Fermented Soy Product Isoflavone Composition, and Role in Gene Responses in C57BL/6 Mice

Thesis

Presented in Partial Fulfillment of the Requirements for the Degree Master of Science
in the Graduate School of The Ohio State University

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2010

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ABSTRACT

Obesity is a problem affecting people around much of the world. Intake of soy may decrease tissue lipid accumulation and adipose tissue mass. We aimed to determine the effect of fermented soy product on weight gain and adiposity in growing male C57Bl/6 mice. Eight week old mice were put in a Casein diet, Soy diet, or Fermented Soy diet (FSoy) group for 17 weeks. Half of the mice fed the Casein diet were switched to the FSoy diet at 8 weeks (CtoFSoy). The FSoy reduced weight gain and adiposity compared to the Soy diet and the Casein diet. At 16 weeks, fasting glucose from the FSoy was significantly lower than the Casein diet or Soy diet. In an insulin tolerance test (ITT), glucose lowering was more dramatic from the FSoy compared to the Soy or Casein diet. Fasting insulin was significantly lower in the FSoy and Soy diet compared to the Casein, and the FSoy group was significantly lower than the Soy group. Serum leptin was significantly reduced from the FSoy and Soy diet compared to the Casein diet. The FSoy diet peroxisome proliferator activated receptor gamma (PPAR-γ) mRNA was significantly lowered compared to the Soy diet. FSoy reduced adipose mass, plasma insulin and glucose, serum leptin, insulin tolerance, and adipose tissue responses in fatty acid-binding protein-4 (FABP4), hormone-sensitive lipase (HSL) and F4/80 gene transcription. These data suggest that the beneficial weight and insulin responsiveness observed in the C57Bl/6 mice from the FSoy study diet are largely due to PPAR-γ-independent mechanisms, and may, in part, be due to PPAR-α-dependent mechanisms.
DEDICATION

This thesis is dedicated to my baby (boy or girl). Thank you for being so cooperative during this writing process. I am looking forward to meeting you soon.
ACKNOWLEDGMENTS

I would like to thank Dr. Belury, who has been very supportive as an employer, advisor, and mentor during all of my years at Ohio State. I admire and am struck by the way she is able to juggle many projects and commitments with grace, competence, and confidence combined with the utmost degree of professionalism.

My family and my husband, Jamison, have believed in me from day one, and I thank them greatly for their support.

I would like to thank my lab associates Michelle Asp, Larissa Brophy, Rachel Cole, Bethany Combs, Dr. Li-Fen Liu, Leigh Norris, Kathleen Nemer, Dr. Aparna Purushotham, Michael Stout, Min Tian, Dr. Angela Wendel, and Saebom Won for the laborious feeding assistance and other mice procedures. I would like to extend special thanks to Dr. Kazunori Koba for his assistance and collaborative feedback.

I would especially like to thank Ajinomoto Corporation specifically Hiroyuki Tanimoto, Yuki Okabe, and Yoshiyuki Kumazawa for their collaborative and financial assistance with this project.
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FIELDS OF STUDY

Major Field: Human Nutrition
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<tr>
<td>A1c</td>
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<tr>
<td>FXR</td>
<td>Farnesoid X receptor</td>
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<tr>
<td>GH</td>
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<td>H&amp;E</td>
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<td>LDL</td>
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<tr>
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<td>LPL</td>
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<td>Liver X receptor</td>
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<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
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<td>NEFA</td>
<td>Non-esterified fatty acids</td>
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<td>PDA</td>
<td>Photodiode array detector</td>
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<td>PPARs</td>
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<tr>
<td>PXR</td>
<td>Pregnane X receptor</td>
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<tr>
<td>RT-PCR</td>
<td>Real-time reverse transcription polymerase chain reaction</td>
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<td>Stearoyl-CoA desaturase 1</td>
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<td>SOC3</td>
<td>Suppressor of cytokine signaling 3</td>
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<tr>
<td>SREBP</td>
<td>Sterol regulatory element binding protein</td>
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<td>TNF-α</td>
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CHAPTER 1
INTRODUCTION

1.1 General Background

Obesity is a problem now affecting people around much of the globe. Effects from obesity and overweight are not only social; physical effects from obesity can increase risks for type 2 diabetes, cardiovascular disease, musculoskeletal disorders (such as osteoarthritis), and endometrial, breast and colon cancers (1). According to a 2006 report from the World Health Organization, there were an estimated 1.6 billion overweight, and 400 million obese adults (age 15+) in 2005 (1). By 2015, there may be 2.3 billion people who are overweight and 700 million adults who are obese worldwide (1). Finding effective dietary options for weight reduction may be a key step in combating this epidemic. Intake of soy protein has been shown to have beneficial effects on lipid metabolism and decreased lipid accumulation in animal studies (2, 3).

Soy and fermented soy products differ in their content of and forms of isoflavones. Isoflavones can occur in four different forms: aglycone, glucoside, malonylglucoside, and acetylglucoside (4). Fermented soy products on the market, such as miso and tempe, are rich in isoflavones, and contain higher concentrations of aglycones than soy foods (5). Aglycones are unconjugated isoflavone forms, whereas glycosides are conjugated (6). During the fermentation process, bacteria use beta-glucosidases to hydrolyze the glycosidic conjugates: thus producing large concentrations of aglycones. Additional heat processing steps in preparations of soy also increase the concentrations of acetylglucosides (5, 6). It is possible that due to the distinct forms and content of isoflavones in fermented soy products, important metabolic and bioavailability characteristics of dietary fermented soy could contribute to more potent action than that of non-
fermented soy food products. This prevention or reversal of overweight would be beneficial slowing the progression of diseases related to obesity, including type 2 diabetes. This research investigates the effects of fermented soy product to determine its role in adiposity and metabolic changes.

1.2 Hypothesis
Fermented soy product suppresses and reduces weight gain and adiposity and preserves or restores insulin sensitivity in growing male C57Bl/6 mice compared to the soy product.

1.3 Aims
1. To determine the extent that fermented soy product reduces weight gain and adiposity and preserves or restores insulin sensitivity in growing male C57Bl/6 mice.
2. To determine, in part, the key gene products that fermented soy product modulates in growing C57Bl/6 mice.
2.1 Animal Models for Obesity

There are a number of rat and mouse animal models used to study obesity, including the Zucker fa/fa rat, the Wistar rat, the C57Bl/6 mouse, and the Lep\textsuperscript{Ob} mouse model. Both the Zucker fa/fa rat and the Lep\textsuperscript{Ob} mouse model have no circulating leptin due to a point mutation in the gene coding for leptin. The absence of leptin has multiple effects that are separate from obesity and diabetes (7). In addition to developing obesity dramatically and quickly, these animals are hyperphagic, and have little or no control of food intake. Wistar rats and C57Bl/6 mice are two animal models less prone to obesity because they can develop obesity given certain diets, but like most humans do have feeding restraint. Both species produce enough blood and tissues to study experimentally, but mice eat considerably less food than rats: about 2.5 grams per day as opposed to 25 grams per day. Instead of gaining weight due to extreme hyperphagia, as observed by Lep\textsuperscript{Ob} mice, C57Bl/6 mice experience weight gain due to an increased feed efficiency when fed a high-fat diet (7).

Not all mouse models have genetic vulnerability to high-fat diet-induced obesity and obesity related diseases. Other strains, such as, A/J mice, are physically resistant to the effects of a high-fat diet (7). C57Bl/6 mice, especially male mice, have been studied extensively as models for metabolic syndrome and diabetes, because in addition to obesity they develop insulin resistance, hyperglycemia, and hypertension when given free access to high-fat diets (7). Epidemiological studies in human populations are supportive of the belief that humans are also genetically vulnerable to high-fat diets. The consumption of high-fat diets correlates to the
development of insulin resistance and type 2 diabetes, whereas the consumption of carbohydrates negatively correlates to these disorders (7). C57Bl/6 mice are an especially good model for humans, because they remain lean and physically normal when restricted to low-fat diets (7). However, there is a difference in responsiveness of C57Bl/6 mice when they are placed on a high-fat diet. C57Bl/6 mice are also prone to hair and skin problems as well as malocclusion. These factors affect the number of mice needed for experimental accuracy.

2.2 Soy Review

Soybeans have a composition of 50% protein, 24% carbohydrates, and 25% oil (8). Besides containing a higher content of protein than most plants, its amino acid composition is comparable to the protein found in milk and meat, and the quantities of these amino acids are in sufficient quantities to support human life (9, 10). Soybeans and soy products are the richest sources of isoflavones in the human diet (5). There are 3 isoflavones that occur in soybeans: genistein, daidzein, and glycitein. These isoflavones occur in four possible forms, the aglycone, the glucoside, the malonylglucoside, and the acetylglucoside (5). All soybean proteins and foods currently available for human consumption contain significant amounts of the isoflavones daidzein and genistein, either as the aglycone or as different types of glycoside conjugates (5). Isoflavones are classified as phytoestrogens which are polyphenolic compounds from plants. They are mostly derived from soy, but other beans, lentils, peas, and clover contain small amounts of isoflavones (5). The amount of soy isoflavone levels found in soybeans varies by the geographic location that the plants were grown, the type of soybean, and the year of harvest (5).

Soy may be effective in reducing risk for breast cancer, diabetes, diabetic nephropathy, diarrhea, hyperlipidemia, kidney disease, menopausal symptoms, and osteoporosis (11, 12, 13, 14, 15, 16, 17, 18). Soy inhibits platelet aggregation, lowers blood pressure, increases bile acid excretion, and up-regulates LDL receptors in humans (19, 20, 21). Soy may also affect asthma, endometrial cancer, hypertension, lung cancer, mastalgia, memory, prostate cancer, thyroid cancer, and weight loss (22, 23, 24, 25, 26, 27, 28, 29, 30). Preliminary evidence suggests that
after 6 months of consumption, soy protein in conjunction with a low-fat, calorie restricted diet, seems to help reduce weight in obese and overweight people more than a high-protein, low-fat, calorie restricted diet alone (30).

Some research conducted in animal models for insulin resistance and type 2 diabetes suggests improvements in insulin resistance with soy protein consumption. Obese Wistar fatty rats with type 2 diabetes were fed either casein or soy protein and saturated or polyunsaturated fat. Rats fed soy protein and saturated fat had higher concentrations of insulin receptor messenger RNA in liver and adipose tissue (31). Soy protein seemed to reduce insulin resistance when consumed with a low level of polyunsaturated fatty acids. This indicated that the type of protein and the type of fat may play a role in energy metabolism (32).

Soy has gained negative attention as being possibly unsafe in certain situations. While people who consume a high-level of soy foods in their diet have a reduced risk of endometrial cancer, some postmenopausal women who consumed soy isoflavone extracts orally in high doses (150mg/day) for 5 years experienced endometrial hyperplasia (33). However, no adverse effects are associated with soy extract use when used for up to 6 months at isoflavone doses between 35-120 mg/day (34). There is no evidence for serious adverse effects when consuming high levels of soy from the diet (35). However, consumption of large amounts of fermented soy products might increase the risk of stomach cancer (35). The results of this association are unclear due to too many confounding factors (35).

2.3 Isoflavone Properties

Soy protein contains about 1-3 mg of isoflavones per gram of protein, but soy products vary in the amount of isoflavones they contain (36). The major sources of phytoestrogens in the human diet are isoflavones from soybeans and soy foods. Isoflavones are also found in alfalfa and clover seeds, usually consumed as sprouts, chick peas, or garbanzo beans (38). Isoflavones play important biological functions; the major bioactive isoflavones are genistein and daidzein. Genistein and daidzein are both aglycones (5). Aglycones are derived by bacterial ß-glucosidase
hydrolysis of conjugated soy isoflavones (glucosides), and are the two most heavily researched isoflavone compounds (5). Bacterial β-glucosidase is used during the process of soy fermentation and is found in the gut. The glucoside forms of these soy isoflavone compounds found in soy foods, genistin and daidzin, are hydrolyzed to the biologically active aglycone forms in the gastrointestinal tract (37). The hydrolysis of inactive glucosides to active aglycones is required for effective biological activity of these compounds.

Isoflavones have beneficial effects on obesity and blood glucose levels in studies of animals and humans (3, 30). High dietary intake of the isoflavone genistein by postmenopausal women reduces BMI, waist circumference, and fasting insulin levels compared to postmenopausal women who consume less genistein in their diet (3). Other effects of isoflavones have been reported as well. Genistein inhibits oxidation of LDL particles and protects cultured human vascular cells from oxidized LDL (39, 40). It also inhibits DNA topoisomerases I and II which may affect the cell cycle (39).

When genistein is injected or fed to ovariectomized C57Bl/6 mice adipose tissue decreases even when fed at levels that produce blood genistein levels within the range of consumption by some humans (4). Ovariectomized mice injected with estrogen (5ug/kg BW*d) showed 45% less fat mass than vehicle (DMSO) injected mice (4). Mice injected with genistein showed 23% and 37% less fat mass than vehicle injected groups with injections of 80 and 200 mg/kg BW*d respectively (4). Estrogen and genistein injections decreased LPL mRNA by 60-70% compared with the vehicle DMSO (4).

Mice fed diets supplemented with 500, 1000, or 1500 ppm genistein had 37%, 40%, and 57% less parametrial fat pad weight than mice fed a casein based phytoestrogen free diet (4). Fotsis et al. (1995) found that in addition to estrogenic effects, genistein may affect adipose tissue deposition through protein tyrosine kinases apoptosis, cell proliferation, and in vitro angiogenesis (4).

In humans soy benefits obesity as well as other diabetic factors (3). Postmenopausal women with high isoflavone intake had significantly lower BMI, glucose levels, and fasting insulin
than women with low or no isoflavone consumption (3). They were also less resistant to insulin which was likely a direct effect of the reduced body mass (3). This was an observational study, however, and isoflavone consumption did not vary by age or lifestyle behavior including physical activity, cigarette smoking, or alcohol intake (3).

2.4 Phytoestrogens

Isoflavones are phytoestrogens that are structurally similar to 17-β-estradiol, and bind to alpha- (ERα) and beta-estrogen (ERβ) receptors (4). Genistein is the major phytoestrogen which is in high concentration in soy and soy products (4). Soy protein may have either an anti-estrogen effect or an estrogenic effect depending on the endogenous levels of circulating estrogen (41). In premenopausal women with normal estrogen levels, isoflavones may bind to estrogen receptors, and prevent estrogen from binding (41). Isoflavones may also cause endogenous estrogen levels to fall (42). In contrast, in postmenopausal women with low circulating estrogen, isoflavones have been shown to have a weak estrogenic effect (43). Genistein binds to the estrogen receptor with lesser affinity than estrogen itself (44). It then initiates gene transcription of estrogen receptors (44). Genistein can also alter expression of the progesterone receptor, the androgen receptor, and the oxytocin receptor, and may effectively alter estrogen metabolism (44). It is unknown whether genistein affects sex hormone-binding globulin, but it inhibits tyrosine kinases, and 17 beta-hydroxysteroid dehydrogenase (44).

Isoflavones are thought to have phytoestrogen effects on bone mineral density (45). They may contribute to relief of menopause symptoms such as hot flashes (45). However, in addition to the phytoestrogen effect, other components of the soy may also be contributing to relief of these menopausal symptoms (45).

Estrogen treatment decreases adipocyte size and adipose tissue weight in male rats (2). High levels of adipose tissue increases obesity and risks for obesity-related diseases such as type 2 diabetes, and the estrogen receptor plays an important role in the development of atherosclerosis and adiposity (2). Since adipose tissue metabolism is partly under the control of
estrogen, isoflavones may act to decrease adipose tissue (46). Indeed, one study reports decreased adipose deposition in C57Bl/6 mice due to intake of the isoflavone genistein, apparently dependent on ERα (2). Previous studies by McElroy and Wade (1987), and Hamosh and Hamosh (1975) showed that estrogen may have some ability to modulate adipose tissue indirectly by modulating appetite, energy expenditure, and lipoprotein lipase which regulates lipid uptake of adipocytes (4).

2.5 Other Active Components of Soy

Soy protein has many active components other than protein and isoflavones that contribute to its beneficial effects. Other active components in soy include saponin, oils, calcium, folic acid, protease inhibitors, phytosterols, phytic acid, fiber, and the composition of amino acids in the protein. These components may act individually, or in combination to contribute to the various effects of soy.

Saponins

Saponins are compounds commonly associated with protein from plant origins (47). When added to the diet, many saponins reduce blood cholesterol in animals through a mechanism that increases bile excretion (48). Saponins present in a soy protein diet could play a role in lowering cholesterol. This mechanism would not occur in animal sources of protein that do not contain saponins (47).

Some researchers have tried to determine if saponins from soy play a role in cholesterol lowering action (49). While some animal studies have suggested a role, human studies have not been convincing (47). Normocholesterolemic human subjects fed intact soy flour with or without saponins, experienced no blood cholesterol or excreted bile acid changes (50). When the chemical interactions between saponin and soy or casein proteins were studied, saponins interacted much more rapidly with soy protein compared with casein (50). In general, studies have shown that saponin added to a casein-based diet has the effect of lowering blood
cholesterol and increasing bile acid secretion, but soy protein–based diets with saponins added do not elicit these effects (47).

**Amino Acid Composition**

Dietary protein alters LDL cholesterol, but the mechanism or components responsible are not known. Investigators originally put a great deal of effort on the effects of amino acid components of soy in cholesterol metabolism (47, 51). One theory is that the ratio of lysine to arginine in protein is a determinant of atherogenic effects.

Researchers observed significant increases in plasma glucagon levels, and slightly decreased plasma insulin levels of patients on a soy-protein diet (52). The plasma glucagon returned to original levels after the consumption of the soy-protein diet stopped (52). The hypothesized mechanism for this is that a low ratio of lysine to arginine in the soy causes a decrease in blood insulin and an increase in blood glucagon (51).

In rabbits, amino acids in a diet patterned after the composition of amino acids found in soy appear important compared to an amino acid diet patterned after casein protein. However, results showed that rabbits and other animal models fed intact soy had even lower cholesterol levels than those fed only the representative amino acids from soy in their diet (53, 54). These studies, however, showed little correlation with the lysine:arginine ratio and plasma cholesterol (55). It has also been reported that serum cholesterol levels were lower in rabbits fed an enzymatic hydrolysate of soy protein isolate than in rabbits fed intact soy protein (56). However, the serum cholesterol was increased when a diet patterned after soy amino acids was fed to the rabbits (51, 56). So, while amino acid composition may play a role, the hydrolysis of the protein seems to remove, free, or alter something that also plays a role in lowering cholesterol (47, 51). Contenders in current hypotheses are saponins, isoflavones, or a peptide sequence that affects intestinal absorption of cholesterol and bile acids (47, 51).

**Insulin:Glucagon Ratio**

It has been proposed that variations in hormone secretion are responsible for the hypocholesterolemic effect of soy protein. Different effects have been noted in concentrations of
insulin and glucagon, but in general, studies suggest that the insulin:glucagon ratio decreases after feeding soy protein (57, 58). Plasma amino acids may alter the secretion of hormones such as glucagon and insulin, which in turn may affect serum cholesterol levels by altering the activity of HMG CoA reductase, the rate limiting enzyme in the synthesis of cholesterol (59). High ratios of insulin:glucagon stimulate lipogenesis, and are linked to cardiovascular disease (60).

Phytic Acid

Phytic acid chelates minerals such as Fe, Ca, Zn, and Mg, and decrease their absorption in the gut (47). For example, phytic acid and calcium in the soy food product, Tofu, may help to inhibit lead absorption (61). The zinc to copper ratio in the blood, which has been associated with plasma cholesterol regulation in humans, can be regulated by phytic acid (47). Zinc and copper compete for the same carrier in the gut, but more zinc is chelated than copper, and the ratio is lowered. A lowered Zn:Cu ratio may result in reduced plasma cholesterol (62). In rats, phytic acid appears to lower serum lipids, but the mechanism does not necessarily involve the Zn:Cu ratio (63).

Folic Acid, Phytosterols, and Fiber

The folic acid found in soy protein may be related to the beneficial effects soy has shown in preventing cardiovascular disease, perhaps by lowering homocysteine levels (21). The phytosterols found in soy help to inhibit cholesterol absorption and therefore contribute to lower circulating LDL levels (19). Finally, studies on fiber in soy indicate that the fiber itself may lower blood lipids, but that it does not lower blood cholesterol when it is associated with various protein preparations (50, 64).

Soybean Oil

One component of the oil from soybeans is stearic acid (C:18) (65). Instead of increasing total plasma and LDL-cholesterol, stearic acid is a saturated fatty acid that has different properties than most saturated fatty acids (65). It has a neutral effect or reduces plasma total and LDL-cholesterol with little effect on HDL-cholesterol levels when replacing carbohydrates (by affecting intestinal cholesterol absorption) (66). In rats, dietary tristearin has been shown to
reduce fatty acid and cholesterol absorption compared to tripalmitin, trisafflower, trmyristin, and
trilaurin (67, 68). In rats, stearic-rich oils reduced the intestinal absorption of cholesterol into the
thoracic duct lymph, however, no studies have demonstrated this effect in humans (69, 70).

2.6 Fermented Soy

Many of the soy foods consumed in the Far East are highly fermented soybean products. Fermented soy products available on the market include some soy sauces, miso, tempe, natto, and touchi. Tempeh contains more isoflavones than all common soy products on market, and tempeh and miso contain the largest concentration of aglycones resulting from the hydrolysis of glycosidic conjugates by the fermentation organisms (5). Compositional differences in the isoflavones may be important with regard to metabolism and bioavailability (5).

Touchi extract may be beneficial for diabetes patients. Preliminary clinical research in patients with type 2 diabetes showed that it modestly lowers hemoglobin A1c, triglycerides, and glucose levels in a dose-dependent manner (12). One mechanism through which it may work is by inhibiting intestinal alpha-glucosidase (71).

One animal study that showed an association between consumption of soy containing isoflavones and reduced adipose mass in juvenile male Wistar rats. Rats were fed a cholesterol-enriched diet for 3 weeks to create hypercholesterolemia and then switched to a fermented soy diet (supplemented with isoflavones) for 3 weeks (72). They had significantly reduced epididymal and retroperitoneal fat pad circumferences compared to mice fed a high cholesterol diet for 3 weeks before being switched to a control (casein protein based) diet for 3 weeks (72). This was likely due to the high isoflavone concentrations in the fermented soy diets (72).

2.7 Other Active Components of Fermented Soy

In addition to having higher aglycone isoflavone levels than soy foods, fermented soy foods contain higher amounts of calcium and tyramine than soy foods that are not fermented (73). Fermented soy foods contain small amounts of tyramine, often less than 0.6 mg per serving, but
levels can range between 0.23 mg to 4.8 mg per serving (73). The amount varies significantly between types of fermented soy foods, storage conditions, and length of time the foods are stored (73).

2.8 Peroxisome Proliferator-Activated Receptor Gamma

Peroxisome Proliferator-Activated Receptors (PPARs) are nuclear hormone receptors that function as transcription regulators. They heterodimerize with the nuclear retinoic acid receptor, and act on DNA response elements. There are three different PPAR receptors, (α, γ, and δ), and they are distributed throughout the different tissues. Natural ligands for PPARs are fatty acids and lipid-derived substrates (74). Because PPARs are closely connected with genes that regulate cellular metabolism, PPARs play important roles in metabolism in various tissues, including liver, muscle, and adipose. PPAR-α and -γ are most important in lipid metabolism and insulin sensitivity.

PPAR-γ is found in a wide variety of tissues, but is most highly expressed in adipose tissue where it is an important energy regulator of that tissue (75). Expression of many adipocytokines including adiponectin, resistin, IL-6, and TNFα is regulated by PPAR-γ activation (76). Some PPAR-γ agonists called thiazoladinediones (TZDs) or glitazones, are pharmaceutical drugs which cause adipocytes to secrete insulin-sensitizing hormones to improve blood glucose, so they are used to improve hepatic and peripheral insulin resistance and prevent the progression of insulin resistance to diabetes (77). However, PPAR-γ activation can be associated with weight gain. This could be from increased adipocyte formation and fluid retention (78).

PPAR-γ activation induces changes in adipocyte morphology and fat distribution. For example, PPAR-γ activation has converted brown adipose tissue to white adipose tissue in cell culture (79). Ex vivo rodent studies also show that activated PPAR-γ up-regulates key genes involved in lipogenesis and triglyceride storage (80, 81). Further, ex vivo data shows that activated PPAR-γ stimulates triglyceride storage, improves hepatic steatosis, and induces differentiation of preadipocytes (in subcutaneous, but not visceral cells), and causes apoptosis of
large adipose cells in visceral and subcutaneous fat depots (74). Treating Zucker rats for 14 days with either the TZD pioglitazone or vehicle showed that TZDs remodeling visceral adipocytes to a smaller size with higher lipid storage potential (82).

2.9 Peroxisome Proliferator-Activated Receptor Alpha

PPAR-α is expressed in liver, heart, kidney, intestine, and skeletal muscle (83, 84). Synthetic PPAR-α ligands called fibrates, are pharmaceutical drugs which are used to improve lipid levels by stimulating the oxidation of fatty acids, synthesizing ketones, and promoting glucose sparing (74). PPAR-α activation improves insulin sensitivity in rodents fed high-fat diets (85). PPAR-α activation induces the expression of fatty acid transport proteins (86, 87). PPAR-α activation also impedes fatty acid metabolism by also inducing the expression of long-chain acyl-CoA synthase in the liver (86). Acyl-CoA oxidase and several other key enzymes involved in peroxisomal β-oxidation are direct targets of PPAR-α (86, 87). One effect of peroxisomal β-oxidation is the shortening of long-chain fatty acids for mitochondrial β-oxidation (86).

PPAR-α actions extend beyond the liver where it induces mitochondrial β-oxidation. In the intestine, PPAR-α may help control cellular fatty acid flux in enterocytes by inducing liver type fatty acid-binding protein (L-FABP) (88). In human and rodent skeletal muscles, PPAR-α may upregulate pyruvate dehydrogenase kinase 4 (PDK4) gene expression and activity (89). Inactivation of pyruvate dehydrogenase by PDK4 redirects glucose carbons from oxidation to lactate synthesis, therefore preventing higher levels of hepatic glucose production (89). However, PPAR-δ may also play a large role in the regulation of PDK4 (90).

2.10 Phytofibrates/Phytoglitazones

More recent research has suggested that isoflavones may also exert an antidiabetic effect, as in-vitro studies and gene profiling have established that soy isoflavones are able to activate both PPAR-α and PPAR-δ mediated gene expression (91, 92). Thus, in-vivo, they may act as “phytofibrates” or “phytoglitazones” (93).
The chemical structure of isoflavones is similar to fibrates, thus improvements in liver lipid levels associated with soy intake could be caused by PPAR-α activation by isoflavones (93). However, isoflavone intake resembles action of fenofibrate on PPAR-α, but less robustly than fenofibrate (91). Soy protein with isoflavones (1.82g aglycone equivalent/kg diet) fed to male 129S4/SvJae mice, significantly decreased liver lipid profiles compared to a soy protein diet without isoflavones (91). When PPAR-α knockout 129S4/SvJae male mice, were fed soy protein diets with and without isoflavones, and injected or not injected with a known agonist of PPAR-α (fenofibrate), results indicated PPAR-α dependent and independent actions of soy protein with isoflavones (91).

One candidate for the PPAR-α independent mechanisms involved is PPAR- ligand binding. In a series of Gal4 luciferase systems studied, luciferase reported bioactivity of 140% by unconjugated soy isoflavones compared to DMSO on the PPAR-α ligand binding domain. On the PPAR- ligand binding domain, unconjugated soy protein had 320% more bioactivity than DMSO, and significantly more bioactivity than PPAR-α (93). These data warrant further investigation into the roles of PPARs as ligand binders of isoflavones, specifically unconjugated isoflavones, which are found in fermented soy.

However, multiple mechanisms are likely involved, as demonstrated through one Zucker Diabetic Fatty rat study on soy feeding and measures of adiposity. The rats were fed either a casein based protein diet, a soy protein diet free of isoflavones, a soy protein diet high in isoflavones, or a casein diet plus the TZD rosiglitazone (rosi) (2). While the low isoflavone and control + rosi diets decreased plasma lipids, only the high isoflavone diet decreased plasma lipids, liver weight, body weight and carcass adiposity (2).

2.11 Possible Mechanisms of Action (Besides PPARs and ERs)

Alternative mechanisms may be involved in effects observed with consumption of soy protein based diets including possible regulation by other sterol-regulated element binding protein
(SREBP) pathways (besides PPARs), pregnane X receptor (PXR), thyroid receptor, liver X receptor (LXR), farnesoid X receptor (FXR), and estrogen-related receptors (91).

2.12 Gender

Estrogen is known to be an important regulator of female adipose deposition in humans, rodents, and other species. Extensive literature documents that ovariectomy leads to increases in adipose tissue in rats and mice (94). Additionally, female ER-α knockout mice have large increases in adipose mass (95). Ovariectomy increases lipoprotein lipase (LPL), an enzyme that regulates the uptake of lipid by adipocytes. However, restoring estrogen in ovariectomized mice can decrease the activity of LPL, and directly inhibit adipose deposition by decreasing lipogenesis (96). Estrogen also induces hormone sensitive lipase (HSL) (a lipolytic enzyme), and can thus indirectly affect lipolysis (97).

Males also circulate estrogen, and have estrogen receptors. ER-α knockout mice and aromatase knockout (ArKO) mice (these mice are enzymatically unable to make estrogen) have demonstrated that estrogen does play a role in adipose development and function. Both of these mouse models have age-dependent increased body weights and fat pad mass compared to control animals (98, 99).

2.13 Adipose Tissue and Adipocytokine

Adipose tissue is not only an energy storage tissue that controls fatty acid flux. It is also a metabolic organ that regulates hormones known as adipocytokines. This is demonstrated in fatless mice, because they are insulin insensitive, glucose intolerant, hyperphagic, and prone to weight gain, fatty liver, high triglycerides, and high plasma nonesterified fatty acid (NEFA) levels (100). However, these effects are partially reversed by transplanting fat into the mice (100). In obesity related disorders such as metabolic syndrome or type 2 diabetes, the normal function and morphology of adipose tissue is impaired. Different depots of adipose tissue have different metabolic functions. Visceral white adipose tissue is a key endocrine organ in obesity related
disorders, and only this depot has direct access to the portal system (75). Because visceral adipose tissue is linked to delivery of NEFA to the liver, increased plasma NEFA levels can cause fatty liver and hepatic insulin resistance (75). Obesity is associated with chronic low-grade inflammation (101). Early in the disease process, adipose tissue dysfunction begins with infiltration by macrophages (102). As insulin insensitivity develops, lypolysis is inhibited, and NEFAs are released into the plasma at an increased rate because the adipose cell cannot effectively buffer the excess fatty acids (103). Altered endocrine function of the tissue follows.

Adiponectin, leptin, and interleukin-6 (IL-6) are three of the more than 100 adipocytokines that have been identified so far (76). Adiponectin secretion affects metabolic function, leptin secretion affects endocrine function, and IL-6 affects immune and inflammatory processes (104). IL-6 is released in higher concentrations in visceral adipose tissue, however leptin and adiponectin are generally released in higher concentrations in subcutaneous adipose tissue (105).

Adiponectin

In obese and insulin-resistant states, adiponectin is the only adipocytokine that is down-regulated (106). High adiponectin levels correlate to a reduced likelihood of developing type 2 diabetes (108). Adiponectin is important in glucose and lipid metabolism in skeletal muscle and liver, and acts as an insulin sensitizer (107). In obese rats, adiponectin mRNA is only reduced in visceral adipose tissue, not in other depots of adipose tissue, but plasma adiponectin levels reflect this drop (109). In rats, weight loss due to food restriction increases adiponectin mRNA to normal levels, but in humans, there is a reverse correlation between adiponectin levels and BMI (108, 110).

There are indications that AMPK activation is likely for adiponectin function, but mechanisms controlling adiponectin have not been clearly determined (109). Adiponectin excretion appears to be controlled by things that ameliorate insulin sensitivity, including weight loss, caloric restriction, and TZD treatment (110, 111, 112). These things increase gene expression, as well as adipose and plasma levels of adiponectin (110, 111, 112). Adiponectin
may help regulate the release of other adipocytokines, including IL-6 (75). However, there may be an autocrine-paracrine inhibition of adiponectin release by TNF-alpha and IL-6-induced insulin resistance (113). Adiponectin and leptin may work together to sensitize peripheral tissues to insulin. In studies of insulin resistant lipoatrophic mice, insulin resistance reversed with physiological doses of both adiponectin and leptin, but not either one separately (114).

Leptin

Leptin, the product of the ob gene, is thought to play a key role in the regulation of body weight (115, 116). Leptin is primarily excreted by mature adipocytes, and is secreted in a greater proportion from subcutaneous adipose tissue than from visceral adipose (117). It acts on the central nervous system, in particular, it binds to a receptor in the hypothalamus, thereby suppressing food intake and stimulating energy expenditure (118). It is a metabolic signal for sufficiency rather than excess (117). Leptin receptors are found ubiquitously in the body indicating a general role of leptin, which is currently not fully understood (119).

Besides its metabolic effects, leptin was shown to have a strong influence on a number of endocrine axes (120, 121). In male mice, it blunted the starvation-induced marked decline of LH, testosterone, thyroxine and the increase of ACTH and corticosterone (120). In female mice, leptin prevented the starvation-induced delay in ovulation (120). Ob/ob mice, which are leptin deficient due to an ob gene mutation, are infertile (121). This infertility can be corrected by administration of leptin, but not through weight loss caused by fasting (121). Therefore, leptin seems to be relevant for reproductive functions (117).

All these actions may, at least in part, be explained by the suppressive effect of leptin on neuropeptide Y (NPY) expression and secretion by neurons in the arcuate nucleus (122). NPY is a strong stimulator of appetite and is known to be involved in the regulation of various pituitary hormones (123). The most important variable that determines circulating leptin levels is body fat mass (124). Under conditions of regular eating cycles, leptin exponentially reflects the proportion of adipose tissue (117). Stimulators in both rodents and humans are overfeeding, insulin, and
glucocorticoids (117). Other factors that regulate expression and secretion are TNF-α, estrogens, decreased beta 3-adrenergic activity, androgens, GH, or PPAR- agonists (117).

**Interleukin-6**

IL-6 plasma levels correlate with body mass and the degree of insulin resistance (125). It induces suppressor of cytokine signaling-3 (SOC-3), which inhibits insulin-dependent insulin receptor autophosphorylation (126). In this way, IL-6 plays a direct role in insulin signaling. An increase in secretions of proinflammatory cytokines, such as IL-6, from visceral adipose tissue in obese states is suggestive of insulin resistance (75).

2.14 Target Genes Regulated in Adipose Tissue

In order to begin understanding how fermented soy product regulates adiposity, it is important to determine the extent that fermented soy product induces changes in accumulation of mRNA that code for enzymes regulating adiposity and lipid metabolism. Because PPAR-γ is the primary PPAR located in adipose tissue, and it plays an important role in positively regulating adipocytokine secretion, it is a key gene of interest for adipose research (75). However, adipocytokine regulation is also largely regulated by insulin. Some of the molecular components most highly involved in the physiological changes as related to PPAR- and responsive genes, include the expression of mRNA levels of the following genes: adipose triglyceride lipase (ATGL), hormone sensitive lipase (HSL), fatty acid binding protein (FABP4), fatty acid synthase (FASN), steryl co-A desaturase 1 (SCD-1), F4/80, and adiponectin. Analysis of PPAR- itself may help shed light on the different mechanisms caused by the FSoy diet in weight and adipose gain in mice.

FABP4 is a PPAR-α responsive gene that facilitates cellular uptake and intracellular transport of fatty acids. HSL and ATGL are genes involved with lipid storage (129). SCD-1 and FASN are target genes of SREBP-1, which is a membrane bound transcription factor that activates the transcription of genes involved in fatty acid synthesis (127). SREBP-1 is increased in response to high insulin levels (127). SREBP-1 activation seems to cause fatty liver in insulin-
resistant states (36). Because SCD-1 and FASN are target genes of SREBP, increases in SCD-1 and FASN mRNA expression levels are indicative of SREBP-1 activation.

Soy protein with isoflavones removed has been shown to decrease the expression of SREBP-1 in rats relative to a casein based diet (127). The soy protein also reduced several SREBP-1 target genes including FAS and SCD-1, and decreased lipogenesis (even in the presence of hyperinsulinemia) (127). However, because the researchers fed a soy diet without isoflavones, this reduction of SREBP-1 was not associated with the presence of soy isoflavones (127).

HSL was originally thought to be only in white adipose tissue, but it is now known to be in brown adipose tissues, mammary glands, steroidogenic tissues, muscle tissues, macrophages, intestinal mucosa cells, and pancreatic beta-cells. It plays varying roles in these different tissues, but the function and regulation of HSL in each of these tissues is still being studied (128). Much study of the regulation of HSL has been done in hydrolysis of triglycerides in white adipose tissue. While lipolysis of triacylglycerides and diacylglycerides is regulated directly by HSL, it is alternatively regulated by adipose triglyceride lipase (ATGL) (129). However, HSL is still thought to be the main rate limiting enzyme in lipolysis (128). Lipolysis is also indirectly regulated by diet, exercise, age, growth hormone, glucocorticoids, atrial natriuretic peptide, TNF-α, IL-6, and adipocytokines like leptin, resistin, and adiponectin (129). In 1998, Raclot, Dauzats, and Langin determined that glucose can cause levels of HSL to increase (130). They incubated cultured 3T3-F442A cells for 32 hours without glucose, and found decreased transcription of HSL. Normal transcription rate of HSL mRNA resumed when glucose was added back to the adipocytes. This led to the hypothesis that transcription of HSL is increased in high levels of glucose (130).

F4/80 is an antigen with a largely unknown function. It has been reported that F4/80 modulates macrophage responses to Listeria monocytogenes (131). Knockout mice that lack expression of F4/80 antigen do not have impaired macrophage development, antimicrobial defense mechanisms, generation of T-cells, or independent B-cell responses, and are healthy and fertile (132).
CHAPTER 3
EXPERIMENTAL DESIGN AND METHODS

3.1 Materials

The leptin enzyme-linked immunosorbent assay (ELISA) kit was purchased from Linco Research (St. Charles, Missouri). The adiponectin ELISA kit was purchased from B-Bridge International Incorporated (Mountain View, California). The interleukin-6 ELISA kit was purchased from Pierce (Rockford, Illinois). Insulin, Humulin® was purchased from Eli Lily and Co. (Indianapolis, IN). The RNeasy Lipid Tissue Mini Kit was purchased from Qiagen (Valencia, CA). The high capacity cDNA reverse transcriptase kit, and primers for RT-PCR were purchased from Applied Biosystems Inc. (Foster City, CA). C57Bl/6 mice were purchased from Harlan Laboratories (Indianapolis, IN).

3.2 Animals and Diets

Male C57Bl6/J mice were purchased from Harlan (Indianapolis, IN), at seven weeks of age, 48 mice arrived. The mice were housed 4 mice per cage in the animal facility at the Dorothy M. Davis Heart and Lung Institute at The Ohio State University, where they were placed on a standard chow diet (22/5 Rodent Diet, Harlan Teklad) for one week to allow them to acclimate to their new environment. All animal procedures were in accordance with institution guidelines and approved by The Institutional Animal Care and Use Committee of The Ohio State University.

The diet intervention was divided into two phases as shown in figure 3.1. For phase I, mice were assigned to one of three diet groups (Casein control, N= 23 mice; Soy control, N=12; FSoy, N= 12). The three diet groups all had equal average body weights. The Casein group was
short one mouse because it died during the ear notching procedure. For phase II, 12 mice from the Casein group were switched to the FSoy group (CtoFSoy). Mice were housed at 22°C ± 0.5°C with a 12/12 hour light-dark cycle, and lights on beginning at 0600. Mice were given free access to water, fed *ad libitum*, food intake was measured semi-weekly, and body weights were measured weekly.

**Figure 3.1: Flow Chart of Study Design**

The diets were prepared and analyzed by Ajinomoto Corporation (Tokyo, Japan). Soybeans for the Soy diet were prepared by soaking and boiling, then cutting them into a paste; the soybeans for the FSoy diet were first fermented with *Aspergillus oryzae*, then made into a paste. Once in the paste form, the other components of the diet were added. Finally the diet was formed into pellets. The isocaloric diets consist of 28% fat, 17% protein, and 55% carbohydrate by calories. Diet composition is shown in table 3.1.
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**Table 3.1: Diet Composition.** The ingredients in each diet are expressed as g/100g.
3.3 *High Performance Liquid Chromatography (HPLC)*

The isoflavone content of the diets was measured by high performance liquid chromatography. Samples of the Casein diet were analyzed for isoflavanoid content with a Waters 2695 HPLC (Milford, MA) and a Waters 2996 photodiode array detector (PDA) set to collect data from 210 to 400 nm. HPLC analysis for the FSoy and Soy diets was provided by Ajinomoto Corporation.

3.4 *Measuring Adipocytokines and Metabolites*

At 1, 8, and 16 weeks after the start of the experimental diets, mice were fasted overnight for 8 hours. Blood glucose levels were analyzed using a One Touch Ultra® Blood glucose meter and strips (Lifescan, Milpitas, CA), and blood was collected with a StatSpin® StatSampler (IRIS International, Inc., Chatsworth, CA) heparinized capillary tubes and blood collection centrifuge tubes that contain a gel barrier between cells and plasma. The blood was spun down via the manufacture protocol, and the plasma was collected. Plasma was stored at 4°C for approximately 2 hours, and then transferred to -20°C until analyzed further. Insulin levels were analyzed from 5 µl of this plasma using a colorimetric Mercodia Ultrasensitive Mouse Insulin ELISA purchased from Alpco Diagnostics (Salem, NH).

Serum from necropsy was analyzed using a colorimetric Leptin ELISA kit (catalog # EZML-82K) purchased from Linco Research (St. Charles, Missouri). Serum from necropsy was analyzed using a colorimetric Adiponectin ELISA kit (catalog # K1002-1) purchased from B-Bridge International Incorporated (Mountain View, California). Serum from necropsy was analyzed using a colorimetric Interleukin-6 ELISA kit (catalog #ER2IL6) purchased from Pierce (Rockford, Illinois).

3.5 *Insulin Tolerance Test.*

Insulin tolerance tests (ITT) were conducted at the beginning of week 17 of feeding. Animals were fasted for 12 hours prior to injection of insulin (0.5 units Humulin ®/kg body weight).
The first glucose reading was taken moments prior to the insulin injection into the peritoneal cavity. Glucose levels were measured at 5, 15, 30, 60, 90, and 120 minutes following the insulin injection. Procedural start times varied throughout the day; therefore, mice were randomized so that start times were equally represented by each diet group. Blood was collected by lateral tail vein prick. The first drop of blood was discarded at each time point, and the second drop of blood was measured by glucometer. The same glucometer was used for each mouse for the duration of the test. Two days after insulin tolerance tests, tissues were collected. The insulin tolerance test was configured as area under the curve (AUC) according to the trapezoidal method (133).

3.6 Necropsy

After 17 weeks on experimental diets, mice were fasted for 12 hours before necropsy was performed. Mice were euthanized by CO₂ for several minutes and cervically dislocated. Blood was collected by heart puncture; liver, spleen, heart, epididymal adipose, and perirenal/pararenal/retroperitoneal adipose tissue were weighed, placed into whirl-paks and snap-frozen in liquid nitrogen. These tissues were stored at -80°C for future analysis. Portions of epididymal adipose, subcutaneous adipose, and liver were removed for histological analysis.

3.7 Histology of Adipose Tissue

Adipose tissue from perimetrial and abdominal subcutaneous depots were collected for hematoxylin and eosin (H&E) stained slides. During necropsy, portions of these two depots were placed into plastic tissue cassettes and fixed in formaldehyde. The tissues were then sent to an on campus facility for H&E staining and slide mounting.

3.8 Analysis of mRNA of Adipose Targeted Genes

Portions of adipose were cut on dry ice from the frozen sample, and immediately homogenized in kit reagent. RNA was isolated using the RNeasy Lipid Tissue Mini Kit from Qiagen (Valencia, CA) according to the manufacturer’s protocol. The optical densities of the
samples were read at 260nm and 280nm. The samples were gel electrophoresed and then exposed to UV-epi-illumination on a Kodak Imager Station 2000RT (Eastman Kodak Company, Rochester, NY). The visible RNA bands were examined for degradation and DNA contamination.

Reverse transcription of RNA to complementary DNA (cDNA) was performed using the High Capacity cDNA Reverse Transcriptase kit, as directed by the manufacturer. The cDNA was then diluted to 2ng/µl. Real time Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) was performed according to the instructions for Taqman® Chemistry and Genes (Applied Biosystems (Foster City, CA). Analysis of mRNA was performed using primers for the genes F4/80, Adiponectin, ATGL, HSL, FABP4, FASN, SCD1, and PPAR- using Applied Biosystems Gene Amp PCR system 9700. Each sample was run in triplicate. For quantifying mRNA, the $2^{-\Delta\Delta CT}$ method was used, and fold inductions were calculated using CT values normalized according to the housekeeping gene product, 18s (134).

3.9 Statistical Analysis

Data are expressed as mean ± standard error. Interactions between Casein, CtoFSoy, and FSoy diet groups: weights of tissues, ITT area under the curve (AUC), average daily intake, average daily gain, Feed:Gain Ratio, serum and plasma protein and glucose levels, and mRNA levels were analyzed by one-way analysis of variance (with Tukey’s family error rate of 5) using Minitab® Software (v14.1) (State College, PA). FSoy and Soy diet groups were analyzed by 2-Sample T-Tests. Differences of P<0.05 were considered significant. Different superscripts among groups indicate statistically significant differences. Three mice were removed as outliers from the entire analysis. One mouse from the FSoy group was an outlier in response to insulin tolerance, body weight and organ weight. A different mouse from the FSoy group was removed from analysis due to a skin infection and an enlarged spleen. Finally, a mouse from the CtoFSoy group was an outlier in body weight and liver weight.
CHAPTER 4
RESULTS

4.1 Body Weight and Food Intake

As demonstrated in figure 4.1, there were no significant differences found in weight between groups for the first four weeks. After feeding diets for five weeks, measurement of weight revealed a significant difference between both of the Casein groups (Casein and CtoFSoy) and the FSoy group. The Casein fed group continued to weigh significantly more than the FSoy group for the remainder of the study. Although the Casein group seemed to weigh more than the Soy group, the only time there was a significant difference was at seven weeks. At week eight, CtoFSoy group was switched from Casein to FSoy for the rest of the experiment. One week later, the CtoFSoy group weighed significantly less than the Casein group. At week nine, the Soy group weighed significantly more than the mice who had been consuming FSoy for the entire time. By week ten, the Soy group also weighed significantly more than the CtoFSoy group. The Soy group continued to weigh significantly more than the FSoy groups (FSoy and CtoFSoy) for the remainder of the experiment. Weights for mice consuming the Casein, Soy, and FSoy diets continued to increase (growth of the animals) during the duration of the study. Periodic drops in weight reveal loss due to experimental fasting for glucose testing and blood collection.

The FSoy diet caused significantly less weight gain than the Casein and Soy diets with significantly more food intake than the Casein and CtoFSoy diets. Food intake was measured semi-weekly by cage. Individual mouse weights were measured weekly and summed by cage. Feed to gain ratios are shown in figure 4.2. The ratios were calculated by the sum of weekly changes in intake (g) (divided by total days): weekly increases in weight (g) (divided by total
days). Week one was not included in these sums, as no weight loss occurred during this time. Average daily dietary intakes and average daily weight gains of the mice are recorded in Table 4.1.

**Figure 4.1: Body Weights.** The FSoy diet reduced weight gain and caused weight loss in mice switched from the Casein to the FSoy diet. All values are expressed as mean ± standard deviation. Significance calculated by one-way ANOVA (p<0.05).
Figure 4.2: Feed to Gain Ratio. The feed:gain ratio was significantly greater in the FSoy diet group compared to the Soy, Casein, and CtoFSoy diet groups. An increased feed:gain ratio could be advantageous for weight loss. All values are expressed as mean ± standard error. The Casein, CtoFSoy and FSoy diet groups with different letters are significantly different (one-way ANOVA). A 2-sample T-Test was used between Soy and FSoy groups. An asterisk denotes significant difference between these two groups. (p<0.05)
<table>
<thead>
<tr>
<th></th>
<th>Casein</th>
<th>Casein-FSoy</th>
<th>FSoy</th>
<th>Soy</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Average Daily Intake</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(g)</td>
<td>2.23 ± 0.14&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.26 ± 0.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.72 ± 0.11&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.54 ± 0.06</td>
</tr>
<tr>
<td><strong>Average Daily Gain</strong></td>
<td>0.13 ± 0.04&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.07 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.05 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.10 ± 0.01&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Table 4.1: *Diet Intake and Weight Gain.* The FSoy diet caused significantly less weight gain than the Casein and Soy diets with significantly more food intake than the Casein and CtoFSoy diets. All values are expressed as mean ± standard deviation. The Casein, CtoFSoy and FSoy diet groups with different letters are significantly different (one-way ANOVA). A 2-sample T-Test was used between Soy and FSoy groups. An asterisk denotes significant difference between these two groups. (p<0.05)

### 4.2 Isoflavone Composition of the Diets

HPLC analysis of isoflavones in the three diets is shown in Table 4.2. The results show that the FSoy diet is composed of only aglycone isoflavones. Of the three types of aglycones (daidzein, genistein, and glycitein), the FSoy diet had the highest level aglycone in the form of genistein (155.56 µmol/100g). The Soy diet does contain a fraction of its isoflavones in the aglycone form (daidzein and genistein), but the majority of the Soy diet is in the glucoside form. Of the three main types of glucosides, (daidzin, genistin, and glycitin) and of those forms that contain an acetyl or malonyl group, genistin was present in the highest level (108.8 µmol/100g). The total amount of isoflavones in the FSoy and Soy diets are very similar, 229.59 µmol/100g, and 261.28 µmol/100g respectively. The type of isoflavones in the FSoy and Soy diets are likely the major, if not only, difference between them. The Casein protein based diet contains no isoflavones.
<table>
<thead>
<tr>
<th>Isoflavone (umol/100g)</th>
<th>FSoy diet</th>
<th>Soy diet</th>
<th>Casein diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Daidzin</td>
<td>0</td>
<td>76.92</td>
<td>0</td>
</tr>
<tr>
<td>Genistin</td>
<td>0</td>
<td>108.80</td>
<td>0</td>
</tr>
<tr>
<td>Glycitin</td>
<td>0</td>
<td>2.69</td>
<td>0</td>
</tr>
<tr>
<td>Acetyldaidzin</td>
<td>0</td>
<td>7.64</td>
<td>0</td>
</tr>
<tr>
<td>Acetylgenistin</td>
<td>0</td>
<td>13.71</td>
<td>0</td>
</tr>
<tr>
<td>Acetylglycitin</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Malonyldaidzin</td>
<td>0</td>
<td>17.73</td>
<td>0</td>
</tr>
<tr>
<td>Malonylgenistin</td>
<td>0</td>
<td>21.24</td>
<td>0</td>
</tr>
<tr>
<td>Malonylglycitin</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Glucoside TOTAL</td>
<td>0</td>
<td>248.73</td>
<td>0</td>
</tr>
<tr>
<td>Daidzein</td>
<td>70.87</td>
<td>5.51</td>
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</tr>
<tr>
<td>Genistein</td>
<td>155.56</td>
<td>7.04</td>
<td>0</td>
</tr>
<tr>
<td>Glycitein</td>
<td>3.17</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Aglycone TOTAL</td>
<td>229.59</td>
<td>12.55</td>
<td>0</td>
</tr>
<tr>
<td>TOTAL Isoflavones</td>
<td>229.59</td>
<td>261.28</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 4.2: Composition of Isoflavones in Experimental Diets. While the Soy diet contains few aglycones, the FSoy diet contains no glucosides. The Casein diet contains no isoflavones.
4.3 Fasting Glucose and Insulin

Mice were fasted for 8 hours overnight at the end of weeks 1, 8, and 16, and their glucose levels were measured with a blood glucose meter. The results are shown in Figure 4.3. The glucose values show a trend towards lower levels in the FSoy diet group. Early glucose levels of animals on the FSoy diet were significantly lower (p<0.05) than the Casein diet group. The FSoy group also had significantly lower fasted glucose levels than the Soy and Casein groups by week 16. Significance is determined within each measurement week.

The FSoy group only increased significantly between weeks 1 and 8. The FSoy group did not show any significant change between week 8 and 16. By week 8, the FSoy diet group had significantly lower fasted insulin levels than the Casein and CtoFSoy groups. By week 16 both FSoy insulin levels were significantly lower than the Soy and FSoy diet groups. The CtoFSoy insulin levels were no longer significantly higher than the FSoy group (that had been eating the same diet throughout the whole study) by week 16. Fasted insulin results are demonstrated in Figure 4.4.
Figure 4.3: Fasted Glucose. Serum from mice fasted overnight for 8 hours at the end of week 1, week 8, and week 16 of the study. Levels are expressed in mg/dl. The FSoy diet significantly decreased fasting glucose levels after feeding diet for 16 weeks. All values are expressed as mean + standard deviation. The Casein, CtoFSoy and FSoy diet groups with different letters are significantly different (one-way ANOVA). A 2-sample T-Test was used between Soy and FSoy groups. An asterisk denotes significant difference between these two groups. (p<0.05)
Figure 4.4: Fasted Insulin. Serum from mice fasted overnight for 8 hours at the end of week 1, week 8, and week 16 of the study. Levels are expressed in µg/l. Fasting insulin levels were significantly reduced after feeding FSoy for 8 weeks compared to Soy and Casein. FSoy insulin levels remained low, while Soy and Casein insulin levels continued to increase for the duration of the study. All values are expressed as mean ± standard deviation. The Casein, CtoFSoy and FSoy diet groups with different letters are significantly different (one-way ANOVA). A 2-sample T-Test was used between Soy and FSoy groups. An asterisk denotes significant difference between these two groups. (p<0.05)

4.4 Enzyme-Linked Immunosorbent Assays

Serum collected during necropsy was used to measure protein levels of IL-6, adiponectin, and leptin using colorimetric ELISA kits. Results for levels of IL-6 (figure 4.5), adiponectin (figure 4.6), and leptin (figure 4.7) from the circulation are shown. There were no significant differences in IL-6 or adiponectin levels measured between mice fed the Casein, CtoFSoy or FSoy. Nor were
there differences between the FSoy and Soy diet groups for serum IL-6 or adiponectin. Mice fed the Casein based diet for 17 weeks had significantly higher serum leptin levels than mice fed any of the soy-containing diets.

Figure 4.5: Interleukin-6 from Necropsy Serum.

Serum from necropsy was analyzed using a colorimetric Interleukin-6 ELISA kit. No significant differences were measured between mice fed the Casein, FSoy, Soy, CtoFSoy diets. All values are expressed as mean + standard error. The Casein, CtoFSoy and FSoy diet groups are compared with one-way ANOVA. A 2-sample T-Test was used between Soy and FSoy groups. (p<0.05)
Figure 4.6: Adiponectin from Necropsy Serum.

Serum from necropsy was analyzed using a colorimetric Adiponectin ELISA kit. No significant differences were measured between the diet groups. All values are expressed as mean ± standard error. The Casein, CtoFSoy and FSoy diet groups are compared with one-way ANOVA. A 2-sample T-Test was used between Soy and FSoy groups. (p<0.05)
Figure 4.7: Leptin from Necropsy Serum.

Serum from necropsy was analyzed using a colorimetric Leptin ELISA kit. Mice fed the Casein based diet for 17 weeks had significantly higher serum leptin levels than mice fed any of the soy-containing diets. All values are expressed as mean ± standard error. The Casein, CtoFSoy and FSoy diet groups with different letters are significantly different (one-way ANOVA). A 2-sample T-Test was used between Soy and FSoy groups. An asterisk denotes significant difference between these two groups. (p<0.05)

4.5 Insulin Tolerance Test

After 16 weeks of feeding, mice were fasted for 12 hours and injected with (0.5U/kg of body weight) insulin (see figure 4.8 A). Glucose values (mg/dl) were measured over time (0, 5, 15, 30, 60, 90, 120 min.), in response to the insulin. For net area under the curve (AUC) results, see figure 4.7 B. AUC was set to zero, and calculated using the trapezoidal rule. The glucose values recorded at time 5 minutes represent a stress response, and are not calculated in the AUC or represented on the ITT graph. The FSoy and CtoFSoy diet groups responded more acutely to
the insulin than the Casein and Soy groups. The slight improvement in insulin sensitivity seen in the FSoy mice was significantly different compared to the Soy group by 2 sample T-Test. Statistics by ANOVA calculated at individual time points reveals a statistically significant difference between FSoy and Casein at 15, 30, 60, and 120 minutes. Soy and FSoy glucose values in response to insulin were statistically different at times 15, 30, and 60 minutes after injection of insulin.

A.

![Graph showing glucose levels over time for Soy, FSoy, Casein, and Casein to FSoy groups](image)

**Figure 4.8:**

**A. Insulin Tolerance Test.** Results of Glucose. After 0.5U/kg injection of insulin, glucose values were measured at 0, 15, 30, 60, 90, and 120 minutes after injection.
B. Area Under the Curve (AUC). Areas were calculated according to the trapezoidal method. All values are expressed as mean ± standard error. The Casein, CtoFSoy and FSoy diet groups with different letters are significantly different (one-way ANOVA). A 2-sample T-Test was used between Soy and FSoy groups. An asterisk denotes significant difference between these two groups. (p<0.05)

4.6 Organ Weights and Histology

Comparing the weights of livers, hearts, and spleens revealed no significant differences between mice consuming the different diets (Table 4.3). Epididymal fat was collected and weighed, as was remaining abdominal fat (pararenal, perirenal, and retroperitoneal). Comparing the sum of both depots to the different diet groups revealed significant differences between the Casein and both FSoy groups, and Soy and both FSoy groups. The epididymal tissue weight...
itself revealed the same significant differences when compared to the diet of the mice. The remaining abdominal fat weight failed to show significant differences between the Soy and the CtoFSoy group. It did, like the epididymal tissue, show a significant increase in weight in the Soy group compared to the FSoy group and the Casein group compared to both FSoy groups.

Observational analysis of the epididymal adipose H&E stained histology slides did not reveal apparent differences between diet groups (figure 4.9). There is observable variation between animals within the same group. Analysis of subcutaneous adipose H&E stained histology slides also did not reveal obvious apparent differences between diet groups. However, there may be a trend toward smaller adipocytes in the CtoFSoy group (figure 4.10). More analysis is needed to determine significant differences.
<table>
<thead>
<tr>
<th></th>
<th>Casein</th>
<th>Casein-FSoy</th>
<th>FSoy</th>
<th>Soy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Final Body Weight (g)</td>
<td>35.3 ± 4.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>29.8 ± 2.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>27.8 ± 01.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>33.1 ± 2.9</td>
</tr>
<tr>
<td>Visceral Intraabdominal Adipose (g)</td>
<td>2.448 ± 0.814&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.355 ± 0.441&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>0.942 ± 0.284&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.955 ± 0.504&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>Adipose:Body Weight Ratio</td>
<td>0.072 ± 0.020&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.049 ± 0.011&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.038 ± 0.010&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.063 ± 0.013</td>
</tr>
<tr>
<td>Liver Weight (g)</td>
<td>1.157 ± 0.267</td>
<td>1.091 ± 0.276</td>
<td>1.094 ± 0.106</td>
<td>1.034 ± 0.138</td>
</tr>
<tr>
<td>Spleen Weight (g)</td>
<td>0.098 ± 0.024</td>
<td>0.143 ± 0.072</td>
<td>0.114 ± 0.049</td>
<td>0.102 ± 0.031</td>
</tr>
<tr>
<td>Heart Weight (g)</td>
<td>0.155 ± 0.030</td>
<td>0.152 ± 0.014</td>
<td>0.149 ± 0.014</td>
<td>0.152 ± 0.015</td>
</tr>
</tbody>
</table>

**Table 4.3: Organ Weights and Body Weights.** Adipose tissue mass was significantly reduced in mice fed FSoy. In other tissues, there were no significant differences in weights between groups. All values are expressed as mean ± standard deviation. The Casein, CtoFSoy and FSoy diet groups with different letters are significantly different (one-way ANOVA). A 2-sample T-Test was used between Soy and FSoy groups. An asterisk denotes significant difference between these two groups. (p<0.05)
Figure 4.9: Epididymal Adipose. Representative images from 40x amplification of random sections from H&E slides.
4.7 Epididymal Adipose mRNA Gene Expression

The mRNA expression of epididymal adipose genes was measured through real time RT-PCR. Results are expressed as fold induction and normalized to 18s. There were no significant differences in the expressions of ATGL, FASN, PPAR-, or Adiponectin among the Casein, CtoFSoy, and FSoy diet groups (figure 4.11). Among these groups, HSL and FABP4 were significantly lower in the FSoy group, SCD1 was significantly higher in the CtoFSoy group, and F4/80 expression was significantly higher in the Casein group.

There were no significant differences in the expression of FASN, SCD1, or ATGL mRNA from epididymal adipose genes comparing tissues from the Soy and FSoy diet groups by 2-sample T-Test. Between these groups, mRNA expression was significantly higher in the Soy group for HSL, FABP4, PPAR-, F4/80, and Adiponectin.
Figure 4.11: mRNA Expression of F4/80, Adiponectin, ATGL, HSL, FABP4, FASN, SCD1, and PPAR-α. All values are expressed as mean ± standard error. All gene expressions were normalized to 18s. The Casein, CtoFSoy and FSoy diet groups with different letters (within the same gene) are significantly different (one-way ANOVA). A 2-sample T-Test was used between Soy and FSoy groups. An asterisk denotes significant difference between these two groups. (p<0.05)
During the growth phase of the mice, the diets separated out in a manner consistent with the hypothesis. The mice fed the Casein diet gained weight at the fastest rate, the Soy group gained weight less substantially, and the FSoy group gained the least weight. After eight weeks of consumption, the FSoy diet group weighed significantly less, had a significantly smaller change in weight, had a significantly greater feed:gain ratio (data not shown) and had lower glucose levels than all of the other diet groups. The FSoy group also had significantly lower insulin levels than the Casein fed groups. These initial data suggest that the FSoy diet does have the effect of reducing weight, and alleviate weight related conditions such as fasted plasma glucose and insulin levels.

Beginning phase II of the study, half of the Casein diet fed mice were switched to the FSoy diet. In addition to continuing to observe these suppressive effects relative to the effects of the Casein and Soy diets as the mice transgressed, the FSoy diet had the effect of weight loss in the animals previously fed the Casein based diet. That is, the FSoy diet reversed weight gain in a period of about two weeks. Additionally, measurement of the tissues during necropsy revealed that adipose tissue mass was significantly reduced in mice fed FSoy. In other tissues, there were no significant differences in weights between groups. This suggests that the weight difference was due to the adipose tissue. As proposed, the FSoy diet provided by Ajinomoto Corporation does suppress and reduce weight gain and adiposity and preserve insulin sensitive in growing
C57Bl/6 mice. Perhaps this is through the ability of the specific is flavones present to act as ligands for PPARs.

Visceral adipose tissue expression of adiponectin mRNA levels were significantly higher in the Soy diet group compared to all of the other diet groups. None of the other three diet groups were significantly different from one another. It is unclear why the soy group adiponectin gene would be up-regulated compared to the other diet groups, or more likely, why the Soy group would be the only group in which the gene is not down-regulated in the visceral adipose tissue. However, serum adiponectin levels were not significantly different among diet groups. In obese states, visceral adipose tissue mRNA levels are down-regulated in rodents and humans, and the plasma adiponectin levels reflect this drop (8, 48). However, the animals in this study were not fed a diet excessively high in fat, and as a result the mice may not have reached the level of obesity as animals referred to as obese in other studies. Perhaps adiponectin may be more closely linked to insulin resistance and hyperinsulinemia than to the level of obesity (12). It is possible, therefore, that even though the mice in the Casein group were obese and demonstrated insulin resistance, they did not undergo detectable dysregulation of adiponectin compared to the other diet groups.

Serum IL-6 levels were also not significantly different among the diet groups. Adiponectin may help regulate the release of other adipocytokines such as IL-6 (12). The fact that serum adiponectin and IL-6 levels were not different among diet groups may indicate that the function of the adipose tissue may not have been impaired to a large degree. Serum IL-6 is representative of IL-6 secretions from tissues other than adipose. This adipocytokine will need to be measured at the level of the adipose tissue and liver to determine whether dietary difference could cause significant differences in IL-6 expression among diet groups. Higher levels of IL-6 at the level of the adipose tissue could be representative of increased macrophage infiltration. For the IL-6 data, there were a number of outliers, and some missing data due to large serum requirements, so the validity of this data may be diminished.
Measurements of leptin appeared to reflect the visceral fat mass. Previous research has indicated that leptin may be more representative of subcutaneous adipose levels than visceral levels, because expression and release of leptin tends to be higher in subcutaneous adipose tissue than visceral tissue (136). While we did not measure subcutaneous mass, our data do support a relationship of leptin with visceral fat mass.

Subcutaneous adipose, visceral adipose, and liver samples were collected for histological analysis of morphological differences at the cellular level. Visual inspection of the slides reveals that the heaviest mice from the Casein group have the largest adipocytes in both the subcutaneous and visceral tissues, as well as the greatest fat deposition in the liver (figure not shown). It is likely that analysis will reveal a link between body weight and adipocyte size. However, the adipocyte size of the mice in the CtoFSoy group may be reduced reflecting their recent weight loss.

Analysis of mRNA expression changes in genes regulating adiposity and lipid metabolism from visceral adipose tissue of the mice in each of the diet groups may help elucidate the mechanisms involved in the regulation of adipose by the FSoy diet. FASN and SCD1 are both genes regulated by the membrane bound transcription factor, SREBP-1, which activates the transcription of genes involved in fatty acid synthesis. However, there was no difference in the mRNA levels between the FSoy and Soy for either gene. Because previous researcher found a reduction in SREBP-1 in rats fed a soy diet without isoflavones, the observed SREBP-1 reduction was not associated with the presence of soy isoflavones (36). Therefore, a difference in the expression of FASN and SCD-1 between the FSoy and Soy diet was not expected in the present study. While soy protein with isoflavones removed has been shown to decrease the expression of SREBP-1 in rats relative to a casein based diet, neither the Soy nor FSoy diets in the present experiment had reduced expression of FASN or SCD1 compared to the Casein diet (36).

It deserves mentioning that the CtoFSoy diet did produce significantly higher expression of the SCD-1 gene than the FSoy diet group (and the Casein group) did in the visceral adipose tissue. This suggests that the CtoFSoy diet had increased expression of SREBP-1. SREBP-1 is
increased in response to high insulin levels (such as in found in an insulin resistant state), and seems to cause fatty liver (36). The CtoFSoy mice exhibited both reduced fasted plasma insulin levels and an increased insulin response to glucose during the insulin tolerance test. However, the lack of significant expression levels of FASN mRNA between the CtoFSoy group and the other diet groups suggests that perhaps SREBP-1 expression did not vary among the diet groups.

F4/80 is an antigen that is associated with macrophage infiltration (52). While it is poorly understood, it could be indicative of inflammation. The F4/80 mRNA was significantly lower in the FSoy and CtoFSoy diet groups, so in this study it seems to be lower in diet groups with less adipose tissue. This association may reflect a relationship, but more information is needed to determine this.

HSL is a gene involved with lipid storage, and is still thought to be the main rate limiting enzyme in lipolysis (49). ATGL is also involved in lipolysis. Glucose can cause levels of HSL to increase (50). The FSoy diet group had significantly lower glucose levels than the Casein and Soy diet groups, and expression of HSL mRNA was also significantly lower in the FSoy group compared to the Casein and Soy groups (as well as the CtoFSoy group). ATGL mRNA expression, on the other hand, did not significantly differ between FSoy and Casein or CtoFSoy or between FSoy and Soy. Statistical analysis of adipose histology will help to reveal more information about lipid storage at the level of the adipocyte.

While the primary focus of this thesis is focused on FSoy regulation of adipose tissue, possibly through the regulation of PPAR-α, PPAR-α mRNA expression in visceral adipose tissue was significantly up-regulated in the Soy diet group compared to the FSoy group. This suggests that the antilipogenic effects of the FSoy diet were largely independent of the PPAR-α receptor in adipose tissue. Indeed, one study reports decreased adipose deposition in C57Bl/6 mice due to intake of the isoflavone genistein, apparently dependent on ERalpha (29). Future investigation of the tissues collected during this study will involve a closer inspection of PPAR-α regulated genes.
FABP4 is a PPAR-α responsive gene that facilitates cellular uptake and intracellular transport of fatty acids. If the FSoy diet works through regulation of PPAR-α, one would expect levels of FABP4 to decrease in the FSoy diet group compared to the Soy and Casein groups, and that is exactly what the results reflected. However, the mice that lost weight when switched from the Casein diet to the FSoy diet did not have a significant change in expression of FABP4 compared to the mice from the Casein group, and the expression of this gene increased moderately in the CtoFSoy group compared to the FSoy group. So while the effect of the FSoy diet may work through PPAR-α-dependent mechanisms, it likely works through PPAR-α-independent mechanisms as well. Perhaps the mechanisms of FSoy in weight prevention and weight reversal are different.

Additional investigation into protein expression levels of PPAR-α regulated genes such as FABP4 will elucidate more information into part of the mechanism involved in the research. Measurements and statistical analysis of adipose and liver histology samples may also provide insight into adipose regulation at the cellular level. Future study designs should focus on isolated isoflavones as opposed to soy diets containing isoflavones in order to better isolate the effects of the isoflavones without the beneficial effects of the other soy components.
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Conclusions

These data suggest that there are beneficial weight and insulin responses observed in the C57Bl/6 mice from the FSoy study diet, and that these are largely due to PPAR- independent mechanisms, and may, in part, be due to PPAR-α-dependent mechanisms.

Limitations

There are many chronic diseases that are now understood to be associated with dietary intake which could be avoided with dietary modifications or interventions. Growing evidence suggests that soy may have an effect in reducing the risk of many common chronic diseases commonly associated with a "Western diet", such as certain cancers, diabetes, hyperlipidemia, high blood pressure, and possibly obesity (8). Soy foods are traditional staple foods in countries like China, Japan, and Indonesia (137). Many of these traditional soy foods are highly fermented, and thus differ from soy foods that have been incorporated into the "Western diet" because they contain higher levels of aglycone isoflavones (138). In these Far East countries, incidence of hormone-dependent diseases are low relative to Western countries, but the incidence is increasing as a more Westernized diet is being incorporated into the cultures (137, 139, 140). The protective effects of the traditional diet from these hormone-dependent diseases are attributed to nonnutritive components of the Far East diet, including isoflavones from soy foods, which can contribute toward significant health benefits (138). While most studies involving soy
Isoflavones show beneficial effects, some experimental studies in animals have shown that there is a distinct possibility of risk associated with the use of isoflavones (141, 142, 143). These experimental studies and epidemiological studies suggest that risks from isoflavones seem to be associated with the consumption of very high levels for long periods of time (35, 141,142,143). Estrogens have been shown to exhibit biphasic responses that are highly dose-dependent, so pharmacological consumption of isolated soy components may present the possibility of risk (138). It may also be possible to consume high enough levels of isoflavones from the diet to pose a risk for negative health effects, but this would be very difficult (138). Studies have shown that positive biological effects from dietary estrogens can be attained by consuming as little as 30-50 mg/day, so even a modest incorporation of soy foods into a “Western diet” could be beneficial. Additionally, consuming up to 120 mg/day of isoflavones for up to 6 months has no evidence of adverse effects (34, 138).

**Future Directions**

The analyses in this present study were conducted on adipose tissue and blood hormones. A more comprehensive, whole body response, from the study diets could be determined by comparing the data herein to other work done in our lab on liver and blood lipids. This comprehensive analysis will be completed in future publications. Further, statistical analysis of the histology of the liver and adipose tissue will be completed to determine differences in cell size. If further morphological differences are noted between groups, those will also be analyzed for further insight into the cellular responses from the study diets on adipocytes and hepatocytes.

The mRNA analysis shows differences in gene expressions, but this does not always correlate to increases or decreases in protein expression, and observed changes in protein expression do not always reflect the degree of change seen at the mRNA level. Therefore, more insight could be gained through future analysis of protein expression changes of PPAR modulated genes.