The Pennsylvania State University

The Graduate School

Department of Food Science

THE REVERSAL AND PREVENTION OF METABOLIC SYNDROME BY

(-)-EPIGALLOCATECHIN-3-GALLATE IN HIGH FAT-FED MICE

A Thesis in

Food Science

by

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Submitted in Partial Fulfillment
of the Requirements
for the Degree of

Master of Science

August 2010
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ABSTRACT

Tea (Camellia sinensis, Theaceae) has been shown to have disease preventive effects in laboratory studies. We investigated the effect of the major green tea polyphenol, (-)-epigallocatechin-3-gallate (EGCG), on high fat diet induced obesity in male C57BL/6J mice. We hypothesized that the supplementation of a high fat diet with EGCG would reverse or prevent metabolic syndrome in this model.

Obese mice were treated with high fat diet containing 0.32% EGCG for 6 weeks. There was a significant decrease in body weight gain in EGCG-treated mice compared to high fat-fed obese mice (44.0% decrease, p<0.01). In addition, obese mice fed high fat diet containing EGCG had significantly decreased fasting blood glucose levels (16.7% decrease at week 14, p<0.05), fasting plasma insulin levels (22.7% decrease, p<0.05) and insulin resistance as measured by homeostasis model assessment of insulin resistance (HOMA-IR) (29%, p<0.05) compared to high fat-fed controls. Short-term EGCG treatment increased plasma high-density lipoprotein (HDL) cholesterol (28.6% increase, p<0.05) and the ratio of HDL to non-HDL cholesterol (46.1% increase, p<0.05) compared to high fat-fed mice. In addition, plasma adiponectin levels were increased (14.3% increase, p<0.05) compared to high fat mice.

Treatment of high fat-fed lean C57BL/6J mice with 0.32% EGCG for 15 weeks reduced final body weight compared to those fed a high fat diet (9.4% decrease, p<0.05). EGCG treatment also reduced fasting blood glucose (18.5% decrease at week 14, p<0.01) and plasma insulin (25.3% decrease, p<0.05) compared to high fat-fed controls. Insulin resistance as estimated by HOMA-IR was also significantly reduced by EGCG treatment (33.9% decrease, p<0.05).
In both studies, EGCG treatment ameliorated the development of fatty liver disease. A 49-51% decrease in plasma alanine aminotransferase levels and a 21-23% decrease in liver weights were observed. EGCG treatment of high fat-fed lean mice decreased final liver triglycerides (27% decrease, p<0.05) and severity of hepatic lipidosis (27.2% decrease, p<0.05) compared to the high fat-fed control; however these effects were not observed following short-term treatment of obese mice with EGCG.

Because EGCG treatment increased fecal lipid content by 20 to 29.4%, we assessed the possible role of inhibition of pancreatic lipase (PL) as a mechanism for EGCG-mediated anti-obesity effects. PL is a key enzyme in lipid digestion. In vitro, EGCG dose-dependently inhibited PL with 50% inhibition at 7.5 µM. Kinetic analysis showed that EGCG inhibition was non-competitive with respect to substrate concentration. Black tea compounds were also examined. Theaflavin-3,3’-gallate (TFdiG) was the most potent inhibitor with an IC\textsubscript{50} of 0.45 µM, and also exhibited a non-competitive inhibition with respect to substrate concentration. In conclusion, EGCG supplementation reverses metabolic syndrome in obese mice and reduces development of metabolic syndrome in high fat-fed lean mice. Some of these effects may be explained by the observed inhibition of PL by EGCG.
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ACKNOWLEDGEMENTS

I would like to express my gratitude to The Pennsylvania State University for giving me the opportunity to pursue a Master of Science degree. The excellent facilities and talented professors were a great asset to my education. This work was supported in part by a grant from the National Center for Complimentary and Alternative Medicine (AT004678). Salary and tuition support came from the Department of Food Science.

I am very grateful to my advisor, Dr. Joshua Lambert, for his guidance and patience throughout our first years in the department. I would also like to thank Dr. Ryan Elias, Dr. Mary Kennett and Dr. Donald Thompson for serving as members of my committee and their guidance along the way.

In addition, I would like to thank the students and staff of the Food Science Department for their friendships and support. Most importantly, I would like to show my appreciation to my lab mates for their friendship and help with research projects. The animal experiments could not have been pulled off without everyone’s help.

Lastly, I would like to thank my family and friends, especially my parents, sister and fiancé for their never ending support, always believing in me and their words of encouragement. I am forever grateful to my loved ones and want to thank them for always being there for me. I would not be the person I am today without them.
Chapter 1: Literature Review

1.1 Tea

Tea, brewed from the leaves of *Camellia sinensis* (Theaceae), is one of the world’s most popular beverages. Currently, the average world consumption of tea is 28 gallons per capita while consumption in the United States is 8 gallons per capita (1). It is consumed as green, black or Oolong tea, which represent 20%, 78% and 2% of world consumption, respectively (2). Black tea is mainly consumed in Western countries, while green and Oolong teas are consumed in Asia.

These teas differ in the degree of processing. Green tea is produced by steaming freshly harvested leaves without any further processing. Black tea is the most processed variety. The leaves are crushed and undergo enzymatic oxidation (2). This process called fermentation allows oxidation of catechin monomers to produce catechin dimers and polymers such as theaflavins (1). Oolong tea is produced by only partially fermenting the leaves.

The major polyphenolic compounds in green tea are known as catechins. The four major catechins in tea are (-)-epigallocatechin-3-gallate (EGCG), (-)-epigallocatechin (EGC), (-)-epicatechin-3-gallate (ECG) and (-)-epicatechin (EC) (Figure 1).
During processing of black tea, most of the catechins are oxidized to theaflavins (Figure 2) and thearubigin. Theaflavins provide the orange-brown color characteristic of black tea and account for 3 to 6% of the dry weight of the black leaves (2).
Other compounds found in tea include flavonols, caffeine and amino acids (3). A typical 8 ounce cup of green tea contains 50 to 540 mg of total catechins, whereas black tea contains 50 to 370 mg total catechins and 4 to 18 mg theaflavins (4). The average total catechin content of Oolong tea ranges from 50 to 460 mg/g (5, 6).

Tea has been shown to have beneficial effects with many diseases such as cancer, neurodegenerative disease, cardiovascular disease (CVD) and metabolic syndrome (7-10).

**Figure 2:** Chemical structures of theaflavins found in black tea

R₁=R₂= H  Theaflavin
R₁=H, R₂ = galloyl  Theaflavin-3-gallate
R₁= galloyl, R₂ = H  Theaflavin-3’-gallate
R₁=R₂= galloyl  Theaflavin-3,3’-digallate
1.2 Metabolic Syndrome

Metabolic Syndrome is defined by The American Heart Association as a complex condition manifesting three or more of the following symptoms: elevated waist circumference, elevated triglycerides, increased blood pressure, increased fasting glucose and reduced high-density lipoprotein (HDL) cholesterol (11). The criteria for metabolic syndrome in animals are not definitively established. In the present work, we consider metabolic syndrome in mice to be the combination of three or more of the following symptoms: body weight greater than or equal to 40 g, elevated triglycerides, increased blood pressure, increased fasting glucose and reduced HDL cholesterol. Because waist circumference is difficult to measure uniformly in mice, body weight is used instead. Triglycerides, blood pressure, fasting glucose and HDL cholesterol can all be measured in mice similar to humans; however the values of these biomarkers are not comparable between species.

Obesity is a major risk factor for metabolic syndrome and is defined as a body mass index (BMI) of 30.0 kg/m² or higher (12). Overweight is defined as BMI of 25.0 to 29.9 kg/m². In 2007, 32.2% of American adult males and 35.5% females were obese according to The National Health and Nutrition Examination Survey (NHANES) (13). By contrast, the NHANES rates in 1999 were 27.5% of American adult males and 33.4% females were obese (13). However, these obesity rates are much higher compared to The Center for Disease Control and Prevention’s (CDC) Behavioral Risk Factor Surveillance System. According to the CDC, obesity rates in men have increased from 19.9% to 27.2% from 1999 to 2007, while these rates have increased from 19.7% to 25.9% in
women (14). Despite discrepancies in obesity rates, both NHANES and CDC statistics show alarming rates of obesity in the United States.

Childhood obesity is defined as BMI at or above the 95th percentile in a given age group (15). Between 1999 and 2007, the rate of childhood obesity increased from 13.7% to 16.9% of children between 2 and 19 years old (16).

Obesity has been reported to account for a relatively large portion of United States health care costs. In 1998, medical expenditures linked to excess body weight were 9.1% of total US medical expenditures (78.5 billion dollars) (17). In 2008, this figure rose to approximately 147 billion dollars or roughly 10% of total health care costs (18). Wang et al., estimate that by 2030, US health care costs linked to excess body weight would increase to 860.7 billion dollars (16-18% of total US health care costs) (19).

Insulin resistance is an early marker of type II diabetes (T2D) and is associated with obesity (20). Insulin is an anabolic hormone involved in glucose, lipid and amino acid/protein synthesis and storage (21). It indirectly increases the activity of enzymes that catalyze synthesis of glycogen while inhibiting the activity of enzymes that catalyze the breakdown of glycogen (21). Normally, when blood glucose levels are high, insulin is released by β-cells from the pancreas and stimulates the uptake of glucose by muscle and adipose tissue and inhibits synthesis of glucose by the liver (21). With insulin resistance, the pancreas needs to release more insulin to maintain normal blood glucose levels. Elevated insulin levels and insensitivity can result in elevated fasting blood glucose and glucose intolerance. Muscle tissues lose their ability to stimulate glucose uptake. After time, the body is unable to produce enough insulin to make up for the resistance and because of this the pancreas may reduce or even stop the production of insulin (22).
T2D represents 90 to 95% of all diabetes cases (20). This disease is associated with insulin resistance in adipose and muscle tissue. The primary cause is believed to be associated with an interrupted insulin signal to the cell’s glucose transporters reducing the rate of glucose uptake (21). As a result, blood glucose levels are raised along with insulin levels. It is predicted that the number of people with both diagnosed and undiagnosed diabetes will increase from 23.7 million in 2009 to 44.1 million in 2034 (23). Pathologies related to T2D can be observed in the eyes, kidneys, heart and vasculature (20).

Other conditions that make up metabolic syndrome include elevated serum cholesterol and triglycerides. High cholesterol and elevated triglycerides are risk factors associated with CVD (24). Low density lipoprotein (LDL) transports cholesterol to tissues where it can be used for membrane construction or conversion into other metabolites (21). Elevated levels of LDL-associated cholesterol are defined as greater than 130 mg/dL and represent a risk factor for atherosclerosis, which in turn can result in a heart attack or stroke (25). HDL cholesterol is known as the reverse cholesterol transport. Its function is to remove unesterified cholesterol from cells and other lipoproteins, where it may have accumulated, and then return it to the liver to be excreted in the bile (21). Reduced serum HDL cholesterol levels (less than 50 mg/dL) are also a risk factor for atherosclerosis and CVD (26). Elevated serum triglyceride levels, greater than 200 mg/dL, also contribute to plaque formation in arteries and can lead to heart attack and stroke (12).

Hypertension, or high blood pressure, is another component of metabolic syndrome (25). Systolic blood pressure is the pressure in the arteries as the heart contracts, whereas, diastolic blood pressure is the pressure in the arteries as the heart
relaxes after the contraction. Hypertension in adults is defined as systolic blood pressure of 140 mm Hg or higher and diastolic blood pressure of 90 mm Hg or higher (27). Chronic hypertension can lead to heart disease, kidney disease and stroke (28).

Another pathology often associated with metabolic syndrome is fatty liver disease. Fatty liver disease is a disorder where fat accumulates in hepatocytes due to increased lipid synthesis, decreased lipid removal and increased dietary intake of fat. Fatty liver disease can be caused by excessive alcohol consumption (alcoholic fatty liver disease or ALFD) or by other causes including obesity. Fatty liver disease caused by these other conditions is known collectively as non-alcoholic fatty liver disease (NAFLD). Our laboratory has coined the term obesity related fatty liver disease (ORLFD) to more specifically address obesity as an etiology. Simple fatty liver or simple steatosis is the mildest form, followed by steatohepatitis, fatty liver with inflammation and damage to hepatocytes (29). Finally damage to the liver can progress into cirrhosis and hepatocellular carcinoma or liver failure (30).

Fatty liver disease is associated with insulin resistance and obesity. Insulin resistance increases the free fatty acid flux to the liver due to the decreased inhibition of lipolysis (29). Furthermore, insulin resistance and obesity decrease levels of adiponectin, a hormone released from adipocytes that regulates metabolism of glucose and lipids. Decreases in adiponectin hinder fatty acid oxidation and thus increase fat accumulation in the liver (29).

A liver biopsy is needed in order to diagnose fatty liver disease because a validated noninvasive biomarker has not been established. Elevated liver enzyme alanine aminotransferase (ALT) suggest that the liver is damaged; however it is not sufficient to
diagnose fatty liver disease. Because of the difficulty in diagnosing fatty liver disease, many cases go undiagnosed. This may account for the fact that fatty liver disease is considered a criteria for establishing metabolic syndrome, but is only an associated condition. There is no established treatment for ORFLD, except for weight loss and treating each component of metabolic syndrome (29).

Because of the many negative health and financial effects of metabolic syndrome, development of effective strategies for treatment and prevention are imperative. Possible sites of intervention include altering lipid absorption and metabolism, increasing energy expenditure and/or reducing insulin resistance.
1.3 Pancreatic lipase as a target for prevention of metabolic syndrome

Pancreatic Lipase (PL) is an enzyme secreted into the duodenum that plays a key role in digestion and absorption of fats. The pancreas also secretes at least two other lipolytic enzymes, carboxyl ester hydrolase and phospholipase A2. Colipase is an essential cofactor for PL in vivo because it is required along with bile salts and calcium ions to activate PL (31). The activity of PL is 100 to 1000 fold in excess of that needed for complete hydrolysis of triglycerides in the upper small intestine (32). At this concentration, PL can hydrolyze 250,000 to 500,000 long-chain triglyceride molecules per min and has been reported to cleave 50-70% of ingested fats (31, 33). The enzyme first rapidly hydrolyzes triglycerides to diglycerides. In a much slower reaction, another fatty acid is cleaved off yielding a monoglyceride (34). Finally, the monoglyceride is converted to glycerol and a fatty acid. Given the importance of PL for lipid digestion, it represents an attractive target for obesity prevention.

Currently, [(1S)-1-[(2S,3S)-3-hexyl-4-oxo-oxetan-2-yl]methyl]dodecyl (2S)-2-formamido-4-methyl-pentanoate (orlistat) is a Food and Drug Administration (FDA) approved PL inhibitor that is marketed for weight loss (Figure 3). Orlistat, which has a systemic bioavailability of less than 1%, inhibits fat absorption by 30% (35). A 4 year double-blind placebo study found that orlistat reduced body weight by 2 to 7 kg in obese subjects in Sweden (n = 3,305) (36, 37). In addition to weight loss, subjects had a decrease risk of T2D (HR = 0.63), reduced fasting blood glucose (50% decrease), reduced fasting serum insulin (35.6% decrease) reduced diastolic and systolic blood pressure (26.9% and 30.6% decrease, respectively), reduced total cholesterol (70.9% decrease), reduced LDL cholesterol (60.2% decrease) and elevated HDL cholesterol
(28.6 increase) compared to placebo. No significant difference was observed in plasma triglyceride or HDL cholesterol levels.

![Chemical structure of Orlistat](image)

**Figure 3**: Chemical structure of Orlistat

Natural products like grape seed extract (33) and berry polyphenols (38) have also been shown to inhibit PL. Nakai *et al.*, have recently reported that Oolong tea polyphenols inhibit PL *in vitro* (39). The IC\textsubscript{50} values ranged from 0.068 µM (oolongtheanin 3’-O-gallate) to >20 µM (EGC). However, no inhibitory kinetic analysis was performed.

Ikeda *et al.*, have looked at the inhibition of PL by catechins *in vitro* and have found that only the galloyl containing compounds inhibited PL (40). (-)-Catechin gallate and (-)-gallocatechin-3-gallate inhibited PL to a greater extent than ECG and EGCG, but all compounds tested had low potency with inhibition observed only at 1 and 2 mM.
(-)-Catechin, EC, (-)-gallocatechin and EGC had no inhibitory activity. These results suggest that the galloyl moiety is critical for PL inhibitory activity. Again, kinetic analysis to determine mode of inhibition was not performed.
1.4 Prevention of Metabolic Syndrome by Tea

1.4.1 Obesity and Tea

Bose et al., have reported that treatment with 3.2 mg/g dietary EGCG for 15 weeks reduced body weight (33 – 41%) in high fat-fed male C57BL/6J mice compared to high fat-fed controls (10). In addition, the EGCG-treated mice had significantly lower adipose tissue weight, fasting blood glucose, fasting plasma cholesterol and plasma ALT levels. The EGCG-treated mice had higher fecal lipid concentrations than the high fat-fed control mice; there was a strong inverse correlation between fecal lipid content and body weight gain. This suggests that EGCG-mediated modulation of dietary fat could account for the decrease in body weight observed in the EGCG-treated mice.

The same group reported that short-term (4 wks) dietary EGCG treatment (3.2 mg/g) of obese C57BL/6J mice tended to reduce body weight gain compared to high fat-fed controls. Although the decrease in body weight gain was not statistically significant, significant reductions in mesenteric adipose tissue weight (36%) and fasting blood glucose (22%) were observed in the EGCG-treated mice compared to high fat-fed controls. This treatment regimen represents a more realistic obesity-related application of EGCG or green tea supplementation since the most likely consumers of these products would have a pre-existing weight problems. Given the small sample size (n = 10) and limited mechanistic data of this short-term study, further studies are warranted.

In another study, Lee et al., found that high fat-fed obese mice treated with EGCG (5 mg/g) for 8 weeks had decreased body weight (8.5% decrease) compared to the high fat-fed control mice (41). In addition, a significant decrease in adipose tissue weight, plasma triglycerides and cholesterol, and plasma leptin was observed. However,
significant differences in fatty liver disease parameters were not observed. Gene expression was examined from adipose tissue and significant decreases in expression were found with adipogenic transcription factors like peroxisome proliferator-activated receptor γ (PPAR-γ), CCAAT/enhancer-binding protein (CEBP-α) and sterol regulatory element binding protein 1c (SREBP-1c) between high fat control and EGCG group. There were also significant increases in gene expression of genes related to lipolysis, β-oxidation and thermogenesis in EGCG treated mice when compared to high fat control.

Wolfram et al., have demonstrated the effect of TEAVIGO (a proprietary green tea extract that contains 90% EGCG) on obesity (42). Treatment of high fat/high sucrose-fed C57BL/6J mice with 10 mg/g dietary TEAVIGO resulted in decreased body weight gain, fed state plasma glucose, plasma triglycerides, and plasma leptin compared to high fat/high sucrose-fed controls. In the same study, the authors reported that 4 week treatment with 10 mg/g dietary TEAVIGO could reduce body weight gain and body fat weight in obese Spague-Dawley rats (n = 8). Gene expression studies revealed that TEAVIGO treatment decreased adipose levels of fatty acid synthase (FAS) and acetyl coA carboxylase-1 mRNA, both key enzymes in de novo fatty acid synthesis. These observations suggest that tea polyphenols may modulate obesity by inhibiting de novo lipogenesis. Several studies have examined the effects of tea polyphenols on FAS in vitro. It has been shown that EGCG and ECG, but not EC and EGC, inhibit FAS with IC₅₀ = 52 and 42 μM, respectively (43, 44). In addition, one study showed that black tea extract inhibited FAS activity to a greater extent than green tea extract (GTE); however, the mechanisms of this inhibition still unclear (45).
Bruno et al., compared the effects of treatment with diets containing 10 mg/g and 20 mg/g GTE for six weeks on B6.V-Lep\textsuperscript{ob}/J (ob/ob) mice (n=24) and C57BL/6J lean littermates (46). Ob/ob mice cannot produce leptin, which results in obesity, glucose intolerance and elevated plasma insulin levels. Leptin is a hormone produced in adipose tissue that regulates appetite. Lean and ob/ob mice treated with GTE weighed less than their respective non-GTE treated controls. Furthermore, the GTE was more effective in the ob/ob mice than the lean C57BL/6J mice. The obese mice weighed 23 to 25% less than controls, while the lean mice were only 11 to 20% less than lean controls. In addition to reduced weight gain, this study found that GTE –treatment reduced the extent of hepatic steatosis in ob/ob mice compared to control ob/ob mice. No dose-response relationship was observed for GTE, which suggests that 10 mg/g GTE may be the maximally-effective dose. This study did not state whether the GTE contained caffeine, an omission which limits interpretation of the results.

In most obesity studies of tea in animals, the diets contain very high concentrations of fat (40 – 60% kcal) which may not represent realistic consumption patterns. A recent study by Yuko et al., is an exception (47). In this study, the effect of tea catechins (1 and 5 mg/mL in the drinking fluid) administered for 3 weeks was determined in male Wistar rats fed a normal fat diet (10% kcal). The 5 mg/mL catechins-treated group had significantly decreased body weight compared to the water-treated control. Both tea catechin groups had lower levels of serum cholesterol, serum triglycerides, and bile acids compared to the control: these values were dose dependent. Mesenteric and liver lipids were also dose-dependently reduced compared to the water-treated group. By incorporating tea catechins into a normal diet, this group suggests that
catechins can modulate lipid metabolism in non-obese rats. This is a very important finding and should be confirmed with additional studies.

A more limited number of studies have examined the obesity preventive effects of Oolong and black tea. Yang et al., compared the effect of GTE, Oolong tea extract, and black tea extract (10 mg/mL in drinking fluid) in rats fed a high sucrose diet. Both Oolong and black tea extract-treated rats, but not green tea extract-treated rats, had decreased body weight gains (28.8 – 35.0%) and feeding efficiency (19.8 – 32.2%) (48). In all of the tea-treated groups, plasma and liver triglycerides were reduced. It is interesting that GTE had no significant effect on body weight gain in this study. The reasons for this are unclear, but may be related to the polyphenol composition, particularities with the model, or some other unknown factors.

In order to induce a negative energy balance, exercise in combination with dietary changes has been the most common recommendation for human weight loss. Few studies, however, have systematically examined the interaction between the tea polyphenols and exercise. Murase et al., have shown that mice consuming a 20% kcal fat diet supplemented with 1.0 – 5.0 mg/g GTE and put on an exhaustive swimming regimen for 10 weeks had increased swimming times compared to Balb/c mice that only had the swimming regimen (49). Mice treated with GTE also had decreased adipose tissue weight, increased oxygen consumption and increased β-oxidation activity in muscle compared to mice given the swimming regimen only.

In a second study, high fat-fed C57BL/6J mice were treated with 5 mg/g dietary catechins, 30 minute swimming three times per week or the combination for 15 weeks (50). Body weight gain was reduced by 18% in the high fat plus catechins group and 14%
in the high fat plus exercise group compared to the high fat diet-treated mice not given catechins or exercise. The combination of high fat plus catechins and exercise group resulted in the greatest difference, body weight gain decreased by 33% compared to the high fat-treated group. Mice on the catechins plus swimming regimen had higher muscular β-oxidation activity and higher lipid oxidation as determined by indirect calorimetry compared to the high fat and the high fat plus catechins group. Future studies should be conducted to determine the different dose-response relationships for tea catechins and exercise time, as well as the effect of different types of exercise (forced vs. voluntary, running wheel, etc.). These studies should also be extended to include obese as well as lean mice.

In all of the above studies, there was no difference between energy intake between the high fat and high fat plus tea dietary groups, except for the following. Murase, et al., observed a decrease of 5.6% between energy intake in the 5 mg/g tea catechins fed mice compared to the high fat-fed mice; however, this trend was not significant. With Yang et al., there was a significant difference between food intake with the Oolong tea group compared to the other groups. The results from this paper show a decrease in body weight gain from Oolong tea extract, which could be effected by this difference in food intake.

The papers reviewed above demonstrate the potential efficacy of tea as an anti-obesity agent. However, further studies are needed, particularly on the efficacy and mechanisms of action of black and Oolong teas, and the interaction between caffeine and the tea polyphenols with regard to weight loss. A potential problem with the current body of literature is related to the amount of fat used in the experimental diets. The typical American diet contains 30% kcal fat, whereas most of the reported animal studies used
diets with 40 – 60% kcal fat (51). Studies with lower fat diet (30 – 40% kcal) might, therefore, produce data more relevant to the human situation. Similarly, weight loss regimens recommended to obese and overweight individuals often include reduced calorie intake and exercise. Future studies should incorporate these factors in examining the efficacy of tea and tea polyphenols.

A limited number of epidemiological studies have examined the impact of tea on body weight and other markers related to obesity. A 2003 cross-sectional epidemiological study of 1103 Taiwanese adults found that habitual tea drinkers (defined as someone who consumed tea at least once per week for six months) who consumed tea for more than 10 years had lower percentage body fat (19.6% decrease) and waist:hip ratio (2.1% decrease) compared to non-habitual consumers (52). In this study, green tea and Oolong tea were consumed more frequently than black tea (41.3% vs. 1.6%). A longitudinal analysis within The Netherlands Cohort study of 4280 adults found an inverse relationship between catechin consumption over the 14 year study period and BMI increase (53). The BMI increases for the lowest quintile and highest quintile of catechin consumption were 0.77 kg/m² and 0.31 kg/m², respectively.

A larger number of intervention studies have been conducted on the effect of tea on markers of obesity (reviewed in (54, 55). In 2005, Nagao et al., examined the effect of supplementing Oolong tea with GTE (22 mg (low) or 690 mg (high) catechins) in 35 healthy, Japanese men once daily for 12 weeks (56). Following treatment, subjects consuming high catechin tea had lower body weight (2.4 kg decrease), BMI (0.8 kg/m² decrease), waist circumference (3.4 cm decrease), and body fat mass (1.4 kg decrease) compared to baseline. This experiment was repeated with 240 obese, Japanese subjects
treated with a catechin-enriched green tea beverage (583 mg catechins) or a control green tea beverage (96 mg) once daily for 12 weeks (57). Participants consuming the high catechin beverage had a significant decrease in body weight (2.3% decrease), total fat area (4.9% decrease), and visceral fat (9.4% decrease) compared to baseline values. Percent body fat, waist and hip circumference, and LDL cholesterol were also significantly decreased compared to baseline. In both studies by Nagao et al., the amount of caffeine in the tea beverages were held constant (21 – 23 mg) while the catechin concentration was varied. In addition, these decreases in symptoms were significantly greater in the high catechin tea-treated subjects than in the low catechin tea treated subjects for both experiments. This strongly supports the hypothesis that catechins can affect markers of obesity.

The results of studies on tea and body weight and body fat have not been universally positive. For example, in a trial of 60 overweight or obese Thai subjects, ingestion of green tea capsules (250 mg each containing 33.6 mg of EGCG, 3 times per day) for 12 weeks resulted in a significant decrease in body weight (3.9%) compared to baseline and placebo control, but there was no significant effect on BMI, waist:hip ratio, or percent body fat (58). A similar result was observed in a study of 46 overweight women in the Netherlands (59). Treatment with GTE (375 mg catechins) for 87 days in combination with reduced calorie diet resulted in no significant effect on BMI, waist:hip ratio, or fat mass. The doses of tea catechins used in these negative studies were somewhat lower than those used in the studies that reported positive doses. So the observed differences may be a dose-effect, such an issue will be resolved only with carefully designed dose-response studies in a well-selected population.
Another critical question regarding the potential efficacy of tea as an anti-obesity agent relates to its ability to aid in weight maintenance following weight loss. A recent meta-analysis of 11 studies on green tea and weight loss showed that habitual consumption of green tea had a beneficial effect on weight loss (mean weight loss of -1.3 kg) and aided weight maintenance following weight loss (60). The effects of green tea were modulated by chronic, high (> 300 mg/d) intake of caffeine. Westererp-Plantenga et al., reported that treatment of overweight or moderately obese subjects from the Netherlands (n = 76) with EGCG/caffeine capsules (250mg/150mg total daily dose) for 3 months following weight loss resulted in further decrease in body weight and increased fat oxidation in people who habitually consumed less than 300 mg caffeine/d (61). In individuals that chronically consumed more than 300 mg caffeine per day, these weight maintenance effects were lost. This is somewhat counter-intuitive given that increased caffeine consumption should facilitate weight loss and energy expenditure. The mechanism by which high chronic caffeine consumption impacts weight maintenance by EGCG plus caffeine is unclear but could be related to modulation of either EGCG or caffeine metabolism. The results of this study should be confirmed and the potential underlying mechanisms examined.
1.4.2 Diabetes and Tea

There are two forms of diabetes, type I and II. Type I diabetes (T1D) begins early in life and occurs as a result of autoimmune destruction of pancreatic β-cells resulting in insulin deficiency. By contrast, T2D is a slow developing disease where insulin is produced but the insulin response system is defective (20). T2D usually occurs in conjunction with obesity and insulin resistance and is believed to be caused by an interrupted insulin signal to the cell’s glucose transporters preventing the body from metabolizing glucose (21). In the United States, rates of T2D in adults has risen from 3% in the 1970s to 6% in 2009 (62). In 2007, about 186,300 people younger than 20 years have diabetes (T1D or T2D), which represents 0.2% of all people in this age group (63). Worldwide, T2D is the fifth leading cause of death (64). Tea has been shown to help reduce blood glucose levels and increase insulin production in both humans and animal models (55). However, a definitive mechanism of action is still uncertain.

Roghani et. al., examined the beneficial effects of EGCG on chemically induced T1D (65). Male Wistar albino rats (n=32) were injected with 60 mg/kg streptozotocin (STZ), a cytotoxicant that inhibits insulin secretion by damaging pancreatic β-cells, and after one week the rats with serum glucose higher than 25 mg/mL were placed into 3 groups: EGCG control, diabetic control and EGCG diabetic. EGCG was administered through a daily gavage of 25 mg/kg body weight in saline for 8 weeks. The EGCG diabetic group had decreased serum glucose levels compared to control and baseline values. In addition, the EGCG diabetic group had a 30.8% decrease in malondialdehyde (MDA), a marker of lipid oxidation, levels and a 21.3% increase in superoxide dismutase (SOD), an enzyme that reduces oxidative stress, levels compared to the diabetic control.
Black tea hot water extract was shown to have a regenerative effect on damaged pancreatic β-cells (66). Wistar albino mice (n = 12) were injected with STZ and then given either 12.5 mg/mL of black tea extract or water daily for 2 weeks. Histological analysis indicated a regeneration of β-cells with treatment of black tea. The water-treated control mice had intense uniform staining for inducible nitric oxide synthase (iNOS) throughout the pancreatic section. iNOS is an enzyme found in the immune and cardiovascular system and converts arginine to produce nitric oxide (NO) (67). High levels of NO can react with superoxide leading to cell toxicity. Black tea-treated mice, however, had reduced staining for iNOS and expression was localized to the acinar regions, where the β-cells are secreted. Reduced staining of iNOS in the tea treatment group indicates there is less damage to the β-cells. This suggests that black tea can help regenerate damaged β-cells in diabetes induced mice.

The effect of tea on T2D has been examined in both diet-induced and genetic models. HF-fed male Sprague-Dawley rats (n = 21) treated with EGCG (1 mg/mL in drinking water) for 6 weeks had a significantly decreased plasma triglyceride, insulin and glucose levels compared to high fat-fed controls (68). These liver and plasma MDA levels of EGCG-treated rats were also decreased compared to the high fat control.

Wolfram et al., examined the effects of EGCG in two rodent genetic models of T2D (69). Zucker Diabetic Fatty (ZDF) rats are an inbred rat model with ineffective leptin receptors thus prone to T2D, glucose intolerance and obesity. Similarly, BKS.Cg-m +/- Lepr<sup>db/db</sup>/J (db/db) mice have a leptin receptor mutation and are obese, have elevated insulin and elevated blood glucose levels. Db/db mice treated with 2.5- 10 mg/g EGCG for 7 weeks, (n=9/group) had dose-dependently improved oral glucose tolerance and
decreased fasting glucose. EGCG-treated ZDF rats showed similar decreases in fasting blood glucose. In addition, mechanistic experiments showed that EGCG dose dependently increased glucokinase (70) expression and decreased the expression of PEPCK in the liver of db/db mice. The former enzyme enhances glycolysis and glucose uptake in the liver, whereas the latter enzyme functions in gluconeogenesis. EGCG increased expression of acyl-CoA oxidase-1 (ACO-1) and carnitine palmitoyl transferase-1 (CPT-1) in liver and adipose tissue in db/db mice. Both enzymes are involved in fatty acid catabolism and increased expression of these genes indicates that EGCG increases this process. These alterations in glucose and lipid metabolism may explain the observed improvement in glucose homeostasis.

There are several cohort studies that have used food frequency questionnaires to determine the association between tea drinking and diabetes. In the Dutch Contribution to the European Prospective Investigation into Cancer and Nutrition (EPIC study) tea consumption was found to be inversely related to risk of T2D (hazard ratio = 0.63 for 5 or more cups of tea per day) (71).

In the Singapore Chinese Health Study (n = 36,908), consumption of greater than 1 cup black tea per day was associated with reduced risk of diabetes (RR = 0.86) (72). However, no association was seen with green tea consumption.

A cohort study of 5823 British male and female subjects with 11.7 y follow-up found an inverse association between daily tea consumption of greater than 3 cups of tea and risk of T2D (hazard ratio = 0.66) (73). However, the study did not differentiate between varieties of tea that was consumed.
An intervention study of Taiwanese subjects (n = 20) with T2D found that treatment with 1500 mL of caffeinated Oolong tea for 30 days resulted in lower plasma glucose compared to baseline (29% decrease), whereas no change was observed in the water control group (74). In addition, concentrations of fructosamine, a long-term measurement of blood sugar, was decreased by 21% compared to baseline in the tea treated group. No change in body weight or physical activity was observed in either group. A limitation of this study was the use of caffeinated tea because it is difficult to differentiate the effects seen with catechins or caffeine. In previous studies, caffeine has been shown to decrease insulin sensitivity and blood glucose (75).

Nagao et al., examined the effects of a green tea beverage on diabetic subjects. Subjects with T2D (n = 43) ingested 72.3 or 582.8 mg catechins in 1 can of green tea beverage once daily for 12 weeks (76). Subjects treated with high dose of catechins had significantly decreased percent body fat, waist circumference, systolic blood pressure, serum triglycerides and total cholesterol compared to the low dose group. However, treatment with high dose of catechins significantly increased plasma insulin compared to low dose treatment. After adjusting for percent body fat, the statistically significant difference between the two groups disappears. Therefore, the increase in plasma insulin may have been independent of body fat reduction and waist circumference.

In a study of 49 American subjects with T2D treated with 375 or 750 mg/day of green and black tea extract for 3 months, no significant change in glycosylated hemoglobin levels was found (77).

A recent study of 88 overweight or obese men in the United Kingdom found that EGCG (400 mg, twice daily for 8 weeks) had no effect on insulin secretion or blood
glucose (78). EGCG also had no effect on weight, insulin sensitivity, secretion or glucose tolerance. These results beg the question of whether the previous observed beneficial outcomes of tea are from the effect of EGCG or the entire green tea extract. There may be additive or synergistic effects of the multiple components in tea extracts which result in better outcomes than EGCG alone.
1.4.3 Hypertension and Tea

Hypertension, also known as high blood pressure, is another condition linked to metabolic syndrome. Tea has been shown to reduce blood pressure and improve endothelial function in animal and human studies. Endothelial dysfunction is an alteration of endothelial cells, resulting from oxidative stress and impairment in nitric oxide (NO) availability (79). NO has a vasodilatory effect.

The effect of green tea extract was examined with arterial hypertension in Sprague-Dawley rats (80). The animals were treated with angiotensin (Ang) II to induce endothelial dysfunction which leads to the development of atherosclerosis and hypertension. Rats were treated with 6 mg/mL green tea extract along with a low or high dose of Ang II (350 µg/kg/d or 700 µg/kg/d) for 13 days. At the end of the study, Ang II treated rats had increased blood pressure and left ventricle mass, while green tea extract blunted these increases and decreased signs of oxidative stress.

The same group performed a similar experiment with the Ang II model and green tea extract (81). Sprague-Dawley rats were treated with Ang II (700 µg/kg/d) and given either water or green tea extract (6 mg/mL in drinking water) for 2 weeks. Treatment with green tea extract significantly reduced final systolic and diastolic blood pressure by 20% and 24%, respectively. In addition, heart weight and the ratio of left ventricle to body weight were also significantly reduced. These results show that green tea extract blunts the effect of Ang II induced hypertension and cardiac hypertrophy.

Potenza et al., examined the effect of EGCG on spontaneously hypertensive rats (SHR), a model of hypertension, insulin resistance and obesity (82). SHR were treated for 3 weeks with EGCG (200 mg/kg/d) or enalapril (3 mg/kg/d), an angiotensin converting
enzyme inhibitor that is used to treat high blood pressure and congestive heart failure. Treatment with EGCG and enalapril significantly enhanced vasorelaxation in mesenteric vascular beds isolated from SHR *ex vivo*. In addition, there was a significant decrease in systolic blood pressure in both EGCG and enalapril treated rats compared to SHR control (approximately 15% and 20% decrease, respectively). Even though the magnitude of decrease with EGCG was not as great as treatment with enalapril, treatment with EGCG still showed a significant decrease in systolic blood pressure. Potenza *et al.*, also demonstrated that EGCG treatment significantly decreased myocardial infarct size by 30% and improved cardiac function of SHR hearts exposed to ischemia-reperfusion injury.

Another hypertension study examined the effect of green tea catechin extract (Polyphenon E) on malignant stroke-prone spontaneously hypertensive rats (M-SHRSP) (83). Polyphenon E (5.0 mg/g) was administered in the drinking water of M-SHRSP for 10 weeks. At the end of the study, the Polyphenon E group had a significant delay (mean = 10 days) in stroke onset compared to control rats. The study did not find a significant difference in systolic or diastolic blood pressure after 10 weeks, but did observe a slight decrease compared to the control.

In a similar model, spontaneously hypertensive rats (SHRSP) were treated with water, black tea polyphenols (3.5 g/L thearubigins, 0.6 g/L theaflavins, 0.5g/L flavanols and 0.4 g/L catechins) or green tea polyphenols (3.5 g/L catechins, 0.5g/L flavanols and 1 g/L flavonoids) for 3 weeks (84). Transmitters were implanted in the peritoneal cavity to measure blood pressure every 5 min for 24 h. Systolic and diastolic blood pressure of both black and green tea polyphenol groups were significantly lower than the water
control. Negishi et al., also examined plasma NO concentration and found ~80% decrease in black and green tea polyphenol treated SHRSP compared to control. A similar trend was seen in urinary NO excretion with a 66% reduction in black and 52% in green tea polyphenol groups; however, the green tea polyphenol decrease was not significant. Despite lower concentrations of NO in both black and green tea polyphenol groups, the authors suggest that tea polyphenols alleviate oxidative stress, thus enhancing NO-mediated vasodilatory tone and amelioration of hypertension.

An epidemiological study of 1507 Taiwanese men and women reported a significant decrease in blood pressure in habitual tea drinkers (120 mL/d) (27). Subjects consuming 120 to 599 mL of tea per day had a 46% decrease in the risk of developing hypertension compared to non-habitual tea drinkers. The risk of hypertension was reduced by 65% in subjects consuming 600 mL of tea per day compared to non-habitual tea drinkers.

A cross-sectional study of Australian women 70 or older (n = 218) examined the effect of tea intake on blood pressure (28). The study measured tea intake (black or green tea) and also 4-O-methylgallic acid, a biomarker of exposure to tea-derived polyphenols found in urine. They found that higher tea intake (250 mL/d) and 4-O-methylgallic acid excretion was associated with significantly lower systolic and diastolic blood pressures, 2.2 mm Hg and 0.9 mm Hg respectively.

In a randomized, double-blind, controlled cross-over study, black tea consumption dose dependently improved blood pressure and flow-mediated dilation (FMD) (85). FMD is a biomarker of endothelial function. Healthy Italian men (n = 19) were assigned to either to control, 100, 200, 400 or 800 mg tea/d for 1 week. FMD was significantly
increased by 24% in the 800 mg tea group compared to the control. In addition, systolic and diastolic blood pressure was decreased by 3.0 mm Hg and 2.0 mm Hg, respectively, with 800 mg tea/d. Similar decreases were seen with the lower concentrations of black tea as well.

Black tea consumption was also examined with endothelial dysfunction in patients with coronary artery disease (86). American subjects (n = 50) consumed 450 mL black tea (containing 585 mg catechins and 270 mg theaflavins) or water and effects were examined after 2 hours (short-term) or after 4 weeks of daily consumption (long-term). Both short and long-term black tea consumption significantly improved endothelial dependent FMD of the brachial artery compared to the control. However, short-term black tea consumption significantly increased systolic blood pressure by 4 mm Hg (2.8%) compared to baseline levels and no effect was seen with diastolic blood pressure. This suggests that short-term tea consumption actually has a negative impact by raising systolic blood pressure.

Endothelial function was also examined in healthy, post-menopausal, German women (n = 21) (70). Subjects consumed 10 mg/mL black or green tea, or water control and measured FMD and nitro-mediated dilation (NMD) before and after 2 hours of consumption. FMD was determined by measuring the change in brachial artery diameter for two minutes by high resolution vascular ultrasound. Similarly, NMD was measured in the same manner but after sublingual application of nitroglycerine spray. NMD measures the endothelium dependent dilation due to NO. FMD significantly increased 50% with green tea compared to control, 44% with black tea compared to control and there was no significant difference between tea treatments. No significant difference was found with
NMD between all three treatments. In this study, green and black teas were equally effective in improving endothelial function.
1.4.4 Hypercholesterolemia and Tea

Hypercholesterolemia or elevated cholesterol levels are associated with metabolic syndrome and linked to CVD. Many studies have examined the effect of tea on cholesterol in the context of studying obesity. These papers were reviewed in Chapter 1.4.1. Several studies found that tea supplementation decreases plasma cholesterol in high fat-fed mice (10, 41, 47). Additional studies have examined the effect of tea on hypercholesterolemia in a non-obese context.

A decrease in plasma cholesterol was seen in New Zealand White rabbits fed a hypercholesterolemic diet in addition to green tea extract (87). Animals were fed a 2.5 mg/g cholesterol diet for 2 weeks followed by treatment with 0, 5, 10 or 20 mg/g green tea extract for 4 weeks. Treatment of 20 mg/g green tea extract significantly lowered plasma cholesterol by 60%, LDL cholesterol by 80% and liver cholesterol by 25% compared to control treatment. No effect was seen on HDL cholesterol.

A cross-sectional study in Norway examined the effect of tea consumption of cholesterol levels (n = 9,856) (88). Mean serum cholesterol was significantly lower in those consuming 5 or more cups of tea per day compared to less than one cup per day. A 4% decrease in plasma cholesterol was observed in men, while a 3.1% decrease was observed in women.

Lastly, a double-blind, randomized placebo controlled study examined daily intake of theaflavin enriched green tea extract (375 mg/d) or placebo on Chinese hypercholesterolemic subjects (n = 240) (24). After 12 weeks on a low fat diet, treated subjects had an 11.3% decrease in total cholesterol, 16.4% decrease in LDL cholesterol,
2.3% increase in HDL cholesterol and 2.6% increase in triglyceride levels. This study shows that green and black tea compounds can significantly decrease cholesterol levels.
1.5 Purpose and Significance

Incidence of metabolic syndrome and its related conditions is rising among adults and children in the United States (13, 16). This is problematic since metabolic syndrome is a risk factor for CVD, heart attack, stroke, and cancer (24, 25). Reducing or preventing metabolic syndrome would lead to a healthier and longer life.

From the studies reviewed above, tea extracts and polyphenols have shown beneficial effects for prevention of metabolic syndrome, obesity, diabetes and related complications. However, little is known about the efficacy of tea at reversing the symptoms of metabolic syndrome. Likewise, the mechanism of action for these beneficial effects remains unclear. The purpose of this research was to establish the efficacy of EGCG to reverse and prevent the symptoms of metabolic syndrome in high fat-fed mice and determine the role of PL as a possible mechanism of action. In addition, determine the efficacy of black tea compounds to inhibit PL.

The significance of this research if it is successful is to establish the efficacy of EGCG to prevent and reverse metabolic syndrome in a pre-clinical model. The results of this research can be used for selection of dose, treatment time and biomarkers for future human intervention studies of metabolic syndrome.
1.6 Hypotheses and Objectives

Tea has been shown to reduce body weight as well as markers of diabetes, CVD and fatty liver disease (89). One possible mechanism for these effects is via inhibition of pancreatic lipase and inhibiting lipid absorption. Based on these findings, I hypothesize that dietary EGCG can reduce and prevent symptoms of metabolic syndrome in high fat-fed mice, and that these effects are due in part to EGCG-mediated inhibition of pancreatic lipase.

To test these hypotheses, I propose the following objectives:

a. To determine if EGCG supplementation can reverse the symptoms of metabolic syndrome in high fat-fed obese mice.

b. To determine if long-term EGCG supplementation can reduce the development of symptoms of metabolic syndrome in high fat-fed lean mice.

c. To determine the inhibitory potency and inhibitory mechanism of tea polyphenols against pancreatic lipase *in vitro.*
Chapter 2: Materials and Methods

2.1 Mouse Studies

Chemicals and Diet:

EGCG (93% pure) was purchased from Taiyo Green Power Company (Jiangsu, China). Diets were prepared by Research Diets, Inc. (New Brunswick, NJ) and are detailed in Table 1. All other chemicals were of the highest grade commercially-available.
Table 1: Composition of mouse diets

<table>
<thead>
<tr>
<th>Macronutrient Composition</th>
<th>Low Fat</th>
<th>High Fat</th>
<th>High Fat with 0.32% EGCG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein (% of energy)</td>
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<td>20.0</td>
<td>20.0</td>
</tr>
<tr>
<td>Carbohydrate (% of energy)</td>
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<td>20.0</td>
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<tr>
<td>Fat (% of energy)</td>
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<tr>
<td>Energy (MJ·kg⁻¹)</td>
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<td>21.8</td>
<td>21.8</td>
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<tr>
<td>Ingredient</td>
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<td></td>
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<tr>
<td>Casein</td>
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<td>258.4</td>
<td>257.6</td>
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<tr>
<td>L-Cystine</td>
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<tr>
<td>Vitamin mix (cat# V10001)</td>
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</tr>
<tr>
<td>EGCG</td>
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</tr>
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</table>

Animals and Treatment- Long-term EGCG

Male C57BL/6J mice 5 weeks old from Jackson Laboratories (Bar Harbor, ME) were maintained on 12 h light/dark and had access to food and water *ad libitum*. Mice were housed in shoebox cages on corn cob bedding. All experiments were approved by the Institutional Animal Care and Use Committee at The Pennsylvania State University.
Mice were divided into 3 treatment groups, low fat (LF, 10% kcal fat, n = 16), high fat (HF, 60% kcal fat, n = 22) and high fat plus 0.32% EGCG (HFE, n = 22). The treatment lasted 15 weeks and body weight and diet consumption were recorded each week. Feces (24 h total cage sample) were collected during weeks 8, 10, 12 and 14 of the study. At the end of the study, mice were fasted for 7 h and blood was taken by cardiac puncture from anesthetized mice. Livers, epididymal fat and retroperitoneal fat were harvested, rinsed and weighed. Sections of livers were fixed in 10% formalin. The remaining liver sample was frozen at -80°C for biochemical analysis. Muscle samples were collected from the rear leg, washed with saline, and frozen at -80°C for biochemical analysis. Plasma samples were isolated by centrifugation at 700 x g for 15 min. All samples were stored at -80°C prior to analysis.

Animals and Treatment- Short-term EGCG

Male C57BL/6J mice 5 weeks from Jackson Laboratories (Bar Harbor, ME) were maintained on 12 h light/dark and had access to food and water ad libitum. Mice were housed in shoebox cages on corn cob bedding. All experiments were approved by the Institutional Animal Care and Use Committee at The Pennsylvania State University (IACUC #28962). Mice were divided into 2 treatment groups, low fat (LF, 10% kcal fat, n = 16) and high fat (HF, 60% kcal fat, n = 44). After 9 weeks of treatment, the HF mice were randomized into two groups based on body weight. One group was maintained on HF diet for the duration of the experiment. The other group was switched to HF diet containing 0.32% EGCG (HF-HFE) and maintained on this new diet for the remainder of the experiment. The experiment lasted 15 weeks and body weight and diet consumption
were recorded each week. Feces (24 h total cage sample) were collected during weeks 8, 10, 12 and 14 of the study. At the end of the study, mice were fasted for 7 h and blood was taken by cardiac puncture from anesthetized mice. Livers, epididymal fat and retroperitoneal fat were harvested, rinsed and weighed. Sections of livers were fixed in 10% formalin. The remaining liver sample was frozen at -80°C for biochemical analysis. Muscle samples were collected from the rear leg, washed with saline, and frozen at -80°C for biochemical analysis. Plasma samples were isolated by centrifugation at 700 x g for 15 min. All samples were stored at -80°C prior to analysis.

**Blood Glucose:**

Fasting blood glucose measurements were recorded at weeks 0, 4, 8, 10, 12 and 14 for each treatment group using a hand-held Contour glucose monitor (Bayer Healthcare, Tarrytown, NY). Mice were fasted for 7 h after the cage bedding was changed (to prevent coprophagy) and blood was sampled from the tail vein.

**Biochemical Analysis of Liver and Plasma Samples:**

Hepatic triglycerides were determined by homogenizing liver tissue (50-100 mg) in 2 mL isopropanol. The homogenate was then centrifuged at 2000 x g for 10 min and the supernatant was collected. The triglycerides in the supernatant were analyzed with L-Type Triglyceride M kit and normalized to tissue wet weight (Wako Diagnostics, Richmond, VA). Plasma triglycerides were also determined using L-Type Triglyceride M kit (Wako Diagnostics, Richmond, VA). Plasma alanine aminotransferase (ALT) levels were determined using a spectrophotometric method ($\lambda_{max} = 340$ nm) from Catachem
Plasma total cholesterol and plasma HDL cholesterol were determined using the Cholesterol E kit (Wako Diagnostics, Richmond, VA) and L-Type HDL kit (Wako Diagnostics, Richmond, VA), respectively. Plasma monocyte chemotactic protein-1 (MCP-1) was determined using an ELISA for Mouse CCL2/JE/MCP-1 (R&D Systems, Minneapolis, MN). Plasma insulin was determined using an ELISA for Rat/Mouse Insulin (Millipore, Billerica, MA). Plasma adiponectin was determined using an ELISA for Mouse Adiponectin/Acrp30 (R&D Systems, Minneapolis, MN).

**Homeostasis Model Assessment of Insulin Resistance:**
The homeostasis model assessment of insulin resistance (HOMA-IR) was used to estimate insulin resistance at the end of the experiment. Using the final fasting blood glucose and fasting plasma insulin values, HOMA-IR was determined using the following formula (90):

\[
\text{HOMA-IR} = \frac{\text{glucose (mmol/L) } \times \text{ insulin (mU/L)}}{22.5}
\]

**Fecal Lipid Analysis:**
Feces were collected for 24 h at week 4, 8, 10, 12 and 14. Fecal samples were combined with deionized water (1:2, w:v) and incubated overnight at 4°C. The samples were vortexed and extracted twice with equal volume of methanol:chloroform (2:1, v:v). The organic phase was filtered through 0.45 µm PTFE membrane and dried under vacuum. The residue was weighed and normalized to fecal weight.
Liver Histopathology:

Formalin-fixed liver sections were dehydrated and embedded in paraffin blocks. Sections (6 µm) were cut and stained with hematoxylin and eosin. Samples were blinded and read by a veterinary pathologist (MJK). Hepatic lipidosis, vacuolization and focal necrosis were determined as criteria for liver disease. Severity of lipidosis was determined semi-quantitatively and based on percent of the liver section showing elevated lipid levels. Lipidosis was scored on a scale of 0 = normal, 1 = minimal (1 – 20%), 2 = slight (21- 40%), 3 = moderate (41- 60%), 4 = marked (61 – 80 %), 5 = severe (81- 100 %).

Statistical Analysis:

All plots show the mean ± standard error of the mean (SEM). One-way ANOVA with Tukey’s post-test was used to compare BW gain, fasting plasma insulin, HOMA-IR, plasma and liver triglycerides, plasma ALT, cholesterol, HDL cholesterol, adiponectin, MCP-1, fecal lipids, hepatic lipidosis and hepatomegaly. Two-way ANOVA with Bonferroni’s post-test was used for BW, food consumption and blood glucose comparisons over the course of the study. Statistical significance was achieved at p < 0.05. All analyses were performed using GraphPad Prism (San Diego, CA).
2.2 *In vitro* Pancreatic Lipase Inhibition by Tea Polyphenols

**Chemicals:**

EGCG (93% pure) was purchased from Taiyo Green Power Company (Jiangsu, China). Pancreatic lipase (type II, from porcine pancreas), 4-nitrophenyl butyrate (4-NPB), orlistat, (-)-epicatechin-3-gallate (ECG, 98% pure), (-)-epigallocatechin (EGC, >95% pure) and theaflavin (TF, >90% pure) were purchased from Sigma Chemical Company (St. Louis, MO). Theaflavin-3-gallate (TF3G, 98% pure) and theaflavin-3′-gallate (TF3′G, 90% pure) was purchased from Quality Phytochemicals LLC (Edison, NJ). Theaflavin-3,3′-digallate (TFdiG) was a gift from Dr. Shengmin Sang (North Carolina Central University, Kannapolis, NC).

**Measurement of Pancreatic Lipase Activity:**

The reaction between PL and the substrate 4-NPB was used to measure PL activity. PL hydrolyzes the ester bond in 4-NPB to release butyrate and 4-nitrophenyl (4-NP) (*Reaction 1*). Subsequently, the concentration of 4-NP can be measured at 400 nm because 4-NP emits a yellow color.

\[
\text{Reaction 1: Reaction between PL and 4-NPB.}
\]

Several reactions were tested before the present method was established. Type VI PL was dissolved in water; however the enzyme had no activity after reconstitution. Type
II PL was successfully reconstituted in water and retained activity. Various substrates for this assay were also examined. The substrate 4-nitrophenyl palmitate was initially analyzed; however, it had low solubility under the present reaction conditions. 4-NPB, with a shorter chain fatty acid, could be solubilized and successfully hydrolyzed by PL in vitro.

PL was suspended in water (10 mg/mL). The solution was then centrifuged for 10 min at 1050 g and the supernatant was used as the enzyme source for the experiment. For each experiment, 0.1 mg PL and the test inhibitor were combined in 10 mM Tris-HCl (pH=8.0). The reaction was started by addition of 4-NPB (final concentration of 190 µM). The tube was then vortexed for 10 s, incubated at room temperature for 10 min, and the absorbance was measured at 400 nm using a spectrophotometer. Absorbance values were normalized with respect to the vehicle control and dose-response curves were prepared.

Enzyme inhibition kinetic analysis was performed in an analogous manner using 6 different concentrations of 4-NPB (0 - 0.50 mM). For each inhibitor, two concentrations were selected based on data obtained from dose-response studies. The maximum velocity (V$_{\text{max}}$) and Michaelis-Menten constant (K$_{\text{m}}$) were determined by fitting the initial velocity data at each concentration of 4-NPB.

**Statistical Analysis:**

Non-linear regression analysis was used to fit initial velocity data to Michaelis-Menten plots. All plots show the mean ± standard error of the mean (SEM). One way ANOVA with Tukey’s Post-test was used to compare K$_{\text{m}}$ and V$_{\text{max}}$ values. Statistical
significance was achieved at $p < 0.05$. All analyses were performed using GraphPad Prism (San Diego, CA).
Chapter 3: Results

3.1 Short-term Mouse Study

*Food Consumption and Body Weight with Short-term treatment of EGCG:*

Throughout the study, the food intake did not significantly differ between groups (Figure 4). There is some variability between food consumption in the high fat diets, but this may be attributed to the loose texture of the diet.

![Figure 4](image_url)

*Figure 4:* Average food intake per mouse throughout the study. Values represent the mean, error bars were omitted for clarity. Values are statistically significantly different by two-way ANOVA with Bonferroni’s post-test, p < 0.05.

At week 9, HF mice had an average body weight of 40.3 g and were randomized into two groups. One group (HF-HFE) was put on high fat diet supplemented with 0.32% EGCG. The remaining group was put on HF diet. After 6 weeks on the EGCG diet, HF-
HFE mice had a final body weight 5.4% less than that of the HF control (Figure 5, p < 0.05). EGCG treatment reduced body weight gain over the course of the experiment by 44% compared to HF treated control mice (p < 0.05). No significant difference in final retroperitoneal or epididymal adipose tissue weight was observed between EGCG treated and HF fed control mice (data not shown).

Figure 5: Effect of EGCG on body weight gain in high fat-fed obese mice. Values represent the mean, error bars were omitted for clarity. Values with * are statistically significantly different from HF groups by two-way ANOVA with Bonferroni’s post-test, p < 0.05. Slopes with different superscripts are statistically significantly different by one-way ANOVA with Tukey’s post-test, p < 0.05.

**Blood Glucose, Insulin and HOMA-IR with Short-term treatment of EGCG:**

After week 8, HF-fed mice had a mean fasting blood glucose value of
199.6 mg/dL which was significantly higher than LF controls (Figure 6). Within one week of switching mice from HF to HFE diet, there was a noticeable, but not statistically significant decrease in blood glucose. By contrast, 6 wks after beginning EGCG treatment HF-HFE mice had 16.7% lower fasting blood glucose than HF mice (172.3 mg/dL vs. 206.7 mg/dL, p < 0.05).

Figure 6: Effect of EGCG on fasting blood glucose in high fat-fed obese mice. Values represent the mean, error bars were omitted for clarity. Values with different subscripts are statistically significantly different by two-way ANOVA with Bonferroni’s post-test, p < 0.05.

High fat-fed mice had significantly greater fasting plasma insulin levels than LF fed controls (6.6 vs. 1.2 ng/mL). This was determined from the final fasting plasma samples. Plasma insulin was decreased by 22.7% in HF-HFE group compared to HF group (Figure 7, p < 0.05).
Figure 7: Effect of EGCG on fasting plasma insulin concentration in high fat-fed obese mice. The dotted line represents the value for the low fat control mice. Values represent the mean ± SEM. Values with * are statistically significantly different from HF group by one-way ANOVA with Tukey’s post-test, p < 0.05.

HOMA-IR is a method used to quantify insulin resistance and β-cell function (90). The formula was developed using equations to describe glucose regulation. HOMA-IR was calculated using final fasting blood glucose and plasma insulin values. HF mice had an 88.6% increase in insulin resistance compared to LF fed mice. Treatment with EGCG significantly decreased insulin resistance by 29% compared to high fat-fed mice as measured by HOMA-IR (Figure 8, p < 0.05).
Figure 8: Effect of EGCG on insulin resistance as estimated by HOMA-IR in high fat-fed obese mice. The dotted line represents the value for the low fat control mice. Values represent the mean ± SEM. Values with * are statistically significantly different from HF group by one-way ANOVA with Tukey’s post-test, p < 0.05.

Liver Pathology with Short-term treatment of EGCG:

Fatty liver disease was assessed both biochemically and histopathologically. Plasma ALT values were measured to assess liver damage. Elevated levels of this enzyme in the blood correlate with an increase in liver cell damage. HF treatment increased plasma ALT levels 30-fold compared to LF. Treatment with EGCG reduced plasma ALT levels by 51% compared to HF (Figure 9, p < 0.05).
Figure 9: Effect of EGCG on plasma ALT in high fat-fed obese mice. The dotted line represents the value for the low fat control mice. Values represent the mean ± SEM. Values with * are statistically significantly different from HF group by one-way ANOVA with Tukey’s post-test, p < 0.05.

Similarly, treatment with HF diet increased liver weight by 35% compared to LF mice. Treatment with 0.32% EGCG reversed this effect (Figure 10, p < 0.05).

Interestingly, there was no difference in liver triglycerides between HF and HF-HFE determined either biochemically or histopathologically (data not shown).
**Figure 10:** Effect of EGCG on liver weight in high fat-fed obese mice. The dotted line represents the value for the low fat control mice. Values represent the mean ± SEM. Values with * are statistically significantly different from HF group by one-way ANOVA with Tukey’s post-test, p < 0.05.

**Plasma Cholesterol with Short-term treatment of EGCG:**

Total cholesterol represents the sum of LDL cholesterol, HDL cholesterol, very low density (VLDL) cholesterol, and intermediate density (IDL) cholesterol. Treatment with EGCG had no effect on plasma total cholesterol (Figure 11, p > 0.05). However, there was a 28.6% increase in plasma HDL cholesterol in HF-HFE compared to the high fat-fed control (Figure 12, p < 0.05).
**Figure 11:** Effect of EGCG on plasma total cholesterol in high fat-fed obese mice. The dotted line represents the value for the low fat control mice. Values represent the mean ± SEM. Values are not statistically significantly different by one-way ANOVA with Tukey’s post-test, $p > 0.05$.

**Figure 12:** Effect of EGCG on plasma HDL cholesterol in high fat-fed obese mice. The dotted line represents the value for the low fat control mice. Values represent the mean ± SEM. Values with * are statistically significantly different from HF group by one-way ANOVA with Tukey’s post-test, $p < 0.05$. 
Non-HDL cholesterol was found by subtracting total cholesterol by HDL cholesterol. This represents an estimate of VLDL, IDL and LDL cholesterol levels combined. There was a 17.1% decrease with EGCG treatment compared to high fat control mice; however this decrease was not significantly different from the high fat-fed mice, (Figure 13, p > 0.05).

![Figure 13](image.png)

**Figure 13**: Effect of EGCG on plasma Non-HDL cholesterol in high fat-fed obese mice. The dotted line represents the value for the low fat control mice. Values represent the mean ± SEM. Values are not statistically significantly different by students t-test, p = 0.11.

The ratio of HDL cholesterol to non-HDL cholesterol can further illustrate the difference between cholesterol values amongst treatment groups. High values of this ratio represent a decreased risk of cardiac events (91). The ratio of HDL to non-HDL cholesterol was significantly increased by 46.1% in HF-HFE mice compared to HF mice (Figure 14, p < 0.05). It is interesting that short-term EGCG treatment had a beneficial effect on cholesterol values whereas long-term treatment with EGCG did not.
**Figure 14**: Effect of EGCG on plasma HDL to non-HDL cholesterol ratio in high fat-fed obese mice. The dotted line represents the value for the low fat control mice. Values represent the mean ± SEM. Values with * are statistically significantly different from HF group by one-way ANOVA with Tukey’s post-test, p < 0.05.

*Adiponectin with Short-term treatment of EGCG:*

Adiponectin is a protein secreted from adipocytes that regulates metabolism of glucose and lipids. Adiponectin influences insulin response and is reduced in patients with CVD, obesity and diabetes (92). In type II diabetics, adiponectin levels were found to be positively correlated with HDL cholesterol values (93). Plasma adiponectin values of HF-HFE treated mice increased by 14.3% compared to high fat-fed control (**Figure 15**, p < 0.05).
Figure 15: Effect of EGCG on plasma adiponectin levels in high fat-fed obese mice. The dotted line represents the value for the low fat control mice. Values represent the mean ± SEM. Values with * are statistically significantly different from HF group by one-way ANOVA with Tukey’s post-test, p < 0.05.

**Triglycerides, and Inflammation with Short-term treatment of EGCG:**

MCP-1 is a cytokine synthesized and secreted from adipocytes that recruits monocytes to sites of injury and infection. Obesity causes chronic, low levels of inflammation which increases levels of MCP-1 (94). However, treatment with EGCG had no effect on plasma MCP-1 levels (data not shown). EGCG treatment also had no significant effect on plasma triglycerides (data not shown).

**Fecal Lipid Content:**

It is hypothesized that EGCG affects lipid absorption. If so, an increase in fecal lipid excretion following EGCG treatment would be expected. To determine if short-term treatment with EGCG can increase fecal lipid content, and thus play a role in the observed effects on body weight gain, we determined fecal lipid content gravimetrically.
EGCG treatment increased the fecal lipid content from $8.75 \pm 0.18 \text{ mg/g}$ (HF control) to $11.01 \pm 0.40 \text{ mg/g}$ (Figure 16, $p < 0.05$).

**Figure 16:** Effect of EGCG on fecal lipid content in high fat-fed obese mice. The dotted line represents the value for the low fat control mice. Values represent the mean ± SEM. Values with * are statistically significantly different from HF group by one-way ANOVA with Tukey’s post-test, $p < 0.05$. 
3.2 Long-term Mouse Study

Food Consumption and Body Weight with Long-term treatment of EGCG:

Throughout the study, the food intake did not significantly differ between groups, except at week 5, 7 and 9 when HFE treated mice consumed more diet than LF or HF treated mice (Figure 17). In the high fat diets, the diet was loose enough to fall into the cage bedding and would be subsequently removed when the cages were changed. This could account for the variation between diet consumption throughout the study.

Figure 17: Average food intake per mouse throughout the study. Values represent the mean, error bars were omitted for clarity. Values with * are statistically significantly different from LF and HF groups by two-way ANOVA with Bonferroni’s post-test, \( p < 0.05 \).

After 4 weeks of treatment, the body weight of HF mice was significantly different than LF mice and this trend continued throughout the study (Figure 18, \( p < 0.05 \).
0.05). At the end of the study, HF mice weighed 49.6 g while LF mice weighed 31.7 g, a 36% increase. Body weight gain was also significantly higher in HF compared to LF mice (98.3% increase).

At week 6, the mean body weight of the EGCG treated mice was significantly different than the high fat-fed control (p < 0.05). Treatment of high fat-fed mice with 0.32% EGCG for 15 weeks resulted in a 9.4% decrease in final body weight compared to HF treated control mice (p < 0.05). In addition, EGCG treatment slowed the rate of body weight gain by 19.2% compared to HF group (p < 0.05). EGCG did not significantly affect final retroperitoneal or epididymal adipose tissue weight compared to HF-fed controls (data not shown).
**Figure 18:** The effect of EGCG on body weight in high fat-fed mice. Values represent the mean, error bars were omitted for clarity. Values with different lowercase letters are statistically significantly different by two-way ANOVA with Bonferroni’s post-test, p < 0.05. Slopes with different superscripts are statistically significantly different by one-way ANOVA with Tukey’s post-test, p < 0.05.

**Blood Glucose, Insulin and HOMA-IR with Long-term treatment of EGCG:**

High fat diet significantly increased fasting blood glucose values at week 4 and continued for the rest of the treatment (**Figure 19**, p < 0.05). After 14 weeks, LF mice had average fasting blood glucose of 119.1 mg/dL while HF was 206.7 mg/dL (42.4% increase). EGCG treatment blunted the high fat-mediated hyperglycemia. By week 4, HFE mice had significantly lower blood glucose values than HF control (p < 0.05). At the end of the experiment, the fasting blood glucose of HFE mice was 18.5% lower than HF mice.
Figure 19: The effect of EGCG on fasting blood glucose in high fat-fed mice. Values represent the mean, error bars were omitted for clarity. Values with different subscripts are statistically significantly different by two-way ANOVA with Bonferroni’s post-test, p < 0.05.

Treatment with EGCG significantly decreased plasma insulin (25.3% decrease) compared to HF (Figure 20, p < 0.05). This was determined from the final fasting plasma samples. It is interesting that long and short-term treatment with EGCG similarly decreased both fasting blood glucose and insulin.
Figure 20: Effect of EGCG on plasma insulin in high fat-fed mice. The dotted line represents the value for the low fat control mice. Values represent the mean ± SEM. Values with * are statistically significantly different from HF group by one-way ANOVA with Tukey’s post-test, p < 0.05.

To estimate insulin resistance, HOMA-IR was calculated with final fasting insulin and blood glucose values. HF mice had an 88.6% increase in insulin resistance compared to LF fed mice. This increase in insulin resistance was blunted in HFE mice (33.9% decrease compared to HF mice) (Figure 21, p < 0.05).
Figure 21: Effect of EGCG on insulin resistance as estimated by HOMA-IR in high fat-fed mice. The dotted line represents the value for the low fat control mice. Values represent the mean ± SEM. Values with * are statistically significantly different from HF group by one-way ANOVA with Tukey’s post-test, p < 0.05.

Liver Pathology with Long-term treatment of EGCG:

Fatty liver disease was assessed both biochemically and histopathologically. In HF mice, liver weight was increased by 26.1% compared to LF mice (Figure 22, p < 0.05). EGCG treatment blunted the effects of the high fat diet and the liver weight resembles that of the LF-fed mice. The liver weight of the EGCG treated mice was 22% less than the high fat-fed mice (p < 0.05).
Figure 22: Effect of EGCG on liver weight in high fat-fed mice. The dotted line represents the value for the low fat control mice. Values represent the mean ± SEM. Values with * are statistically significantly different from HF group by one-way ANOVA with Tukey’s post-test, p < 0.05.

Plasma ALT values were measured to assess liver damage. Average plasma ALT levels in LF and HF mice were 3.3 and 100.1 U/L, which is a 96.7% increase. Plasma ALT levels were decreased by 50% in HFE compared to HF control mice (Figure 23, p < 0.05).
**Figure 23:** Effect of EGCG on plasma ALT in high fat-fed mice. The dotted line represents the value for the low fat control mice. Values represent the mean ± SEM. Values with * are statistically significantly different from HF group by one-way ANOVA with Tukey’s post-test, $p < 0.05$.

There was also a 27% decrease in the concentration of liver triglycerides in HFE compared to HF (**Figure 24**, $p < 0.05$). This shows that fatty liver disease was less severe with EGCG treatment since it significantly decreased the concentration of liver triglycerides.
Figure 24: Effect of EGCG on liver triglycerides in high fat-fed mice. The dotted line represents the value for the low fat control mice. Values represent the mean ± SEM. Values with * are statistically significantly different from HF group by one-way ANOVA with Tukey’s post-test, p < 0.05.

Cross sections of livers were analyzed by an experienced histopathologist and assessed for severity of hepatic lipidosis. Samples were photographed in low and high resolution (100x and 400x, respectively) (Figure 25). The low fat liver cross section has mild lipidosis with a small amount of lipids. The HF cross section has severe lipidosis with a large accumulation of lipids, while the HFE cross section has visibly less fat accumulation and is a moderate case of lipidosis.
Figure 25: Liver histopathology in high fat-fed mice. Photo micrographs represent liver samples at low resolution (100x) of LF (A) HF (B) HFE (C) and at high resolution (400x) of LF (D) HF (E) and HFE (F).

Severity of hepatic lipidosis was scored on a scale from 0 to 5, 0 being considered normal liver and 5 being severe lipidosis. HF increased lipidosis by 74.6% compared to LF control (Figure 26, p < 0.05). Long-term treatment of EGCG lessened the severity of lipidosis by 27.2% compared to high fat-fed mice (p < 0.05).
Figure 26: Effect of EGCG on hepatic lipidosis in high fat-fed mice. The dotted line represents the value for the low fat control mice. Values represent the mean ± SEM. Values with * are statistically significantly different from HF group by one-way ANOVA with Tukey’s post-test, p < 0.05.

Plasma Cholesterol, Triglycerides and Inflammation with Long-term treatment of EGCG:

Several other plasma markers related to metabolic syndrome were not affected by long-term treatment with EGCG. There was no significant difference in plasma levels of total cholesterol, HDL cholesterol, non-HDL cholesterol, triglycerides, adiponectin and with the inflammation marker MCP-1 between the EGCG treated mice and the high fat-fed mice (data not shown).

Fecal Lipid Content:

We determined fecal lipid content gravimetrically and found an average fecal lipid concentration of 8.6 and 10.8 mg/g in HF and HFE, respectively. In long-term EGCG treatment, the average fecal lipid content significantly increased by 20.4% compared to HF control group (Figure 27, p < 0.05). This shows that there is an effect with EGCG treatment in lipid absorption.
Figure 27: Effect of EGCG on fecal lipid content in high fat-fed mice. The dotted line represents the value for the low fat control mice. Values represent the mean ± SEM. Values with * are statistically significantly different from HF group by one-way ANOVA with Tukey’s post-test, p < 0.05.
3.3 Pancreatic Lipase Inhibition by Tea Polyphenols

Initially the PL assay was tested at 37° C to replicate biological conditions; however, the reaction between PL and 4-NPB was very quick and the assay was not practical for kinetic analysis. At room temperature, the reaction speed was reduced and produced a linear trend. Preliminary studies showed that a 10 min incubation period was within the linear time range for conversion of 4-NPB to 4-NP by PL (Figure 28). Subsequent experiments were therefore performed at room temperature with 10 min incubation.

![Incubation curve of PL at room temperature and 37° C. Values and error bars represent the mean ± SEM.](image)

**Figure 28:** Incubation curve of PL at room temperature and 37° C. Values and error bars represent the mean ± SEM.

Inhibition of PL by EGCG, ECG and EGC was examined. EGCG dose-dependently inhibited PL with 50% inhibition at 7.5 µM (Figure 29). At concentrations greater than 7.5 µM no further increase in inhibitory effect was observed. Both EGC and
ECG are structurally similar to EGCG; however, EGC lacks a galloyl ester group whereas ECG lacks one hydroxyl group on the B-ring. The maximum inhibition of PL by ECG was 40% inhibition at 5 µM. By contrast, EGC had no significant inhibitory effect on PL at concentrations up to 100 µM. It appears that the presence of the galloyl group affects the inhibitory power against PL.

![Inhibition of pancreatic lipase by green tea polyphenols](image)

**Figure 29:** Inhibition of pancreatic lipase by green tea polyphenols. Values and error bars represent the mean ± SEM.

The inhibitory kinetics of EGCG were determined with respect to 4-NPB concentration (**Figure 30**). Analysis of the Michaelis-Menten plot of PL in the presence of EGCG showed that \( V_{\text{max}} \) was significantly concentration dependently decreased by EGCG (**Table 2**). Although there is a trend for increasing \( K_m \) as a function of EGCG
concentration, this effect was not significant. These results suggest that EGCG noncompetitively inhibits PL with respect to substrate concentration.

**Figure 30.** Pancreatic lipase inhibitory kinetics of EGCG. Values and error bars represent the mean ± SEM.

**Table 2:** Effect of EGCG on the kinetic parameter of PL-mediated cleaved of 4-NPB

<table>
<thead>
<tr>
<th>EGCG (µM)</th>
<th>V&lt;sub&gt;max&lt;/sub&gt; (pmol/mg/min)</th>
<th>K&lt;sub&gt;m&lt;/sub&gt; (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>14.6 ± 0.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>116.7 ± 16.2</td>
</tr>
<tr>
<td>1</td>
<td>10.0 ± 0.9&lt;sup&gt;c&lt;/sup&gt;</td>
<td>124.6 ± 27.8</td>
</tr>
<tr>
<td>5</td>
<td>7.8 ± 0.6&lt;sup&gt;c&lt;/sup&gt;</td>
<td>128.1 ± 26.1</td>
</tr>
</tbody>
</table>

<sup>a</sup>Kinetic studies were conducted at room temperature for 10 min using 0.1 mg PL and 50-500 µM 4-NPB. Values represent the mean ± SEM. Values with different superscripts are statistically significantly different by one-way ANOVA with Tukey’s post-test, p < 0.05.
Inhibition of PL by TF, TF3G, TF3’G and TFdiG was also examined. TFdiG was
the most potent PL inhibitor of the black tea polyphenols tested (Figure 31). At 0.45 µM
TFdiG, PL activity was reduced by 50%. TF3G, with only one galloyl group, was
similarly potent with 50% inhibition at 0.75 µM. On the other hand, TF3’G had less of an
inhibitory effect on PL with 50% inhibition at 20 µM. By contrast, TF had reduced
inhibitory potency compared to TFdiG with 50% inhibition observed at 20 µM. As with
EGCG and EGC, the presence of the galloyl moiety appears to be important for inhibition
of PL. Interestingly, although EGC was inactive, TF still retained significant PL
inhibitory activity. It is quite possible, based on the likely concentration of TF achievable
in the intestinal lumen, that theaflavins could play a role in modulating PL in vivo.

Figure 31: Inhibition of pancreatic lipase by black tea polyphenols. Values and error bars
represent the mean ± SEM.
The inhibitory kinetics of TFdiG were also determined with respect to 4-NPB concentration (Figure 32). The kinetic parameters for PL in the presence of TFdiG are shown in Table 3. As with EGCG, the $V_{\text{max}}$ values significantly decreased, and there was a non-significant trend for increased $K_m$ as a function of TFdiG concentration. TFdiG therefore appears to also inhibit PL in a noncompetitive fashion with respect to substrate.

**Figure 32:** Pancreatic lipase inhibitory kinetics of TFdiG. Values and error bars represent the mean ± SEM.
Table 3: Effect of TFdiG on the kinetic parameters of PL-mediated cleavage of 4-NPB

<table>
<thead>
<tr>
<th>Theaflavin-3,3′-digallate (µM)</th>
<th>V$_{\text{max}}$ (pmol/mg/min)</th>
<th>K$_{\text{m}}$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>15.0 ± 1.0$^b$</td>
<td>149.3 ± 24.2</td>
</tr>
<tr>
<td>0.3</td>
<td>11.3 ± 0.9$^c$</td>
<td>203.8 ± 33.0</td>
</tr>
<tr>
<td>0.5</td>
<td>9.6 ± 0.6$^c$</td>
<td>225.9 ± 30.4</td>
</tr>
</tbody>
</table>

$^a$Kinetic studies were conducted at room temperature for 10 min using 0.1 mg PL and 50-500 µM 4-NPB. Values represent the mean ± SEM. Values with different superscripts are statistically significantly different by one-way ANOVA with Tukey’s post-test, p < 0.05

Orlistat is an FDA approved weight loss drug that targets PL. In the present studies, we assessed orlistat as a positive control. When tested in our PL assay in vitro, orlistat had an IC$_{50}$ value of 0.5 µM and continued to inhibit PL by 80% at 5.0 µM (Figure 33).

![Figure 33: Inhibition of pancreatic lipase by Orlistat. Values and error bars represent the mean ± SEM.](image-url)
Chapter 4: Discussion

4.1 Short-term Mouse Study

In the present study, short-term treatment of obese mice with ECGC reversed some of the symptoms of metabolic syndrome. After 6 weeks, ECGC treatment reduced body weight gain by 44% compared to HF treated mice. These results are similar to those of Lee et. al., who noticed a decrease in overall body weight after 8 weeks of 2.0 mg/g and 5.0 mg/g ECGC treatment on obese mice (41). By comparison, Bose et. al., did not find a significant difference in body weight with supplementation of ECGC compared to high fat control (10).

There was no effect on epididymal or retroperitoneal adipose tissue weight. Lee et. al., found a significant difference in subcutaneous tissue weight in obese mice treated with 2.0 mg/g ECGC compared to high fat control; however, there was no significant difference with epididymal and retroperitoneal (41). With 5.0 mg/g ECGC, Lee et. al., found significant decreases in epididymal, subcutaneous, visceral and retroperitoneal tissue weight. In addition, Bose et. al., found no significant difference between mesenteric, epididymal and retroperitoneal adipose tissue weights in short-term treated ECGC mice compared to high fat-fed control (10).

In our study, ECGC treatment also significantly decreased fasting blood glucose (16.7% decrease) compared to the high fat-fed mice. We observed similar effects for markers of insulin resistance. This is the first report of reversal of biomarkers of T2D by ECGC in high fat-fed obese mice. Given that T2D is the fifth leading cause of death worldwide, the effects of ECGC could have significant public health benefit if they can be recapitulated in humans (64).
Fatty liver disease is a common co-morbidity of obesity that increases long-term risk of cirrhosis and liver cancer (95). Short-term treatment with EGCG reduced the liver to body weight ratio by 22.8% and reduced plasma ALT levels by 49.5% compared to HF-fed obese mice. It is interesting to note that liver triglycerides were not changed by supplementation with EGCG. Perhaps changes in liver triglycerides require more time to occur and if EGCG treatment were carried out longer, liver triglycerides would decrease. This hypothesis is suggested by histopathological analysis which did show a decrease in hepatic lipidosis in EGCG-treated mice (8.5% decrease); however the effect was not significant.

Short-term treatment with EGCG had no significant effect on plasma total cholesterol; however, EGCG treatment did increase plasma HDL cholesterol by 28.6% and the ratio of HDL to non-HDL cholesterol was increased by 46.1% compared to the high fat control. As previously mentioned, non-HDL cholesterol represents an estimate of the combined levels of VLDL, IDL and LDL cholesterol. Low levels of HDL and high levels of LDL, VLDL and IDL are risk factors for atherosclerosis (25). More specifically, each increase 0.03 mmol/L in HDL cholesterol is associated with a decrease of 2 to 3% in the risk of coronary heart disease and for every 1 mmol/L increment in LDL cholesterol, coronary heart disease risk is elevated by 40% (96). A lower ratio of HDL to LDL cholesterol in addition to high levels of triglycerides was found to raise the risk of cardiac events (RR = 3.82, 95% confidence interval: 2.20-6.63) (91). Our data suggest that EGCG may modify the CVD risk of a high fat diet.

Plasma adiponectin values increased by 14.3% in HF-HFE mice compared to high fat-fed control. Adiponectin is secreted from adipocytes and regulates metabolism of
glucose and lipids. This protein is inversely correlated with body fat percentage and positively correlated to HDL cholesterol levels (92). HF-HFE mice experienced an increase in the concentration of adiponectin possibly due to the reduction of body weight gain. Bruno et. al., measured serum adiponectin in ob/ob mice and their lean littermates given 0, 1 and 2% green tea extract for 6 weeks and found no significant treatment effect (97). Unlike our short-term study where obese mice were switched to a high fat diet plus EGCG after 9 weeks, the study by Bruno et. al., was a “prevention” directed study with continuous treatment of initially lean mice with EGCG. Changes in adiponectin may be secondary to changes in body weight rather than a mechanistically important biomarker; however, more research is needed in this area.

One key piece of mechanistic data from this project was the observation that EGCG caused a 29.4% increase in average fecal lipid concentration in obese high fat-fed mice compared to HF fed controls. This increase suggests that EGCG affects lipid metabolism by blocking absorption. Decreased lipid absorption has been shown to result in decreased body weight gain and is the mechanism of the weight loss drug orlistat. Other studies have looked at the effect of EGCG on fecal lipid content following long-term treatment (10). To our knowledge, this is the first demonstration of the relationship between fecal lipid concentration and short-term treatment with EGCG. Short-term treatment with EGCG increased fecal lipid energy excretion to 0.10 kcal/d from 0.08 kcal/d in high fat-fed control. Feeding efficiency was determined using the following equation:

\[
\text{Feeding Efficiency} = \frac{A \text{ g diet/day} \times 0.348 \text{ g fat/g diet}}{B \text{ g fecal lipids}} \times 100\%
\]
The value for diet consumed per day (A), 3.0 g diet/day, and was the average diet consumption of HF and HF-HFE throughout the study. In addition, the amount of fat in the diet was based upon the percentage of lard and soybean oil (Table 1). The weight of fecal lipids (B) was 9.17 mg and 11.01 mg for HF and HF-HFE respectively. Based on this calculation, it was determined that short-term EGCG treatment decreased feeding efficiency by 1.1%.

Plasma triglycerides and MCP-1 were not significantly decreased by supplementation with EGCG. Triglyceride levels are associated with fatty liver and CVD while MCP-1 is associated with inflammation. One possibility for the lack of effect was that the treatment time was too short. As with the body weight change, a longer treatment period could reduce some, or all, of the biomarkers of inflammation and plasma hyperlipidemia.

Based on the findings in this short-term experiment, further studies on the reversal of metabolic syndrome by EGCG and tea should be conducted. This EGCG treatment was only for 6 weeks and significant changes in body weight gain and insulin resistance values were observed. Perhaps these effects would be greater if the treatment period or dose were increased. Lee et al., found a decrease in the body weight of obese mice after 8 weeks of treatment with 0.5% EGCG (41). Other reversal of metabolic syndrome experiments could be done to examine the combination of EGCG and decreased calorie diet, or EGCG and exercise.

I would also suggest these EGCG reversal of metabolic syndrome studies be conducted as a lifetime study. Baur et al., examined the effects of resveratrol in mice fed a high fat diet similar to our study (60% of calories from fat) (98). Middle aged mice (1
year-old) were treated with a standard diet, high calorie diet or a high calorie diet with resveratrol (0.0224 mg/g) for the remainder of their lives. Supplementation of resveratrol significantly increased life span, decreased liver weights and increased insulin sensitivity compared to high calorie mice. A similar lifetime study with EGCG in high fat-fed mice would be valuable to establish long-term effects of EGCG in obese mice.

In this study, EGCG was supplemented in the diet and the concentration of 3.2 mg/g corresponds to approximately 10 cups of green tea per day. This assumes a typical cup is 200 mL and is brewed with 2 g of tea leaves per cup. Because 10 cups of tea could be inconvenient to consume every day, it begs the question whether tea consumption or dietary supplementation is a better option. Tea is typically consumed throughout the day, thus giving a low continuous dose of catechins. In addition, tea has a long history of use and no significant adverse effects have been reported in controlled studies of tea consumption.

With tea based supplements, a high bolus dose (2-12 mg/kg/d EGCG in capsule or pill form) is typically taken at one time (99). As more data is collected with green tea supplements in humans, a question of toxicity is raised. Several cases of hepatotoxicity have been reported after consumption of tea extract-containing supplements like Hydroxycut, The Right Approach and Exolise (100). All patients improved after stopping the product and chronic liver injury was not observed. However, long-term use of these supplements could lead to liver failure.

Animal studies also suggest that high doses of tea polyphenols are toxic. One study reported that high bolus doses of EGCG (500-1500 mg/kg) caused toxicity in CF-1
mice (99). These doses correspond to 30 - 90 mg/kg in humans, representing 10.5-32 cups of green tea.

Consumption of green tea beverages likely does not pose as a risk of hepatotoxicity in people with normal liver function, however, high bolus doses of tea supplements could cause damage, especially if exceeding the recommended dose. One study assessed the maximum concentration (C\text{max}) of a single dose of EGCG (0.69 – 22.22 mg/kg) in healthy men (n = 60) (101). They found that plasma C\text{max} values increased dose-proportionately in a range of 130 to 3391.6 ng/mL. A typical cup of tea contains up to 200 mg (2.78 mg/kg) EGCG, which corresponds to a C\text{max} of 332.16 ng/mL (102). The highest dose of EGCG that was administered in this study was 22.22 mg/kg (approximately 8 cups of green tea) and C\text{max} was over 10 times greater than that of a typical cup of green tea, which is very close to the predicted toxicity dose range mentioned above. With all doses, no signs of toxicity were observed.

In summary, short-term treatment of high fat-fed obese mice with EGCG can reverse some of the symptoms of metabolic syndrome. These findings should be confirmed with longer treatment times, expanded to other animal models of obesity and translated to human intervention studies.
4.2 Long-term Mouse Study

Long-term treatment with 0.32% EGCG significantly decreased the development of many of the symptoms associated with metabolic syndrome in high fat-fed mice. Final body weight of HFE mice was 9.4% less than high fat-fed mice. EGCG treatment also decreased body weight gain by 19.2% compared to high fat control mice. These significant differences in body weight illustrate that supplementation with EGCG in the absence of other changes in diet or physical activity can moderate obesity in mice.

HFE had no effect on epididymal and retroperitoneal adipose tissue weight. It is possible that other adipose depots, such as intestinal or subcutaneous fat, were significantly reduced. Unfortunately, these were not quantified; however this hypothesis is supported by the fact that visually the EGCG treated mice appeared to be slimmer than HF mice. In one experiment by Bose et. al., there was no significant difference between total adipose tissue weight in long-term EGCG treated mice compared to high fat-fed mice, although there was a significant difference in mesenteric and retroperitoneal tissue weight (10).

Treatment with EGCG also decreased markers of T2D. At the end of the experiment, fasting blood glucose of HFE was 18.5% less than the high fat control. The difference between HF and HFE mice was significant beginning after week 4 and continued for the duration of the study. Fasting plasma insulin and insulin resistance as estimated by HOMA-IR were also reduced by 25.3% and 33.9%, respectively, in HFE compared to HF treated mice. Insulin resistance is a key biomarker of T2D. With decreasing fasting blood glucose and insulin levels, EGCG shows potential as a beneficial supplement for prevention of obesity related diabetes.
Previous animal studies have shown similar results with decreases in fasting blood glucose and plasma insulin levels observed after treatment with EGCG (68, 69). In addition, human observational and intervention studies have shown that supplementation with tea or tea catechins can reduce fasting blood glucose and fructosamine values (71, 74). These positive results, along with our current data, suggest that tea supplementation may be useful in preventing the development of T2D.

In the present study, EGCG treatment had the greatest effect on fatty liver disease. Fatty liver disease occurs in 31% of adults in the US: 50% of diabetics and 76% of obese individuals suffer from this disease (103). Fatty liver disease is a risk factor for cirrhosis and hepatocellular carcinoma (30). Liver weight and triglycerides were significantly increased by HF treatment and EGCG blunted this effect. EGCG treatment also decreased plasma ALT levels by 2-fold compared to HF treated mice. These markers show that long-term EGCG treatment can decrease the severity of obesity-induced fatty liver disease. In addition, treatment with EGCG decreased the severity of lipidosis by 27.2% compared to high fat-fed control mice. From the images of the liver sections, it is clear that supplementation of EGCG decreases the amount of fat accumulation. These results are similar to those previously reported (10, 104).

There are very few human studies with EGCG or green tea treatment examining the effect on fatty liver disease (105). Imai et. al., surveyed Japanese men for green tea consumption and preformed several biochemical assays with blood samples (106). They found a decrease in plasma ALT and aspartate aminotransferase (AST) with 10 cups of green tea or greater per day. Based on the results of our study and others in animal
models, further studies in human subjects for prevention or treatment of fatty liver disease are warranted.

Unlike in the short-term EGCG treatment, we observed no significant effect of long-term EGCG treatment on plasma levels of total, HDL or non-HDL cholesterol. These results agree with other studies that have also failed to show an effect on cholesterol (48, 68). Perhaps, beneficial effects on cholesterol occur in the early stages of the long-term EGCG treatment, but diminish over time.

Adiponectin, which is synthesized in adipose tissue, was not significantly different between HF and HFE mice. Increases in adiponectin values are associated with increases in HDL cholesterol, and decreases in body weight and insulin resistance. HFE mice weighed significantly less than HF-fed mice and also had lower insulin resistance values; however, there was no difference in cholesterol levels. Perhaps the weight difference along with low HDL cholesterol levels in HFE mice was not great enough to cause a difference in adiponectin concentration.

Mechanistically, it appears that the anti-obesity effects of EGCG are largely due to modulation of fat absorption. Long-term treatment with EGCG resulted in a 20% increase in fecal lipid excretion compared to high fat treated controls. EGCG increased fecal lipid energy excretion from 0.10 kcal/d compared to 0.08 kcal/d in high fat-fed control. Feeding efficiency was found with the same equation as the short-term EGCG treatment. In long-term EGCG mice, feeding efficiency was decreased by 1.0%. Previous studies found similar significant increases in fecal lipid content with treatment of EGCG (10). Those authors found that EGCG treated mice had higher fecal lipid content than the high fat-fed control mice and a strong inverse correlation was observed between fecal
lipid concentration and body weight gain. The previous and current work suggesting that EGCG partially modulates body weight by decreasing absorption of dietary fat led us to study pancreatic lipase as a potential target (Chapter 3.3).

Although the high fat-fed mouse model used in this experiment is beneficial because it aims to replicate human conditions, there are some draw backs. First, the concentration of fat in the diet is very high, 60% kcal from lard. This allows the mice to become obese very quickly, but the average human diet contains 30% kcal from fat or less (51). In addition, the source of fat in a typical human diet is not solely saturated fats such as lard. Since obesity in our model is based on consuming large amounts of fat, it is assumed that all mice will consume equal amounts of diet. This may not be the case; therefore, different levels of body weight gain and the related pathologies of diabetes and fatty liver disease may occur.
4.3 Pancreatic Lipase Inhibition by Tea Polyphenols

We found that tea polyphenols dose-dependently inhibit PL. EGCG shows the highest inhibition of the green tea catechins with an IC$_{50}$ value of 7.5 µM. ECG was slightly less potent, whereas EGC, which lacks a galloyl ester group, was inactive. The black tea theaflavins were more potent inhibitors than the green tea catechins. TFdiG was the most potent with an IC$_{50}$ value of 0.45 µM. TF3G and TF3’G, with one less galloyl group, had IC$_{50}$ values of 1.0 µM and 20 µM, whereas TF, which has no galloyl groups, had an IC$_{50}$ value of 20 µM.

The position of the galloyl group in TF3G and TF3’G has a large impact on the inhibitory potency of these compounds. It is interesting to note that TF3’G was only 90% pure; the 10% impurity was due to TF3G. TF3G was much more potent at inhibiting PL compared to TF3’G. The inhibition of PL from 90% pure TF3’G could actually be due to the TF3G impurity. A plot of the concentration of the TF3G impurity versus inhibition corresponds to the inhibition of equivalent concentrations of pure TF3G. Further experimentation should be completed to determine if TF3’G has any inhibitory power against PL.

In silico modeling studies of the binding of TF3G and TF3’G would also be useful in understanding the differences in potency of these positional isomers. This corresponds with previous work by Ikeda et al., demonstrating that only galloyl containing compounds inhibited PL (40). Interestingly, TF shows significant inhibitory activity without any galloyl groups. Perhaps this molecule interacts with PL by a different mechanism. Further studies are needed to better understand this.
In the work of Nakai et al., similar PL inhibition trends were observed for tea polyphenols; however, the IC\textsubscript{50} values were very different from those reported here. These differences may be due to the use of a different enzyme preparation (Type VI vs Type II) or a different substrate (4-methylumbelliferone vs 4-NPB). Type II PL is a more crude preparation than Type VI, which may result in the decrease in potency observed in our study compared to those reported in the study using Type VI. These differences point out some of the limitations of this \textit{in vitro} approach. Still, these data provide some potential mechanistic insight into our \textit{in vivo} studies, and extend previously reported enzymology data.

Orlistat is a weight loss drug that targets PL inhibition. In the present study, orlistat was used as a positive control and exhibited an IC\textsubscript{50} value of 0.5 µM which is very similar to that of TFdiG. These data would suggest that TFdiG could have effects on weight \textit{in vivo} and possibly with fewer side effects. Although orlistat has been reported to have significant gastrointestinal side effects like fatty and oily stool, fecal urgency and oily spotting, the reported side effects of black tea consumption are limited (35). Future studies should examine the effect of TFdiG or black tea extracts on weight loss and inhibition of PL \textit{in vivo}.

The effective concentrations of the test compounds observed here are comparable to those achievable \textit{in vivo}. After oral administration of 75 mg/kg EGCG to mice, the peak concentration of EGCG in the small intestinal tissue was reported to be approximately 20.9 µg/g (107). The concentration found in the intestinal contents is likely much higher. Since PL is secreted into the intestinal lumen, it is likely that ingested green and black tea compounds would be present at sufficiently high doses to elicit an
inhibitory response. Further experiments should be conducted in this area to determine the inhibitory effects of black and green tea polyphenols on PL \textit{in vivo}.

In conclusion, these experiments showed that EGCG and TFdiG potently inhibit PL in a noncompetitive manner. These compounds were more potent than ECG, EGC, TF3G, TF3′G and TF possibly due to the presence and position of the galloyl ester group. EGCG and TFdiG should be further investigated for \textit{in vivo} inhibitory effects against PL and the anti-obesity effects of TFdiG should be determined in animal models.
Chapter 5: Conclusions and Future Work

5.1 Conclusions

We tested the hypothesis that dietary EGCG can reduce and prevent symptoms of metabolic syndrome in high fat-fed mice, and that these effects are due in part to EGCG-mediated inhibition of pancreatic lipase. This was accomplished by the following objectives.

1. To determine if EGCG supplementation can reverse the symptoms of metabolic syndrome in high fat-fed obese mice.

Dietary treatment with 0.32% EGCG has been shown to reduce some of the symptoms of metabolic syndrome in high fat-fed obese mice. EGCG-treated obese mice had significant decreases in body weight gain, markers of T2D, like fasting blood glucose, plasma insulin and insulin resistance values, as well as decreased symptoms of fatty liver disease (decreased liver weight and plasma ALT levels). Short-term treatment with EGCG also significantly increased plasma HDL cholesterol and adiponectin values, effects not found with long-term EGCG treatment.

2. To determine if long-term EGCG supplementation can reduce the development of symptoms of metabolic syndrome symptoms in high fat-fed lean mice.
Long-term treatment of high fat-fed lean mice with 0.32% EGCG had significantly decreased body weight gain, markers of T2D, such as fasting plasma insulin, blood glucose and insulin resistance levels, and fatty liver disease. This shows a reduction in the development of metabolic syndrome symptoms.

3. To determine the inhibitory potency and inhibitory mechanism of tea polyphenols against pancreatic lipase in vitro.

Both long-term and short-term EGCG treatment increased fecal lipid content compared to the high fat-fed control mice. This demonstrates that EGCG supplementation modulates lipid absorption, an effect that may be mediated by inhibition of PL. In vitro, green and black tea polyphenols potently inhibit PL. EGCG and TFdiG were two of the most potent compounds tested and inhibited PL in a noncompetitive manner with respect to substrate concentration. Compared to orlistat, the positive control, TFdiG was equally efficacious and experiments are needed to determine its effectiveness in vivo.

Overall, the results of this work show that EGCG can reduce or reverse some of the symptoms of metabolic syndrome in high fat-fed, and that this may be related to inhibition of PL by green tea catechins. The results from the present work may be useful for establishing appropriate treatment times, doses and biomarkers for future preclinical animal studies and human intervention studies.
5.2 Future Work

Short-term treatment of obese mice with EGCG resulted in reduced metabolic syndrome symptoms after 6 weeks. Future work should examine if a longer treatment period (e.g. 10-15 weeks) would result in a more profound effect. Also in the present study, EGCG was added to the diets, but no changes in calorie or fat content was made, and changes in weight gain were observed. Typically with diet plans, a decrease in fat (or calorie) intake is often involved. A study of short-term treatment with EGCG in combination with decreased dietary fat should also be undertaken. The current study used 60% of calories from fat and a lower percentage of dietary fat (20-30%) would better represent a typical human diet.

An additional element to most weight loss plans is incorporation of exercise. To date, very few animal and human studies have examined the weight loss effects of the combination of tea and exercise on high fat diet. Such combinations could have an additive or synergistic effect on obesity prevention. The type and duration of exercise, however, is important to keep in mind. Forced exercise has been shown to induce stress and may negate the beneficial effects of tea and exercise on metabolic syndrome.

From the in vitro inhibition studies of PL, TFdiG was found to be the most potent inhibitor. If PL is responsible for the observed beneficial in vivo effects on weight loss, TFdiG treatment in the same high fat-fed mouse model could cause even greater preventative effects on body weight gain. TFdiG and orlistat showed similar inhibitory potency in our PL assay and a comparative study in vivo would be interesting. Further, it is important to demonstrate inhibition of PL in vivo in order to establish its importance as a target of EGCG.
The present studies focus on pure EGCG. This compound is just one of the catechins found in green tea. Perhaps the combination of the catechins in green tea would produce greater effects on metabolic syndrome. Likewise the interaction between the catechins and caffeine in tea has yet to be determined.

The beneficial results from our study open the door to many other projects involving tea and metabolic syndrome. These studies will be crucial in defining the potential mechanisms of action, dose-response relationships, and pre-clinical safety of tea for prevention of metabolic syndrome. Ultimately, more human experiments are needed to definitively demonstrate the benefit of tea in the prevention and reversal of metabolic syndrome.
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