]. Not only do fruits and vegetables provide a low-calorie, high-fiber option, but researchers within the past several decades have also identified more than thousands of phytochemicals that naturally occur in the produce we eat [65]. Discovery of bioactive compounds has invited researchers to explore the vast array of beneficial effects these compounds possess. Much interest surrounds the use of bioactive

compounds, through increased consumption and supplementation, to prevent or alleviate symptoms of metabolic syndrome as drug therapy is not only costly, but often elicits negative side-effects [4, 66].

Currently, it is estimated that humans consume about 1-3g of bioactive compounds daily, depending on their level of fruit and vegetable intake [67]. Nearly all bioactive compounds have been shown to possess health-promoting properties, typically in a dose-dependent manner. The American Dietetic Association (ADA) acknowledges the significance of consumption of bioactive compounds and therefore supports the marketing of functional foods, either conventional or modified, when there is substantial scientific evidence of benefit [68]. Flavonoids, a sub group of polyphenols, are currently a top priority of research efforts to The National Institute of Health's National Center for Complimentary and Alternative Medicine (NCCAM) because the health benefits shown. Specifically, the flavonoid quercetin is of great interest because of its suggested ability to alleviate obesity-related conditions [67].

1.6 The Dietary Flavonoid Quercetin

1.6.1 Overview

Flavonoids are a class of phytochemicals, discovered in the 1930s, that generate pigment in plants and play a biological role in other cellular processes. It is estimated that the average dietary intake of flavonoids in humans is several hundred

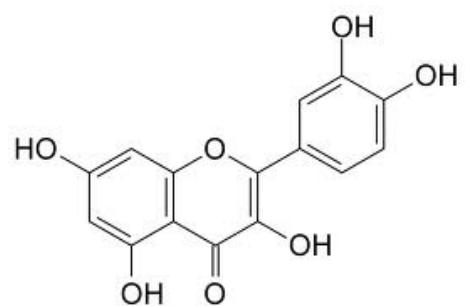


Figure 1. Structure of Quercetin

milligrams per day [65].

Quercetin, the most abundant dietary flavonoid, has been the focus of 30 years of research for its strong antioxidant capabilities. It is a flavonol, one of the five subclasses of flavonoids, and it's ring structure and aglycone configuration of hydroxyl groups, make it one of the most potent flavonoids in terms of antioxidant capabilities [69, 70]. Quercetin is found in numerous fruits and vegetables but the major dietary sources include onion, red apples, red wine, tea, cranberry, kale, hot peppers and broccoli [71].

Determining exact amounts of flavonoid content in any plant is very challenging because flavonoid production is affected by sunlight, environmental factors, degree of ripeness, and species genetic variability [72]. Further quercetin is stored in the

skin of most fruits and vegetables, therefore content can very significantly depending on skin to overall weight ratio. Consequently, estimating quercetin intakes through dietary analysis is particularly limited, making it difficult to complete epidemiological studies relating quercetin intake to chronic disease [65]. Estimated quercetin intakes range from 10-100mg per day depending on an individual's consumption of these fruits and vegetables [67]. It could be speculated that 6-8 servings of fruits and vegetables daily would provide an average daily consumption of 100mg quercetin, however as discussed, exact quercetin content data is limited. Higher levels of intake between 500mg-1200mg can be safely achieved by the use of supplements (this amount would be likely be comparable to consumption of 5-10 times the recommended servings, 6-8,

Table 1. Sources of Quercetin

Food Source	Amount (mg/kg)
Onions	284-486
Kale	110
Asparagus	142
Apple	21-72
Green Beans	39
Cherry Tomatoes	17-200
Broccoli	30
Tea	10-25
Red Wine	4-16

Modified from Manach, 2005 [71]

of fruits and vegetable, and could only be achieved through supplementation).

Researchers are thus trying to utilize the potential of this readily available flavonoid through increased consumption and supplementation for its antioxidant, anti-inflammatory, and anti-proliferative properties [67, 73].

1.6.2 Bioavailability

As with any bioactive compound, bioavailability is of great interest and importance. Quercetin is predominantly found in nature in variations of its glycoside form: either quercetin 3-O- β -D-glucoside (Q3G) or quercetin 4'-O- β -D-glucoside (Q4G) [74]. These forms are useful to the plant because of their water-soluble properties, but do not have the same antioxidant capabilities of the aglycone form, and are thought to be metabolized by the gut microflora before absorption. Three factors that determine the bioavailability of quercetin: (1) metabolism by gut microflora, (2) transport through small intestinal cells, and (3) final circulating metabolites. Although the question of which metabolites are absorbed and circulate remains controversial, advancements in detection methods have come a long way since 1975, when Gugler et al. [75] first gloomily reported the quercetin doses did not alter plasma levels in humans.

1.6.3 Gut Metabolism

It is estimated that 9 out of 10 cells in the body are bacterial [76], which puts in perspective the influence of an individual's gut microflora on digestion and gut metabolism. Specifically, it has been well demonstrated that gut bacteria are capable of degrading quercetin into phenolic acids and CO₂, methylating aglycone compounds,

and hydrolyzing quercetin glucosides [77]. Chen et al. [78] determined in rats that 93.3% of absorbed quercetin had been metabolized by the gut and only 3% by the liver after absorption. They also showed that 60% of total ingested quercetin was absorbed. Earlier studies done by Hollman et al. [79, 80] indicated that a dose of quercetin in glucoside form was better absorbed than the aglycone, as measured by ileostomy effluent content of glucosides. In stark contrast, Walgren et al. [81] showed, using a Caco-2 cell monolayer, that aglycones are more readily transported across the intestinal layer than glucosides. Walle et al. [82] aimed to address this conflict and discovered that glucosides in an onion meal are effectively hydrolyzed to the aglycone form, which can be absorbed. These findings suggest that nearly all excreted quercetin is aglycone and further that we rely on gut bacteria to hydrolyze dietary quercetin for absorption. This explains why significant variation may exist among individuals in their ability to absorb quercetin based on the make up of their gut microflora.

1.6.4 Absorption and Transport

Piskula and Terao [83] were among the first, along with Manach [84], to demonstrate that quercetin doses in rats result in plasma metabolite concentrations of 1-2 μ M. Their findings further suggested that absorption was affected by the solubility of quercetin in the vehicle of administration (quercetin dissolved in propylene glycol resulted in plasma concentration of 50 μ M). Human supplementation studies typically result in a total plasma quercetin metabolite range of 10-200nM [77]. The most common detection method for plasma quercetin digests all the quercetin metabolites and provides an overall concentration. With more sensitive techniques, researchers are

trying to determine which metabolites, specifically aglycone verses glucosides, circulate at higher levels, as this would likely be the metabolite that exerts an effect on tissues. The pertinent body of research remains inconclusive, according to a recent review, as some researchers indicated that quercetin glucoside forms were detected in plasma [85, 86], and others reported that aglycone forms but not glucosides were present [87, 88]. Other studies have shown a wide range of quercetin metabolites detected in the plasma [89]. Because of the significant *in vitro* findings of quercetin aglycone, work is currently underway to develop nanoparticle vehicles that protect quercetin from gut and liver metabolism and therefore deliver it in its potent aglycone form to tissues [73].

Because it is reasonable to postulate that both aglycone and glucoside forms circulate in the plasma, it is of interest to study the effect of both metabolites on adipose tissue. However the scope of research in this study focuses on the aglycone form. Although concentration ranges used in this study are higher than physiological concentrations, as compared to what is observed *in vivo* after supplementation (10-200nM), it is relevant for our work in less sensitive cell culture systems to observe the molecular changes that occur in response to quercetin.

1.7 Quercetin's Effect on Metabolic Syndrome: *In Vivo* Studies

Quercetin is currently of great interest to researchers because of significant *in vivo* animal studies showing its ability to ameliorate metabolic syndrome. Vessal et al. [6] found that in streptozocin (STZ)-induced diabetic rats (a model that is comparable

to type 1 diabetics), doses of 10mg/kg and 15mg/kg per day, began to lower blood glucose levels after only 3 days and had completely normalized blood sugar levels after 10 days. The authors concluded that quercetin may be able to stimulate pancreatic islet cell regeneration. In 2008, Rivera et al. [7], found that quercetin doses (2 or 10 mg/kg) ameliorated high plasma concentrations of triglycerides, total cholesterol, FFA, and insulin in Zucker rats, and further that the higher dose increased plasma concentrations of adiponectin, reduced NOx levels in plasma, and lowered VAT TNF-alpha production. Kobori et al. [8] fed mice a diet high in fat, cholesterol, and sugar for 20 weeks, and supplemented one group with 0.05% quercetin. After the 20-week period, supplemented mice had plasma quercetin levels of 14.16 μ M, and had significantly less fat accumulation in the liver, reduced circulating triglyceride levels, and improved blood glucose and insulin levels. The same group had previously found quercetin-fed (0.1% or 0.5%) streptozotocin (STZ)-induced diabetic mice had improved blood glucose and plasma insulin levels [9]. Bansal et al. [10] showed similar results feeding STZ-induced mice a high-fat diet with *Pileamicrophylla* (PM1), a plant containing quercetin, among other flavonoids. PM1 at a dose of 100 mg/kg/day, for 28 days produced significant ($p<0.05$) reduction in body weight, plasma glucose, triglycerides, and total cholesterol content. It is important to note, due to the complexity of quercetin bioavailability, significant results are not always found with varying quercetin doses [90]. However, the majority of animal studies to date strongly suggest quercetin as an anti-obesity, insulin-sensitizing agent, warranting further research.

Human trials show promising preliminary results. Egert et al., in a randomized double-blinded, placebo-controlled cross-over trial gave overweight and obese subjects

150mg dose of quercetin daily for 6 weeks (resulting in plasma quercetin levels of 71 to 269 nM/l). Quercetin treatment decreased systolic blood pressure (-2.6 mmHg, p<0.001), oxidized LDL levels, and, interestingly, HDL levels, when compared to the placebo group [91]. Further, this group retrospectively characterized the effect of quercetin on serum blood pressure and lipid profile to be linked to variations in apolipoproteinepsilon genotype [92], suggesting an explanation for varied responses to quercetin treatment. Although some supplementation studies have shown no significant effect [93, 94], this is likely due to dosage, study duration, poor bioavailability, and variable gut microflora of each individual. Therefore, optimizing study design is essential in human supplementation trials.

These significant *in vivo* findings command further mechanistic research of quercetin's effect at the cellular level, specifically in the areas of hyperplasia and hypertrophy as both lead to the development of metabolic syndrome. Current research detailing what is known about both quercetin's effect on adipogenesis and adipocyte metabolism, as well as our recent findings, are described below in Chapters 2 and 3, respectively.

CHAPTER 2

EFFECT OF QUERCETIN ON ADIPOGENESIS

2.1 Literature Review

The rise in obesity will significantly decrease life expectancy and continue to burden health care systems because of increased risk for the development of chronic diseases [95]. On a cellular level, obesity is defined as the increase in size (hypertrophy) and number (hyperplasia) of adipocytes. Studies have shown that adipocyte number is determined during childhood and maintained through adulthood, emphasizing the importance of preventing childhood obesity. In adulthood, hypertrophy precedes hyperplasia to accommodate initial excess energy intake, however as excess intake persists, such as in the development of obesity, hyperplasia is accelerated [23, 25]. Further, weight loss results in a reduction in adipocyte volume but not necessarily adipocyte number [2]. Adipocytes are extremely resistant to apoptosis; therefore adipose tissue that has been expanded by hyperplasia will be maintained, making it harder for an individual to sustain weight loss and worsening the prognosis for treatment [26, 27]. Targeting hyperplasia is thus essential for preventing the progression of childhood and adult obesity.

Several bioactive compounds have been shown to inhibit adipogenesis, the process of developing new adipocytes. Quercetin, the most commonly consumed flavonoid, is one such compound. Because quercetin is known to have anti-proliferative and apoptotic properties, it was first hypothesized to inhibit preadipocyte growth. Hsu

et al. [96] ,while comparing the effects of various flavonoids on undifferentiated 3T3-L1 pre-adipocytes, found that quercetin most strongly inhibited cell population growth and induced cell apoptosis through caspase-3 in a dose-dependent manner (50-250 μ M). Quercetin was also found to be anti-proliferative in human stromal cells [97]. Park et al. [11] showed that quercetin, alone or in combination with genistein and resveratrol inhibited lipid accumulation in differentiating 3T3-L1 and human adipocytes. Similar results were also found by Yang et al. [12] who determined in 3T3-L1 cells that quercetin (25 μ M) alone inhibited lipid accumulation by 15.9+/-2.5% (p<0.001), and in combination with resveratrol (25 μ M) inhibited lipid accumulation by 68.6+/-0.7% (p<0.001). Although it is generally agreed that quercetin is anti-adipogenic, Morikawa [98] interestingly showed that quercetin induced apoptotic genes without inhibiting PPAR γ and fatty acid synthase (FAS) during differentiation of human preadipocytes, suggesting quercetin may work through PPAR γ -independent pathways.

One potential mechanism for quercetin's anti-adipogenic effect was recently proposed by Ahn et al. in 2008 [13]. They reported that differentiating 3T3-L1 cells in the presence of quercetin (10-100 μ M) decreased lipid accumulation and expression of adipogenic factors (CEBP α , PPAR γ , SREBP-1, FAS). They further looked at the effects of quercetin during differentiation on AMP-activated protein kinase (AMPK), the metabolic regulator of the cell, and its down stream target acetyl-CoA carboxylase (ACC), and found that phosphorylation of both of these proteins by Day 8 was increased in the presence of higher concentrations of quercetin (50 μ M and 100 μ M). The authors suggested AMPK activation as a potential mechanism for quercetin's anti-

adipogenic effect. The role of AMPK activation during differentiation has been considered anti-adipogenic [49] and potentially regulates mitotic clonal expansion.

Mitotic clonal expansion (MCE) has been implicated as a critical regulator of adipogenesis *in vivo* and is a current targeted pharmacological approach in the fight against obesity [25]. Cell cycle is tightly regulated by transitory cyclin dependent kinases and kinase inhibitors, two of which are Cyclin A and p27, respectively. The abolishment of p27 *in vivo* lead to increased proliferation and recruitment of preadipocytes [35]. MCE has been shown to be necessary for terminal differentiation in vitro in 3T3-L1 preadipocytes [99]. Given quercetin's anti-proliferative properties, and ability to activate AMPK, it is logical to hypothesize that quercetin may inhibit adipogenesis through altering cell cycle events; however this effect has never been explored.

Understanding this mechanism will help develop quercetin as an anti-obesity agent because of its ability to target hyperplasia, a critical process in the development of obesity and metabolic syndrome.

2.2 Purpose of Study

Given the current gap in the literature surrounding quercetin's ability to inhibit adipogenesis through mitotic clonal expansion, and the significance these findings would have on the development of quercetin as an anti-obesity agent, we aimed to fill this gap by determining (1) quercetin's effect on adipogenesis using 3T3-L1

preadipocytes, and (2) quercetin's effect on cell number and cell cycle related genes Cyclin A and p27, key regulators for cell cycle progression from G1 phase to S phase.

Our hypothesis was that quercetin will (1) inhibit lipid accumulation, as measured by Oil Red O staining, and expression of adipogenic factors PPAR γ , C/EBP α , aP2, ACC on the mRNA and protein levels, and (2) inhibit cell number, a measure of mitotic clonal expansion, and alter mRNA expression of Cyclin A and p27 when analyzed at 0-48 hours.

2.3 Materials and Methods

2.3.1 Cell Culture Model and Treatments

For the differentiation of 3T3-L1 preadipocytes, cells were grown to 100% confluence in Growth Media [DMEM (high glucose), 10% Calf Serum, 1% Penicillin/Streptomycin] replaced every two days. At two days post-confluence, growth media was changed to Differentiation Media [DMEM (high glucose), 10% Fetal Bovine Serum, 1% P/S, 1% Insulin, 1% 3-Isobutyl-1-methylxanthine 11.5mg/ml, 0.01% Dexamethasone 3.9mg/ml] in the presence or absence of quercetin aglycone (Sigma-Aldrich Catalog # 6151-25-3). The combination of 3-Isobutyl-1-methylxanthine (MIX), dexamethasone, and insulin is known as the standard MDI adipogenic cocktail. At two days post-MDI induction, media was replaced every two days with post-differentiation media [DMEM (high glucose), 10% Fetal Bovine Serum, 1% P/S, 1% Insulin, +/- quercetin]. 3T3-L1 preadipocytes generally reach 80% differentiation 4 to 5 days post-differentiation.

2.3.2 Oil Red O Lipid Staining

Cells were harvested on desired days and underwent Oil Red O Staining to quantify lipid accumulation, as it is an indirect determiner of cell differentiation. Cells, grown on 24-well and 6-well plates, were treated with 10% formaldehyde in phosphate buffer solution (PBS) for 1 hour, washed with 60% isopropanol, and completely dried. Then, cells were stained with 0.5% Oil Red O solution in 60:40 (v/v) isopropanol: H₂O, for 30 minutes at room temperature. Finally, wells were rinsed with distilled water and dried. Optical density was then measured at 490nm, after eluting with isopropanol, to quantify lipid accumulation.

2.3.3 Cell Viability and Counting

Cells were grown to confluence in 24-well plates. At two-days post-confluence cells were differentiated with MDI in the presence or absence of quercetin (25 and 50μM). Media was replaced, two days later, with post-differentiation media (treatment continued). On day 3, cells were prepared for Trypan Blue staining. Media was removed and wells were washed with PBS. Trypsin was added to each well (0.25ml) and let sit for 10 minutes to ensure cell detachment. PBS (0.75ml) was then added to bring cell suspension volume to 1ml. Trypan Blue (modified) 0.4% solution 1N PBS (from MP Biomedical Inc, cat# 1691049) was added to cell suspension (1:1) in a fresh centrifuge tube for 3 minutes. A hemocytometer was used to count cells from each well twice. Each treatment had 6 replicate wells. Both stained and unstained cells were counted (stained cells are non-viable). Cells were counted in the 4 corners of the hemocytometer, and total cell number was calculated as (total of 4 squares) x (2500) x

(2 dilution factor). Percent viable equaled the number of viable cells divided by total cell number (viable plus non viable counts). Total viable cell number was the average number of unstained cells from each treatment replicate.

2.3.4 Protein Isolation and Western Blotting

Cells were harvested at desired times with RIPA Buffer containing protease inhibitor (500ul per p-100 dish). Samples were stored at -80° C until protein quantification. Samples were thawed on ice, sonicated, and centrifuged. Supernatant, containing whole cell protein, was transferred to a fresh tube for protein quantification using the Bicinchoninic Acid (BCA) assay. A BCA standard curve was established using bovine serum albumin (BSA)[2mg/ml] protein and measured at 570nm. Sample concentrations were then measured against the standard, and prepared with sample buffer to allow for 15 μ g to be loaded to the gel. Samples were run on acrylamide (varying percentages) gels via electrophoresis, and then proteins were transferred to a PVDF membrane through wet transfer. Upon successful transfer, membranes were blocked with 5% non-fat dry milk, and treated with primary and secondary antibodies (purchased from Santa Cruz Biological). HRP detection was done through ECL solution. Chemiluminescent bands were captured on x-ray and developed.

2.3.5 RNA Isolation and Analysis

Cells washed with phosphate buffer solution (PBS), were harvested with Trizol Reagent and stored at -80° C until mRNA isolation. For isolation, samples were thawed and centrifuged with 200 μ L chloroform. Supernatant was transferred to a fresh tube

and centrifuged with isopropanol (1:1) to precipitate RNA. Isopropanol was removed and the pellet was washed three times with ethanol by centrifugation. The pellet was then resuspended in DEPC water and quantified using a spectrophotometer at 260nm. 8 μ g of RNA was used along with SuperScript III reagents to make cDNA which was stored at -4°C. Polymerase Chain Reaction was performed with primers for PPAR γ , C/EBP α , aP2, Cyclin A, and β (see Table 2). Samples were run on an agarose gel containing Ethidium Bromide, and detected using Ultra Violet light. Band intensity was determined using Image J analysis and adjusted to β -actin.

Table 2. Primer Information

Primer	Forward Sequence	Reverse Sequence	Temp/Cycles
ACC α	5'-CCCAGCAGAATAAAGCTACTTTGG-3'	5'-TGAGCATGGCATCCGGCGACT-3'	58°C / 40
Adiponectin	5'-AAGGACAAGGCCGTCTCT-3'	5'-TATGGGTAGTTGCAGTCAGTTGG-3'	56°C / 35
aP2	5'-GACCTGAAACTCGTCTCCA-3'	5'-CATGACACATTCCACCACCA-3'	56°C / 30
β -actin	5'-AGGCTGTGCTGCCCTGTATGC-3'	5'-ACCCAAGAAGGAAGGCTGGAAA-3'	61°C / 35
C/EBP α	5'-GGTGCAGAACAGCCGAGATAAAG-3'	5'-AGTTCACGGCTCAGCTGTTCCAC-3'	61°C / 35
Cyclin A	5'GCAGTTGAATCACCACATGCATGCCGGCACCTCGAGGCAT-3'	5'-TGGCTGCCCTTCATGTAACCTCACACACTTAGTGTCTGGTG-3'	63°C / 35
GAPDH	5'-CAAGGTATCCATGACAACTTG-3'	5'-GCCCATCCACAGTCTCTGG-3'	60°C / 35
NOX4	5'-GAAGCCCATTGAGGAGTCA-3'	5'-GGGTCCACAGCAGAAAACTC-3'	56°C / 35
PGC-1 α	5'-TGGACGGAAGCAATTTC-3'	5'-TTACCTGCGCAAGCTTCTCT-3'	56°C / 35
PPAR γ	5'-CCAGAGTCTGCTGATCTCG-3'	5'-GCCACCTTTGCTCTGATC-3'	58°C / 40
UCP-1	5'-ATACTGGCAGATGACGTCCC-3'	5'-GTACATGGACATCGCACAGC-3'	59°C / 35
UCP-3	5'-ACTCCAGCGTCGCCATCAGGATTCT-3'	5'-TAAACAGGTGAGACTCCAGCAACTT-3'	59°C / 35

2.3.6 Statistical Analysis

Samples were collected in at least duplicate or triplicate, and differences between the means were determined by student's T-test. P-values were considered significant at <0.05.

2.4 Results

2.4.1 Quercetin inhibits lipid accumulation and adipogenic transcriptional factors

3T3-L1 pre-adipocytes were stimulated to differentiate at Day 0 in the presence of increasing concentrations of quercetin (0-100 μ M). By Day 8 of differentiation quercetin dose-dependently inhibited lipid accumulation (Figure 2). (Troglitazone, a PPAR γ ligand, was used as a positive control to demonstrate the ability of our model system to be stimulated toward differentiation.) Further we determined quercetin inhibited mRNA expression of PPAR γ (1.1 fold), C/EBP α (17.6 fold), aP2 (2.1 fold) (Figure 3) and protein expression of PPAR γ , and ACC (Figures 4). These results indicate that quercetin inhibits adipogenesis in our model system through the inhibition of critical transcriptional adipogenic factors.

2.4.2 Quercetin's inhibitory effect is limited to early time points

Mitotic clonal expansion (MCE) occurs in 3T3-L1 preadipocytes between Day 0 and Day 3 after MDI stimulation. Because quercetin is known to be a strong anti-proliferative agent and AMPK activator, we tested the hypothesis that quercetin's effect on lipid accumulation is limited to treatment during early time points of differentiation. Quercetin treatment was staggered, with treatments starting at 0, 6, 12, 18, 24, 36, 48, 72 hours after MDI stimulation at time 0 hour. All treatments were continued until Day 6 of differentiation when cells were harvested for Oil Red O staining. Quercetin's strongest inhibitory effect was observed when added between 0-18hours (Figure 5). Quercetin treatment starting at 24-36 hours still significantly inhibited lipid

accumulation as compared to the control, however after 48 hours quercetin had no significant effect. This finding indicates quercetin's effect is limited to early time points of adipogenesis.

2.4.3 Quercetin may inhibit adipogenesis through regulation of early cell cycle events

We further investigated quercetin's effect on mitotic clonal expansion by looking at cell number and cell cycle related genes. Figure 6a shows that between Day 0 and 3, cell number dramatically increases. Quercetin significantly inhibited total cell number in this trial. We next repeated this experiment using Trypan Blue staining to detect viable and non-viable cells, and only looked at Day 3 counts since they reflect the end of MCE. Quercetin treatment did not alter the % of viable cells indicating quercetin's effect is not cytotoxic. Although not significant, there was a clear trend indicating quercetin may decrease viable cell number at Day 3. This suggests quercetin may inhibit MCE. We next looked at cell cycle genes Cyclin A, a positive regulator of cell cycle, and p27, an inhibitory regulatory of cell cycle. In preadipocytes stimulated to differentiate at time 0 hour, Cyclin A mRNA rose at 12 hours and fell by 24 hours in the control. However quercetin (50 μ M) treatment inhibited Cyclin A expression at 12 and 24 hours, delaying Cyclin A induction until 36 and 48 hours (Figure 7). Quercetin treatment also altered the expression of p27. Preadipocytes stimulated to differentiate at time 0 hour transiently decreased protein expression of p27 between 18-24 hours. Quercetin (50 μ M) sustained p27 expression through the mitotic clonal expansion period (Figure 8).

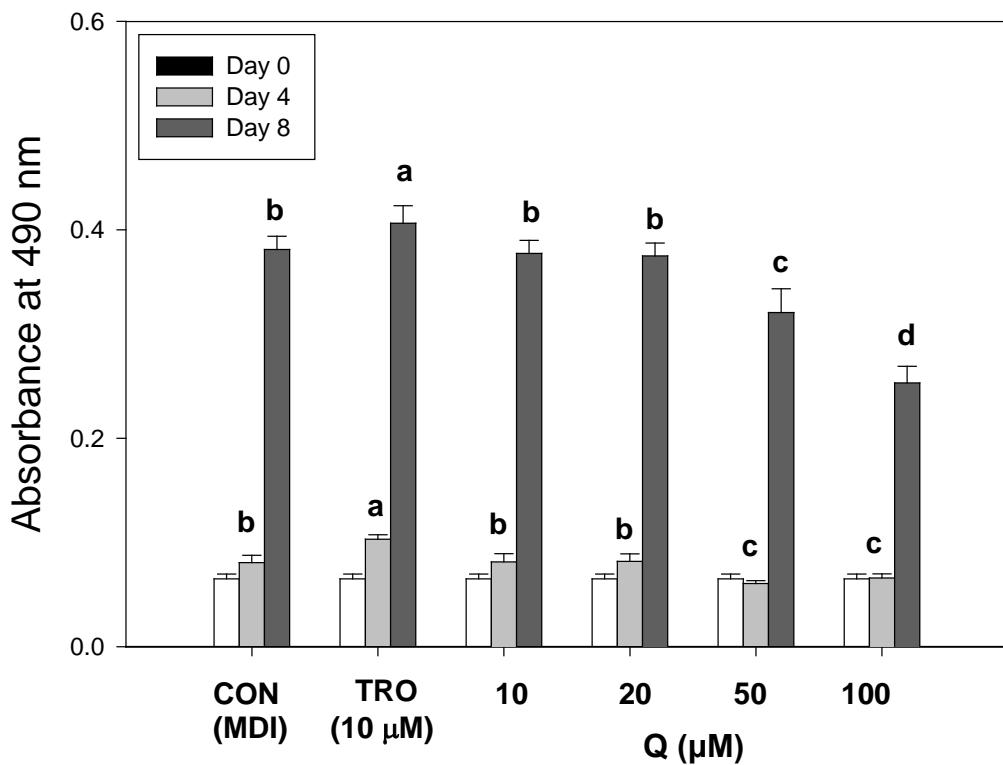


Figure 2. Quercetin dose-dependently inhibits lipid accumulation. 3T3-L1 preadipocytes were differentiated in the presence or absence of quercetin (10–100 μ M), and harvested at Day 0, 4, and 8 for Oil Red O staining to measure lipid accumulation. Troglitazone (Tro), a PPAR γ ligand, was used as a positive control. Compared to the control (CON) with just MDI differentiation media, quercetin treatment 50 and 100 μ M significantly decreased lipid accumulation dose-dependently at Day 4 and Day 8 of adipogenesis.

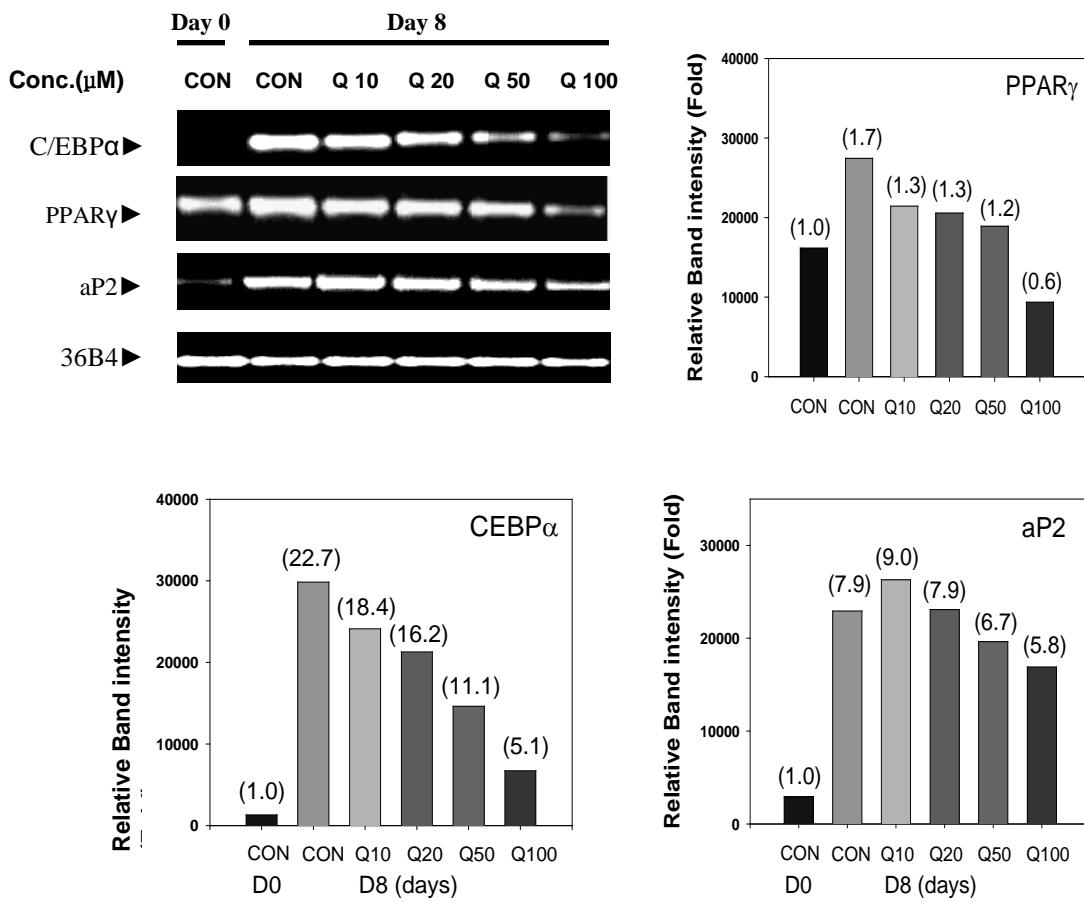


Figure 3. Quercetin inhibits adipogenic factors at the mRNA level. 3T3-L1 preadipocytes were differentiated in the presence or absence of quercetin (10-100 μ M), and harvested at Day 8 for mRNA analysis, by qualitative PCR. The major adipogenic regulators, PPAR γ , C/EBP α , and their target gene aP2 all increased in the control from Day 0 to Day 8. Quercetin treatment dose-dependently inhibited expression of these factors by Day 8, indicating quercetin inhibits adipogenesis through the down regulation of adipogenic transcription regulators.

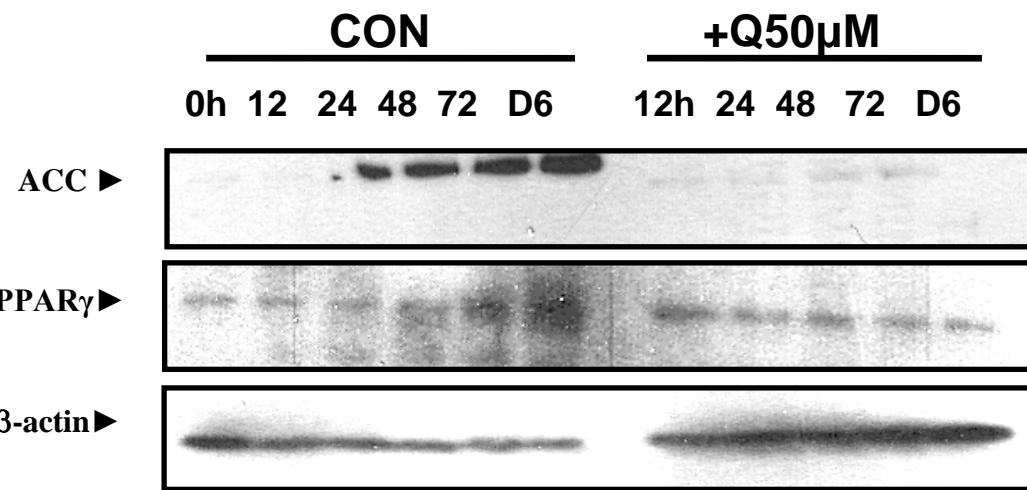


Figure 4. Quercetin inhibits adipogenic factors at the protein level. 3T3-L1 preadipocytes were differentiated in the presence or absence of quercetin (50μM), and harvested at sequential time points (0, 12, 24, 48, 72 hours, and Day 6) for protein analysis. Acetyl-CoA Carboxylase (ACC), an enzyme responsible for lipid synthesis, and PPAR γ , the transcriptional regulator of adipogenesis both increased in the control over the course of adipogenesis. Quercetin treatment (50μM) inhibited the increase of expression in both these adipogenic factors, especially the induction around 24 and 48 hours.

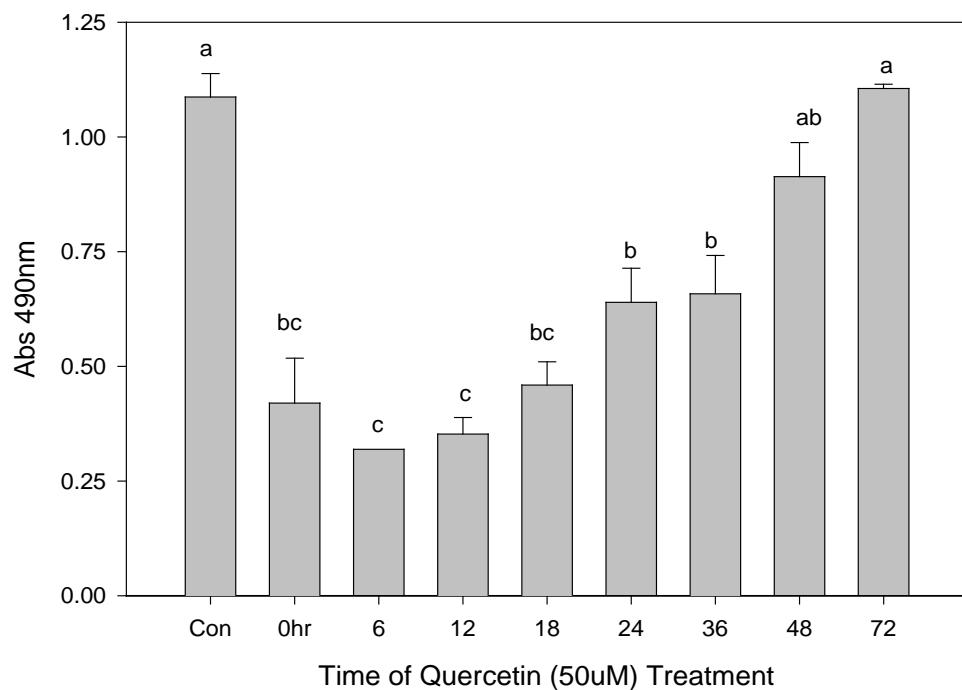


Figure 5. Quercetin's inhibitory effect on lipid accumulation is limited to early time points. 3T3-L1 preadipocytes were stimulated to differentiate at time 0 hour. Quercetin was added to differentiating media at subsequent times (0, 6, 12, 18, 24, 36, 48, and 72 hours) and continued through the duration of the experiment until Day 6 when all cells were harvested for Oil Red O staining to measure lipid accumulation. Quercetin inhibited lipid accumulation strongest when added between 0-18 hours, less but still significant at 24-36 hours, and no effect if added after 48 hours. These results indicate quercetin's anti-adipogenic effect is limited to early events of adipogenesis.

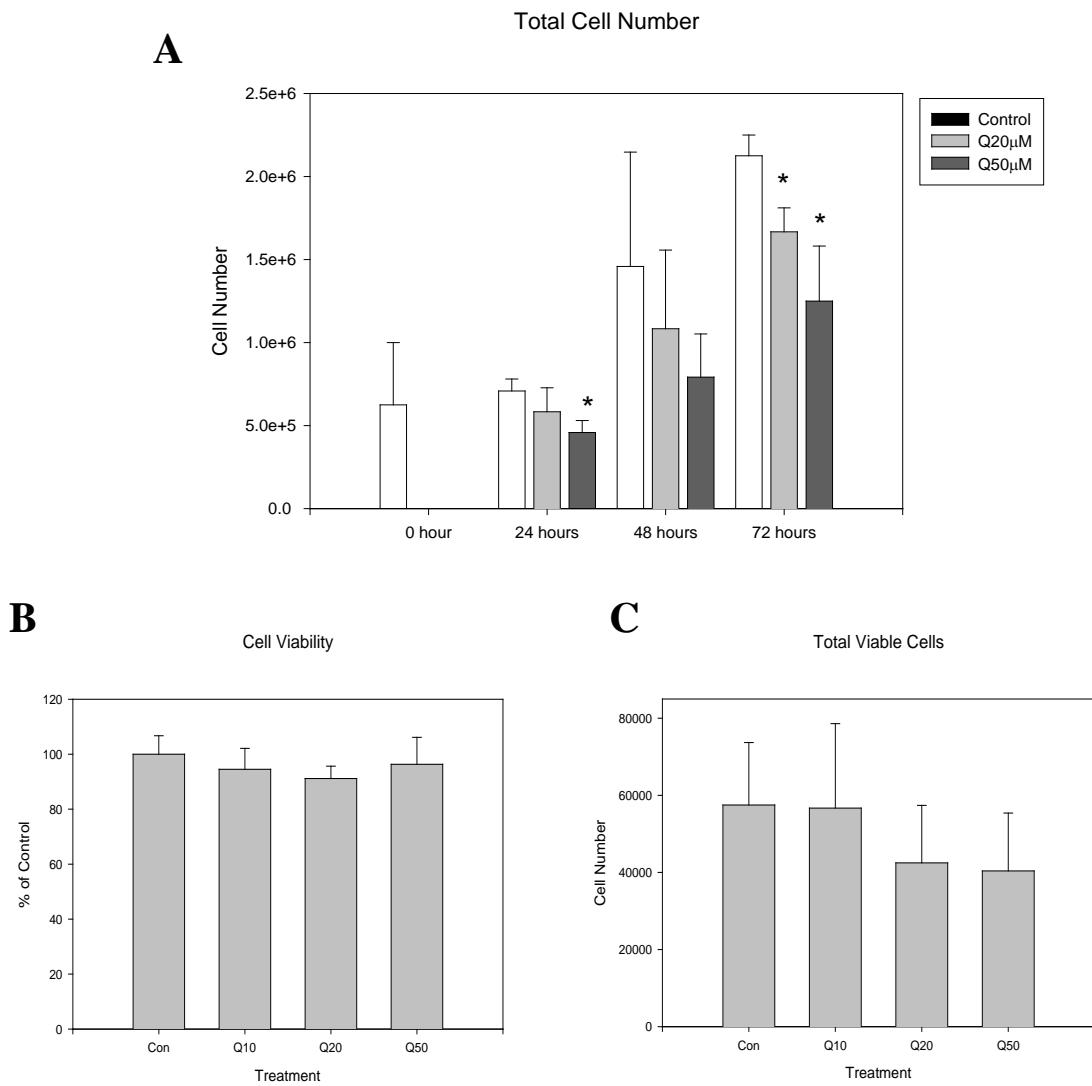


Figure 6. Quercetin does not affect cell viability but may inhibit cell number. Mitotic clonal expansion (MCE) occurs between Day 0-3 of differentiation in 3T3-L1 preadipocytes, dramatically increasing the number of cells in a well 2 or 3 fold by Day 3. (A) Trial 1: Cells were differentiated and harvested at 0, 24, 48, and 72 hours for cell counting by hemocytometer ($n=3$). The control clearly increased over 72hours. Quercetin significantly inhibited cell number at 24 and 72 hours. (B and C) Trial 2: Cells were differentiated in the presence or absence of quercetin ($50\mu M$) and harvested at Day 3 for Trypan Blue staining and counting by hemocytometer ($n=6$). Viable and non-viable cells were counted and % viable was determined by # of viable cells/ #of total cell, indicating the percent of living cells at Day 3. Quercetin treatment did not alter the % viable, indicating quercetin's effect is not cytotoxic. Although not significant, there was a clear trend indicating quercetin may decrease cell number at Day 3. Trypan Blue staining allows for the detection between viable and non viable and was thus employed during the second trial.

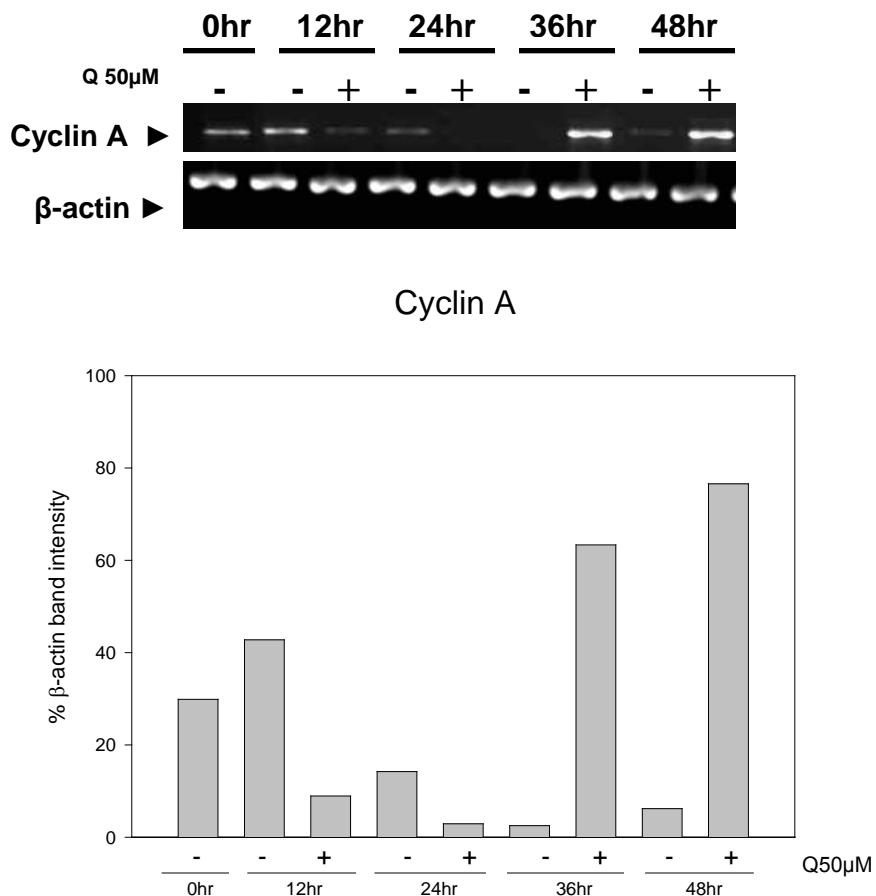


Figure 7. Quercetin delays mRNA expression of Cyclin A during early stages of adipogenesis. During mitosis, Cyclin A accumulates to regulate the transition from G0 to S phase. In 3T3-L1 preadipocytes stimulated to differentiate at time 0 hour, Cyclin A mRNA rose at 12 hours and fell by 24 hours. However quercetin (50µM) treatment, at time 0 hour, inhibited Cyclin A expression at 12 and 24 hours, delaying Cyclin A induction until 36 and 48 hours. A possible explanation is that quercetin is able to blunt reactive oxygen species signaling that is required for the stimulation of cell cycle during the first 24 hours but losses its anti-oxidant capacity by 36 hours allowing Cyclin A to accumulate. Thus, this data suggests quercetin is able to alter normal cell cycle events.

P 27

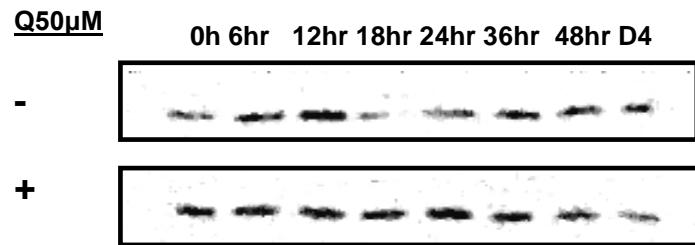


Figure 8. Quercetin sustains protein expression of P27 during the early stages of adipogenesis. P27 is a cyclin dependent kinase inhibitor that inhibits cell cycle progression. Cells undergoing mitosis transiently down regulate this protein to allow the transition from G0 to S phase. Preadipocytes stimulated to differentiate at time 0 hour transiently decreased protein expression of p27 between 18-24 hours. Quercetin (50 μ M) sustained p27 expression through the mitotic clonal expansion period. This data suggests quercetin inhibits the progression of cell cycle.

2.5 Discussion

Adipogenesis has been identified as a critical target in the progression of obesity and metabolic syndrome. In our model system, quercetin inhibited adipogenesis as seen by the inhibition of lipid accumulation and adipogenic factors at the mRNA and protein level. Our findings further suggest that quercetin may inhibit adipogenesis through altering early mitotic clonal expansion (MCE) events, as (1) quercetin's effect was limited to the early stages of adipogenesis (0-36 hours), (2) quercetin treatment tended to inhibit cell number, and (3) quercetin treatment altered the normal expression pattern of cell cycle related genes Cyclin A and p27.

During normal MDI stimulation, cells undergo several rounds of replication between Day 0 and Day 3 and dramatically increase cell number on the plate, as seen in Figure 6a. Quercetin significantly inhibited total cell # counts, but total viable cell counts from our second trial using Trypan Blue staining did not show significance. Although not significant, cell number counts showed a clear trend that quercetin inhibited cell number while not altering cell viability. We attribute the lack of significance to the limitation of counting by hemocytometer, which is a rough estimate of cell number. To obtain more accurate cell counts an automated device such as NucleoCounter, should be used.

Cyclin A and p27 regulate the transition from G0 to S phase in the cell cycle. Our findings indicate that quercetin altered this cell cycle regulation particularly between 6-18 hours post-MDI induction. Quercetin treatment delayed Cyclin A expression until 36 hours, where expression is then markedly increased. One possible explanation for this late onset is that over the first 24 hours of treatment quercetin was

able to quench reactive oxygen species (ROS) signaling necessary for the induction of cell cycle events [37], however, by the end of 24 hours quercetin's antioxidant capacity was depleted allowing the cell to mount one final attempt at cell cycle progression. We have further shown in preliminary data (see Appendix 1) that quercetin inhibits the mRNA expression of NADPH oxidase 4 (NOX4), a mitochondrial protein responsible for producing ROS required for differentiation [100]. Nonetheless, it is clear that quercetin prevented early induction of Cyclin A which facilitates the transition from S phase into G2 phase. To further determine quercetin's effect on cell cycle, Flow Cytometry should be employed to provide a quantitative profile of the number cells in each cell cycle stage.

MCE has been implicated to be required for terminal differentiation in 3T3-L1 adipocytes. 3T3-L1 pre-adipocytes in the presence of a mitotic inhibitor fail to differentiate [99]. However, other adipogenic models (10T1/2 MSCs, and Human MSCs) have been shown to differentiate independent of MCE, and reflect *in vivo* stimulation of precursors cells one step prior to preadipocyte commitment. This raises the question as to whether or not compounds previously characterized anti-adipogenic in 3T3-L1 systems are able to inhibit adipogenesis in models less dependent on MCE events. To pursue this question we tested quercetin in 10T1/2 cells, and found that quercetin (50 μ M) significantly inhibited lipid accumulation and that this effect was not time dependent (see Appendices 2 and 3). Although further work needs to be done in this model system, our preliminary data suggest that quercetin's effect is not limited to the 3T3-L1 model system, affirming that quercetin may inhibit adipogenesis *in vivo*.

Because AMPK has been implicated to inhibit differentiation and cell cycle progression, we investigated whether Compound C, an AMPK inhibitor, mediates quercetin's effect on adipogenesis, testing the hypothesis that quercetin may inhibit adipogenesis via AMPK activation. Both strong activation and strong inhibition of AMPK has been shown to inhibit adipogenesis suggesting AMPK is tightly regulated during these early stages. Compound C inhibits adipogenesis at high concentrations ($>1\mu\text{M}$), therefore lower concentrations were used (0.1-1 μM) in combination with quercetin (25 and 50 μM). Preadipocytes were stimulated to differentiate in the presence of both of these compounds. Lipid accumulation was measured at Day 6 (see Appendix 4). Compound C did not mediate quercetin's effect at any concentration, suggesting that quercetin may inhibit adipogenesis through AMPK-independent pathways. Protein analysis confirming Compound C's ability to block AMPK activation is needed to further demonstrate that Compound C is still effective at lower concentrations.

The physiological impact of inhibiting cell cycle and thus adipogenesis could be viewed positively or negatively: (1) it would be beneficial to mediate the accelerated rate of adipogenesis seen in childhood obesity, early stages of obesity, or in patients taking TZD drugs used for type 2 diabetes, or (2) it would be harmful to alter the body's natural response to increase fat storage, and potentially accelerate the hypertrophy of mature adipocytes leading to their dysfunction. Both views are valid at this point because of our limited knowledge of *in vivo* regulation of adipogenesis, however quercetin should not be thought to completely block adipogenesis but rather mediate an accelerated, irreversible, over-stimulation of adipogenesis. It is also

important to consider quercetin's effect on mature adipocytes, to gain a holistic view of quercetin's potential as an anti-obesity agent.

CHAPTER 3

EFFECT OF QUERCETIN ON ADIPOCYTE METABOLISM

3.1 Literature Review

The rise in obesity will significantly decrease life expectancy and continue to burden health care systems because of increased risk for the development of chronic disease [95]. Therefore, feasible, large-scale prevention and treatment options must be made available to the public in order to reverse this growing epidemic. At the cellular level, obesity is defined as the increase in size (hypertrophy) and number (hyperplasia) of adipocytes. Hypertrophy of adipocytes leads to their dysfunction as the endoplasmic reticulum and mitochondria are unable to keep up with increased metabolic demands. Dysfunctional adipocytes become insulin resistant and elicit macrophage induction to the adipose tissue further promoting the development of chronic disease [3]. Therefore understanding adipocyte metabolism and preventing adipocyte hypertrophy is a critical approach in the fight against obesity.

Adipocytes handle lipid storage through the synthesis (lipogenesis) and breakdown (lipolysis) of triglyceride. Naturally, there is much interest in factors that inhibit lipogenesis and promote lipolysis. However, adipocytes are also capable of burning stored fatty acids through β -oxidation, providing an exciting approach against hypertrophy. AMP-activated protein kinase (AMPK), the major metabolic regulator of all cells, has been shown to induce partial lipolysis and β -oxidation [57]. Therefore AMPK activators are one such way to metabolically alter adipocytes.

Several bioactive compounds have been identified as AMPK activators [16]. One such compound is quercetin, the most commonly consumed dietary flavonoid in the western diet. Not only has quercetin been implicated as an AMPK activator [13], *in vivo* studies have shown quercetin has the ability to improve hyperglycemia, dyslipidemia, and blunt excess weight gain in animal models of obesity and diabetes [7-10]. However the role of quercetin on adipocyte metabolism remains largely unknown.

Several reports have identified quercetin's effect on mature adipocytes to be lipolytic [101, 102], apoptotic [11, 13, 96, 98], and anti-inflammatory [103]. In the early 1990s, Kuppusamy et al. carried out experiments with several flavonoids and showed that quercetin, among others, inhibited phosphodiesterase break down of cAMP and increased lipolysis in the presence or absence of a lipolytic agent [102]. They further found that this effect was mediated through activation of β -adrenergic signaling, as the effect was inhibited by an β -adrenergic antagonist [101]. Furthermore, Ohkoshi et al. [104] found extract from Nelumbonecifera containing quercetin metabolites to also stimulate lipolysis in white adipose tissue. Thus, quercetin is generally recognized as lipolytic, however the mechanism remains unknown.

Currently, no studies have characterized quercetin's ability to activate β -oxidation, which is a likely hypothesis given quercetin's ability to activate AMPK.

3.2 Purpose of Study

Quercetin, a widely consumed dietary flavonoid, holds enormous potential as an anti-obesity agent. Recently quercetin has been implicated as an AMPK activator, bringing into question its ability to induce lipolysis and β -oxidation in adipocytes. We

therefore aimed to fill this gap of knowledge by testing quercetin's effect on lipolysis and fatty acid oxidation, specifically through the activation of AMPK. To test this, in mature 3T3-L1 adipocytes we investigated (1) quercetin's action on AMPK and downstream target ACC at the protein level, (2) quercetin's action on free fatty acid content in the media, and (3) quercetin's effect on mRNA expression of PGC-1 α , UCP-1 and UCP-3.

We hypothesized that (1) quercetin would increase protein levels of phosphorylated AMPK and the downstream target ACC, and that Compound C, an AMPK inhibitor, would inhibit this effect, (2) quercetin would increase free fatty acid (a lipolytic effect) and that Compound C would block this effect, and (3) quercetin would increase mRNA expression of PGC-1 α , UCP-1 and UCP-3 (genes involved in fatty acid oxidation) and that Compound C would block this effect.

3.3 Materials and Methods

3.3.1 Cell Culture and Treatments

For the differentiation of 3T3-L1 preadipocytes, cells were grown to 100% confluence in Growth Media [DMEM (high glucose), 10% Calf Serum, 1% Penicillin/Streptomycin] replaced every two days. Two days post-confluence, growth media was changed to Differentiation Media [DMEM (high glucose), 10% Fetal Bovine Serum, 1% P/S, 1% Insulin, 1% 3-Isobutyl-1-methylxanthine 11.5mg/ml, 0.01% Dexamethasone 3.9mg/ml] in the presence or absence of quercetin aglycone (Sigma-Aldrich Catalog # 6151-25-3). Three days post-differentiation, media was replaced with

Post-Differentiation media [DMEM (high glucose), 10% Fetal Bovine Serum, 1% P/S, 1% Insulin, +/- quercetin]. 3T3 L1 preadipocytes generally reach 80 -90% differentiation 8 to 9 days post MDI induction. At this point cells were washed with PBS to remove residual serum and insulin, and media was replaced with serum and insulin free DMEM supplemented with 0.5% bovine serum albumin (BSA) in the presence or absence of treatment. (Quercetin : 25, 50 , 100 μ M, AICAR: 0.5, 1 mM, and Compound C: 10 μ M). Treatment lasted 15-48 hrs at which point cells and media were harvested for analysis.

3.3.2 Oil Red O Lipid Staining

Cells were harvested on desired days and underwent Oil Red O Staining to quantify lipid accumulation, as it is an indirect determiner of cell differentiation. Cells, grown on 24-well and 6-well plates, were treated with 10% formaldehyde in PBS for 1 hour, washed with 60% isopropanol, and completely dried. Then, cells were stained with 0.5% Oil Red O solution in 60:40 (v/v) isopropanol: H₂O, for 30 minutes at room temperature. Finally wells were washed with distilled water and dried. Optical density was then measured at 490nm, after eluting with isopropanol, to quantify lipid accumulation.

3.3.3 Protein Isolation and Western Blotting

Cells were harvested at desired times with RIPA Buffer containing protease inhibitor (500ul per p-100 dish). Samples were stored at -80° C until protein quantification. Samples were thawed on ice, sonicated, and centrifuged. Supernatant, containing whole

cell protein, was transferred to a fresh tube for protein quantification using the Bicinchoninic Acid (BCA) assay. A BCA standard curve was established using bovine serum albumin (BSA) [2mg/ml] protein and measured at 570nm. Sample concentrations were then measured against the standard, and prepared with sample buffer to allow for 15 μ g to be loaded to the gel. Samples were run on acrylamide (varying percentages) gels via electrophoresis, and then proteins were transferred to a PVDF membrane through wet transfer. Upon successful transfer, membranes were blocked with 5% non-fat dry milk, and treated with primary and secondary antibodies (purchased from Santa Cruz Biological). HRP detection was done through ECL solution. Chemiluminescent bands were captured on x-ray and developed.

3.3.4 RNA isolation and Analysis

Cells washed with phosphate buffer solution were harvested with Trizol Reagent and stored at -80° C until mRNA isolation. For isolation, samples were thawed and centrifuged with 200 μ L chloroform. Supernatant was transferred to a fresh tube and centrifuged with isopropanol (1:1) to precipitate RNA. Isopropanol was removed and the pellet was washed three times with ethanol by centrifugation. Pellet was resuspended in DEPC water and quantified using a spectrophotometer at 260nm. 8 μ g of RNA was used along with SuperScript III reagents to make cDNA which was stored at -4°C. Polymerase Chain Reaction was performed with the primers for PGC-1 α , UCP-1, UCP-3, and adiponectin (see Table 2). Samples were run on an agarose gel containing Ethidium Bromide and detected using UV light. Band intensity was determined using Image J analysis and adjusted to β -actin.

3.3.5 Free Fatty Acid and Glycerol Assays

Media was collected from wells and stored at 4°C. *Free Glycerol Assay* (K630-100) and *Free Fatty Acid Quantification Kits* (K612-100) were purchased from BIOVISION. Product protocols were followed. In brief, reagents were warmed to room temperature, standard curves and samples (undiluted) were added to a 96-well plate. Reagent mix was added and plates were incubated for the appropriate time. Absorbance was taken at 570nm.

3.3.6 Statistical Analysis

Samples were collected in at least duplicate or triplicate, and differences between the means were determined by student T-test analysis. P-values were considered significant at <0.05.

3.4 Results

3.4.1 Quercetin phosphorylates ACC, a downstream target of AMPK

Quercetin has been implicated as an AMPK activator; therefore we tested in our model system whether or not quercetin activates AMPK and its downstream target, acetyl-CoA Carboxylase (ACC). Quercetin treatment (50μM) increased activated AMPK as compared to standard MDI treatment over the course of differentiation (Figure 9A). Further, quercetin treatment (25-100μM) increased phosphorylated ACC, and this effect was blocked by the AMPK inhibitor Compound C (Figure 9B). This confirms that we have a working model to study quercetin's action dependent on AMPK activation.

3.4.2 Quercetin induces partial lipolysis

We next looked at quercetin's ability to induce lipolysis by measuring release of free fatty acids in the media of mature adipocytes treated with serum and insulin-free media. Our data pooled from 3 separate trials (total n = 7) show that quercetin (100 μ M) significantly increased the release of free fatty acids significantly and Compound C (10 μ M) blocked this effect (Figure 10).

3.4.3 Quercetin induces oxidative pathways

Because AMPK is known to induce β -oxidation, we looked at quercetin's ability to induce the expression of PGC-1 α , a transcriptional regulator of oxidative genes, Uncoupling Protein 1 (UCP-1) and Uncoupling Protein 3 (UCP-3), two of the uncoupling proteins expressed in the mitochondria of muscle and brown adipose tissue to uncouple oxidation from ATP synthesis. Mature adipocytes were treated with quercetin (25-100 μ M) for 24 hours in serum-free insulin-free media. Quercetin increased mRNA expression of PGC-1 α , and this effect appeared to be attenuated by Compound C treatment (Figure 11). Further, quercetin treatment induced mRNA expression of UCP-1 and UCP-3 (Figures 12 and 13). Upregulation of UCP-3 by quercetin appeared to be attenuated by Compound C treatment. These results suggest quercetin is able to induce uncoupled fatty acid oxidation through the activation of AMPK.

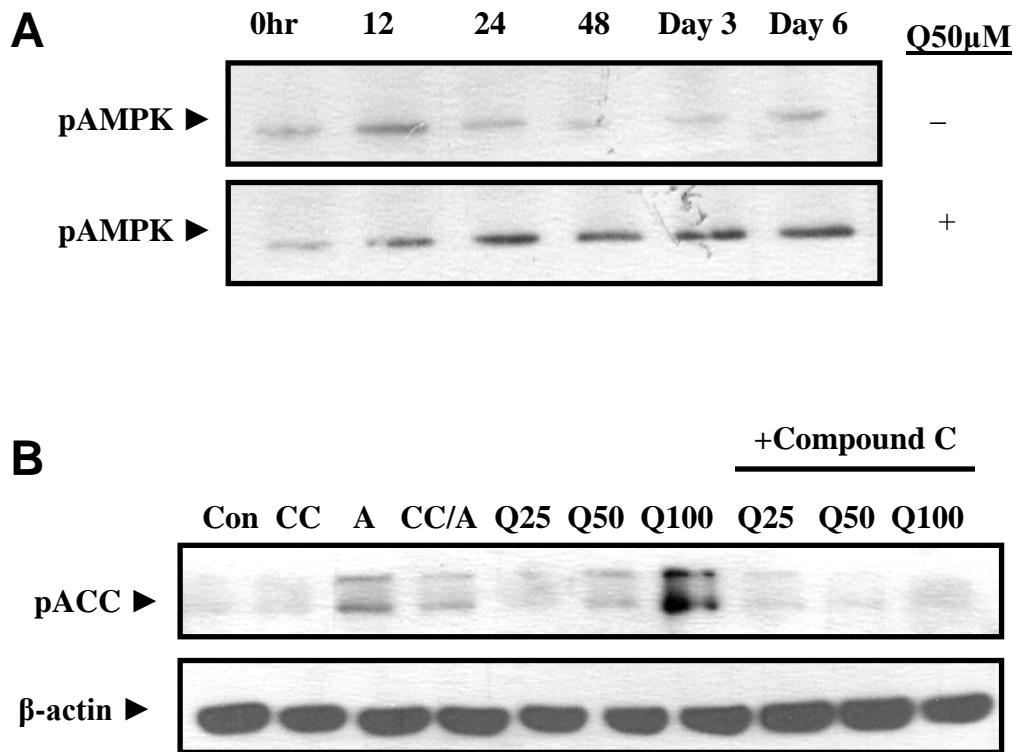


Figure 9. Quercetin increases protein expression of pAMPK and pACC.
 Protein analysis of pAMPK and downstream target pACC. (A) 3T3-L1 preadipocytes were differentiated in the presence or absence of quercetin ($50\mu\text{M}$) and harvested at subsequent time points throughout the course of adipogenesis to determine AMPK activation. In the control pAMPK increased at 12 hour but then decreased to a basal level from 24 hour to Day 6. Quercetin treatment sustained pAMPK levels through the course of adipogenesis. (B) Mature adipocytes were treated for 48 hours in serum-free insulin-free %0.5 BSA DMEM in the presence or absence of Compound C (CC) $10\mu\text{M}$, AICAR (A) 1mM , or quercetin (Q) $25-100\mu\text{M}$. AICAR treatment increased pACC protein levels and CC attenuated this effect. Quercetin treatment dose-dependently increased pACC expression and CC also attenuated this effect. These results indicate quercetin activates AMPK pathways and CC, and AMPK inhibitor successfully blocked AMPK's downstream action.

Free Fatty Acid Release

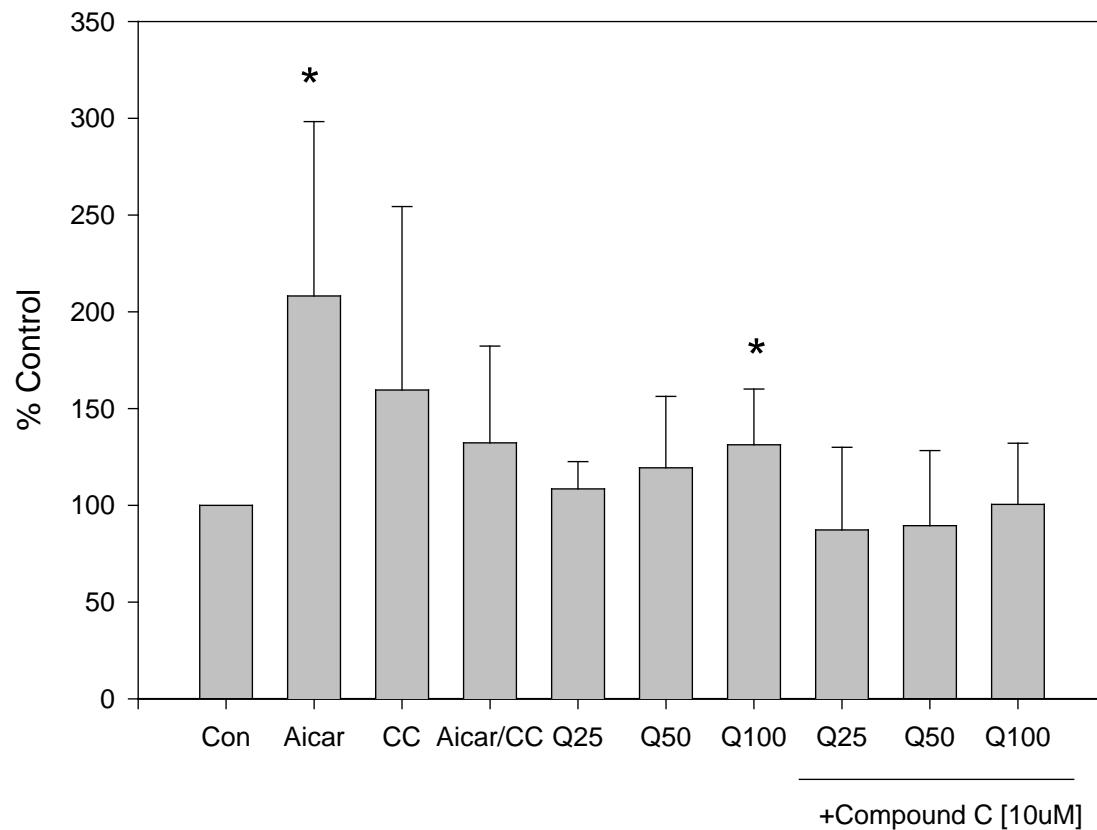


Figure 10. Quercetin 100 μ M increases free fatty acid content in media.

Mature adipocytes were treated with Compound C (CC) 10 μ M, Aicar (A) 0.5 and 1mM, and quercetin (Q) 25-100 μ M for either 24 or 48hrs and media was collected and analyzed for free fatty acid content. Data presented here reflects pooled data from 3 separate trials each slightly varied by length of treatment or AICAR concentration (n=7). AICAR treatment significantly increased FFA content in the media while CC attenuated this effect. Likewise quercetin (100 μ M) significantly increased FFA content and CC attenuated this effect suggesting quercetin is able to stimulate FFA release through AMPK activation.

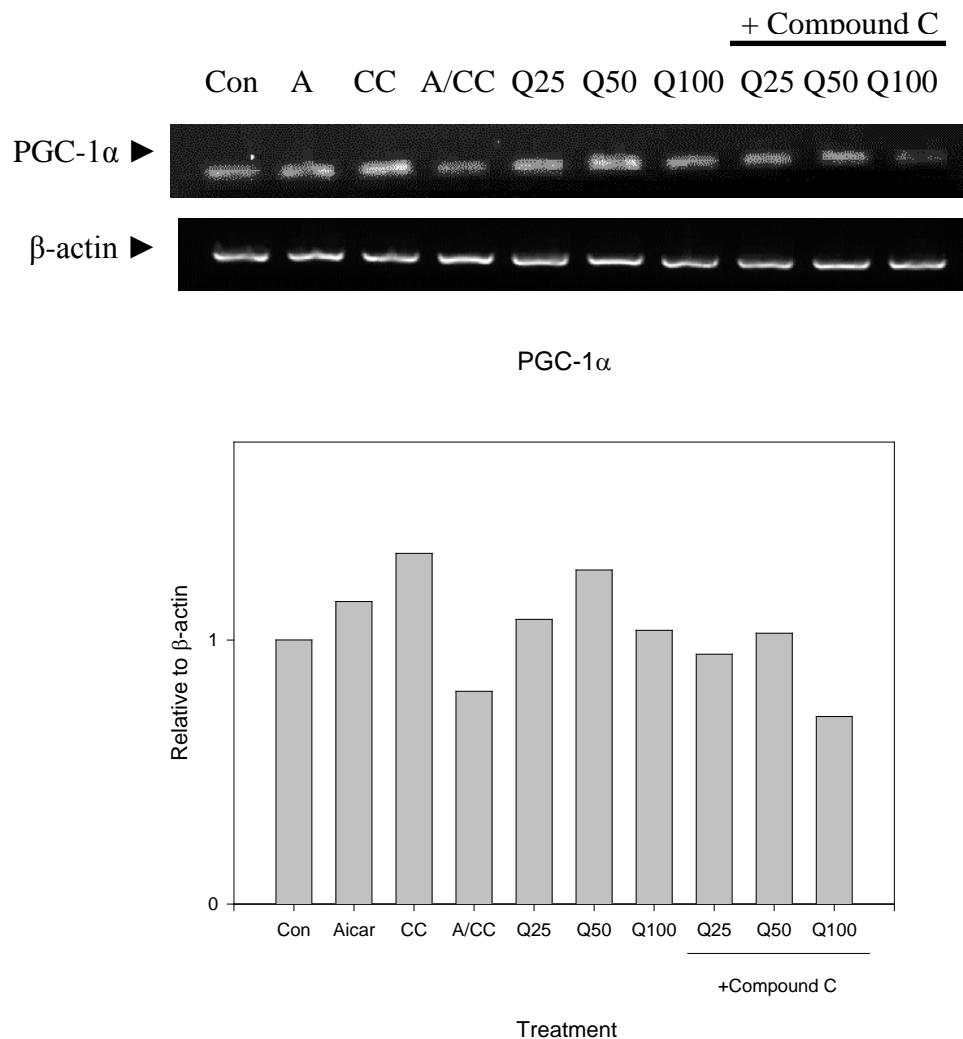


Figure 11. Quercetin up-regulates mRNA expression of PGC-1 α . PGC-1 α is a transcriptional regulator of β -oxidation among other metabolic pathways. 3T3-L1 mature adipocytes were treated with AICAR (A) 1mM, Compound C (CC) 10 μ M, and quercetin (Q) 25-100 μ M for 24 hours and harvested for mRNA analysis by qualitative PCR. AICAR increased PGC-1 α expression and CC blunted this effect. Quercetin also increased PGC-1 α expression and CC attenuated this effect. This data suggests quercetin is able to increase PGC1 α mRNA through an AMPK dependent pathway.

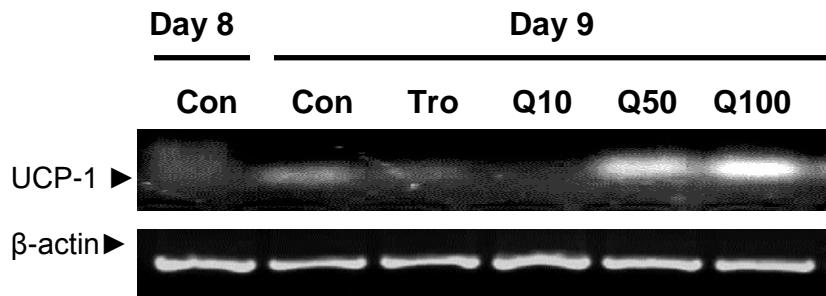


Figure 12. Quercetin up-regulated mRNA expression of Uncoupling Protein 1. Uncoupling Protein 1 (UCP-1) is the major thermogenic gene expressed predominantly in muscle and brown fat. UCP1 uncouples ATP synthesis from mitochondrial oxidation. 3T3-L1 mature adipocytes were treated with quercetin (10-100 μ M) for 24 hours. Slight UCP-1 expression was induced in the control between Day 8 and 9, however quercetin 50 μ M and 100 μ M significantly increased UCP-1 expression. Troglitazone (Tro 10 μ M), a diabetic drug and PPAR γ ligand had no effect on UCP-1 expression.

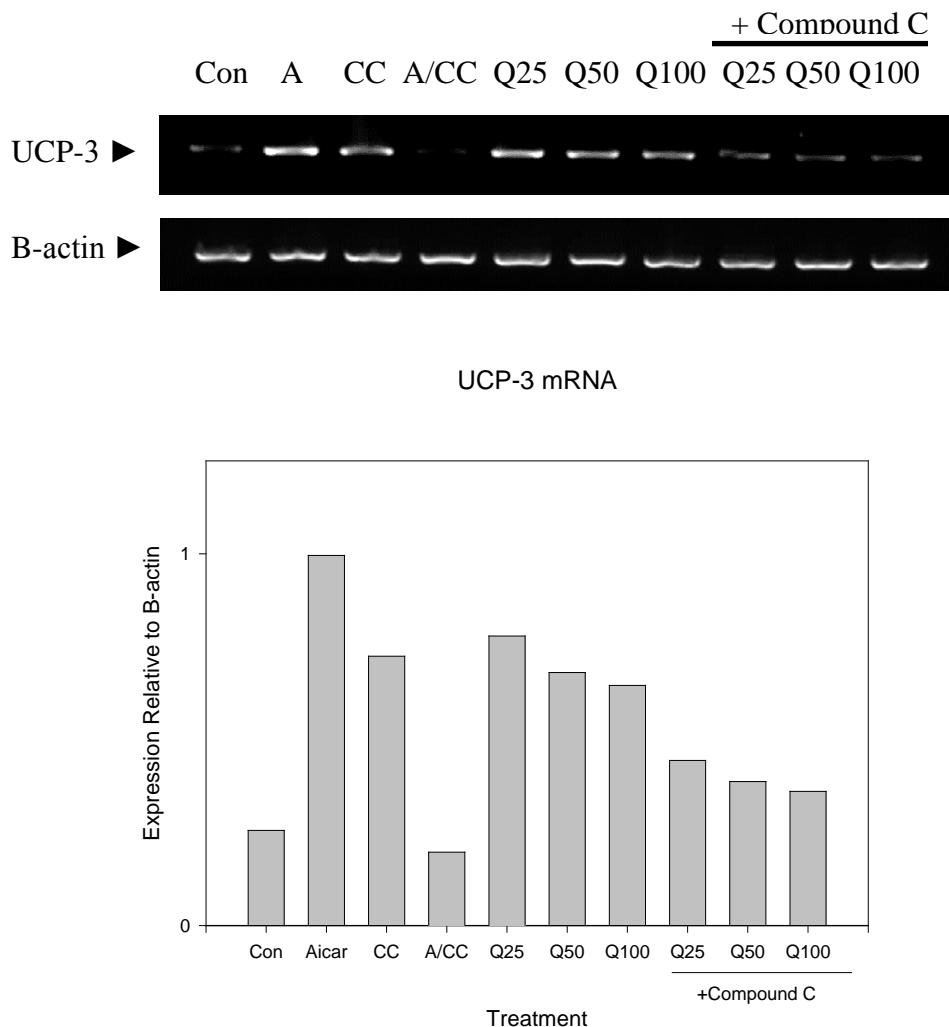


Figure 13. Quercetin increased mRNA expression of Uncoupling Protein 3. Uncoupling Protein 3 (UCP-3) is part of the group of uncoupling proteins that uncouples ATP synthesis from oxidation in the mitochondria and is a transcriptional regulator of β -oxidation among other metabolic pathways. 3T3-L1 mature adipocytes were treated with AICAR (A) 1mM, Compound C (CC) 10 μ M, and quercetin (Q) 25-100 μ M for 24 hours and harvested for mRNA analysis by qualitative PCR. AICAR increased PGC-1 α expression and CC blunted this effect. Quercetin also increased PGC-1 α expression and CC attenuated this effect. This data suggests quercetin increases PGC-1 α mRNA through an AMPK dependent pathway.

3.4 Discussion

There is great potential in the use of bioactive compounds to alter adipocyte metabolism and thus thwart the progression of adipocyte dysfunction and metabolic syndrome. Our preliminary findings suggest quercetin is one such compound.

In our model system we clearly showed quercetin's ability to activate AMPK, a target in the treatment of metabolic syndrome.

AMPK is the major metabolic regulator of the cell, regulated by the level of AMP/ATP. As AMP levels rise, more AMP binds to the gamma subunit of AMPK causing a conformational change protecting AMPK from being dephosphorylated. Thus a rise in AMP results in increased phosphorylated AMPK. It has been determined that AMPK is activated by low glucose, hypoxia, ischemia, adiponectin, leptin, and alpha adrenergic receptor. It would be of interest to further investigate the mechanism by which quercetin activates AMPK. It is possible that quercetin creates a hypoxic environment because of its antioxidant capacity. However, it has also been suggested that quercetin activates adrenergic receptors to mediate lipolysis, thus it is plausible that quercetin may interact with membrane bound receptors [101]. It has been also been shown that quercetin is able to penetrates the membrane through passive diffusion and significantly accumulates in the mitochondria [105]. In fact, Gledhill et al. determined that quercetin binds and inhibits the rotary mechanism of the F1-ATPase, required for ATP synthesis in the mitochondria [106]. This action may suggest quercetin activates AMPK by lowering the ATP/AMPK ratio in the cell; however, this mechanism would need to be further tested.

AMPK has been implicated to induce partial lipolysis and β -oxidation. We therefore investigated quercetin's effect on both of these pathways. As shown, quercetin stimulated free fatty acid release into the media, and this effect was blunted by the AMPK inhibitor, Compound C. Figure 10 consists of pooled data from 3 separate trials, each with a slightly different experimental design. Therefore these trials should be repeated with a consistent design. From our experience, the best design would be to treat mature adipocytes (80% differentiated) in serum-free, insulin-free DMEM supplemented with 0.5% Bovine Serum Albumin (BSA) for 48hrs using AICAR (1mM) and Compound C (10 μ M) concentrations.

Interestingly, quercetin significantly reduced glycerol release in the media, and Compound C did not consistently reverse this effect in all treatments (see Appendix 5). This finding suggests quercetin induces partial lipolysis (the release of free fatty acids but not glycerol), and this effect may be through AMPK-independent pathways, as AICAR treatment not alter glycerol release. It has been implicated that AMPK deactivates HSL the major lipolytic enzyme, however ATGL is still available in the cytoplasm to begin TG breakdown by releasing the first free fatty acid (FFA) [52]. Therefore, if HSL is inactivated by AMPK incomplete lipolysis occurs, which explains why free fatty acid is released but not glycerol. However, this explanation does not account for the significant decrease in glycerol release seen with quercetin treatment; therefore quercetin may act independently of AMPK on glycerol metabolism. It would be of great interest to explore the mechanism behind this observation, specifically by looking at the activity of glycerol-3-phosphate dehydrogenase in the mitochondria, and

aquaporin 3 and 9, which are membrane bound glycerol transporters, all of which regulate glycerol release during lipolysis.

Quercetin increased PGC-1 α , UCP-1 and UCP-3 mRNA expression, suggesting quercetin induces uncoupled oxidation in the cell. These preliminary findings should be repeated, as results here only reflect one trial and qualitative RT-PCR is not as precise as real-time PCR analysis. Interestingly, it appears that Compound C blunted quercetin's ability to induce PGC-1 α , and UCP-3, suggesting that AMPK activation may be the mechanism by which quercetin induces oxidation. Quercetin's effect on UCP-1 expression in the presence of Compound C was not investigated here and is worth exploring. Other researchers found that chronic AMPK stimulation through AICAR induced mitochondrial biogenesis and fatty acid oxidation , but did not stimulate UCP-1 expression [48, 107].Therefore it is likely that quercetin may stimulate UCP-1 through an AMPK-independent pathway. These exciting preliminary findings showing quercetin ability to stimulate uncoupled β -oxidation would have far reaching effects if translated into human cells lines and further into *in vivo* models. Potentially quercetin could enhance β -oxidation in hypertrophied adipocytes enabling them to prevent or recover from metabolic dysfunction, from the burden of excess lipid.

Although mRNA expression seen in this research suggests quercetin induces uncoupled fatty acid oxidation, further research needs to be done to confirm this effect. In particular it would be of interest to determine whether or not quercetin increases oxygen consumption, which would be an indication of increased mitochondrial function. Quercetin did not significantly decrease triglyceride content in mature adipocytes over a 48hr period in our model system (see Appendix 6). In animal studies, quercetin doses

did result in weight loss [7] suggesting prolonged treatment of quercetin might be necessary to see dramatic changes in adipocyte lipid content. Increased uncoupled oxidation of free fatty acids in adipose tissue provides a possible mechanism for quercetin's effect.

Quercetin's ability to stimulate partial lipolysis and uncoupled fatty acid oxidation *in vivo* could potentially prevent and/or alleviate the progression of hypertrophied adipocytes. It is carefully noted that releasing free fatty acids into circulation is not ideal given cardiovascular risk; however partial lipolysis would evoke a mild effect *in vivo*, because essentially only a small fraction of the broken down triglyceride would be released. The remaining diglyceride would be further metabolized in the cell, likely through uncoupled β -oxidation. Therefore, these findings suggest that quercetin may improve signs of metabolic syndrome, specifically hyperglycemia and insulin sensitivity, *in vivo* by preventing or ameliorating the lipid burden in hypertrophied adipocyte through AMPK mediated metabolic changes.

CHAPTER 4

CONCLUSION & FUTURE DIRECTIONS

4.1 Summary

Obesity is one of the most pressing concerns of health care officials because of its increasing prevalence and significant link to the development of type 2 diabetes, cardiovascular disease and cancer. At the cellular level obesity is defined as the increase in cell number (hyperplasia) and cell size (hypertrophy), resulting in the irreversible expansion of adipose tissue in unwanted depots and the dysfunction of adipocytes leading to the development of metabolic system. Our findings here demonstrate that quercetin, the most widely consumed dietary flavonoid, shows promising potential as an anti-obesity agent because of its ability to target both hyperplasia and hypertrophy in our *in vitro* model.

We found that quercetin inhibited adipogenesis in 3T3-L1 preadipocytes through altering cell cycle events, specifically cell number, and expression of cell cycle regulators Cyclin A and p27. These findings are significant as *in vivo* hyperplasia has been implicated to be tightly controlled by cell cycle [35]. In 10T1/2 mesenchymal stem cells, a cell line one step removed from preadipocyte commitment, quercetin significantly inhibited adipogenesis, further supporting quercetin's potential as an anti-adipogenic agent *in vivo*. Quercetin has been implicated as an AMPK activator [13], and further either strong inhibition or activation of AMPK inhibits adipogenesis through MCE events. However our preliminary findings did not indicate that quercetin's anti-adipogenic effect is AMPK dependent.

In mature adipocytes, quercetin induced lipolytic and oxidative pathways. Quercetin significantly increased free fatty acid content in the media, and up-regulated expression of PGC-1 α , Uncoupling Protein 1 (UCP-1) and 3 (UCP-3). Compound C, an AMPK inhibitor, blunted these effects suggesting quercetin's action is AMPK dependent. These initial findings indicate quercetin may be able to shift adipocytes toward energy burning pathways, which could alleviate adipocyte hypertrophy *in vivo*.

4.2 Limitations

There are a few limitations of the work presented here that are important to discuss. First is the concentration of quercetin used in our *in vitro* model. Physiological levels generally approach the 200nM range, however the concentration used in our experiments were 25, 50, and 100 μ M. These *in vitro* levels have never been reached in humans, and would likely only result from enhanced supplementation such as nanoparticle delivery of quercetin. Nonetheless, *in vitro* studies allow researchers to understand mechanistic aspects of quercetin's action.

A second limitation we encountered was the use of Compound C to investigate whether quercetin's anti-adipogenic effect was AMPK dependent. Compound C at high doses (5-10 μ M) inhibited adipogenesis (data not included), therefore lower concentrations (0.1-1 μ M) were used in combination with quercetin. However, this brings into consideration Compound C's effectiveness to inhibit AMPK at such a low

dose. Therefore, other strategies for determining whether quercetin anti-adipogenic effect is through AMPK activation need to be developed.

A third limitation was sample size, due to time constraints. Most of the work presented here is in the preliminary stage and needs to be repeated in order to gain statistical significance. However we were able to gain valuable insight from these initial findings.

Finally a major limitation not encountered here but commonly encountered with human supplementation trials of quercetin is bioavailability. To date, human studies have only shown quercetin supplementation to reduce systolic blood pressure [91]. Quercetin has been shown to be safe at high doses [108] therefore pharmacological doses can be used. However absorption is still variable from individual to individual because of the gut microflora's ability to metabolize quercetin. One possible way to overcome this limitation is through the use of nanoparticle quercetin delivery, which is currently being developed [73, 109].

4.3 Significance of findings in the context of obesity and metabolic syndrome

In vivo animal studies have shown quercetin to ameliorate metabolic syndrome, specifically insulin resistance, hyperglycemia, and weight gain. Our findings here of quercetin's effect on adipocytes help speculate on the mechanisms behind quercetin's *in vivo* effects.

4.3.1 Weight Management

Quercetin's ability to stimulate oxidative pathways, specifically thermogenesis, through AMPK activation is an exciting new finding that suggests quercetin may be able to attenuate weight gain and help promote weight loss in obese individuals. Currently a major focus of anti-obesity drug designers is targeting thermogenesis to increase resting metabolic rate [110]. It is known that those with lower daily energy expenditure are more susceptible to obesity [111]. As an individual's body mass increases, their resting metabolic rate will increase to maintain the excess tissue, however it has been shown that after weight loss, post-obese individuals have a reduced metabolic rate [112]. Further fatty acid oxidation is impaired in obese individuals, even after weight loss [110, 113]. The only uncoupling drug used in humans, dinitrophenol, resulted in weight loss up to 3kg per week, but caused sweating and worse hypoxia [114]. Therefore milder thermogenic agents are currently being pursued to obtain similar weight loss effects at a safer level.

Quercetin's ability to increase metabolic rate has been determined in animals but not yet in humans. Stewart et al. [115] looked at energy expenditure, as measured by indirect calorimetry, in C57BL/6J mice fed a high-fat diet with quercetin (0.8g/kg) for 8 weeks. At week 3, energy expenditure was significantly higher ($p<0.05$) in the quercetin group over the control, however at week 8 there was no difference observed between the two groups. Based on this finding in animals, and quercetin's proposed ability to stimulate oxygen consumption in muscle cells [116], Egert et al. conducted a pilot study in humans to test quercetin's ability to increase metabolic rate. Six healthy normal-weight women were given 150mg doses of quercetin and respiratory

consumption of oxygen was measured 5 min – 3 hours post-ingestion, however no significant difference was found with quercetin treatment. This result is likely due to the small sample size (n=6) and lower dosage of quercetin (300 times lower than Stewart et al. dosage in rats), therefore plasma quercetin concentrations may have been below the threshold for any observed metabolic effect. Our findings that quercetin increases mRNA expression of UCP-1, UCP-3 and PGC-1 α in 3T3-L1 adipocytes strongly supports continued efforts to determine quercetin's metabolic effects *in vivo*.

4.3.2 Insulin Sensitivity

Quercetin has repeatedly been shown to improve hyperglycemia and insulin sensitivity in animal models. Our findings that quercetin stimulates AMPK, induces β -oxidation, and increases adiponectin mRNA expression all suggest potential mechanisms for quercetin's *in vivo* effect. AMPK is a major target for drug companies AMPK has been shown to improve glucose uptake *in vitro* and improve hyperglycemia *in vivo*. Furthermore, the commonly prescribed class of diabetic drugs, Thiazolidinediones (TZDs), have been shown to activate AMPK [117]. Chronic use of these drugs has been associated with negative side-effects therefore milder activators of AMPK are wanted to allow for long-term use [66, 118]. We clearly showed quercetin's ability to activate AMPK in 3T3-L1 preadipocytes. Therefore, quercetin holds great potential as an anti-diabetic agent, and AMPK activation is likely the mechanism behind quercetin's *in vivo* effect. So far quercetin has been shown to improve glucose uptake in adipocytes [119] and in muscle cells [120], and AMPK has been implicated in the mechanism.

One area that warrants future research is quercetin's ability to increase adiponectin levels and the anti-diabetic effects associated with that. Adiponectin levels are negatively correlated with obesity and type 2 diabetes [121], and further administration of adiponectin in rats improves glucose regulation [122]. Quercetin treatment has been shown to increase circulating adiponectin levels in animal models [7, 8, 123]. To our knowledge no human studies have explored quercetin's ability to increase circulating adiponectin levels and our preliminary findings that show quercetin's ability to increases mRNA expression of adiponectin in mature adipocytes certainly warrant this exploration.

4.4 Application of Quercetin from a Public Health Perspective

Estimating quercetin content in plants is extremely limited due to the variability in flavonoid production based on environmental as well as genetic factors [65], therefore limited data exists on the correlation between quercetin intake and chronic disease. Because of this missing link, it is too soon for public health officials to develop feasible recommendations to the public for the use of quercetin as an anti-obesity agent.

Our working knowledge, based largely on supplementation studies done by Egert et al., is that 150mg supplemental doses (roughly the equivalent of 12-15 servings of fruits and vegetables) may not be sufficient to elicit the powerful health benefits found in animal models using nearly 200 to 300 times the dosage amount. Therefore it can be speculated that the future use of quercetin as an anti-obesity agent will be

through supplementation or product enhancement, from which a quercetin intake of around 1000mg could be reached.

This gap in our current knowledge should not however detract from the recommendation of health officials to increase consumption of quercetin through intake of fruits and vegetables, specifically those high in quercetin (onions, apples, kale, etc.). It is likely that sustained lower doses (50-100mg, the equivalent of roughly 5-8 servings of fruits and vegetables) would have a preventative effect, while higher doses would be required to use quercetin therapeutically to treat conditions related to obesity.

4.5 Direction of Future Research

4.5.1 Quercetin's effect on adipogenesis

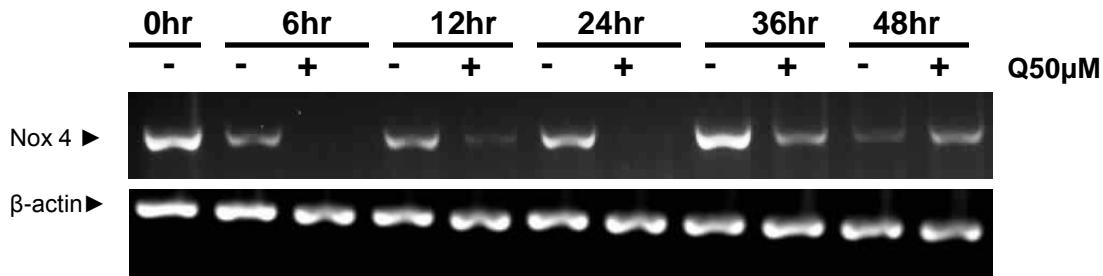
Although our findings here suggest quercetin inhibits adipogenesis through altering cell cycle events, this effect should be further confirmed *in vitro* through the use of (1) a more accurate cell counting technique, NucleoCounter, (2) Flow Cytometry, to quantify the number of cells in each cell cycle stage, and (3) human cell lines that may behave differently than 3T3-L1 preadipocyte because of pluripotency stage. Once quercetin's effect has been confirmed *in vitro*, animal studies should be employed in order to determine quercetin's ability to inhibit hyperplasia in various adipose depots.

4.5.2 Quercetin's effect on mature adipocytes

Our preliminary findings suggest quercetin may induce partial lipolysis and shift the cell toward uncoupled β -oxidation. To further confirm this effect *in vitro* (1) our experiments should be repeated at least two times to gain validity, and (2) oxygen

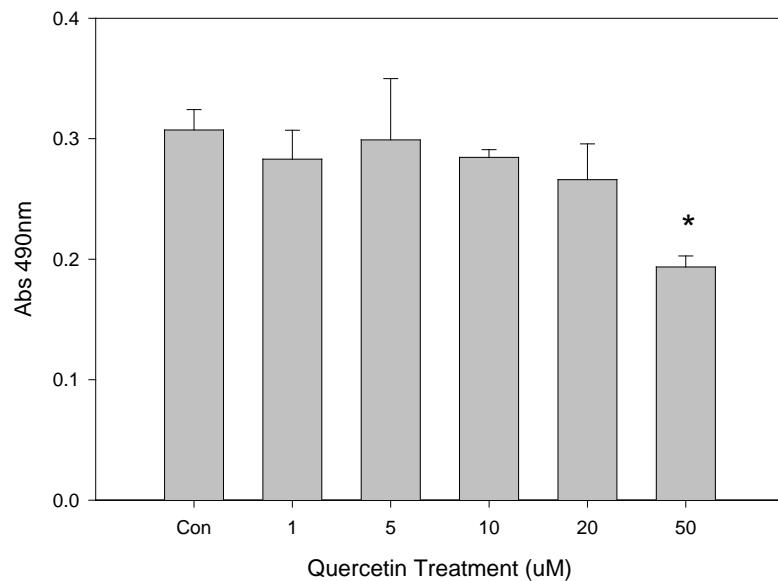
consumption should be analyzed to determine quercetin's ability to increase mitochondrial oxidation. Next it would be of interest to determine whether quercetin decreases adipocyte size in an obese animal model. This would confirm quercetin's ability to ameliorate hypertrophied cells and the consequences associated with them. It should also be determined whether quercetin improves adiponectin levels *in vivo* as our findings indicate quercetin increases mRNA expression of adiponectin.

APPENDIX 1
QUERCETIN MAY INHIBIT NOX4 EXPRESSION DURING THE EARLY
STAGES OF ADIPOGENESIS



Appendix 1. NADPH oxidase 4 (NOX4) is a major producer of reactive oxygen species (ROS) in the cell. During mitotic clonal expansion NOX4 has shown to be required for ROS signaling that promotes cell cycle events. Here we investigated whether quercetin treatment alters NOX4 mRNA expression. Our hypothesis was that quercetin would inhibit or alter NOX4 expression, thus inhibiting mitotic clonal expansion (MCE), a process required for terminal differentiation. 3T3-L1 preadipocytes were differentiated in the presence or absence of quercetin ($50\mu M$) and harvested at subsequent time points (0,6,12,24,36,48 hours) for mRNA analysis to characterize NOX4 mRNA expression during MCE. In the absence of quercetin Nox 4 expression rose steadily from 6 hours to 36 hours and then was down-regulated by 48 hours. However in the presence of quercetin NOX4 mRNA expression was completely blunted until slight stimulation came on a 36 and 48 hours. Similar to Cyclin A (see Figure 9) results, it is possible that quercetin's anti-oxidant capacity is responsible for blunting the expression of NOX4 and that by 36 hours quercetin's anti-oxidant capacity is quenched, allowing the cells to signal a delayed response to the adipogenic cocktail (MDI). The implications of these findings would be that quercetin may inhibit cell cycle progression by blunting the signaling pathways involved in initiating and sustaining cell cycle events. It has also been shown that strong antioxidant treatment inhibit cell cycle, therefore the mechanism by which quercetin inhibits cell cycle is likely through it's anti-oxidant capacity. It would be of interest to determine ROS levels during early time points to confirm quercetin ability to quench ROS.

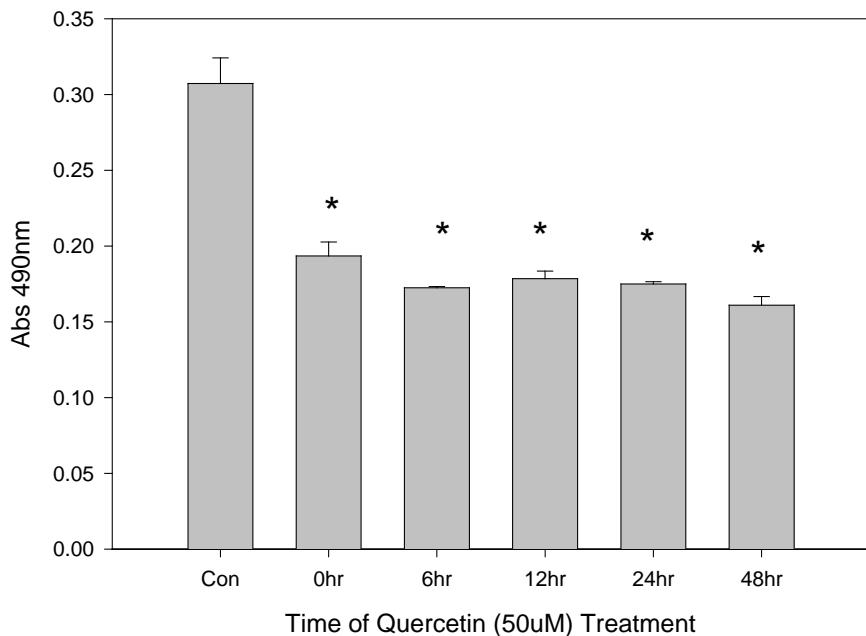
APPENDIX 2
QUERCETIN INHIBITS LIPID ACCUMULATION OF 10T1/2
MESENCHYMAL STEM CELLS



Appendix 2. 10T1/2 mesenchymal stem cells are an adipogenic cell line model one-step removed from preadipocytes commitment, they therefore more closely model adipogenesis as it occurs from the initial recruitment of stromal vascular stem cells to preadipocytes to mature adipocytes. This cell line has been determined to be less reliant on mitotic clonal expansion (MCE) for terminal differentiation therefore it was of interest to test quercetin's effect on their differentiation. Our hypothesis was that if quercetin inhibits adipogenesis through MCE events then quercetin would have no effect on 10T1/2 cell differentiation. 10T1/2 cells were differentiated in the presence or absence of quercetin (1, 5, 10, 20, 50 μM) and harvested at Day 8 to measure lipid accumulation, an indirect measure of adipogenesis, by Oil Red O staining. Quercetin (50 μM) significantly inhibited lipid accumulation by Day 8, indicating that quercetin is able to inhibit adipogenesis in adipogenic models less depending on MCE, further affirming quercetin potential to inhibit adipogenesis *in vivo*. It should be noted that lower concentrations of quercetin did not significantly inhibit lipid accumulation. 10T1/2 cells are thought to be more sensitive than 3T3-L1 preadipocytes to treatments, therefore the fact that lower concentrations had no effect may suggest that quercetin's effect in 10T1/2 cells is indeed weaker and only very strong doses has an effect.

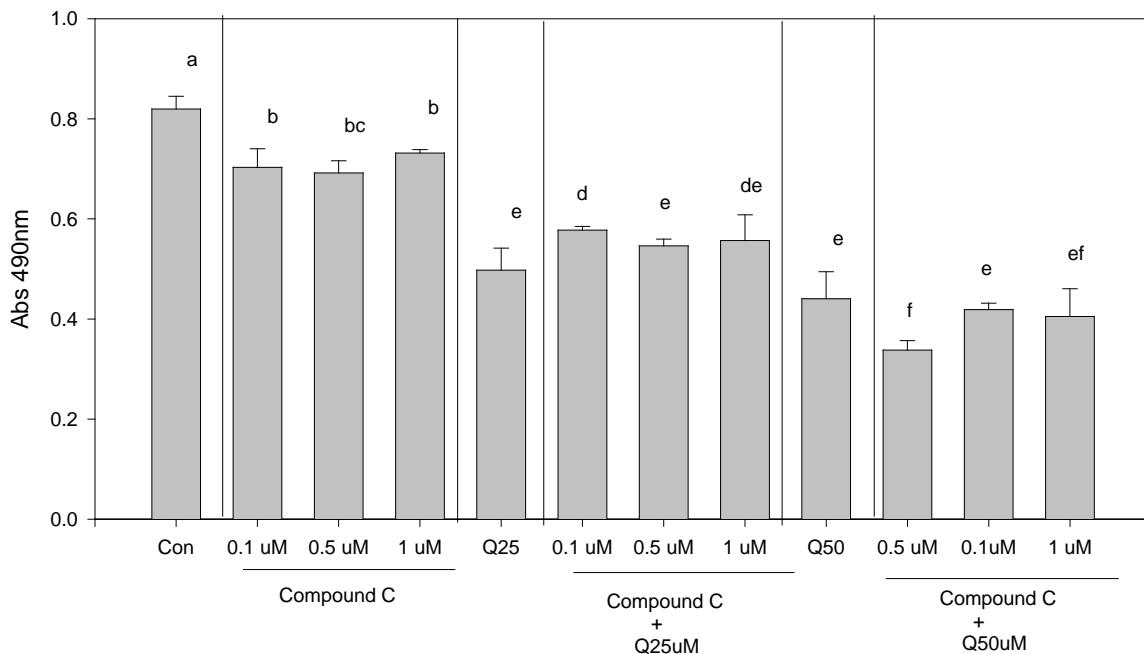
APPENDIX 3

QUERCETIN'S INHIBITORY EFFECT IS NOT LIMITED TO EARLY TIME POINTS IN 10T1/2 MESENCHYMAL STEM CELLS



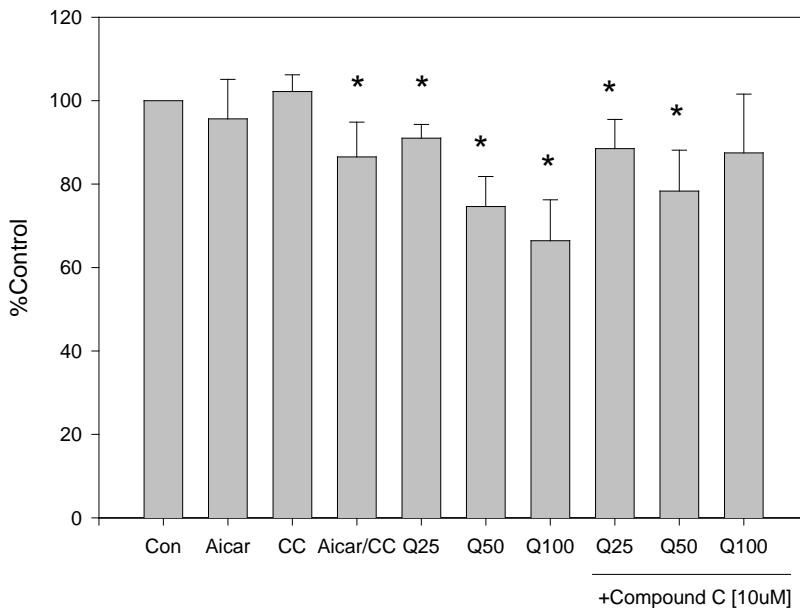
Appendix 3. Our results indicate that quercetin's inhibitory effect in 3T3-L1 preadipocytes is limited to the first 36 hours of differentiation, when mitotic clonal expansion (MCE) is occurring. 10T1/2 mesenchymal stem cells model an uncommitted model vascular stromal stem cell differentiation, and have been determined to be less dependent on MCE events for terminal differentiation. Previous findings indicate quercetin (50 μ M) inhibits lipid accumulation (appendix 2), therefore we hypothesized that if quercetin is able to inhibit adipogenesis in 10T1/2 cells independent of MCE, quercetin's effect will not be time-dependent. 10T1/2 cells were differentiated at time 0 hour and quercetin (50 μ M) was added to differentiating media at subsequent times (0, 6, 12, 24 and 48 hours) and continued until Day 8, when all cells were harvested for Oil Red O staining to determine lipid accumulation. Quercetin treatment consistently inhibited lipid accumulation regardless of treatment time. Interpretation of this data is difficult because of our limited understanding of mitotic clonal expansion events in 10T1/2 cells. It is possible that MCE events last longer in 10T1/2 cells therefore it would be of interest to test the effect of quercetin treatment given past 48 hours to see if at some time point quercetin has no effect. It is also important to characterize quercetin's effect on adipogenic factors (PPAR γ , C/EBP α , aP2 and ACC).

APPENDIX 4
COMPOUND C DID NOT REVERSE THE ANTI-LIPOGENIC EFFECT OF
QUERCETIN



Appendix 4. AMP-activated protein kinase (AMPK), the major metabolic regulator of the cell, has been demonstrated to control mitotic clonal expansion (MCE) in 3T3-L1 preadipocytes, as either inhibition or activation inhibits MCE and differentiation. Our preliminary findings demonstrate quercetin activates AMPK during MCE events and throughout the course of differentiation (see Figure 8A), therefore we hypothesized that the AMPK inhibitor, Compound C, will attenuate quercetin's anti-adipogenic effect by counteracting quercetin's activation of AMPK. 3T3-L1 preadipocytes were differentiated in the presence or absence of quercetin (Q) 25-50 μ M and Compound C (CC) 0.1-1 μ M and harvested at Day 6 for Oil Red O staining to measure lipid accumulation ($n=4$). As expected, quercetin treatment alone inhibited lipid accumulation. CC treatment slightly inhibited lipid accumulation; however this inhibition was not detectable by microscope observation. CC, at any concentration, did not improve lipid accumulation in the presence of quercetin. These preliminary results suggest quercetin inhibits adipogenesis independent of AMPK pathways. To confirm this hypothesis, pAMPK protein levels should be determined throughout the course of adipogenesis to determine whether CC at low doses (0.1-1 μ M) effectively inhibited AMPK activation. CC at higher doses (5 -10 μ M) inhibited lipid accumulation comparable to quercetin 50 μ M treatment (data not shown); therefore lower concentrations were selected for this experiment to avoid a confounding effect.

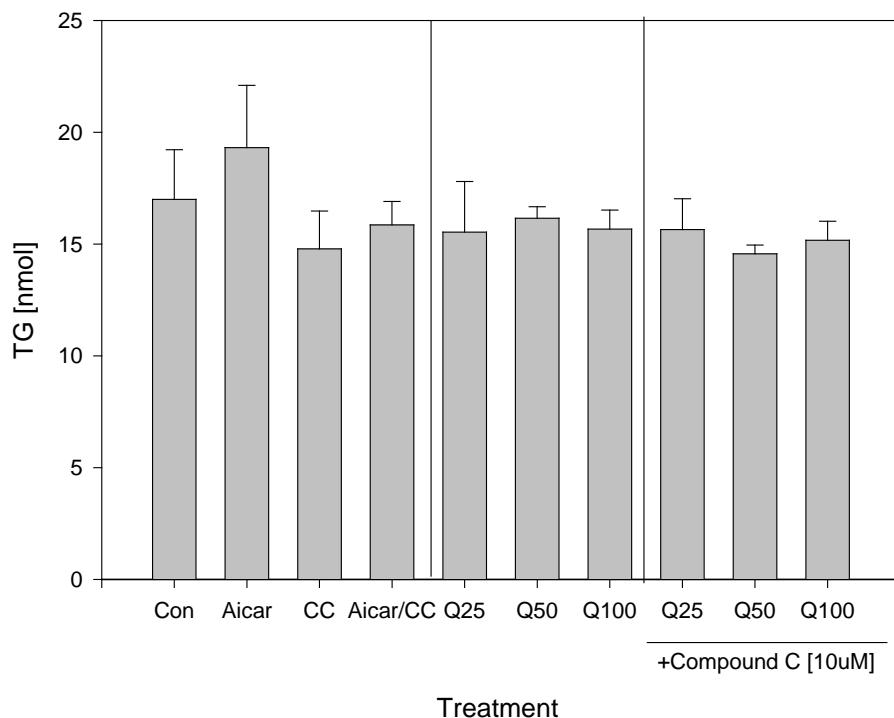
APPENDIX 5
QUERCETIN TREATMENT IN MATURE ADIPOCYTES RESULTS IN
DECREASED GLYCEROL CONTENT IN THE MEDIA



Appendix 5. Lipolysis is the breakdown of triglyceride into free fatty acids and glycerol, which are released from the cell. Researchers have previously determined quercetin enhances lipolysis however the mechanism remains unknown. Our findings and others indicate quercetin activates AMP-activated protein kinase (AMPK), the major metabolic regulator of the cell. AMPK has been implicated to induce partial lipolysis, the release of free fatty acid, but not glycerol. We therefore tested quercetin's effect on glycerol release in the presence or absence of an AMPK inhibitor, Compound C (CC) to determine whether quercetin's effect on glycerol release was dependent on AMPK activation. Our hypothesis was two fold (1) if quercetin induces lipolysis, then quercetin treatment will increase glycerol release independent of CC treatment, and (2) if quercetin induces partial lipolysis, as seen with AMPK activation, then quercetin treatment will not alter glycerol release. Mature adipocytes were treated with AICAR (0.5-1mM), CC (10 μ M) and quercetin (Q) (25-100 μ M) for 15-48hrs in serum free, insulin free media. Media was collected and analyzed for glycerol content. Data from 3 trials ($n=7$) was pooled and shown here. Each trial had a slightly different experiment design, either varied by AICAR treatment (0.5 or 1mM) or length of treatment time (15, 24, or 48 hours). The pooled data showed quercetin treatment significantly decreased glycerol content in the media, indicating quercetin's effect is neither lipolytic nor partially lipolytic through AMPK activation. Interestingly CC only blunted the effect of the strong quercetin treatment (100 μ M). These findings suggest quercetin treatment alters the normal metabolism of glycerol, and therefore future studies should look at quercetin's effect on glycerol metabolism, specifically glycerol-3 phosphate dehydrogenase (GPDH) in the mitochondria. Insight here will illuminate quercetin's overall metabolic effect on mature adipocytes.

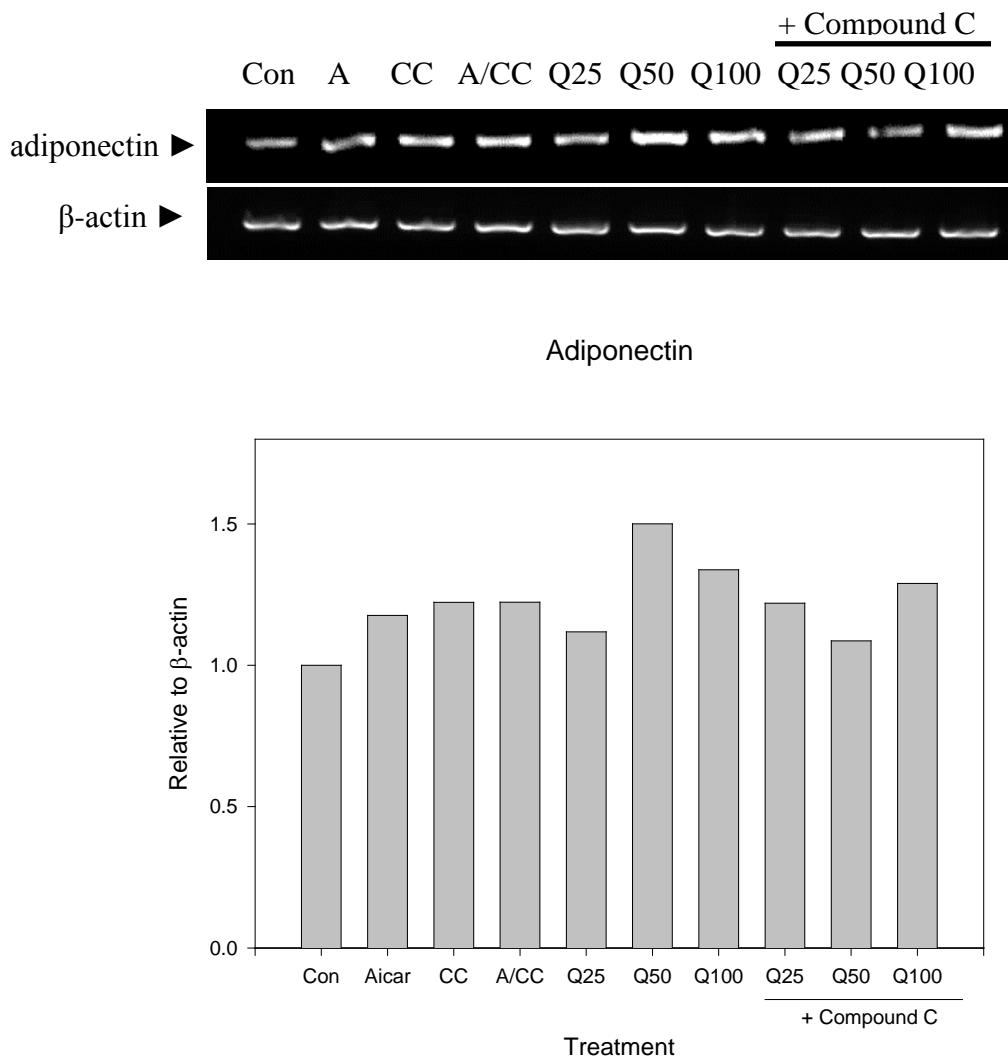
APPENDIX 6
QUERCETIN TREATMENT IN MATURE ADIPOCYTES FOR 48 HOURS DID
NOT ALTER INTRACELLULAR TRIGLYCERIDE LEVELS

Intracellular Triglyceride Content



Appendix 6. Adipocytes undergo lipolysis to breakdown stored triglyceride for use in the body. Quercetin has been implicated to stimulate lipolysis. Further quercetin has also been implicated to activate AMPK, which is known to stimulate partial lipolysis. We therefore tested whether quercetin treatment significantly reduced the amount of store triglyceride content and whether the AMPK inhibitor, Compound C mediated this effect. Mature adipocytes were treated with Compound C (CC) 10 μ M, Aicar (A) 0.5 and 1mM, and quercetin (Q) 25-100 μ M for either 24 or 48hrs and media was collected and analyzed for free fatty acid content. Data presented here reflects 1 trial (n=3). No treatment significantly altered triglyceride content in the cell. This is likely because any quantifiable change in triglyceride content would take a longer treatment period, since mature adipocytes are resistant to purging lipid.

APPENDIX 7
QUERCETIN UPREGULATES mRNA EXPRESSION OF ADIPONECTIN



Appendix 7. Adiponectin, an adipokine known to improve insulin sensitivity, is negatively associated with obesity. Therefore increasing adiponectin levels in obese individuals may be beneficial. Mature adipocytes were treated with AICAR (A) 1mM, Compound C (CC) 10µM, and quercetin (Q) 25-100µM for 24 hours and harvested for mRNA analysis by qualitative PCR. AICAR and CC treatment equally stimulated adiponectin mRNA expression, however quercetin treatment dramatically up-regulated expression. CC attenuated this effect suggesting quercetin may increase adiponectin mRNA expression through an AMPK dependent mechanism.

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