

THE EFFECT OF EPIGALLOCATECHIN-GALLATE ON ADIPONECTIN EXPRESSION  
AND THE INSULIN SIGNALING PATHWAY

By

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Para mi familia

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## LIST OF ABBREVIATIONS

ADIPOR1	Adiponectin receptor 1
ADIPOR2	Adiponectin receptor 2
AMPK	Adenosine monophosphate-activated protein kinase
ANOVA	Analysis of variance
C	(+)-catechin
COMT	Catechol-O-methyltransferase
DAG	Diacylglycerol
DMSO	Dimethyl sulfoxide
DMEM	Dulbecco's Modified Eagle Media
ELISA	Enzyme-linked immunosorbent assay
EC	(-)-Epicatechin
ECG	(-)-Epicatechin 3-O gallate
EGC	(-)-Epigallocatechin
EGCG	(-)-Epigallocatechin gallate
Fatty acyl-CoA	Fatty acyl-coenzyme
FBS	Fetal bovine serum
FFA	Free fatty acids
GA	Gallic acid
GK	Glucokinase
G6Pase	Glucose-6-phosphatase
H	Hour
HMW	High-molecular weight
INS	Insulin
INSA	Chronic insulin treatment

INSA5	Chronic insulin and EGCG treatment
IR	Insulin Receptor
pIR	Insulin receptor phosphorylation
IRS-1	Insulin receptor substrate-1
pIRS-1	Insulin receptor substrate-1 phosphorylation
IL-6	Interleukin-6
IBMX	3-Isobutyl-1- methylxanthine
KO	Knockout
LMW	Low-molecular weight
µg	Microgram
µL	Microliter
µM	Micromolar
MIN	Minute
mRNA	Messenger RNA
MRP2	Multidrug resistance-associated protein-2
nM	Nanomolar
NEFA	Non-esterified fatty acid
NO	Nitric Oxide
PEPCK	Phosphoenolpyruvate carboxykinase
PI3K	Phosphatidylinositol 3- kinase
PPAR-γ	Peroxisome proliferator- activated receptor
PTP	Protein-tyrosine phosphatase
qRT-PCR	Quantitative reverse transcriptase PCR
ROS	Reactive oxygen species
SEM	Standard error of measurement

SNP	Single nucleotide polymorphism
SDS	Sodium dodecyl sulfate
SULT	Phenolsulfotransferases
TBS	Tris-buffered Saline
TZD	Thiazolidinedione
TNF- $\alpha$	Tumor necrosis factor- $\alpha$
T2DM	Type 2 diabetes mellitus
UGT	UDP-glucuronosyltransferases
WT	Wild Type

Abstract of Thesis Presented to the Graduate School  
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Adiponectin, a hormone secreted from adipocytes, in low circulating levels has been epidemiologically associated with obesity, insulin resistance, type 2 diabetes, and cardiovascular disease; it is also thought to be the link between adipose tissue and insulin sensitivity. In 3T3-L1 adipocytes, insulin has been shown to increase adiponectin release and accumulation. Tea has been consumed by humans for thousands of years and is currently a worldwide consumed beverage, coming in second to water. Green tea constitutes of 20% of the amount of tea produced. It is rich in polyphenols, principally flavanols, which include catechins. Green tea's most abundant catechin, (-)-Epigallocatechin-3-gallate (EGCG), accounts for 30-50% of catechins and has been shown to have an effect on factors such as the prevention of metabolic syndrome, inflammation, weight loss, fat oxidation, and glucose control. Recent studies have suggested EGCG anti-diabetic effects could be through the increase of adiponectin accumulation and release.

In order to understand a possible underlying mechanism in adipocytes, we investigated the effect EGCG had on adiponectin expression in 3T3-L1 adipocytes. We hypothesized EGCG would increase intracellular and extracellular protein expression of

adiponectin through enhanced insulin stimulation and phosphorylation of insulin signaling proteins. Adiponectin protein secretion was measured by enzyme-linked immunosorbent assay (ELISA). EGCG significantly increased adiponectin protein secretion when compared to control in 3T3-L1 adipocytes. Adiponectin intracellular protein expression was also significantly increased in EGCG treated cells, measured by western blot analysis. We performed qRT-PCR in order to measure adiponectin mRNA expression and also found a significant increase in the mRNA levels of EGCG treated cells.

In a previous study hepatoma cells treated with EGCG showed an increase in the tyrosine phosphorylation of the insulin receptor, the insulin receptor substrate-1, and Phosphatidylinositol 3- kinase (PI3K), suggesting EGCG mimics insulin when incubated in hepatoma cells, lowering hepatic glucose production. We investigated whether the same mechanism is involved in 3T3-L1 adipocytes treatment with EGCG; therefore increasing adiponectin protein expression through the insulin signaling pathway. We measured the tyrosine phosphorylation of Insulin Receptor (IR) and found no significant changes in EGCG treated adipocytes.

These results suggest that EGCG anti-diabetic role and improved glucose control effects could be through the increased accumulation and secretion of adiponectin from adipocytes. EGCG does not enhance insulin stimulation of insulin receptor phosphorylation, suggesting its mode of action might not work through the insulin signaling pathway. However, the increase in messenger RNA (mRNA) expression of EGCG treated cells could suggest EGCG is enhancing adiponectin secretion through an increase of unknown transcription factors or enhanced mRNA stability.

## CHAPTER 1 INTRODUCTION

Obesity has been shown to be the primary cause of metabolic syndrome, which includes several risk factors such as clustering of abdominal obesity, dyslipidemia, insulin resistance, elevated blood pressure, and other co-morbidities. Metabolic syndrome is associated with a five-fold increased risk for type 2 diabetes mellitus (T2DM) (1). Type 2 diabetes has become an epidemic worldwide. In the past decade a huge amount of research has been focused on an insulin sensitizing hormone, adiponectin.

Adiponectin knockout mice exhibit severe diet-induced insulin resistance, and a decrease in insulin receptor substrate-1 (IRS-1) phosphorylation and glucose uptake (2, 3). Adiponectin is a very abundant serum hormone secreted almost exclusively from differentiated adipocytes. It is seen in low circulating levels in obesity and type 2 diabetes; it is also thought to be the link between adipose tissue and insulin sensitivity (4, 5). In 3T3-L1 adipocytes, insulin has been shown to increase adiponectin accumulation and release (6). Adiponectin exerts autocrine effects in adipocytes by increasing total GLUT 4 gene expression and transporters recruited in response to insulin (7). Expression of GLUT 4 in adipose tissue is as important as it is in skeletal muscle. Mice with selective reduction of GLUT 4 in adipose tissue showed no change in GLUT 4 expression in skeletal muscle, but still developed insulin resistance in skeletal muscle and liver. (8). With its many benefits involving glucose homeostasis, increased secretion of adiponectin is important in increasing insulin sensitivity.

Green tea's most abundant catechin, (-)-Epigallocatechin-3-gallate (EGCG), has been shown to have the greatest effect when compared to other catechins, on factors

such as the prevention of metabolic syndrome, inflammation, weight loss, fat oxidation, and glucose control (9). A recent review looked at results from epidemiological, cell culture, animal, and clinical studies done in the last decade, showing proposed mechanisms for the EGCG anti-obesity effect and its potential role in the prevention and treatment of type 2 diabetes mellitus (10). EGCG antidiabetic role was shown in animal studies, in which EGCG was injected into lean and obese Zucker rats and significantly lowered blood glucose and insulin levels (11), and where supplementation of EGCG alleviated diabetes in ZDF rats (12). In cell culture studies, a green tea extract increased glucose metabolism in adipocytes (13).

Recent research has begun to investigate the effect EGCG has on adiponectin release and accumulation. In a study conducted on obese women taking capsulated 400 mg/d EGCG supplementation for 12 weeks, subjects showed an increase of circulating adiponectin, when compared to the placebo group (14). Dietary supplementation of EGCG elevated circulating levels of adiponectin in Non-Obese Type 2 diabetic Goto-Kakizaki rats (15). (-)- Catechin, a catechin also found in green tea, has shown an increase in the expression and secretion of adiponectin in 3T3-L1 adipocytes (16). The relationship between EGCG and its dose response on adiponectin release and accumulation needs further investigation, in order to better understand a possible underlying mechanism in adipocytes. Therefore, the present study analyzed whether EGCG increased adiponectin cellular accumulation and release, and whether or not the underlying mechanism was through the insulin signaling pathway.

## CHAPTER 2 LITERATURE REVIEW

### **Type 2 Diabetes**

Type 2 Diabetes Mellitus (T2DM) is a rapid growing public health concern all over the world. In 2010, the prevalence of diabetes worldwide was over 284 million people and projected to grow to a total of 439 million individuals by the year 2030. This rising epidemic is also an economic burden reaching \$376 billion in 2010 and is predicted to reach \$490 billion in 2030 (17). Type 2 diabetes is the non-insulin dependent diabetes mellitus, in which the body does not produce or properly use insulin, it accounts for 90-95% of the cases of diabetes. Both genetic and environmental factors, such as obesity and lack of exercise, can play a role in this disease. A major health complication of this metabolic disease is hyperglycemia, which can lead to long-term damage, dysfunction, and failure of organs, specifically the eyes, kidneys, nerves, heart, and blood vessels. Chronic stress, depression, and trouble sleeping have been shown to be associated with T2DM, as well as an overall reduction of quality of life and life expectancy (18).

A recent review gave a new name to Type 2 Diabetes, terming it “Diabesity.” Diabesity is defined as describing diabetes in the context of obesity (17). Obesity usually constitutes an excess of adipose tissue which has an influence on many different processes. Adipose tissue was once considered a lipid storage compartment, today it is considered to be an endocrine organ with unlimited growing ability (19). Adipose tissue secretes adipose tissue-specific hormones called adipokines, which help regulate carbohydrate and lipid metabolism. The balance of these adipokines is perturbed in obesity. The imbalance of these adipokines may increase fatty acids in other cells, possibly impairing insulin signaling and normal cellular function (20).

Type 2 diabetes and obesity cause a resistance to insulin's action to stimulate skeletal muscle glucose disposal. In skeletal muscle, insulin resistance has several defective steps throughout the insulin signaling pathway including: decreases in the insulin receptor (IR), insulin receptor substrate-1 tyrosine phosphorylation (pIRS-1), and phosphatidylinositol 3-kinase (PI-3kinase) activation, leading to a decrease in GLUT4 translocation and glucose transport (21). The result of having T2DM is not only an obese, insulin resistant individual, but an underlying mechanism involving pancreatic islet  $\beta$ -cells not being able to compensate fully for the decrease in insulin sensitivity. Insulin sensitivity fluctuates throughout a normal life cycle. The most critical factor in insulin sensitivity modulation is the release of Non-esterified fatty acids (NEFAs) from adipose tissue, which induces insulin resistance and impairs  $\beta$ -cell function (23). Lifestyle changes such as increased physical activity, weight loss, and changes in diet can enhance insulin sensitivity by decreasing the size of adipocytes, therefore decreasing the amount of NEFAs released (23).

Treatment of T2DM can also be achieved with drug therapy which may sometimes be necessary. In 2007, drug expenditures for diabetes in the US were \$12.7 billion . As the cost for drug therapy increases, individuals are searching for more cost effective treatments (77). Researchers are studying the many mechanisms involved in T2DM in order to target specific pathways. A good deal of that research has been focused on the adipokines secreted from adipose tissue, specifically, an insulin sensitizing hormone called adiponectin (22).

### **Adiponectin**

Adiponectin is a hormone produced exclusively by adipocytes (adipokine); it is also referred to as ACRP-30, AdipoQ, apM1, and GBP28 (24-27). Adiponectin consists

of an amino terminal signal sequence, a variable region, and a collagenous domain. This protein has a molecular weight of 30 kDa in its most basic form as a homotrimer. The trimers associate through disulphide bonds within the collagenous domains of each monomer, in order to form higher-order structures. These include a low-molecular weight (LMW) hexamer of 180 kDa and a high-molecular weight (HMW) 16-18 mer of 400 kDa. A growing amount of evidence suggest that the HMW complex is the most active form and is responsible for the majority of the adipokine's metabolic effects (23). Adiponectin was discovered by four independent labs in 1995 (24-27). Scherer et al. (24) discovered the secretory protein in adipocytes, named it Acrp30 and described it as being structurally similar to complement factor C1q. In the study, adiponectin mRNA was induced by over 100-fold during adipocyte differentiation, and its secretion was enhanced by the addition of insulin. This was the first study linking an insulin regulatory pathway in adipocytes (24). The second study isolated adiponectin cDNA by performing a large scale random sequencing of the human adipose tissue cDNA library and calling it AdipoQ (25). It was observed exclusively in mature adipocytes and was found to be significantly reduced in the adipose tissues from obese mice and humans (25). The third study analyzed random clones and obtained actively transcribed genes in adipose tissue referring to it as adipose most abundant gene transcript 1 (apM1) (26). The final study isolated adiponectin from human plasma, named it GBP28, and suggested its functions involved lipid catabolism or whole body metabolism (27). Although this adipose tissue specific protein was discovered in 1995, its clinical relevance remained obscure until 2001 when other labs began to suggest its potential as an anti-diabetic, anti-inflammatory, and antiatherosclerotic hormone (28).

## **Proposed Functions of Adiponectin**

Unlike other adipokines, adiponectin mRNA and plasma levels decrease with obesity. It is also inversely correlated with body weight, even in extremely lean individuals with conditions like anorexia nervosa, who eventually suffer from hyperadiponectinemia.

A study was designed using ob/ob mice that were lacking leptin, while over expressing adiponectin. The over expression of adiponectin normalized blood glucose and insulin levels, and positively affected serum triglyceride levels. Although the ob/ob mice with an over expression of adiponectin were heavier, they were healthier. Overall, while these mice showed a massive expansion of subcutaneous tissue, displayed hyperphagia, and early onset obesity, metabolic parameters including hyperglycemia, hyperinsulemia, and dyslipidemia were normalized by the over expression of adiponectin (29). In skeletal muscle, adiponectin increases the tyrosine phosphorylation of the insulin receptor. It has also been reported that adiponectin is closely related to insulin sensitivity, independent of fat mass (30). This result points to a role for decreased adiponectin involvement in the development of insulin resistance and T2DM, but not necessarily only because of body fat. This, in turn, could make adiponectin an extremely good biomarker for metabolic parameters (31).

In order to further investigate adiponectin function, adiponectin knockout mice with different phenotypes were developed by three different groups (3, 32-33). Maeda et al. (3) disrupted the adiponectin gene by replacing exon 2 with the neomycin resistant gene. During development up to 16 weeks, there were no large differences in growth rate and food intake between wild type (WT) and knockout (KO) mice. KO mice showed a delayed clearance of free fatty acids (FFA) in plasma, high levels of Tumor necrosis

factor- $\alpha$  (TNF- $\alpha$ ) mRNA and high plasma TNF- $\alpha$  concentrations, although they exhibited severe diet induced insulin resistance (3). Another group had different results with no significant changes in insulin sensitivity when KO mice were compared to WT mice. They also reported an increase in muscle and liver  $\beta$ -oxidation, suggesting compensatory mechanism activation with the loss of adiponectin (32). Finally, Kubota et al. showed impairment in FFA plasma clearance, high levels of TNF- $\alpha$  mRNA in adipose tissue and high plasma TNF- $\alpha$  concentrations (33). In many studies TNF- $\alpha$  has been shown to have an inverse relationship with adiponectin (34). An increase in fatty acid oxidation by adiponectin decreases free fatty acid influx, which then reduces hepatic glucose output (35).

Other proposed functions for adiponectin include its role in atherosclerosis (36), cancer (37), longevity (38), and metabolic flexibility (39). Recent studies have shown that adiponectin has anti-atherogenic and anti-inflammatory properties. When monocytes adhere to the vascular endothelium and differentiate into macrophages and foam cells, vascular disease develops. Hypoadiponectinemia has been associated with endothelial dysfunction. Adiponectin deficient mice display impaired endothelium vasodilation and NO (Nitric Oxide) production, therefore alleviating obesity associated endothelial function and cardiovascular disease (36). A systemic review on adiponectin and cancer discovered adiponectin's role in different types of cancers. A low amount of circulating adiponectin is associated with cancer risk in obesity. Adiponectin analogues may be effective as anti-cancer treatments, but more research needs to be done in this developing area (37). In a study using centenarians, their offspring, and unrelated participants, it was shown that the centenarians and their offspring had higher levels of

circulating adiponectin. Genotyped AdipoQ variants were analyzed. Two common AdipoQ polymorphisms were associated with higher adiponectin in men. These genetic variants could contribute to the link between adiponectin and longevity (38). Adiponectin is also purported to increase metabolic flexibility in adipose tissue, therefore increasing its ability to maintain proper function under metabolically demanding conditions.

Overall, adiponectin seems to play a major role in glucose and lipid metabolism. A summary of physiological and pathophysiological conditions and their association to adiponectin levels can be seen in Table 2-1.

### **Regulation of Adiponectin**

Circulating levels of adiponectin in plasma are at concentrations of 2-20  $\mu\text{g/ml}$ , which are considered very high for an endocrine hormone. Despite its abundant presence in plasma, it is cleared rapidly, having a half-life of about 75 min (40). Regulation of adiponectin may take place at either the transcriptional, translational, or post-translational level. The mechanism of the degradation and clearance of adiponectin is still inconclusive, which could also prove important in regulation (41). The peroxisome proliferator-activated receptor (PPAR- $\gamma$ ) is a ligand transcription factor thought to be a master regulator of adipocyte differentiation and multiple adipocyte genes. Thiazolidinediones (TZDs) are specific synthetic ligand activators of PPAR- $\gamma$ . The administration of TZDs has been shown to increase plasma adiponectin. Subjects with PPAR- $\gamma$  mutations suffer from insulin resistance and low circulating levels of adiponectin. This suggests that PPAR- $\gamma$  may play a role in regulation of adiponectin synthesis (42). Other adipokines may play a role in the regulation of adiponectin. TNF- $\alpha$  and Interleukin-6 (IL-6) are seen in higher levels in obese subjects. Many studies have shown that TNF- $\alpha$  down-regulates adiponectin mRNA and negative associations have

been shown between TNF- $\alpha$  and adiponectin. Adiponectin and TNF- $\alpha$  seem to regulate each other (43). Adiponectin is depressed during lactation and late gestation; it increases during sexual maturation, as well as with a calorie-restricted diet (44). In a study investigating the effects of sex hormones on the production of adiponectin in human subjects, rodents and cultured cells, investigators found testosterone treatment reduced plasma adiponectin levels in mice and 3T3-L1 adipocytes. In human subjects, plasma adiponectin concentrations were lower in men, when compared to women, whereas women had higher levels of leptin. This study related the high incidence of T2DM and atherosclerosis in men to lower circulating levels of adiponectin (45). The fact that women had higher levels of circulating adiponectin but higher levels of body fat for a given BMI, would indicate the involvement of factors other than fat mass (23). Visceral fat has been shown in many studies to have an effect on metabolic syndrome, glucose intolerance, hypertension, dyslipidemia, and insulin resistance. Adiponectin mRNA and protein levels were found to be reduced when visceral to subcutaneous fat were compared (46). The results are still inconclusive as to whether adiponectin levels are the cause or the effect of visceral fat and its implications on the metabolic state.

In 3T3-L1 adipocytes, Phosphatidylinositol 3-kinase (PI3K) has been shown to be necessary for adiponectin secretion (76). Low levels of adiponectin have been associated with a decrease in PI3K activity and a decrease in the insulin receptor substrate-1. Insulin stimulates the secretion of adiponectin, but is dependent on the PI3K dependent signaling pathway. A study using a PI3K inhibitor and insulin showed that an inhibition in the PI3K pathway completely inhibits the insulin stimulated increase

in adiponectin secretion (76). There may be other insulin-dependent pathways that could affect adiponectin production and secretion, but they have yet to be discovered.

### **Adiponectin Receptors**

Yamauchi et al. discovered adiponectin receptors 1 and 2 (ADIPOR1 AND ADIPOR2) in 2003 (47). Both receptors have been detected in many tissues with high levels being found in skeletal muscle, liver, and pancreatic  $\beta$ -cells: they are also found in macrophages. These two receptors interact directly with adiponectin and mediate many of its effects. Adiponectin receptor expression in the skeletal muscle of T2DM subjects has been shown to be reduced. Obesity does not only decrease adiponectin, but decreases both of its receptors, thus decreasing adiponectin sensitivity (48). Polymorphisms in adiponectin receptor genes have been linked to insulin resistance (49). Lower levels of ADIPOR1 mRNA were found in visceral fat than in subcutaneous fat (50).

### **Adiponectin and Insulin Resistance**

Insulin resistance is one of the main factors contributing to type 2 diabetes. Adiponectin levels have been shown to correlate with insulin resistance. Decreased plasma adiponectin has been associated with a higher incidence of diabetes. Adiponectin's insulin sensitizing effects were discovered in 2001 by three independent groups. Adiponectin was shown to reverse insulin resistance in mice receiving intraperitoneal injections of adiponectin. Yamauchi et al. (51) used KKA $\gamma$  mice fed a high fat diet to induce an insulin-resistant state. When the mice were injected with adiponectin, the insulin resistance was significantly ameliorated, suggesting that adiponectin was acting as an insulin sensitizing hormone (51). Scherer et al. (52) reported that an acute increase in circulating adiponectin triggered a transient decrease

in basal glucose by inhibiting the expression of gluconeogenic enzymes and the rate of endogenous glucose production in wild type mice. In ob/ob mice and streptozotocin-treated WT mice, adiponectin injections abolished hyperglycemia. Administration of adiponectin in rodents has also shown increases in tyrosine phosphorylation of the insulin receptor in skeletal muscle (30, 52).

Studies done in adiponectin-deficient mice showed a decrease in insulin sensitivity and glucose intolerance, as noted in the previous section. Adiponectin-deficient mice fed a high fat diet, developed severe insulin resistance in skeletal muscle in as little as two weeks. One possible mechanism underlying adiponectin insulin sensitizing effects involves the insulin signaling cascade. NEFAs are the single most critical factor affecting insulin sensitivity. Increased levels of NEFAs cause insulin resistance within hours of being released in acute amounts. Increased NEFA delivery increases fatty acid metabolites such as diacylglycerol (DAG), fatty acyl-coenzyme (fatty acyl-CoA), and ceramides. In turn, these metabolites turn on the serine/threonine kinase cascade leading to the serine/threonine phosphorylation of IRS-1/IRS-2. This reduces the ability of IRS-1 tyrosine phosphorylation to activate PI3K and the downstream events in the insulin signaling pathway.  $\beta$ -cell dysfunction occurs with high levels of NEFAs. Overtime, this process moves forward slowly, making the onset of T2DM a slow process that usually takes many years (6, 49).

Adiponectin plays a major role in the clearance of NEFAs from plasma. Full-length adiponectin was found to stimulate AMPK phosphorylation and activation in the liver, suppressing hepatic glucose production; through AMPK phosphorylation in the liver it directly regulates glucose metabolism and insulin metabolism. Adiponectin's ability to

decrease gluconeogenic enzymes could be the mechanism underlying the increase in AMPK phosphorylation (30). In 3T3-L1 adipocytes, adiponectin was shown to increase the ability of insulin to stimulate glucose uptake by increasing GLUT4 gene expression and recruitment to the plasma membrane (53).

The adiponectin gene is located on chromosome 3q27. One single nucleotide polymorphism (SNP) located at position 276 from the translational site has been associated with a higher risk for T2DM, insulin resistance, and lower levels of circulating adiponectin (54). In patients with T2DM and insulin resistance, metabolic syndrome markers were closely related to adiponectin levels (55). Overall, extensive research has proven that adiponectin is an insulin sensitizing hormone involved in glucose and lipid metabolism.

### **Epigallocatechin-gallate**

Tea, known as a plant, leaf, or beverage originating from the perennial evergreen shrub, *Camellia Sinensis*, has been consumed by humans for thousands of years and has now become a worldwide consumed beverage, coming in second only to water (56). Green tea constitutes 20% of the amount of tea produced and has been studied extensively and shown to have beneficial health effects. It is rich in polyphenols, specifically a subclass of flavan-3-ols, the catechins (57). Catechin levels depend on the geographical area the tea was grown in, how carefully it was processed, and the age of the leaf. While the tea is being processed, manufacturers have to be very careful to preserve the catechins (58). Green tea's most abundant catechin, (-)-Epigallocatechin-3-gallate (EGCG), accounts for 30-50% of the catechins found in green tea. Green tea usually contains between 30 and 130 mg of EGCG in one cup (237 mL). EGCG has been shown to have the greatest effect, when compared with other catechins, on factors

such as the prevention of metabolic syndrome, inflammation, weight loss, fat oxidation, and glucose control (9).

## **Introduction to Polyphenols**

Polyphenols are characterized as chemical compounds containing more than one phenol group or building block molecule. The most studied group is the flavanoids whose subclass includes: flavonols, flavones, catechins, flavanones, anthocyanidins, and isoflavonoids. Many of these flavonoids have been shown to exert beneficial effects in humans. Catechins represent about 70% of the polyphenolic content of green tea. The 5 major catechins isolated from in green tea are: (+)-catechin (C), (-)-epicatechin (EC), (-)-epicatechin 3-O gallate (ECG), (-)-epigallocatechin (EGC), (-)-epigallocatechin gallate (EGCG), and gallic acid (GA).

In order to understand their bioavailability, absorption, and metabolism, a number of studies have examined various aspects of these catechins. In human plasma, catechin levels reach their peak 2 to 4 h after ingestion; the highest concentration of catechins that has been measured is about 1  $\mu\text{M}$ . After ingesting a single dose of 800mg of EGCG, human plasma concentrations were found to be 0.96  $\mu\text{M}$  (57). EGCG has very poor bioavailability which might be due to its instability in alkaline or neutral conditions, low cellular uptake, metabolic transformations, and active efflux of polyphenolic compounds by the multidrug resistance-associated protein-2 (MRP2). The same conditions are seen in cell culture models, in which EGCG levels peak at 2 to 4 h and where it is less stable at a neutral pH. EGCG displayed a half-life of 130 min when in the presence of cells (59). Using a pro-drug approach, the use of (-)-EGCG octaacetate (ProEGCG) improved bioavailability and was more stable in a pH=8 solution (57). Metabolic transformations of green tea polyphenols have been looked at

to understand absorption. Valerolactones have been identified as metabolites of catechins in human urine and plasma. In the liver, brain, and gastrointestinal tract of rats and mice, glucuronidation, sulphation, and methylation of catechins were found. A recent study using an *ex vivo* ileostomy model to study the microbial metabolism of green tea catechins, found that 31% of ingested green tea flavanols were absorbed in the small intestine and the rest in preceding parts of the gut (60). Catechins are thought to be metabolized rapidly. EGCG becomes degalloyated in the mouth and esophagus, and saliva has been found to have catechin esterase activity. Enzymes involved in polyphenol metabolism include catechol-O-methyltransferase (COMT), UDP-glucuronosyltransferases (UGT) and phenolsulfotransferases (SULT) (61). This is a developing area of research and results are still not conclusive.

### **Epigallocatechin-gallate Health Benefits**

The health benefits of EGCG have been studied extensively. Thielecke et al. (34) reviewed a large number of *in vitro*, animal, and human studies, and found fairly conclusive evidence that EGCG underlying mechanism and proven benefits in obesity, type 2 diabetes, and cardiovascular factors. Almost all the human studies showed a reduction in body weight and body fat in response to EGCG supplementation, suggesting a possible increase in beta-oxidation (34). A study done in 3T3-L1 adipocytes used concentrations of 0, 50, 100, and 200  $\mu\text{M}$  of EGCG to determine whether EGCG would inhibit adipogenesis and induce apoptosis. Cell viability was not affected during the 24 h incubation period. Results indicated a dose dependent decrease in average lipid droplets and total lipid droplet number after EGCG treatment. However, when the cells were exposed to 200  $\mu\text{M}$  EGCG, very few mature adipocytes

exhibited normal morphology. EGCG had an inhibitory effect on differentiation and the induction of apoptosis (62).

Wolfram et al. (63) further investigated these effects in rodents. Diets for C57BL/6J mice and Sprague-Dawley rats were supplemented with EGCG. The C57BL/6J mice were fed a high fat diet containing 1% EGCG (1 mg) for 5 months; obese Sprague-Dawley rats were fed a high fat diet containing 1% EGCG (1 mg) for 4 weeks. The results showed that EGCG prevented diet-induced obesity in C57BL/6J mice, decreased the body weight of Sprague Dawley rats reversing their diet-induced obesity, and reduced mRNA expression of lipogenic enzymes in both animals. The most significant result was found in the C57BL/6J mice, which suggested that EGCG could be beneficial in the prevention of obesity (63).

Jeukendrup et al. (64) investigated fat oxidation and glucose tolerance in males consuming EGCG during moderate-intensity exercises. Subjects ingested capsules containing about 360 mg of EGCG during the 24 h period before they began exercising. The results showed a 21% increase in fat oxidation in the subjects who were consuming EGCG versus the placebo. Testing showed that EGCG improved glucose tolerance, and that insulin sensitivity increased an average of 13%. This increase in fat oxidation could be due to an increase in lipolysis, as seen in the *in vitro* studies. EGCG has beneficial effects that could modulate obesity (64).

EGCG also plays a role as an anti-oxidant, due to its effective scavenging of free radicals. It has the most potent anti-oxidant effect when compared to the other catechins. EGCG has also been credited as being an immunomodulator, anti-inflammatory, anti-cancer, and anti-atherosclerotic (57). EGCG's capability of preventing

cellular damage by mitigating metabolic production of reactive oxygen species (ROS) could play a role in aging (65). Further investigation into the mechanism of EGCG will be necessary to better understand its exact role in affecting these specific conditions.

### **Epigallocatechin-gallate and Insulin Resistance**

Patients with type 2 diabetes are usually prescribed metformin as first line therapy, and then TZD, sulfonylurea, or insulin are subsequently added if specific target glucose levels are not met (66). Although these drugs ameliorate the condition, they also come with an array of side effects. Promising alternative therapies have used active phytochemicals such as resveratrol, xanthones, and catechins as antidiabetic agents (67, 68, 16). EGCG is a catechin that has been shown to be a promising alternative therapy (57). One of the trademarks of diabetes is the inability of insulin to inhibit hepatic glucose production. Gluconeogenesis is the main reason for increases in hepatic glucose production and insulin's inability to regulate the two gluconeogenic enzymes phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) causing this state. In a study using rat hepatoma cells, cells were incubated in the presence or absence of insulin and/or EGCG. Cells treated with EGCG and insulin showed an increase in the tyrosine phosphorylation of the insulin receptor, the insulin receptor substrate-1, and PI3K, suggesting that EGCG mimics insulin in hepatoma cells, by lowering hepatic glucose production. EGCG also decreased PEPCK and G6Pase mRNA expression, while increasing the production of reactive oxygen species (ROS) in hepatoma cells. Although EGCG acts as an antioxidant in other cells, it appears to act as a pro-oxidant in hepatoma cells. The increase in ROS production could be the mechanism behind EGCG inhibiting protein-tyrosine phosphatase (PTP), which contains an oxidizable cysteine in its active site (2).

In an animal study using db/db (leptin receptor deficient, severely diabetic) mice and ZDF (animal model of T2DM, moderately diabetic) rats, EGCG supplementation was given along with a chow diet. EGCG supplemented db/db mice showed improved oral glucose tolerance and insulin plasma concentrations were increased, when compared to the control animals. The mRNA expression of glucokinase (GK) was also increased in the livers of these mice. Increased activity of GK leads to enhanced glycolysis and hepatic glucose uptake. EGCG could be improving pancreatic function by its ability to inhibit nuclear factor  $\kappa\beta$  activation, thereby improving insulin secretion. In the ZDF rats, blood glucose concentrations were again lower in EGCG supplemented animals and insulin concentrations in the plasma were increased. This study showed EGCG's potential as an anti-diabetic agent (12).

To further investigate the effects of EGCG, a human study was done with T2DM patients who were not receiving insulin treatments. Participants ingested a green tea beverage that contained 583 mg of catechins daily for 12 weeks. Subjects consuming the catechin beverage had a reduced waist circumference, increased levels of plasma adiponectin, and an increase in insulin compared to the control subjects. These results indicate that this catechin-rich beverage improved blood glucose control in these T2DM subjects (69).

Many studies have been done to show the effect EGCG has on insulin resistance and glucose homeostasis. In 3T3-L1 adipocytes, EGCG, at a concentration of 5  $\mu\text{M}$ , was shown to enhance the expression of genes related to insulin sensitivity (70). In skeletal muscle, EGCG was shown to promote GLUT 4 translocation; GLUT 4 plays a pivotal role in insulin stimulated glucose transport (71).

## **Adiponectin and Epigallocatechin-gallate**

EGCG has been shown to suppress fatty acid synthesis and activate fatty acid oxidation in the liver and adipose tissue. EGCG has also been shown to decrease gluconeogenesis, decreasing hepatic glucose production (10). Adiponectin improves insulin resistance by enhancing  $\beta$ -oxidation and decreasing gluconeogenesis in the liver and by increasing glucose uptake in skeletal muscle (12). Recent research has suggested that EGCG could improve insulin resistance and lipid metabolic abnormalities by enhancing adiponectin production. A study was done using non-obese Type 2 diabetic Goto-Kakizaki rats and measuring plasma adiponectin levels (12). The rats were fed a normal chow diet supplemented with 0.2% EGCG for 12 weeks. Plasma adiponectin levels were significantly increased over those in the control group. Plasma triacylglycerol levels of animals treated with EGCG were significantly lower when compared to controls (12). In a study conducted using obese women taking capsulated 400 mg/d EGCG supplementation for 12 weeks, subjects showed an increase of plasma adiponectin, ghrelin, and HDL- cholesterol, when compared to placebo takers (14). Adiponectin secretion from adipocytes is thought to be enhanced by insulin initiating the insulin signaling cascade (6). Insulin stimulates the secretion of adiponectin, but is dependent on the PI3K-dependent signaling pathway (76). Phosphatidylinositol 3-kinase (PI3K) activity has been shown to be necessary for adiponectin secretion; while low levels of adiponectin can be associated with a decrease in PI3K activity and a decrease in the insulin receptor substrate-1 tyrosine phosphorylation (76). It has been suggested that EGCG might be insulin mimetic, activating the insulin signaling cascade in hepatocytes. EGCG could be mimicking insulin in adipocytes, and stimulating the

secretion of adiponectin through the insulin signaling cascade (2). Future studies using adipocytes as a cell model will be necessary to determine whether this is the case.

### **Specific Aims**

**AIM I:** Investigate the effect of EGCG on adiponectin protein expression and secretion in 3T3-L1 adipocytes. A study conducted in obese women taking capsulated 400 mg/d EGCG supplementation for 12 weeks, resulted in an increase of circulating adiponectin when compared to placebo (14). Another study showed dietary supplementation of EGCG elevated circulating levels of adiponectin in Non Obese Type 2 diabetic Goto-Kakizaki rats (16). Studies have not been done to explore EGCG's effect on adiponectin *in vitro* using 3T3-L1 adipocytes as a cell model. 3T3-L1 mature adipocytes will be used in order to measure adiponectin protein and mRNA expression in response to EGCG treatment.

**AIM II:** Investigate whether the mechanism underlying the effects of EGCG on adiponectin protein expression is through the insulin signaling pathway. EGCG's antidiabetic role was shown in an animal study in which EGCG was injected into lean and obese Zucker rats and it significantly lowered blood glucose and insulin levels (35). In order to determine if the insulin signaling pathway has involvement in EGCG's antidiabetic role, this study will determine the effect of EGCG on the phosphorylation of insulin signaling proteins such as insulin receptor (IR) and insulin receptor substrate- 1 (IRS-1) in 3T3-L1 adipocytes.

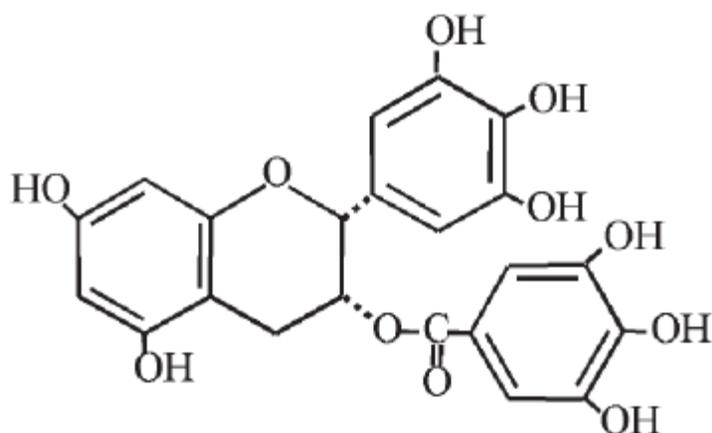


Figure 2-1. Structure and Nomenclature of (-)-Epigallocatechin-gallate

Table 2-1. Conditions or treatments associated with changes in plasma adiponectin

Increase	Decrease
TZDs	Variation in Adiponectin Gene
Heart failure	Obesity
Renal failure	Insulin Resistance
Weight loss	Type 2 Diabetes
Dietary factors:	Metabolic Syndrome
Soy protein	Dyslipidemia
Oils	Cardiovascular disease
	Hypertension
	Sex hormones (androgens, testosterone)
	Oxidative stress
	Carbohydrate-rich diet

## CHAPTER 3 MATERIALS AND METHODS

### **Study Design for Specific Aims I and II**

For specific aim I, 3T3-L1 adipocytes were provided by Dr. Susan C. Frost. The adipocytes were differentiated and grown to mature adipocytes (Day 9). Studies show adiponectin is secreted in higher amounts from mature adipocytes (30). On day 4 after differentiation, cells were started on different treatments showed in Table 3-1. Cells were given treatment every other day until day 9. On day 9, cells were given last treatment for a 6 h incubation. A recent study revealed a 6 h incubation time as an ideal time for adiponectin expression in 3T3-L1 adipocytes (72). For specific aim II, adipocytes were differentiated and grown to mature adipocytes (Day 9). On day 4, after differentiation, cells were started on different treatments showed in Table 3-1. Cells were given treatment every other day until day 9. On day 9, cells were given the last treatment for 30 min. Insulin phosphorylation occurs very quickly. Most studies choose a 30 min incubation as time point to visualize phosphorylation (30).

### **Cell Culture and Treatments**

3T3-L1 cells, a mouse embryonic fibroblast - adipose like cell line (gifts of Dr. Susan C. Frost, UF), were cultured in Dulbecco's Modified Eagle Media (DMEM) containing 4.5 g/L D-glucose and 4 mM glutamine (GIBCO brand, INVITROGEN, Carlsbad, CA) supplemented with Penicillin(100 U/ml)- Streptomycin (100µg/ml) (Sigma, St. Louis, MO), 10% calf serum (Atlanta Biologicals, Lawrenceville, GA) and incubated at 37°C in 7.5% CO<sub>2</sub>. Fibroblasts were grown in 6-well plates under the same conditions for 48 h. Subsequent medium changes were done every 48 h until the fibroblast had reached confluence. When fibroblasts were two days post-confluent they

were cultured with differentiation medium for 48 h (Day 0). Differentiation Medium contained: DMEM containing 4.5 g/L D-glucose and 4 mM glutamine, 10% fetal bovine serum (FBS) (GIBCO brand, INVITROGEN, Carlsbad, CA), 0.5 mM IBMX (3-Isobutyl-1-methylxanthine) (Sigma) , 0.25  $\mu$ M Dexamethasone (Sigma), 1  $\mu$ g/ml (170 nM) Insulin (Sigma). After 48 h fresh media was added containing DMEM with 10% FBS and 170 nM insulin (Day 2). On day 2 of differentiation, cells were started on different treatments of either 0.1% Dimethyl sulfoxide (DMSO) (Fisher Scientific, Fair Lawn, NJ), 100 nM Insulin, or Epigallocatechin-gallate (EGCG) (Sigma) as show in Table 3-1. Subsequent medium changes were given every 48 h (Day 4- 9) containing DMEM, 10% FBS and assigned treatment. On day 9, cells were fully differentiated mature adipocytes. Medium containing DMEM, 10% FBS, and specific treatment was added on day 9 for a 6 h incubation. After 6 h, media from each well was removed and centrifuged at 10,000x g for 10 min, then into -80°C. Cells were washed with cold phosphate buffered saline (PBS) 3 times and cell lysate was processed. Adipocyte induction was as described by Frost et.al (73).

### **Cellular Secretion of Adiponectin Measured by ELISA**

3T3-L1 cells (Day 9) were incubated with 1  $\mu$ M, 2.5  $\mu$ M, 5  $\mu$ M, 10  $\mu$ M EGCG, or 100 nM insulin, 100 nM chronic insulin (INSA), 100 nM chronic insulin plus 5  $\mu$ M EGCG (INSA5) , or 0.1% DMSO for 6 h at 37°C in 7.5% CO<sub>2</sub>. Media was removed from individual wells and centrifuged at 10,000x g for 10 min. The supernatant was then removed and used for the analysis of Adiponectin secretion using a mouse Adiponectin ELISA kit (R&D systems, Minneapolis, MN). Supernatants were diluted 200 fold in order to fit standard curve. Protein content of supernatants were determined by the

Bradford method (Bio-Rad, Hercules, CA). Three independent experiments were conducted.

### **Sample Preparation for Western Blot Analysis**

After incubation, cells were washed with ice-cold PBS 3 times. Lysis buffer containing 20 mM Tris buffer (Sigma) pH to 7.5 with hydrochloric acid (HCl) (Fisher), 150 mM sodium chloride (NaCl)(Fisher), 1% Triton X-100 (Fisher), 0.1% Sodium dodecyl sulfate (SDS) (Fisher), 2 mM Ethylenediaminetetraacetic acid (EDTA) (Fisher), and protease inhibitor cocktail (Roche, Boulder, CO) was added to cells. For lysates used in measuring insulin receptor phosphorylation, a phosphatase inhibitor cocktail (Roche) was also added. Using a cell lifter (Fisher), cells in lysis buffer were transferred into a tube, vortexed, and centrifuged at 13,000x g for 15 min at 4°C in order to pellet nuclei and cell debris. Supernatant was removed and stored in -80°C until analysis. Protein concentrations were analyzed using the Bradford method (Bio-Rad).

### **Adiponectin Sample**

3T3-L1 cells (Day 9) were incubated with 1  $\mu$ M, 2.5  $\mu$ M, 5  $\mu$ M, 10  $\mu$ M EGCG, or 100 nM Insulin, 100 nM chronic insulin (INSA), 100 nM chronic insulin plus 5  $\mu$ M EGCG (INSA5), or 0.1% DMSO for 6 h at 37°C in 7.5% CO<sub>2</sub>. Followed sample preparation protocol mentioned in the sample preparation section.

### **Insulin Receptor and Phosphorylation of Insulin Receptor Sample**

3T3-L1 cells (Day 9) were incubated with 1  $\mu$ M, 2.5  $\mu$ M, 5  $\mu$ M, 10  $\mu$ M EGCG, or 100 nM Insulin, or 0.1% DMSO for 30 min at 37°C in 7.5% CO<sub>2</sub>. Followed sample preparation protocol mentioned in the sample preparation section.

## Western Blotting

### Adiponectin Analysis

Samples containing 45 µg of protein were mixed with Laemmli buffer, heated at 95° C for 5 min, and proteins were electrophoretically separated on a 15% sodium dodecyl sulfate (SDS)-polyacrylamide gel. Molecular weight standards (Precision Plus, dual color, Bio-Rad) were run in parallel, as well as MagicMark XP Western Protein Standard (Invitrogen). Separated proteins on the gel were then transferred to a nitrocellulose membrane (iBlot Dry Blotting System; Invitrogen). Transfer and equal loading were verified by Ponceau staining. Blots were blocked for 1 h in blocking buffer [BB: 5% nonfat dry milk in Tris-buffered saline (TBS), pH 7.4, and 0.01% Tween20 (TBST)], pH 7.4) for 1 h at room temperature (RT). Blots were then incubated overnight at 4°C in rabbit anti-adiponectin (1:5000; Millipore, Billerica, MA). Following primary incubation, blots were washed several times with TBST before being incubated in blocking buffer containing a 1:6000 dilution of peroxidase-conjugated goat anti-rabbit IgG (Pierce, Rockford, IL) for 40 min at RT. Blots were washed in TBST twice for 5 min, and then washed in TBS twice for 5 min. Immunoreactivity was visualized by using enhanced chemiluminescence (SuperSignal WestPico, Pierce) and x-ray film (Fisher). To control for loading, the blot was stripped and re-probed for loading control mouse monoclonal anti-β-Actin (2.5 µg/ 1 mL) and a 1:8,000 dilution of Anti-Mouse IgG (Fc Specific) peroxidase antibody produced in goat (Sigma). Immunoreactivity was visualized by using enhanced chemiluminescence (SuperSignal WestPico) and x-ray film.

## **Insulin Receptor and Phosphorylation of Insulin Receptor**

Samples containing 45 µg of protein were mixed with Laemmli buffer, heated at 95° C for 5 min, and proteins were electrophoretically separated on a 7.5% sodium dodecyl sulfate (SDS)-polyacrylamide gel. Molecular weight standards (Precision Plus, dual color, Bio-Rad) were run in parallel, as well as MagicMark XP Western Protein Standard (Invitrogen). Separated proteins on the gel were then transferred to a nitrocellulose membrane (iBlot Dry Blotting System; Invitrogen). Transfer and equal loading were verified by Ponceau staining. Blots were blocked for 1 h in blocking buffer [BB: 5% nonfat dry milk in Tris-buffered saline (TBS), pH 7.4, and 0.01% Tween20 (TBST)], pH 7.4) for 1 h at room temperature (RT). Blots were then incubated overnight at 4°C in rabbit anti-insulin receptor (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA). Following primary incubation, blots were washed several times with TBST before being incubated in blocking buffer containing a 1:6000 dilution of peroxidase conjugated goat anti-rabbit IgG (Pierce, Rockford, IL) for 40 min at RT. Blots were washed in TBST twice for 5 min, and then washed in TBS twice for 5 min. Immunoreactivity was visualized by using enhanced chemiluminescence (SuperSignal WestPico, Pierce) and x-ray film (Fisher). Blots were then stripped and re-probed for a 1:1000 dilution of the rabbit anti-insulin receptor phosphorylation (pIR). Following primary incubation, blots were washed several times with TBST before being incubated in blocking buffer containing a 1:6000 dilution of peroxidase conjugated goat anti-rabbit IgG (Pierce, Rockford, IL) for 40 min at RT. Blots were washed in TBST twice for 5 min, and then washed in TBS twice for 5 min. Immunoreactivity was visualized by using enhanced chemiluminescence (SuperSignal WestPico, Pierce) and x-ray film (Fisher). To control for loading, the blot was stripped and re-probed for loading control mouse monoclonal

anti- $\beta$ -Actin (2.5  $\mu$ g/ 1 mL) and a 1:8,000 dilution of anti-mouse IgG (Fc Specific) peroxidase antibody produced in goat (Sigma). Immunoreactivity was visualized by using enhanced chemiluminescence (SuperSignal WestPico) and x-ray film.

### **Adiponectin Gene Expression by Quantitative RT-PCR Analysis**

Specific RNA abundance was quantified by quantitative reverse transcriptase PCR (qRT-PCR) analysis. RNA was isolated from cultured 3T3-L1 cells using RNABee (Tel-Test, Friendswood, TX) according to the manufacturer's protocol. Genomic DNA was removed from isolated RNA by treating RNA with DNase(TURBO DNA-Free kit Ambion, Austin, TX) according to manufacturer's protocol. First strand cDNA was synthesized using High-Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA) according to manufacturer's protocol. qRT-PCR was performed by using Power SYBR Green PCR Master Mix (Applied Biosystems) and an Applied Biosystems 7300 real time PCR system. Standard curves were generated by four, 10-fold serial dilutions ( $10^3$ ,  $10^2$ ,  $10^1$ ,  $10^0$ ) of standard cDNA. Quantitation of mRNA was in comparison to the standard curves. Levels of mRNA were normalized to that of RPL13A. The primer sequences used to measure specific mRNAs are shown in Table 3-2.

### **Oil Red O Staining**

3T3-L1 adipocyte cells (Day 9) were stained with Oil Red O as described by Garvey et.al (29). The cells were first fixed in 2% paraformaldehyde for 90 min. They were then washed with deionized water. Cells were incubated with a working solution of Oil Red O staining for 3 h. The staining of the lipid droplets were visualized using a phase contrast microscope at 100X magnification. Pictures were taken with a digital camera (Cannon).

## **Statistical Analysis**

Statistical analyses were performed by using SigmaStat and SigmaPlot 11 software. All data are expressed as means  $\pm$  SD. All data were analyzed by one-way analysis of variance (ANOVA) and Student-Newman-Keuls post hoc test. Differences with  $P < 0.05$  were considered significant.

Table 3-1. Cell Culture Treatments

Treatment	Concentration
DMSO	0.1%100 nM
INSULIN(INS)	100 nM (Acute)
INSA	100 nM insulin given every other day after and during
INSA5	differentiation (Chronic)
	100 nM insulin+ 5 µM EGCG Given every 2 days after
	differentiation (Chronic)
EGCG	1 µM
EGCG	2.5 µM
EGCG	5 µM
EGCG	10 µM
EGCG	20 µM
EGCG	50 µM

INS-A=Chronic insulin treatment    INS-A5= Chronic insulin and EGCG treatment

Table 3-2. Primer sequences chosen for the quantification of gene transcripts

Gene	Forward primers (5'→3')	Reverse primers (5'→3')
<i>mAdiponectin</i>	aaccctggcaggaaagg	tgaacgctgagcgatacacat
<i>RPL13A</i>	cccaccacctgaccaagt	cagctcatgtgcagatctcattt

m=mouse

## CHAPTER 4 RESULTS

EGCG has been shown to decrease gluconeogenesis, decreasing hepatic glucose production, therefore possibly preventing insulin resistance (2). Adiponectin improves insulin resistance by enhancing  $\beta$ -oxidation and decreasing gluconeogenesis in the liver and by increasing glucose uptake in skeletal muscle. The mechanism by which EGCG improves insulin sensitivity is not well known. Recent research has suggested that EGCG could improve insulin resistance by enhancing adiponectin production. A recent study was done using Non-obese Type 2 diabetic Goto-Kakizaki rats fed a normal chow diet along with a 0.2% EGCG supplementation. Plasma adiponectin levels were increased significantly higher than those in the control group (12). This data suggest that EGCG could be improving insulin sensitivity by increasing the production of adiponectin in adipocytes. Adiponectin is mostly secreted by mature adipocytes. Oil red O staining of mature adipocytes can be seen in Figure 4-1. In order to investigate the effect of EGCG on the production of adiponectin we measured adiponectin extracellular secretion, intracellular expression, and mRNA expression.

### **EGCG Stimulates Adiponectin Secretion in 3T3-L1 Adipocytes**

We investigated EGCG effect on adiponectin secretion by ELISA. Cell media was taken from cells after a 6 h incubation with specified treatments. Insulin was used as a control. Insulin stimulates secretion of adiponectin, but stimulation is dependent on the PI3K signaling pathway. Inhibition of the PI3K signaling pathway decreases insulin stimulated adiponectin secretion in 3T3-L1 adipocytes (46). An insulin resistant state was made by adding insulin to cells every other day during and after differentiation (INSA). Cellular protein was determined by the Bradford assay and used to normalize

adiponectin concentration. At the 6 h time point (Figure 4-2), 10  $\mu$ M EGCG significantly increased adiponectin secretion by 50% ( $p < 0.04$ ) compared to control. The other EGCG treatments had no significant effect on adiponectin secretion, but showed a positive linear correlation as treatment increased to 10  $\mu$ M EGCG and then began to show a negative linear correlation after 10  $\mu$ M EGCG. Insulin did not stimulate a significant increase of adiponectin secretion, but insulin resistant cells (INSA), exhibited a decrease in adiponectin secretion that was not shown to be significant. The addition of 5  $\mu$ M EGCG to insulin resistant cell (INSA5) had no effect. A time course was done using 2, 6, and 18 h time points in order to determine if the increase in adiponectin secretion was only seen at the 6 h time point (Figure 4-3). The results indicated no significant differences among different treatments during the 2 and 18 h incubation periods. The 10  $\mu$ M EGCG treatment only significantly increased adiponectin secretion after a 6 h incubation period ( $p < 0.04$ ).

### **EGCG Enhances Adiponectin Protein Expression in 3T3-L1 Adipocytes**

In order to understand the effect of EGCG on adiponectin synthesis, intracellular adiponectin was measured by western blot analysis. Adipocytes were incubated with specified treatments for 2, 6 and 18 h. No significant changes were found during the 2 and 18 h incubation period (data not shown). At the 6 h incubation period, densitometric quantification of immunoreactive band intensities revealed that 10  $\mu$ M EGCG significantly increased intracellular adiponectin by about 50% ( $p < 0.05$ ). Cell treatments including 0.1% DMSO, 100 nM chronic insulin (INSA), or 1  $\mu$ M, 2.5  $\mu$ M, 5  $\mu$ M, 10  $\mu$ M EGCG are shown in Figure 4-4. Cell treatments including 0.1% DMSO, 100 nM Insulin, 100 nM chronic insulin (INSA), 100 nM chronic insulin plus 5  $\mu$ M EGCG (INSA5), or 5

$\mu\text{M}$ , 10  $\mu\text{M}$ , 20  $\mu\text{M}$ , 50  $\mu\text{M}$  are shown in Figure 4-5. The induction of protein expression in the cell relates to the increase in secretion of adiponectin.

### **EGCG Increases Adiponectin mRNA expression in 3T3-L1 Adipocytes**

We measured adiponectin transcript abundance in order to determine if EGCG played a role in increasing adiponectin mRNA levels. Cells were treated with DMSO, or 5  $\mu\text{M}$ , 10  $\mu\text{M}$  EGCG. The rest of the treatments were no longer used due their non significant changes in intracellular and extracellular adiponectin protein expression. After a 6 h incubation; 5  $\mu\text{M}$  ( $p < 0.05$ ) and 10  $\mu\text{M}$  ( $p < 0.04$ ) EGCG treatments showed a 50% increase in transcript abundance, as measured by qRT-PCR (Figure 4-6).

### **EGCG Does Not Stimulate the Insulin Signaling Pathway in 3T3-L1 Adipocytes**

In a previous study, cells treated with EGCG and insulin showed an increase in the tyrosine phosphorylation of the insulin receptor, the insulin receptor substrate-1, and PI3K in hepatocytes, suggesting EGCG mimics insulin when incubated in hepatoma cells, lowering hepatic glucose production (2). Insulin has been shown to increase adiponectin secretion in adipocytes. We hypothesized that EGCG was increasing adiponectin production by increasing the tyrosine phosphorylation of the insulin receptor and insulin receptor substrate-1, therefore mimicking insulin stimulation of the insulin signaling cascade. Western blot analysis was used to measure total insulin receptor (Total IR), phosphorylated insulin receptor (p-IR), and beta-actin after a 30 min incubation with specified treatments. A 30 min time point was chosen due to the rapid speed of phosphorylation; a 10 min time point was also done but showed no significant difference from the 30 min time point (data not shown). EGCG did not increase the phosphorylation of the insulin receptor. Densitometric quantification of immunoreactive band intensities revealed a 14 fold increase of the tyrosine phosphorylation of the insulin

receptor protein with insulin treatment ( $p < 0.001$ ). EGCG does not mimic insulin in the 3T3-L1 adipocyte. Insulin receptor substrate and insulin receptor substrate phosphorylation was measured but results were not clear (data not shown).

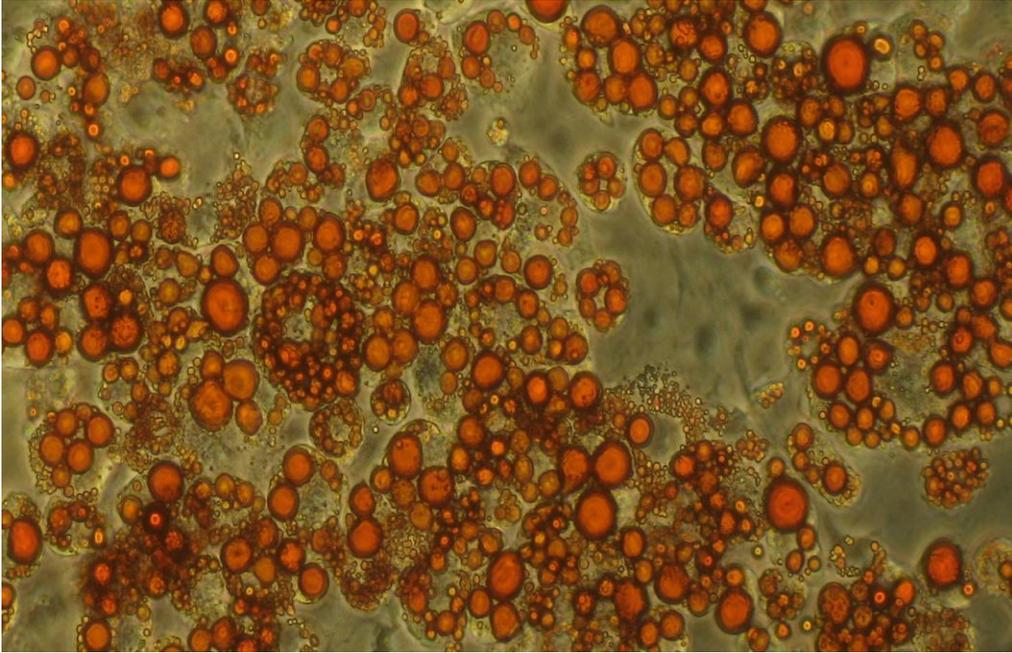


Figure 4-1. Oil Red O staining of 3T3-L1 mature adipocytes (Day 9). The staining of the lipid droplets were visualized using a phase contrast microscope at 100X magnification. Pictures were taken with a digital camera (Cannon).

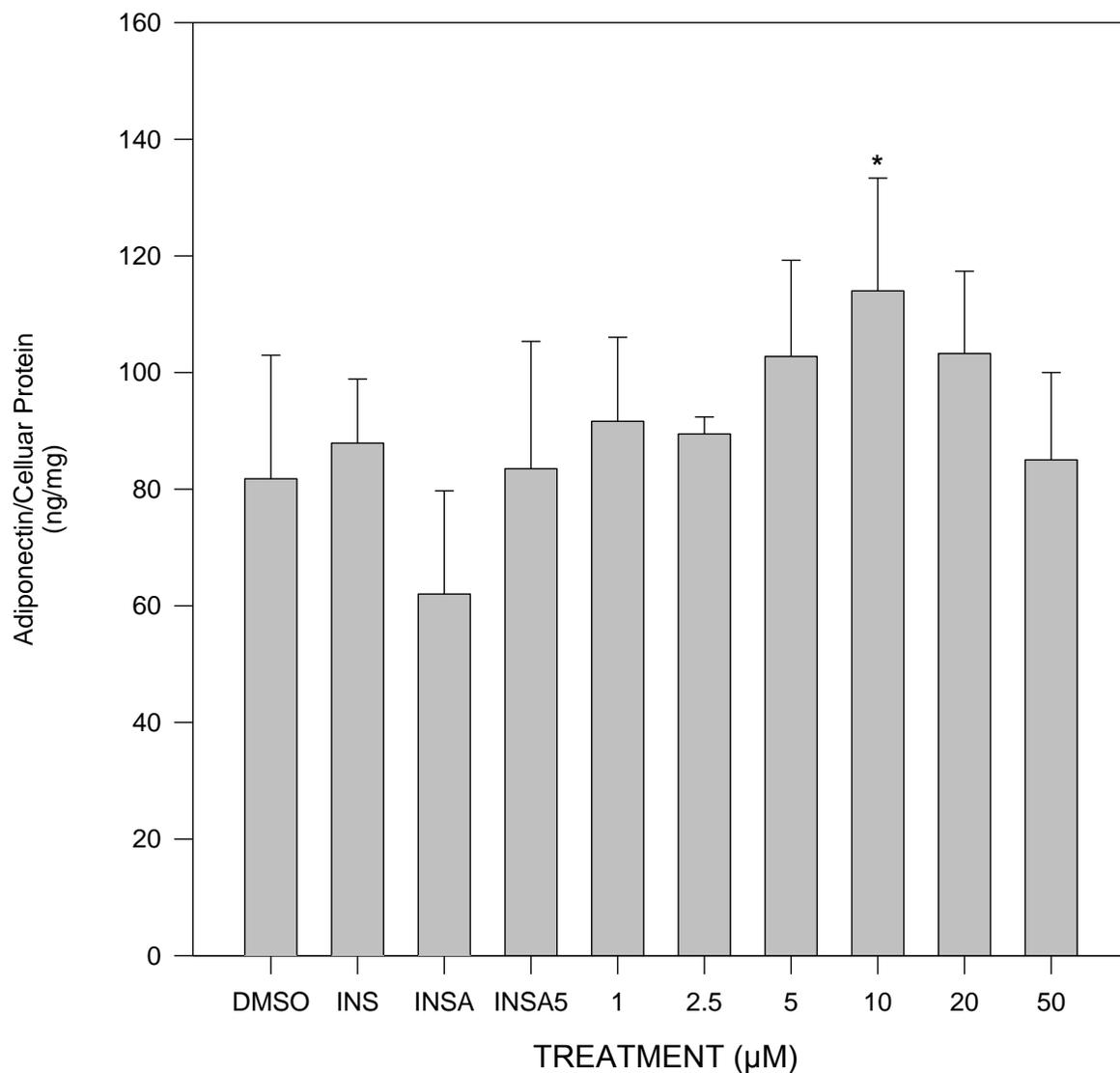


Figure 4-2. EGCG stimulates adiponectin secretion in 3T3-L1 adipocytes.

Mature adipocytes (Day 9) Cells were incubated with 0.1% DMSO, 100 nM Insulin (INS), 100 nM Insulin-Continuous (INSA), 100 nM Insulin + 5 μM EGCG Continuous (INSA5), or 1 μM, 5 μM, 2.5 μM, 5 μM, 10 μM, 20 μM, 50 μM EGCG for 6 h. Cell media was removed and used for analysis of Adiponectin protein secretion using a mouse adiponectin ELISA kit. Protein content was determined by the Bradford method (Bio-Rad, Hercules, CA). Asterisks indicates a significant difference ( $P < 0.05$ ). Values are means  $\pm$  SD from triplicates of 3 independent experiments.

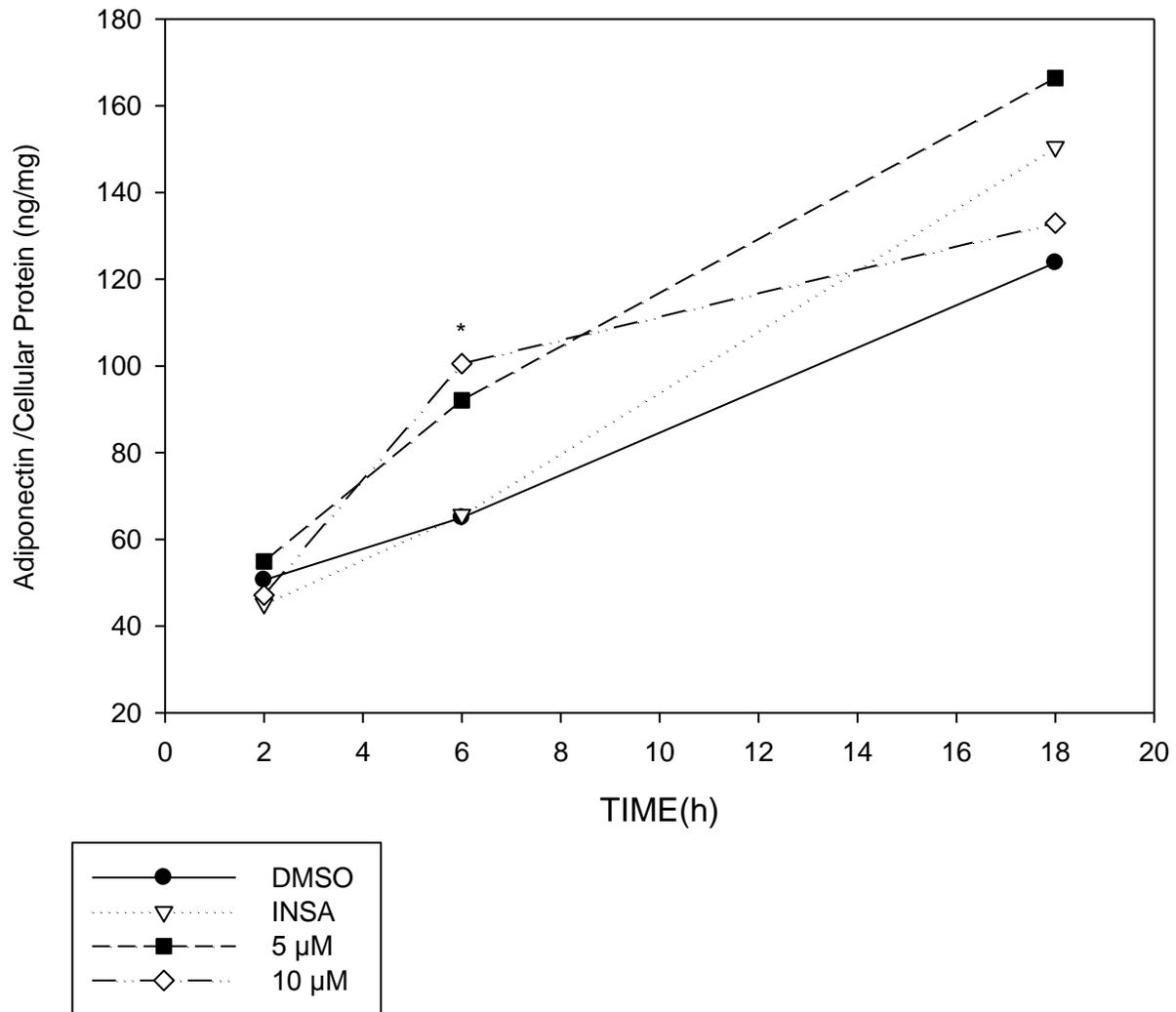
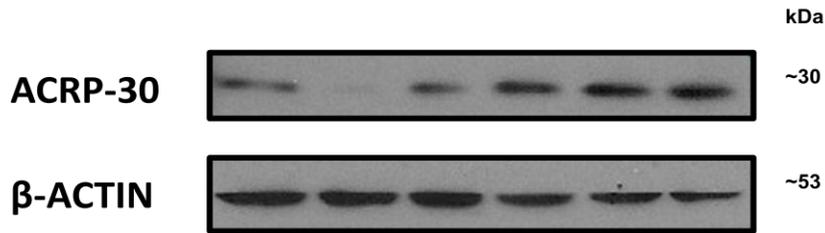


Figure 4-3. Time course on the inducible effect of EGCG on adiponectin secretion in 3T3-L1 adipocytes. Mature adipocytes (Day 9) Cells were incubated with 0.1% DMSO; 100 nM Insulin-Continuous (INSA), or 5 μM, 10 μM EGCG at 2 h, 6h, or 18h. Cell media was removed and used for analysis of Adiponectin protein secretion using a mouse adiponectin ELISA kit. Protein content was determined by the Bradford method (Bio-Rad, Hercules, CA). Asterisks indicates a significant difference ( $P < 0.05$ ). Values are means  $\pm$  SD from triplicates of 3 independent experiments.

A



B

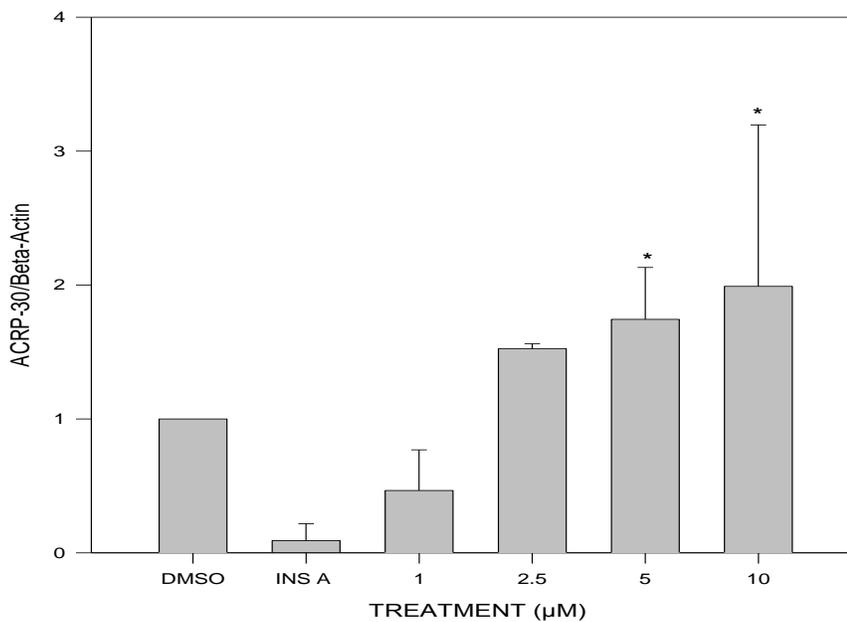


Figure 4-4. EGCG increases intracellular adiponectin expression in 3T3-L1 adipocytes. Mature adipocytes (Day 9) Cells were incubated with 0.1% DMSO, 100 nM Insulin-Continuously (INSA), or 1  $\mu$ M, 5  $\mu$ M, 2.5  $\mu$ M, 5  $\mu$ M, 10  $\mu$ M EGCG for 6hr. A) Western blot analysis of cell lysates. Samples containing 45  $\mu$ g of protein were separated on a 15% sodium dodecyl sulfate (SDS)-polyacrylamide gel. The Blot was probed for anti-adiponectin primary antibody, then stripped and re-probed for loading control anti- $\beta$ -Actin. Protein concentration of lysates was determined by the Bradford method. B) Data represents the densitometric analysis of the relative densities of the means  $\pm$  SD from 3 independent experiments. Asterisks indicates a significant difference ( $P < 0.05$ ).

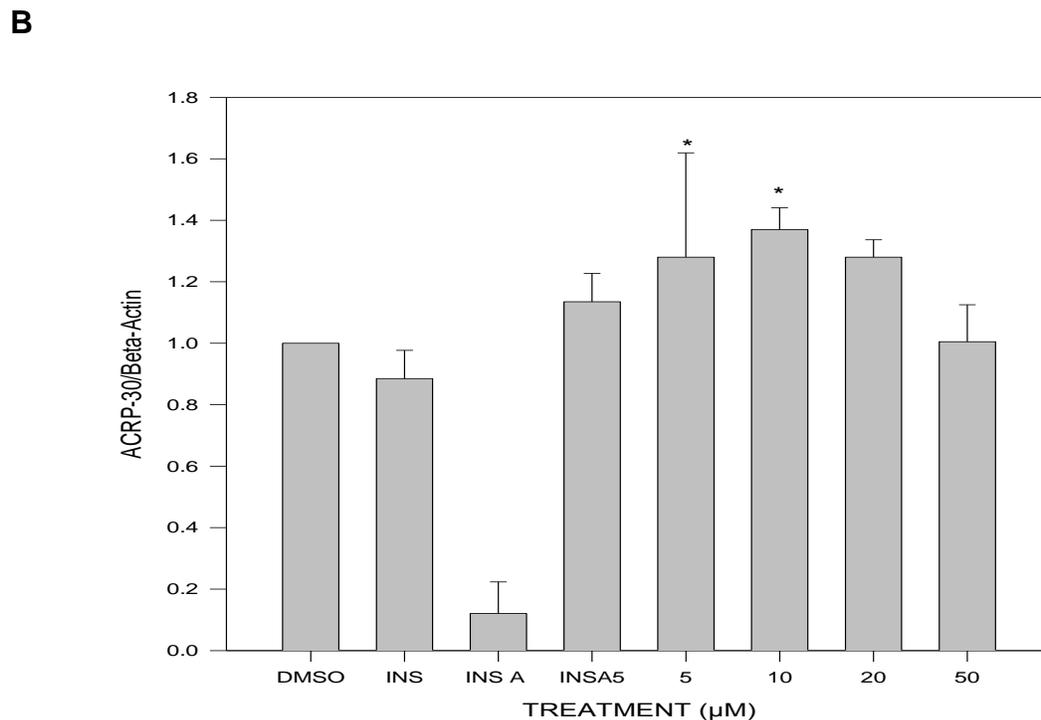
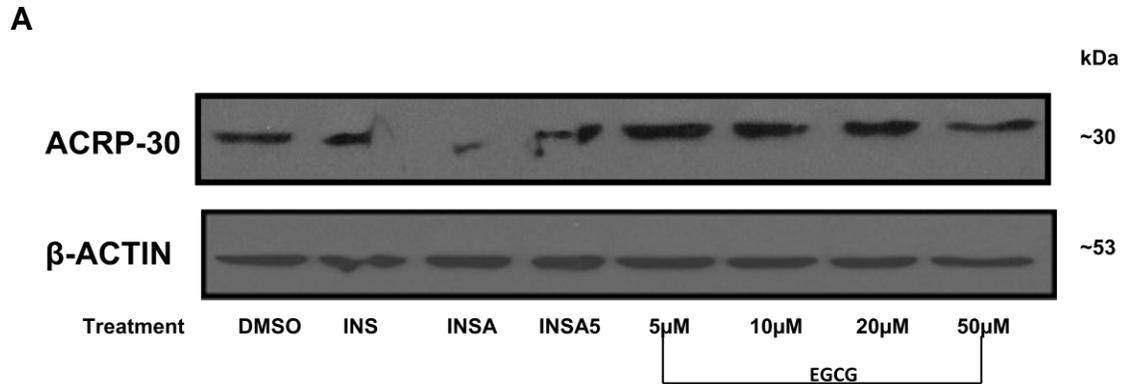


Figure 4-5. EGCG increases intracellular adiponectin expression 3T3-L1 adipocytes (Continued). Mature adipocytes (Day 9) Cells were incubated with 0.1% DMSO, 100 nM Insulin, 100 nM Insulin-Continuously (INSA), 100 nM Insulin-Continuously + 5  $\mu$ M (INSA5), or 5  $\mu$ M, 10  $\mu$ M, 20  $\mu$ M, 50  $\mu$ M EGCG for 6 h. A) Western blot analysis of cell lysates. Samples containing 45  $\mu$ g of protein were separated on a 15% sodium dodecyl sulfate (SDS)-polyacrylamide gel. The blot was probed for anti-adiponectin primary antibody, then stripped and re-probed for loading control anti- $\beta$ -Actin. Protein concentration of lysates was determined by the Bradford method. B) Data represents the densitometric analysis of the relative densities of the means  $\pm$  SD from 3 independent experiments. Asterisks indicates a significant difference ( $P < 0.05$ ).

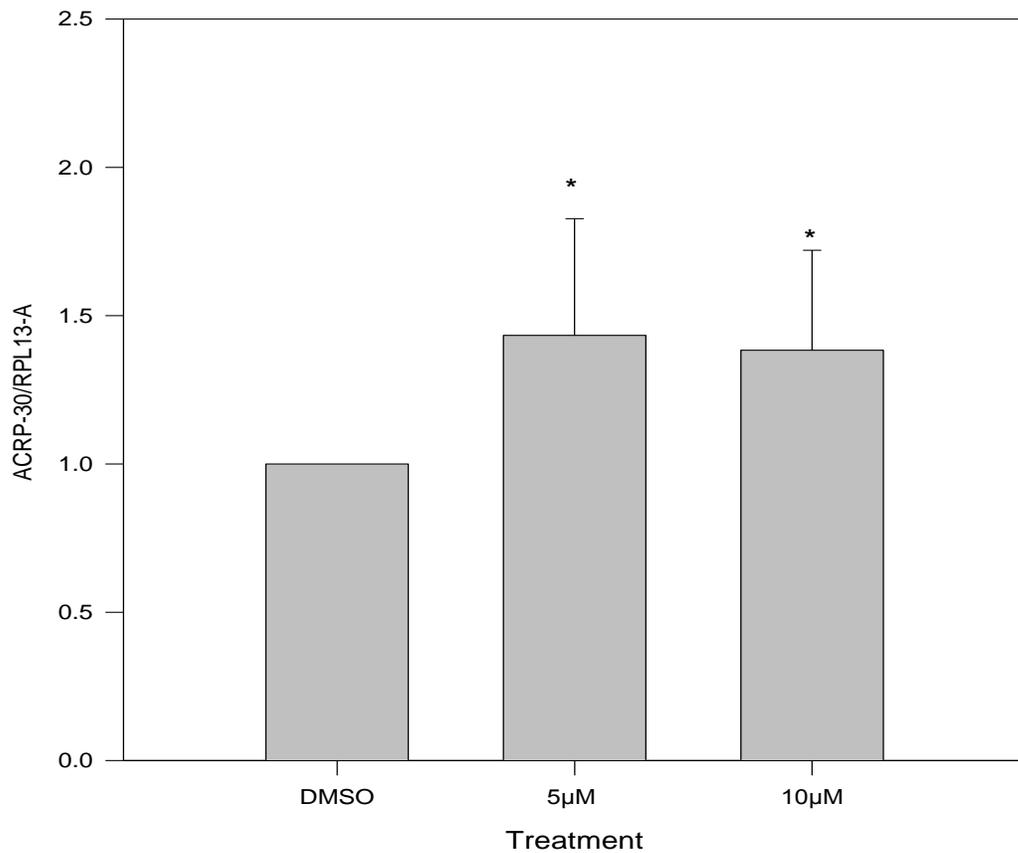


Figure 4-6. EGCG increases adiponectin mRNA expression. Mature adipocytes (Day 9) Cells were incubated with 0.1% DMSO, or 5 µM, 10 µM EGCG for 6 h. Total RNA was isolated from cell lysates and relative ACRP-30 mRNA levels were quantified using qRT-PCR analysis. Data from 3 different experiments are represented as means  $\pm$  SD of the relative mRNA normalized with RPL-13A. Asterisks indicates a significant difference ( $P < 0.05$ ).

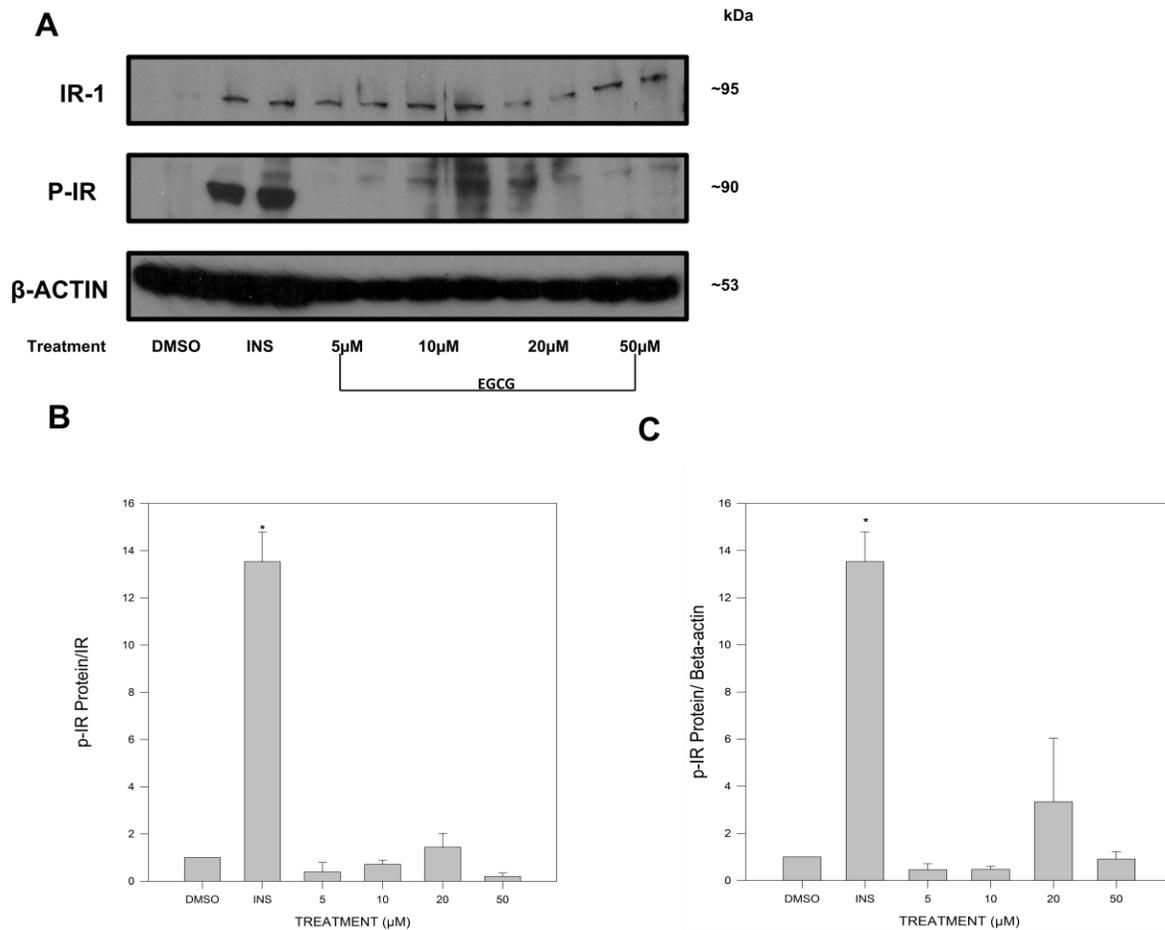


Figure 4-7. EGCG does not stimulate tyrosine phosphorylation of insulin receptor. Mature adipocytes (Day 9) Cells were incubated with 0.1% DMSO, 100 nM Insulin, or 5 μM, 10 μM, 20 μM, 50 μM EGCG for 30 min. A) Western blot analysis of cell lysates. Samples containing 45 μg of protein were separated on a 7.5% sodium dodecyl sulfate (SDS)-polyacrylamide gel. The Blot was probed for anti-insulin receptor primary antibody, then stripped and re-probed for anti-Insulin receptor phosphorylation, then stripped and re-probed for loading control anti-β-Actin. Protein concentration of lysates was determined by the Bradford method. B) Tyrosine phosphorylation of Insulin receptor/ Total insulin receptor relative density. C) Tyrosine phosphorylation of Insulin receptor/ β-Actin relative density. B, C) Data represents the densitometric analysis of the relative densities of the means ± SD from 3 independent experiments. Asterisks indicates a significant difference ( $P < 0.05$ ).

## CHAPTER 5 DISCUSSION

### **Specific Aim I**

In this study, we hypothesized EGCG would increase adiponectin production. We observed that a 10  $\mu\text{M}$  concentration for a 6 h incubation was necessary in order to get a significant increase in adiponectin intracellular and extracellular protein expression. EGCG at a 10  $\mu\text{M}$  concentration also increases adiponectin mRNA levels, possibly indicating EGCG involvement in increasing adiponectin transcription factors like PPAR- $\gamma$ . Subjects with PPAR- $\gamma$  mutations suffer from insulin resistance and low circulating levels of adiponectin. This suggests PPAR- $\gamma$  may play a role in regulation of adiponectin synthesis. The results found in Aim I lead us to believe EGCG is involved in increasing the transcription of adiponectin, therefore increasing adiponectin synthesis in the ER, and secretion out of the cell.

Initially, we believed a higher concentration of EGCG would increase adiponectin. Previous studies exploring EGCG relationship to insulin resistance have been shown to use concentrations above 50  $\mu\text{M}$ . We initially began with concentrations of 5, 10, 50, 100, 200  $\mu\text{M}$  EGCG (data not shown). The higher concentrations seemed to down regulate the production of adiponectin secretion. The data shows that the 5  $\mu\text{M}$  concentration to not be enough to stimulate a significant change and the 20  $\mu\text{M}$  concentration to be toxic, therefore decreasing adiponectin production. Studies have shown that EGCG at high levels could prove toxic to the cell and possibly change the morphology of the cell. The higher concentrations of EGCG would not be physiological in humans. The highest concentration of catechins measured in human plasma is about 1  $\mu\text{M}$ , after a single dose of 800 mg of EGCG (57). Anything above a 1  $\mu\text{M}$

concentration would not be physiologically relevant. EGCG becomes degalloyated in the mouth and esophagus, saliva has been found to have catechin esterase activity. Although 1  $\mu\text{M}$  concentrations are found in the plasma, higher concentrations might have been metabolized, EGCG has very poor bioavailability. Many studies have found high levels of ECG after EGCG ingestion (57). In our study we found a 10  $\mu\text{M}$  concentration to have a significant effect on adiponectin production, which could possibly be relevant in humans.

Our results also indicated a 6 h incubation time was necessary in order to see a significant increase in adiponectin secretion. Several studies have shown EGCG to be very unstable in *in vitro* models. EGCG has been shown to have a 130 min half life in cell media and reach peak activity from 2 to 4 h after being in cell medium. In human plasma, catechin levels reach their peak 2 to 4 h after ingestion as well (59). The reason for the significant increase in adiponectin at the 6 h mark could be due to EGCG peak time in the cell. As seen in Figure 4-3, adiponectin rises up sharply at the 6 h incubation with a 10  $\mu\text{M}$  concentration of EGCG and then plateaus with the rest of the treatments. Further studies could be done to prove this concept by adding EGCG every 2 to 4 h to the media and measuring adiponectin secretion.

Insulin has been shown to increase adiponectin secretion in adipocytes. In our study, insulin did not show any significant increases in adiponectin secretion. *Frost et al.* demonstrated a minimum concentration of 100 pM of insulin to reduce glucose transport activity by 50% and a concentration of 100 nM of insulin could decrease GLUT 4 protein expression in 3T3-L1 adipocytes (73). The concentration of insulin that was used in this study was 100 nM, which could have cause an insulin resistance state in

the cell or changed the morphology of the cell. The cells that were supplemented with insulin continuously were chronically exposed with insulin. Adiponectin in these cells was shown to be secreted in lower levels, but intracellular protein was extremely low. Adiponectin was possibly secreted all at once during the specific time point. Time points of 2 and 18 h show intracellular adiponectin with chronic insulin exposure. Another explanation could be that the cellular protein of the cells exposed to chronic insulin was almost 90% higher than the other treatments (data not shown); the protein concentration used in western blot analysis could have been from a lower number of cells, therefore having a lower amount of cellular adiponectin. The chronic insulin treatment with the addition of EGCG did not show any significant improvements. EGCG might play a role in improving insulin sensitivity, but not improving an already insulin resistant state. Further studies should be done in order to further investigate this.

### **Specific Aim II**

EGCG has been previously shown to increase the tyrosine phosphorylation of the insulin receptor, the insulin receptor substrate-1, and PI3K in hepatocytes, suggesting EGCG to be an insulin mimetic, lowering hepatic glucose production (2). In our study we did not come to the same conclusion as that seen in hepatocytes. In our 3T3-L1 adipocyte model, EGCG does not increase the tyrosine phosphorylation of the insulin receptor, thus indicating EGCG does not stimulate the insulin signaling pathway from the phosphorylation of the insulin receptor. A major regulator in adiponectin secretion is PI3K, but it is highly dependent in insulin in order to stimulate adiponectin secretion. A recent study, observed a tyrosine phosphorylation of the insulin receptor and insulin receptor substrate with 100  $\mu$ M EGCG concentration (74). This could prove toxic to the cell and is not a physiologically relevant concentration. A recently published study has

identified what they think is the EGCG receptor (also known as the 67 kDa laminin receptor) in 3T3-L1 adipocytes (75). In this study it was discovered that EGCG acts by phosphorylating its own receptor and then phosphorylating downstream IRS proteins. It could be through this mechanism that EGCG bypasses the insulin receptor phosphorylation and phosphorylates IRS proteins, eventually increasing the activation of PI3K, in turn increasing adiponectin production (76). Further studies need to be done in order to understand EGCG effect on IRS proteins and PI3K activation.

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## BIOGRAPHICAL SKETCH

Cindy Montero was born in Lima, Peru and moved to the United States when she was just 5 years old. A first generation graduate, Cindy completed her B.S. in nutritional sciences at the University of Florida, becoming the first person in her entire family to receive a bachelor's degree in science. As an undergraduate, Cindy worked under Dr. Susan Percival as an undergraduate researcher. Taking great interest in her field of research of adiponectin, she decided to pursue a graduate degree. In 2008, Cindy was accepted into the M.S. program in nutritional sciences at the University of Florida. She continued working under the guidance of Dr. Susan S. Percival. After graduation Cindy intends on pursuing a doctor of philosophy in nutritional science.