ABSTRACT

METABOLIC EFFECTS OF SHORT-TERM HIGH-FAT DIET FEEDING IN MALE AND FEMALE MICE

by Shiva Priya Dharshan

The lipid profiles and molecular events related to the development of lipid accumulation and insulin resistance were characterized in male and female C57Bl6 mice fed with standard low-fat chow (Chow), high-fat SFA (SFA), high-fat mono-UFA (MUFA), or high-fat poly-UFA (PUFA) for a period of four days. SFA- and MUFA-fed mice displayed triglycerides (TG) accumulation in the liver, with higher calorific intake as well as greater white adipose tissue (WAT) mass compared to mice on Chow and PUFA. However, female mice were well protected against the deleterious effects of diet induced obesity (DIO) particularly SFA females. Interestingly, female mice on MUFA displayed a lower atherogenic index (HDL/LDL) as well as up-regulated gene expression of de novo lipogenic genes such as ACC and SREBP-1c compared to females on standard low-fat chow, SFA and PUFA. In contrast, PUFA fed mice, both males and females, had comparable caloric intake, and plasma LDL and free fatty acid (FFA) levels as same sex chow-fed mice, and had lowest TG accumulation in the liver compared to mice from other groups.
METABOLIC EFFECTS OF SHORT-TERM HIGH-FAT DIET FEEDING IN MALE AND FEMALE MICE

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**Key Words:**
Obesity, insulin resistance, high-fat diets, diet induced obesity, sex difference

**Abbreviations:**
ACC – Acetyl Co-A carboxylase
DIO – diet induced obesity
E2 – estradiol
ER – estrogen receptor
EWAT – epididymal white adipose tissue
FAS – fatty acid synthase
GWAT – gonadal white adipose tissue
HDL – high density lipoprotein cholesterol
HFD – high-fat diet
HPV – Hepatic portal vein
LDL – low density lipoprotein cholesterol
LFD – Low fat diet
MUFA – monounsaturated fatty acids
NAFLD – nonalcoholic fatty liver disease
NEFA – non-esterified fatty acids
OVX – ovariectomized
PUFA – polyunsaturated fatty acid
PGC1α – Peroxisome proliferator-activated receptor gamma coactivator 1-α
PEPCK – phosphoenol pyruvate carboxykinase
RQ – Respiratory quotient
SFA – saturated fatty acids
SREBP – Sterol regulatory binding protein
TG – triglycerides
vLDL – very low density lipoprotein
WAT – white adipose tissue;
PWAT – parametrial white adipose tissue
CHAPTER I

HIGH-FAT DIET-INDUCED OBESITY AND METABOLIC DISEASES

Obesity is a major health problem. Obesity dramatically increases the risk of developing type 2 diabetes, dyslipidemia, cardiovascular disease and even some types of cancer. Dietary high-fat content in many Western diets is considered one of the most important environmental factors leading to obesity (French et al., 2001). Dietary high-fat content also results in accumulation of triglyceride (TG) in the liver and development of nonalcoholic fatty liver disease (NAFLD). NAFLD leads to marked hepatic dysfunction and is an important contributor to obesity-induced insulin resistance, thus NAFLD is the hepatic manifestation of the metabolic syndrome (Marchesini et al., 2003).

The Three HFDs with different types of fatty acids: SFA, MUFA and PUFA

Obesity having reached its pandemic status has been an area of active research for which many rodent models have been devised to study the pathology of this metabolic disease. DIO models were probably used first by Masek and Fabry in 1959; from then on DIO models have been utilized in studies that evaluate physiology of metabolic tissues, such as the muscle and the liver, as well as their insulin resistance (Buettner et al., 2004).

As far as fatty acids are considered, their chain length and level of saturation dictate many of the physical properties of the lipids that constitute them. In a broader sense, fatty acids affect the fluidity and membrane properties as they form a major portion of the cell membrane, thereby affecting receptors and other signaling molecules harbored at the membrane. A study in rodents (Leyton et al., 1987) showed that a major portion of the ingested fatty acids are incorporated into storage as complex lipids or as membrane lipids. Most animals along the evolutionary tree have lost the ability to synthesize polyunsaturated fatty acids (PUFA), and they rely on plant sources or their gut bacteria to produce them, whereas monounsaturated fatty acids (MUFA) and saturated fatty acids (SFA) can be synthesized de novo. Hence it has been shown that the amount of PUFA in an animal’s diet certainly affects the composition of fatty acids in its cell membrane (Hulbert et al., 2005). PUFA diet with its membrane changing effect has been hypothesized to
increase metabolic rate, as the major portion of a cell’s energy traffic is through membrane
associated processes. This is evidently seen in nature where sea birds, whose primary diet
is fish (rich in PUFA) have been observed to have a higher metabolic rate due to rich PUFA
diet compared to termite eaters with relatively less PUFA content (Hulbert et al., 2005).

With an increase in understanding of the progression and development of obesity,
prevention and reversal studies using alternate type of fat content have been reported.
These studies have guided the emergence of healthy food supplements containing omega
fatty acids, such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), that are a
current focus of research. Studies with these diets would help in a better understanding of
the pathophysiology of the disease. Hence the crux of this MS thesis is to analyze how the
different high-fat diets SFA, MUFA and PUFA prove deleterious or beneficial from the
metabolic perspective.

**Long-term homeostatic regulation vs. short-term hedonic regulation of energy balance: Time
Scale for Obesity Studies**

It is customary in any DIO study to feed animals with a high-fat diet for at least 8
weeks, some last 15-16 weeks, in order to get them obese, and animals are studied after
that time period. Even though the energy intake, body weight, and circulating metabolic
parameters (such as levels of adiposity signals, other hormones, and glucose) could be
recorded longitudinally without sacrificing animals, it is important to study any
physiological changes however small during the initial phases of the diet feeding to
understand disease development. A recent study by Kleemann et al. (2010) tried to map
the development of insulin resistance in male ApoE3Leiden transgenic mice on a HFD, a
mouse model that mimics human lipid metabolism, through a time resolved
metabolonomic approach. The study discovered the rise of inflammatory markers in
certain metabolic tissues, including the liver, white adipose tissue (WAT), and muscle,
within 7 days. From this study it was evident that the alteration in dietary lipid
composition plays a role in the induction of hepatic insulin resistance, and importantly,
from early days of initiation of a high-fat diet (Kleemann et al., 2010). This recent evidence
begs for further probing through a short-term study. In the same study, a change in the
composition of circulating FFAs was observed with an increase in MUFAs and decrease in
PUFAs using, during the progression of insulin resistance (Kleemann et al., 2010). In another study, PUFA diet (EPA emulsion by gavage) reduced the increased hepatic TG in high-fat high-sucrose diet (only appears once, so do not need abbreviation) fed mice. This suppression of TG accumulation correlated with the decrease in MUFA levels in the liver (Kajikawa et al., 2009). This recent evidence questions the credibility of a “healthy” Mediterranean diet, i.e. rich in MUFAs.

**Mechanism of Insulin Action**

Insulin is a peptide anabolic hormone secreted from β-cells of the pancreas. Its primary role is to maintain glucose homeostasis, but also has an important part to play in the body’s metabolism and energy status, pushing glucose from circulation into liver glycogen, lipid into adipose tissue, and decreasing glucose production in hepatic cells. Insulin mainly targets the liver, skeletal muscle, and WAT. Insulin signaling is through membrane receptors on cells leading to autophosphorylation and internalization of the receptors, followed by activation cascade of receptor kinases, cell signaling secondary messengers that finally allow the translocation of Glut4 molecules to the cell surface that allow entry of glucose molecules into the cell.

Of the secondary cell messengers, phosphorylation of Akt, is deemed to play an important role in the translocation of Glut4. Three isoforms of Akt have been isolated, Akt1, Akt2 and Akt3. Insulin downstream actions on these isoforms are different in various tissues (Walker et al., 1998). Overall, Akt has been attributed to be phosphorylated in the liver, muscle, and WAT with proper insulin signaling, which is evident in obese and type 2 diabetics subjects who show impaired Akt-activated insulin signaling.

**Sexual Dimorphism**

Estrogens are important multifunctional hormones regulating sexual function, reproduction, maintenance of bone turnover (Gruber et al. 2002), neurological function (Sherwin 2012), energy homeostasis (Shi et al. 2009), adiposity and fuel partitioning (D'Eon et al. 2005), and glucose metabolism (Shi and Senthil Kumar 2012). Ovariectomized rodents with low level of endogenous estrogens (Kumagai 1993) or aromatase knockout mice with a genetic impairment in estrogen synthesis (Takeda 2003) exhibit insulin
resistance, suggesting that estrogens contribute to maintain insulin sensitivity and their deficiency leads to development of insulin resistance. Premenopausal women have fewer metabolic disorders than men; however after menopause women are as susceptible to glucose- and lipid-related diseases as men with similar ages (Ford 2005). This has resulted in the use of estrogen hormone replacement therapy (Salpeter 2006) or administration with estradiol (E2), the most physiologically active estrogen (Rochira 2007), for the treatment of insulin resistance in postmenopausal women (Salpeter 2006) or in men with congenital aromatase deficiency (Rochira 2007).

Data from previous literature suggest that E2 certainly does have a beneficial role in improving insulin sensitivity. E2 exerts its biological function through classic genomic actions initiated by binding to its nuclear estrogen receptors (ER) or through recently defined non-genomic actions initiated by binding to its membrane ER. Its nuclear receptors ERα and ERβ are present in most metabolic tissues both peripherally and centrally (Shi et al., 2013). There have been contradicting reports regarding the roles of the specific ERs. Previously it was thought that ERα was responsible for the metabolic actions and ERβ for the sexual characteristics. Barros and colleagues (2011) reported that both the receptor isoforms are necessary for maintaining energy homeostasis, the ratio of distribution playing an important role (Barros et al., 2011). Whereas in another study, ERα absence caused insulin resistance and glucose intolerance (Heine et al. 2000; Bryzgalova et al. 2006), and ERβKO mice demonstrated normal glucose tolerance and insulin sensitivity (Bryzgalova, Gao et al. 2006).

At present the main focus of E2 research from a metabolic perspective is on both central (Pratcayakul et al., 2011; Xu et al., 2011) and peripheral (Bryzgalova et al., 2008; Riant et al., 2009) metabolic tissues. In vitro studies have showed that E2 enhances insulin signaling through ERα in 3T3-L1 adipocytes (Nagira et al. 2006) and E2 stimulates Akt and AMP kinase in soleus muscle (Rogers et al. 2009), suggesting that the beneficial effects of E2 on glucose metabolism and insulin sensitivity may be exerted via a direct action in peripheral tissues. Other studies have identified central actions of E2 in the regulation of energy homeostasis on proopiomelanocortin (POMC) neurons (Gao et al. 2007) and insulin sensitivity on ventromedial nucleus (VMN) of hypothalamus (Musatov et al. 2007). A comparative study addressing the metabolic state in males and females with a short term
exposure to different high-fat diets have not been done yet. In this thesis, I compared development of lipid profile and insulin signaling in male and female mice fed with different diets.

**Rationale and Hypothesis**

The major intent of my thesis is to study the development of the obesity phenotypes particularly at an early stage, with our lab focusing mainly on the cellular and molecular events of the progression of the disease. Another area of study is to find out how beneficial the PUFA diet is when compared to MUFA and SFA. We have previous data (unpublished observation) from our long term study in males that provide evidence showing better metabolic parameters in mice fed with PUFA diet than those fed with either the SFA or MUFA. Body weight of the mice fed with PUFA was less compared to those fed with MUFA or SFA \([F_{(3,36)} = 29.91, P < 0.0001]\) (Fig 1A). Also even though each group had comparable lean mass \([F_{(3,36)} = 1.59, P = 0.21]\) (Fig 1B), their total fat mass \([F_{(3,36)} = 52.77, P < 0.0001]\) (Fig 1C) and epididymal white adipose tissue (EWAT) mass \([F_{(3,36)} = 63.64, P < 0.0001]\) (Fig 1D) were different; again, the group fed with PUFA had lower adiposity and fat mass compared to SFA and MUFA groups.

As far as lipid profile was considered, MUFA- and SFA-fed mice displayed a lower HDL/LDL ratio compared to the PUFA group \([F_{(3,36)} = 5.89, P = 0.002]\) (Fig 2A), suggesting that PUFA fed mice maintained healthy lipid profile, whereas MUFA- and SFA-fed mice did not. Interestingly, when looked at the triglyceride (TG) levels, both MUFA and SFA groups developed signs of fatty liver with liver TG levels higher in both the SFA and MUFA groups compared to Chow group, whereas PUFA-fed mice had similar liver TG levels as chow-fed mice \([F_{(3,36)} = 4.26, P = 0.01]\) (Fig 2C). The plasma TG levels of PUFA-fed mice were significantly lower than Chow mice. Interestingly we also observed a 30% lowered circulating TG levels in the SFA- and MUFA-fed mice compared to Chow-fed groups \([F_{(3,36)} = 5.52, P = 0.003]\) (Fig 2B).

**Specific Aim 1:** *To test the hypothesis that SFA, MUFA and PUFA have distinct effects on metabolic tissues, which contributes to long-term obesity and diabetes phenotypes.*
The first hypothesis is regarding the short term studies. Based on previous data on effects of long-term HFD feeding on lipid accumulation in the liver, I would hypothesize the short term studies to mirror the long term studies. I will be focusing majorly on the changes in the hepatic lipid profiles, because from a previous short-term study (Kleemann et al., 2010) showed changes in the liver first. I will be looking at plasma lipid profiles, hepatic TG accumulation, and expression of genes involved in hepatic de novo lipogenesis and β-oxidation. With respect to molecular changes, I will be looking at pAKT levels to infer insulin sensitivity in the liver, muscle and gonadal WAT. I expect the PUFA diet, but not MUFA diet, show ‘healthier’ conditions compared to SFA, such as less lipogenesis and non-toxic lipid levels. This could give some insights about how insulin resistance progresses with regards to different diets.

**Specific Aim 2: To test the hypothesis that female mice would be better protected against the effects of SFA and to compare the effects of PUFA and MUFA with males**

As discussed above, the second area of interest is if females would be different in their short term metabolic parameters given these different high-fat diets. There is a plethora of data pointing towards protective roles of estrogens against metabolic dysfunction. Additionally, it has been shown that female mice are more sensitive to insulin, displaying a greater decrease in plasma glucose compared to their male counterparts (Shi et al. 2008; Shi et al. 2010). In addition female adipocytes have been shown to be more insulin sensitive, showing higher insulin-induced lipogenic properties (Macotela et al., 2009). In light of this evidence we expect female mice to be more adapted to the high fat diet, thus presenting a more stable lipid profile and glucose tolerance at least in the short term.

The findings from my thesis would allow us to map how the disease progresses from the starting stage, and to dissect how each diet is perceived by body of each sex. By knowing this we can certainly come up with better and specific treatment schemes against obesity and its complications, particular for males and females.
Chapter II

METABOLIC EFFECTS OF SHORT-TERM EXPOSURE OF HIGH-FAT DIETS IN MALE MICE

Introduction

Diet-induced obesity (DIO) through chronic high-fat diet (HFD) feeding has been well documented, with different hypothesis tested and mechanisms put forth. However there are still a lot of grey areas with respect to the initial phases during the development of the disease. Recently, the pathophysiology of an acute HFD feeding has been receiving attention. Several studies have been trying to delineate the occurrence of events that lead to the various established metabolic dysfunctions such as insulin resistance, hyperinsulinemia, ectopic fat accumulation and chronic low-grade inflammatory status to name a few. Wright et al (2009), through feeding C57Bl6 mice a high-fat and high-sucrose diet for 2 weeks, observed a decrease in insulin stimulated glycolysis rates and oxidation via faulty GLUT4 translocation. They attributed this observation the stepping stone for the development of insulin resistance.

One missing piece to the puzzle is the primary mechanism involved in the development of obesity and the metabolic syndrome. There have been two well established ideas – metabolic dysfunction and inflammation. Although each idea has been separately tested with compelling results, recently the interplay between the metabolic and inflammatory organ systems has been implicated. Therefore a good place to start looking would be at the initial phase of the disease. This is one of the reasons why acute feeding regimen studies have received recognition lately. In this study, metabolic changes will be characterized in animals fed with a diet rich in lipids for 4 days.

Lipids play an essential role in metabolism and are a major component of cell membranes. Lipids circulate in the plasma as non-esterified fatty acids (NEFA)/free fatty acids (FFA) bound to albumin. Their primary area of storage is in the adipose tissue as triglycerides (TG) from where they are catabolized through lipolysis to FFA and transported to the liver and muscles for utilization. This storage and release of FFA are under tight regulation by hormones such as insulin and adrenaline, physiological states such as stress, pathological states such as diabetes, and nutritional status such as
overfeeding and starvation. Although diets rich in lipids have been deemed unhealthy, the type of fat is very important. In a comprehensive review, Hulbert et al. (2005) illustrated the importance of the different dietary lipids and their implications in the body’s metabolism standpoint. A mention of all their implications is beyond the scope of this study, therefore only those concerning with developing insulin resistance will be addressed in this thesis.

The number of double bonds and the length of the acyl chains govern the physical properties of lipids and can be used to classify lipids into saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids. The nature of the dietary lipid ingested governs the composition of membrane lipids and hence fluidity and physical properties of the plasma membrane. This mechanism has been well received, with many groups showing proof of changes in membrane composition in liver plasmalemma (Clamp et al., 1997), cardiac sarcolemma (Vajreswari and Narayanareddy, 1992), cerebral synaptosomes and myelin (Srinivasarao et al., 1997). Any change, chemical or physical, to the plasma membranes would change the functioning of membrane bound receptors or ion channels as well. Membrane associated processes such a H⁺ gradient and proton leak are responsible for determining the metabolic state of the cells (Hulbert et al., 2005). Therefore, in retrospect, the composition of the membrane is directly related to the metabolic output of the cell. This theory is also called the ‘membrane pacemaker’ theory (Hulbert et al., 2000).

In addition to the short time scale for feeding, how different HFDs affect the metabolic characters such as circulating lipid profiles, hepatic TG as well as hepatic expression of genes involved in hepatic de novo lipogenesis and β-oxidation, and insulin sensitivity of metabolic tissues (liver, muscle and EWAT) through measuring pAKT levels will be investigated.

**Methods**

**Animals:**

Adult male C57Bl6 mice were maintained on standard 12-h light-dark cycle (lights out at 1700 h). The animals were fed with either a low-fat diet (LFD), which was a standard pelleted laboratory rodent chow (Teklad, Madison, WI), or one of three high-fat diets (HFD)
with matched macro- and micro-nutrient contents for 4 days. Respective diets and water were provided *ad libitum* unless otherwise mentioned. All procedures were approved by the Institutional Animal Care and Use Committee of Miami University Ohio and were in strict accordance with the Guide for the Care and Use of Laboratory Animals.

**Experimental Design:**

Metabolic syndrome is caused by obesity, and it includes hyperlipidemia and insulin resistance. Change of body adiposity leads to related changes in the blood lipid. Circulating leptin, a hormone secreted proportionally to body fat mass, is an indicator for total adiposity. HDL/LDL ratio is a useful indicator for health risks related to obesity and cardiovascular diseases. Insulin resistance is indicated at both whole body level and tissue level. At the body level, hyperinsulinemia is caused by increased secretion of insulin in response to continued elevated blood glucose. At the tissue level, metabolic tissues are resistant to the effects of insulin to uptake glucose via Akt/GLUT pathway. Plasma glucose and insulin levels are net effects contributed by the major metabolic tissues, the liver, adipose tissue, skeletal muscle. To specifically identify the contribution of each of these tissues, analysis of insulin signaling of each tissue was performed.

Total of 32 male C57Bl6 mice were used in this study with 8 animals allotted to each diet group for Chow, SFA, MUFA and PUFA. The mice were allowed to eat their respective diet and water was given *ad libitum* for 4 days. Their daily food intake and body weights were measured. Fresh food was given each day to the animals. At the end of 4 days the animals were food deprived for 2 hr, either injected intraperitoneally with insulin (1 U / kg BW) or saline, and then sacrificed with isofluorane overdose 15 min after injection (Foster, Shi, et al 2011 Diabetologia). Blood from the hepatic portal vein were collected, to be centrifuged to separate plasma and stored at -20°C for lipid profiling and leptin measurement through ELISA assays. The liver, soleus muscle, and abdominal EWAT were collected and immediately frozen in isobutene cooled by dry ice. The frozen tissues were stored at -80°C for western blotting for pAKT, liver TG extraction, and RNA extraction.

**Diets:**
All HFD are formulated by Research Diets, Inc. (New Brunswick, NJ). LFD and HFD contain similar amounts of proteins (0.243 g per gram of LFD vs. 0.237 g per gram of HFD) and carbohydrates (0.402 g per gram of LFD vs. 0.414 g per gram of HFD), but quite different amounts of fat (0.047 g per gram of LFD vs. 0.236 g per gram of HFD). The energy content of the LFD was 3.00 kcal/g and 14% calories from fat, whereas that of all three HFD enriched in different sources of dietary fat, lard oil (SFA), olive oil (MUFA), and fish oil (menhaden oil PUFA) was 4.73 kcal/g and 45% calories from fat. The fatty acid in the lard oil diet were mainly SFAs, palmitic and stearic acid (C16:0 and C18:0), in the olive oil diet was mainly MUFA oleic acid (18:1, N-9), and in the menhaden oil diet was mainly N-3 PUFA (EPA, 20:5; docosahexaenoic acid, 22:6). For all three HFDs, the protein content was 20%, and was composed of casein and L-cystine (in the ratio of 1: 0.015, by weight) and the carbohydrate content was 35%, and was composed of corn starch, maltodextrin 10, sucrose, and cellulose (in the ratio of 1: 1.4: 2.4: 0.7, by weight). HFD also contained 5.2% minerals and 1.4% vitamins, and 2.9% soybean oil to ease compounding.

**Tissue collection and Assays:**

Animals were overdosed with isofluorane and blood samples were collected from hepatic portal vein in heparin coated tubes. Blood samples were centrifuged to obtain the plasma and stored at -20°C until further assays was performed. Subsequently liver, bilateral soleus muscle, and bilateral epididymal white adipose tissues (EWAT) were collected in 2 ml tubes separately, frozen using isobutane and dry ice, and stored at -80 °C. Plasma concentrations of FFA, TG, HDL and LDL were measure using specific kits (Wako diagnostics, Richmond VA). Insulin and leptin levels were measure by ultrasensitive ELISA kits provided by CrystalChem Inc. (Downers Grove, IL)

**Liver TG Measurement:**

Liver TG extraction was adapted from the methods described by Storlien et al. (1991) and Maycock et al. (1980). In short, about 50mg of liver tissue (weight of tissue recorded) was lysed in 1 ml of 2:1 (v/v) mixture of chloroform and methanol. After 2 hours of gentle shaking, 0.3 ml of water is added to the lysed mixture for phase separation. The samples are then centrifuged at room temperature for 35 min at 2000 rpm. The top
two layers (cell debris) were discarded and the bottom organic layer (containing dissolved lipids) was transferred to a new tube. The new tubes were left open overnight to allow evaporation of the organic layer.

Next day, the solute present in tubes after evaporation were redissolved in 225 µl of chloroform and mixed thoroughly to ensure complete mixing. Triplicates were prepared using the chloroform mixture along with the standards (Multi Calibrator lipids: TG = 107 mg/dl, Wako Chemicals, Richmond VA). The contents were evaporated in an oven at 65 °C. 25 µl of 0.9% NaCl was added, followed by 200 µl of TG or cholesterol enzyme reagent to each well. The absorbance of the contents was read at 600 nm.

Liver gene expression (quantitative RT-PCR):

Liver samples were homogenized in trizol TRI reagent® (MRC, Cincinnati, OH) with consequent addition of bromoanisole BAN reagent ® (MRC, Cincinnati, OH) for phase separation, and centrifuged at 12,000 g for 15 mins. Total RNA was extracted from the clear supernatant layer using RNeasy mini kit (Qiagen). cDNA was subsequently synthesized using the iScript kit (Bio-Rad, Hercules, CA) and subjected to gene expression analysis for particular target genes. The PCR efficiencies were calculated through the measured threshold cycles (C_T).

Quantitative PCR was performed for the following genes whose primers have been specified in Table 1. The Genes of interest are those implicated in hepatic lipogenesis, including lipogenic enzyme fatty acid synthase (FAS), acetyl co-enzyme carboxylase (ACC) and sterol-regulatory binding protein-1c (SREBP-1c), a pivotal regulator of expression of ACC (page 23); implicated in hepatic mitochondrial β-oxidation of fatty acids, including peroxisome proliferator-activated receptor gamma coactivator 1-α (PGC1α) which is involved in the activity of mitochondrial β-oxidation of fatty acids; and implicated in hepatic gluconeogenesis, including gluconeogenic enzyme phosphoenol pyruvate carboxykinase (PEPCK), by which insulin inhibits to suppress hepatic glucose production and gluconeogenesis.
### Table 1. qPCR primer sequences

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>GenBank accession number</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>SREBP-1c</td>
<td>NM_011480</td>
<td>F: 5’-GGCATAAGTGCCCTCAACCT-3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: 5’-GCCACATAGATCTCTGCCAGTGT-3’</td>
</tr>
<tr>
<td>FAS</td>
<td>NM_007988</td>
<td>F: 5’-TCACCACCTGTGGGCTCTGCGAGAAGCGAG-3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: 5’-TGTCATTGGGCTCAAAGGGCGTCCA-3’</td>
</tr>
<tr>
<td>PGC1α</td>
<td>NR_027710</td>
<td>F: 5’-ATGCTGCGCCTTCTTGCTCT-3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: 5’-ATCTACTGCTGGGGACCT-3’</td>
</tr>
<tr>
<td>PEPCK</td>
<td>NM_011044</td>
<td>F: 5’-CACCTCCTGGAAGAACAAGG-3’</td>
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<td></td>
<td></td>
<td>R: 5’-CTACGGCCACCAAGATGAT-3’</td>
</tr>
<tr>
<td>ACC</td>
<td>NM_133360</td>
<td>F: 5’-CCCAGAGAGAATAAGCTACTTTTG-3’</td>
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<td></td>
<td></td>
<td>R: 5’-TCCTTTTGTGCAACTAGGAAG-3’</td>
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GAPDH was used as the reference gene (Accession number NM_008084 (F: 5’-TGCGACTTCAACAGCAACTC-3’, R: 5’-GCCTCTCTTGCTGAGTGTCC-3’). Expression of GAPDH was not different among groups. Quantitative RT-PCR was run with a Bio-Rad iCycler (Bio-Rad, Hercules, CA) with iQ SYBR Green Supermix (Bio-Rad). The set program was 2 step cycle - amplification (95 °C for 10s) and annealing (58 °C for 30s) for a total of 40 cycles. The amplified products were confirmed through gel electrophoresis and melt curve analysis. The samples were run in triplicates and the PCR efficiencies were calculated from the slope of the threshold cycle (Ct).

The relative fold changes between the target and reference gene GAPDH were calculated using the Delta-Delta CT (ΔΔCt) method. In this approximation method the average difference of the Ct between GAPDH and the target gene (ΔCt) of the control is subtracted from each ΔCt of each sample (ΔΔCt) from the experimental group to obtain the relative fold change. The results were graphed using Chow group as 100% and the other groups were calculated relative to the animals.
Western Blotting:

Total protein was extracted by homogenizing tissues using RIPA lysis buffer (Santa Cruz Biotechnology; kit includes PMSF, Sodium orthovanadate, Protease inhibitor) and phosphatase inhibitor cocktail (Sigma). The homogenate was centrifuged at 10,000g with repeated transfer of supernatant to purify the supernatant of cell debris. The final supernatant was collected and stored at -80°C until use. Protein concentration will be measured using the standard BCA assay (Quick Start™ Bradford protein assay).

Western blots were run on precast 10 or 15 well gels (Mini-PROTEAN® TGX™, Bio-Rad). The samples were prepared to be loaded using the Laemmli method. The following buffers were used: running buffer (TGS) (25 mM Tris, 192 mM glycine and 0.1% SDS (w/v)), transfer(blotting) buffer (20% v/v methanol in 1X TGS), wash buffer (TGST) (10 mM Tris, 100 mM NaCl, 0.1% Tween 20), 10% milk blocking buffer (in 1X TGST). Primary antibodies are rabbit monoclonal antibodies (pAKt, pMAPK, tAKt and tMAPK) and secondary antibodies are goat anti-rabbit IgG HRP-linked (Cell Signalling Technologies®). Antibodies were used as 5% milk buffer preparations.

The samples were run at 120 V for 5 min and at 180 V for 1 h. The gels were then separated from the cassettes to be transferred onto a nitrocellulose membrane, Trans-Blot® transfer membrane (Bio-Rad) at 80 V for 1.5 h. The transferred membrane is blocked with 10% milk for 1 h. Then the membrane was probed with the primary antibody with overnight incubation at 4°C. The primary antibody was then removed and membrane washed with wash buffer for about 4-5 times with 10 min each time. The membrane was then incubated with the secondary antibody at room temperature for 2 h followed by 4-5 washes. Finally the membrane was incubated with Amersham™ ECL™ Prime (GE Healthcare) for substrate detection. The membrane was placed in an autoradiography cassette along with a X-ray film (GeneMate, Bioexpress) and the film was developed in a dark room to visualize the bands. The densities of the bands in the films were quantified using the ImageQuant TL software (Amersham Biosciences) for 1D electrophoresis gel analysis.
Statistical analysis:

Data analysis was performed using Prism Statistical Software 5 (La Jolla, CA). Data were expressed as mean ± SEM. A one-way ANOVA followed by Tukey’s multiple comparison test was used to analyze body weight, caloric intake, plasma and liver lipid profile, plasma glucose level, and hepatic gene expression among four dietary groups. A two-way ANOVA followed by Bonferroni posttest was used to analyze insulin signaling by comparing dietary factor and treatment factor (insulin or saline injections). A test with $P$ value less than 0.05 (i.e. $P < 0.05$) was considered statistically significant. Post hoc multiple comparison tests were carried out with computing the 95% confidence interval (CI) of the difference between two group means and the test was considered statistically significant if this CI did not include zero.

Results

Body weight, food and caloric intake and adiposity:

The body weights and food intake were measured daily for 4 days between 1000 h and 1030 h. On the 4th day, the epididymal adipose tissue (EWAT), which is the male gonadal fat on top of each testis, was collected and weighed, and daily average of food and calories consumed were calculated. Although the mice fed with SFA, MUFA and PUFA ate less food based on the weight than chow-fed mice [$F(3, 28) = 42.52, P < 0.0001$](Figure 3A), the average daily caloric intake of SFA and MUFA mice were significantly higher than chow and PUFA mice [$F(3, 28) = 30.99, P < 0.0001$] (Figure 3B).

Despite their greater total caloric intake over the four days, the SFA and MUFA mice did not show any significant difference in their body weight gain, at the end of 4 days compared to Chow [$F(3, 28) = 2.60, P = 0.07$] (Figure 3C). In contrast, the SFA and MUFA groups had significantly greater EWAT weights compared to Chow [$F(3, 28) = 5.51, P = 0.004$] (Figure 3D). This increase in adiposity has been reflected to significantly higher plasma leptin levels in SFA and MUFA animals [$F(3, 28) = 15.33, P < 0.0001$] (Figure 3E). In the case of PUFA mice, their daily average food intake (Figure 3A) and calorific intake (Figure 3B) were significantly lower than SFA and MUFA groups. However, there was no significant difference in their body weights. As expected, the EWAT weights (Figure 3C) and
corresponding plasma leptin levels (Figure 3E) of PUFA-fed mice were comparable to those of the chow group, which were significantly lower than SFA and MUFA groups.

**Hepatic Lipid Accumulation:**

Plasma levels of high density lipoprotein cholesterol (HDL) and low density lipoprotein (LDL) cholesterol were measured. The LDL levels of SFA group were significantly higher than Chow and PUFA groups \( [F(3, 28) = 6.43, P = 0.002] \) (Figure 4A). Although the HDL level of the three HFD groups were not different compared to Chow group \( [F(3, 28) = 2.37, P = 0.09] \) (Figure 4B), HDL level of PUFA mice was significantly greater than HDL level of SFA group only \( [t = 2.85, P = 0.01] \). A more useful annotation is the HDL/LDL, called the atherogenic index that denotes possible risk of a cardiovascular incident (Leenen et al., 1993). The SFA group resulted in having a lower HDL/LDL ratio compared to Chow and PUFA mice \( [F(3, 28) = 3.64, P = 0.04] \)(Figure 4C). The increase in LDL levels was found to accompanied with greater TG levels in the liver in SFA as well as MUFA groups \( [F(3, 28) = 21.46, P < 0.0001] \). Liver TG levels of MUFA mice were greater than all three other groups, and the liver TG levels of SFA mice were greater than Chow and PUFA mice, whose liver TG levels were comparable (Figure 4D). To analyze the hepatic lipid environment, blood feeding the liver from the hepatic portal vessel was analyzed for FFA and plasma TG levels. FFA level was highest in MUFA-fed mice, which was significantly greater than that of PUFA mice \( [F(3, 28) = 2.92, P < 0.05] \) (Figure 4E). Interestingly, the circulating plasma TG levels for the SFA and PUFA mice were significantly lower than Chow group \( [F(3, 28) = 4.38, P = 0.01] \) (Figure 4F).

**Expression of genes involved in hepatic lipid oxidation and lipogenesis:**

Expression of genes involved in the regulation of lipogenesis (SREBP-1c, ACC and FAS) and oxidation (PGC1-α) were analyzed. Although SFA- and MUFA-fed groups showed about 50% decreased in FAS expression \( [F(3, 28) = 2.39, P = 0.09] \), only PUFA group reached significance \( [t = 2.16, P < 0.05] \) (Figure 5A). There were no significant differences in SREBP-1c levels among the groups \( [F(3, 28) = 0.12, P = 0.95] \) (Figure 5B). SREBP-1c is major transcription factor for the up-regulation of ACC. Similarly, the ACC mRNA levels were not different among all groups \( [F(3, 28) = 1.17, P = 0.34] \) (Figure 5C). Opposite to the lipogenic
genes, the expression of oxidative gene PGC1-α increased in these HFD-fed mice. MUFA displayed higher levels of PGC1-α levels compared to Chow and PUFA groups \([F_{(3, 28)} = 3.38, P = 0.03]\). Although not significant, it should be noted that SFA mice showed about 100% increase in their PGC1-α expression compared to Chow mice \([t = 1.80, P = 0.09]\) (Figure 5D).

*Circulating glucose level and Hepatic Gluconeogenesis:*

Two hours prior to euthanization, food was removed from cages so that mice were food deprived for two hours and their blood glucose concentrations were measured by the tail clip method. qRT-PCR analysis was run from collected liver samples for PEPCK which is a major enzyme involved in hepatic gluconeogenesis. 2 h fasting glucose levels were not significantly different among all four groups \([F_{(3, 28)} = 2.26, P = 0.10]\), although the SFA and MUFA groups showed about 11% higher glucose levels than Chow and PUFA groups (Figure 6A). PEPCK expression was not different among groups \([F_{(3, 28)} = 0.51, P = 0.68]\) (Figure 6B).

*Insulin Signaling in Metabolic Tissues:*

Sensitivity to insulin signaling was analyzed by measuring the levels of AKT activation through phosphorylation (pAKT) in the major metabolic target tissues for insulin, i.e. liver, soleus muscle and EWAT. Within each group, animals were divided into two subgroups with respect to their final weights (matched body weights on day 4), one group receiving insulin (i.p.) (1 U / kg BW) and the other saline.

In the EWAT tissue, diet had significant effects \([F_{(3, 24)} = 6.24, P = 0.003]\), but insulin/saline treatment did not \([F_{(1, 24)} = 2.24, P = 0.15]\) affect pAKT/tAKT levels. Additionally, there was no interaction between diet and insulin treatment \([F_{(3, 24)} = 1.41, P = 0.26]\). Bonferroni posttest indicated that insulin-injected Chow mice had significantly greater pAKT/tAKT than saline-injected Chow mice \([t = 2.59, P = 0.04]\). However, insulin injection did not change pAKT/tAKT in any of HFD groups \([P > 0.05]\). It’s interesting that although not significant, all three saline-treated HFD groups had slightly elevated pAKT/tAKT baseline levels compared to Chow mice \([P > 0.05]\). When pAKT/tAKT levels of all four insulin-injected groups were compared, there was significant difference in the
levels of pAKT/tAKT from the PUFA group compared to the mice fed with Chow \(t = 3.68, P < 0.01\), SFA \(t = 2.99, P < 0.05\), or MUFA \(t = 3.50, P < 0.01\) (Fig 7A). Although the pAKT/tAKT level of insulin-injected PUFA mice was greatest among four groups, it was not different from the saline-injected PUFA mice, suggesting that the EWAT tissue of PUFA-fed mice was not insulin sensitive.

In the muscle, diet did not \(F(3, 24) = 1.38, P = 0.27\), whereas insulin/saline treatment affected \(F(1, 24) = 6.61, P = 0.017\) pAKT/tAKT levels. Additionally, there was no interaction between diet and insulin treatment for pAKT/tAKT levels \(F(3, 24) = 1.22, P = 0.32\). Insulin-injected Chow mice had significantly greater pAKT/tAKT than saline-injected Chow mice \(t = 2.92, P < 0.05\), whereas insulin injection did not change pAKT/tAKT in any of HFD groups \(P > 0.05\) (Figure 7B). All saline-treated groups had similar baseline levels of pAKT/tAKT. Muscle tissue of insulin-treated MUFA mice had significantly lower level of pAKT/tAKT than muscle of insulin-treated Chow mice.

In the liver, both diet \(F(3, 24) = 5.37, P = 0.0057\) and insulin/saline treatment \(F(1, 24) = 9.07, P = 0.0060\) significantly affected pAKT/tAKT levels, but there was no interaction between diet and insulin treatment \(F(3, 24) = 0.34, P = 0.80\). Saline-treated HFD mice had similar pAKT/tAKT basal levels compared to saline-treated Chow mice \(P > 0.05\). Saline-treated MUFA mice had elevated pAKT/tAKT basal level compared to saline-treated SFA mice \(t = 2.72, P < 0.05\). Insulin-treated MUFA mice had greater pAKT/tAKT levels than insulin-treated Chow mice \(t = 2.58, P < 0.05\). Surprisingly insulin-injection failed to significantly elevate pAKT/tAKT level in the liver tissue of Chow mice \(t = 2.18, P > 0.05\), possibly due to small group size and large variance between the samples from each group. However, insulin significantly increased pAKT/tAKT level in the liver tissue of SFA mice \(t = 3.88, P < 0.01\). The pAKT/tAKT levels of insulin-treated MUFA or PUFA mice were similar compared to those of saline-treated mice on the same diet \(MUFA: t = 1.70; PUFA: t = 0.87; P > 0.05\), indicating that the liver tissues were not insulin sensitive.

Discussion

There have been long term studies, investigating the effects of HFDs with different fatty acids (Woods, D’Alessio et al. 2004). A few recent short term studies have been reported but have addressed specific factors such as inflammation (Yun sok Lee et al.,
2011; Yewei Ji et al., 2012) and metabolomics mapping of progression of insulin resistance (Kleeman et al., 2010). This is the first study to our knowledge that evaluated the effects of different HFDs on metabolic tissues in C57Bl6 male mice on a short term (4 days) feeding regimen.

Since this was a short term study the different groups did not show any changes in their body weights, however the SFA and MUFA groups were observed to have a greater EWAT mass compared to PUFA and Chow groups. Lean tissue mass of adult animals usually does not change within a short-term. Therefore the lean to fat mass ratio or the body fat percentage should be higher in animals fed with the SFA and the MUFA diet compared to chow and PUFA. The SFA and MUFA animals have an increased adiposity and visceral obesity have been related to insulin resistance and metabolic syndrome (Wronska and Kmiec, 2012).

Delany et al. (2000) in their study using carbon isotopes in humans, observed that the amount of dietary fatty acids oxidized is directly related to level of unsaturation and inversely related to chain length. This was done by measuring the labeled CO₂ expired after 4-6 h after diet ingestion. Therefore a diet rich in PUFA tend to be oxidized more readily rather than compartmentalized for storage. Similar results were also observed in rats whose levels of labeled CO₂ measurements were taken after 24 h (Leyton, Drury and Crawford, 1987). Therefore besides caloric intake, difference in fat mass among PUFA, MUFA, and SFA groups could also be attributed to (i) increased energy expenditure or (ii) increased fat utilization/decreased lipid storage by PUFA groups. As a result PUFA animals would accumulate less fat mass compared to SFA and MUFA groups. Increased fat tissue mass could be better addressed using whole body composition analyzer, and alterations in energy expenditure and fuel utilization could be accessed using indirect calorimetry to indicate energy expenditure and respiratory quotient to indicate substrate utilization.

We previously performed a long-term (12-week) HFD (SFA, MUFA and PUFA) feeding study and measured oxygen consumption and carbon dioxide production using indirect calorimetry metabolic chambers (unpublished observation). We observed that PUFA-fed mice had significantly lower body fat mass with similar lean tissue mass; additionally PUFA-fed mice had greater oxygen consumption but similar RQ as MUFA- and SFA-fed mice, indicating that PUFA contributes to energy balance by burning more calories
than SFA and MUFA. We could extend this inference to explain the lower fat pad mass in our current short term studies, but certainly this hypothesis needs to be tested.

An important finding was that the levels of leptin were significantly elevated in the SFA and MUFA compared to PUFA and Chow groups (Figure 3E). Leptin is an adiposity signal peptide hormone produced by adipocytes and secreted at levels proportional to amount of white adipose tissue present in the body. Thus, leptin levels indicate the differences in adiposity among groups.

Kleemann et al. (2010) in their time scaled study of development of insulin resistance, reported that in the span of 7 days of his study, of the metabolic tissues the liver was the one that started developing signs of lipid accumulation and insulin resistance. In this study, liver of MUFA- and PUFA-, but not SFA-, fed mice developed insulin resistance after 4 days of HFD feeding. Additionally, EWAT and soleus muscle of all three HFD groups developed insulin resistance after 4 days of HFD feeding. Lipid analysis was done on blood collected from the hepatic portal vein (HPV), instead from systemic circulation. The HPV bathes the liver in blood lavage from the GI tract and omentum; therefore the liver directly receives the contents of the diet absorbed from the GI tract. Also the portal venous system carries effluents of lipid metabolites from visceral omentum fat which includes mesenteric fat of rodents. Therefore analysis of blood from the HPV would depict a better picture of the effects of different nutritional diets and the metabolic talk between the liver and the visceral fat.

The risk of cardiovascular disease (CVD) is very high in obese individuals. High LDL and low HDL levels are an indicator of such a risk. However, it is the relative values (HDL/LDL), known as atherogenic indices that are medically used in CVD prognosis (Leenen et al., 1993). It was evident that although MUFA had higher levels of LDL, the HDL/LDL ratio was at similar levels compared to Chow group. However within a meager span of 4 days, the mice on SFA displayed lower HDL/LDL ratio signifying higher risk for CVD. In contrast, PUFA had similar HDL/LDL as Chow animals, indicating healthy blood cholesterol levels and no increased CVD risk.

The SFA and MUFA groups displayed elevated levels of plasma FFA compared to PUFA and Chow groups. In both muscle and WAT, FFA are utilized, for energy production in the muscle whereas in the WAT depots these FFAs are re-esterified to TGs for storage.
During exercise or starving, TG is hydrolyzed to FFA by lipoprotein lipase (LPL) to be transported to respective sites for utilization. There have been reports for a “spillover” effect (Evans K et al., 2002), where 2 h after a high-fat meal, some FFAs leak into the plasma to join the plasma FFA pool, during their uptake into the adipocytes. But this might not be the case here, because (i) food was removed from the mice 2 h before HPV blood collection, thus FFA measurement from this study was not due to a “spillover” effect which occurs immediately after a meal. It’s possible that FFA level is affected by lipid metabolism during postprandial conditions between meals; (ii) the animals that were fed with PUFA (a HFD with similar fat content as SFA and MUFA, except for the type of fatty acids) did not show any increase in their levels of FFA, thus characteristics of different lipid may affect lipid metabolism. However Karpe et al. (2011) in a systematic study from 1064 articles with human data suggested that the observed levels of elevated plasma FFA concentrations are not related to the WAT masses. If this is also true in mice, the increase in FFA could not be because these animals had larger WAT depots. One plausible explanation would be that it is known that during postprandial state the levels of FFAs are at their maximum to enable tissue utilization for energy between meals. Where the animals on the PUFA diet were able to maintain their FFA levels at a similar level as the animals on Chow, the SFA and MUFA animals showed elevated levels that led to increased TG accumulation in the liver, as observed in animals that were on the SFA and MUFA diets. This liver TG accumulation would lead to reduced hepatic insulin clearance (Boden G et al., 1994) with defective glucose tolerance (Kruszynska et al., 1997) leading to insulin resistance and a fatty liver.

An interesting finding is that MUFA diets that have been deemed healthy mirrored the same detrimental effects as seen in animals on SFA diets. However we need to keep in mind that these observations are made from a span of 4 days and there could be some compensatory mechanisms in place to check the stress of a high-fat diet. For instance, we did observe that in response to the high-fat content in the liver, the animals on MUFA diet showed significantly increased mRNA expression of PGC1-α compared to SFA as well as PUFA groups (Figure 5D). This indicates that these animals are driving more lipids into β-oxidation as a compensatory mechanism for their elevated liver TG levels.

Although the animals on MUFA diet have compensatory mechanisms, certain results that we observed could possibly lead to an increased risk of developing obesity and type II
diabetes. According to the “glucose-fatty acid cycle” (Fig 8A) proposed by Randle et al. (1963) and reviewed by Louis and Heinrich Taegtmeyer (2009), with an increase in lipolysis during fasted state and with the availability of increased FFAs for oxidation, these FFAs become the fuel of preference instead of glucose, leading to a glucose sparing effect. This was initially shown in muscles (Randle et al., 1963) but later on extended to liver (Berry et al., 1993; Hue et al., 1988) and β-cells of pancreas (Randle, 1998). This stalling of glucose oxidation is through inhibition of pyruvate dehydrogenase (PDH) (Depre et al. 1998; Randle et al., 1964) and phosphofructokinase (PFK) (Depre et al., 1998; Hue and Rider 2007). This leads to the preservation of pyruvate that is a potent gluconeogenic precursor. As shown in Fig 8B, an increase in β-oxidation leads to increase in levels of acetyl Co-A and citrate. Acetyl Co-A has been shown to particularly inhibit glucokinase, specifically in the liver, (Tippett et al., 1982) that regulates glucose uptake (Iynedjian, 2008) and citrate has been observed to allosterically inhibit PFK-2 in liver (Depre et al., 1998).

Although the “Randle cycle” has had opposing views and counter hypothesis to explain the lipid induced insulin resistance (Varman et al., 2010), it holds well for our observations. The mice fed with MUFA have increased liver TG levels and increased expression of PGC1-α to increase β-oxidation of the accumulated lipids. Following the Randle cycle, the current sequence of events will probably lead to accumulation of Acetyl Co-A and citrate, followed by inhibition of PDH and PFK, leading to the glucose sparing effect, i.e. reduced glucose uptake signifying decreased sensitivity to insulin. This is supported by our results that MUFA-fed mice showed trends to increased plasma glucose levels and to increase mRNA levels of PEPCK signifying accumulation of pyruvate (gluconeogenic precursor), and liver insulin resistance.

The animals on SFA diet clearly showed higher risk of cardiovascular complications with higher levels of LDL and lower levels of HDL, consequently a significantly low HDL/LDL ratio. The levels of FFAs were observed to be elevated, but the liver TG accumulation was significantly lower than those animals that were fed the MUFA diet. Although TG accumulation of SFA-fed animals was significantly higher than those fed with PUFA and Chow, the animals fed on SFA did not increase β-oxidation, indicated by similar liver PGC1-α mRNA levels between Chow and SFA groups, which was contrary to the MUFA
group, as a compensation mechanism. Indeed these SFA animals did not show signs of insulin resistance in the liver with increased circulating glucose levels but those mice on MUFA diet showed insulin resistance in the liver together with increased glucose levels. Interestingly although MUFA and PUFA groups developed liver insulin resistance, neither group increased mRNA levels of PEPCK. Considering the Randle cycle discussed above, this developing insulin resistance in the liver does not seem to be as a result of impaired metabolic feedback. A possible mechanism is considering an inflammatory component, which suggests that increased inflammatory cytokines / microphage infiltration due to the fatty liver contributes to liver insulin resistance. Although this premise has been accepted in many reports (Kleemann et al., 2010; Yewei Ji et al., 2012), there have been reports contradicting this hypothesis (Yun Sok Lee et al., 2011). Therefore further experiments are required to address this conclusively.

There are some observations from our data that do require further probing and continuation of our research to enhance understanding. First was the decrease in plasma TG levels with the HFDs. We expected the plasma TG levels in HFD-fed animals to be higher than Chow-fed counterparts. Paradoxically, we observed quite the opposite for SFA and PUFA groups, with those on the PUFA diet with lower levels of plasma TG than Chow and MUFA groups (Figure 4F). These results have been observed in many previous studies, all of which were long term studies (>12 weeks of HFD). We are the first to report this phenomenon in a short term study, therefore implying that this observation is not an artifact of the time scale used in the study. Juen Guo et al., (2009) observed lower plasma TG levels in C57Bl6 mice fed on a SFA for 19 weeks. However, when these mice were reverted to a chow diet after 7 week of HFD, their TG levels rose to levels comparable to the chow group. The authors attributed this change to increase in carbohydrate or fat content in the diet that could either decrease TG production and/or increase TG clearance. Meugnier and colleagues (2007) observed similar phenomenon in humans and reported similar decrease in plasma TG levels in lean men when additional saturated fat was added to their diet. This HFD induced lowering of plasma TG was not strain specific. Sudha et al. (2005) showed that both 129S6/SvEvTac, an obesity resistant mouse strain, as well as C57Bl6, an obesity prone mouse strain showed decreased plasma TG levels compared to their respective chow controls after they have been on SFA HFD for 18 weeks. Although
none of the present literature could provide a solid interpretation, the current hypothesis is that reduced VLDL-TG hepatic secretion leads to the lowered plasma TG levels. Juen Guo et al. hypothesized that this reduction in VLDL levels was the result of hyperinsulinemia, followed by insulin’s energy storage functions, i.e., decrease TG secretion leads to increased TG storage. However, the authors did not take into consideration that long term HFD would render the liver and adipose tissue insulin resistant and therefore any further physiological changes are not due to the actions of insulin. Osterveer et al., (2010) in their 6 weeks HFD study reported that fatty diets could decrease VLDL production; additionally there was also a decrease in the TG levels carried by these VLDL particles. Also they reported a significant decrease in VLDL particle size and particle volume of HFD-fed mice compared to chow-fed counterparts. The percentage ratio of ApoB48 to ApoB100 making up the VLDL particles in the HFD group was reported to be 92%: 8%, signifying that most of these particles are of intestinal origin tying the current observation at hand to the type of diet ingested (Osterveer et al., 2009). With our recent finding that these developments do not need long term HFD feeding and they do appear within four days of HFD, it certainly does shed light on the current outlook of the progression of the disease.

Although PUFA mice had significantly lower FAS expression than Chow mice, it was not different among the HFD groups, and so were SREBP-1 or ACC expressions which were not different among 4 groups (Figure 5). Because with an increase in influx of lipids, i.e. FFAs in the HPV, seen in the animals fed with MUFA (Figure 4E), there would be no need of any de novo lipogenesis required in the tissue. Different from what was observed in mice that fed with HFD for a short-term of 4 days, Oesterveer et al., (2009) observed an increase in expression of hepatic lipogenic genes (SREBP-1, ACC, FAS, and many others) in C57Bl6 mice on HFD for 6 weeks. However through $^{13}$C quantification of lipids the authors conclusively provided evidence that this increase in expression of lipogenic genes did not translate to increase in de novo lipogenesis and hence was not the cause of hepatic lipid accumulation.

**Conclusions**

With the countless years of human existence, obesity seems to haunt the human race relatively of late. Hence environmental issues, particularly the availability of palatable
foods and high energy diets along with unhealthy lifestyles have largely impacted the spread and existence of obesity and ensued metabolic syndrome. Therefore there is always a search for finding alternatives to substitute diets for both reversal and prevention of the different facets of metabolic syndrome. As discussed in the previous chapter there has been a long standing difference of opinions on the acceptability of MUFA diets as a diet alternative.

With the mixed reviews in the literature about the credibility of the MUFA diets (Piers et al. 2002; Schroder et al. 2004), we critically analyzed the sequence of events that are involved in ingesting a MUFA diet. The two hit hypothesis by Day et al. (1998) reviewed by Gentile and Pagliassotti (2008) is the current working model for the development of non-alcoholic fatty liver disease. We, for the first time, provided an idea for the minimum time required (4 days) to instigate that “first hit” that leaves the liver susceptible to further second hits to cause full blown non-alcoholic fatty liver disease.

Finally we observed the metabolically advantageous properties of PUFA diet even from a short term feeding perspective. With the same calorific value as the SFA and MUFA diets, PUFA diets seem to induce more energy expenditure compared to SFA and MUFA. The animals on MUFA or PUFA diet showed higher levels of HDL and lower levels of LDL leading to a relatively higher HDL/LDL ratio compared to HFD with SFA and similar HDL/LDL ratio compared to low-fat standard diet, signifying CVD risk free high-fat diets. The lipid profiles of PUFA via liver TG and plasma FFA were much lower than SFA and MUFA diets denoting lower hepatic lipid accumulation of PUFA mice. Their circulating glucose levels were also at levels comparable to the Chow group.

The short term feeding model that we studied has allowed us to dissect to an extent the mechanisms that are involved in the development of insulin resistance with novel insights. We demonstrated that even from a short span of 4 days, there were pronounced changes in the metabolic profile, particularly in the liver. We provided for the first time evidence that suggests (i) diets rich in MUFA have the potential to be as predisposed to cause obesity and insulin resistance as SFA diets and (ii) SFA and MUFA diets could lead to insulin resistance through different pathways (iii) PUFA diets as in previous reports show better prospects for a healthy alternate, however understanding its molecular mechanisms responsible for its beneficial qualities is essential.
Chapter III

METABOLIC EFFECTS OF SHORT-TERM EXPOSURE OF HIGH-FAT DIETS IN FEMALE MICE

Introduction

Most previous metabolic studies involving obesity and type II diabetes have been done using male subjects. During the last few decades, estrogens have been investigated for their roles besides reproductive and sexual development, particularly their role in regulation of metabolism. Characteristic metabolic changes have been observed in postmenopausal women compared to premenopausal women such as energy expenditure with accompanied increased total body fat and body mass (Lovejoy et al., 2008). These changes have been duplicated in rodent models such as ovariectomized (OVX) mice (Carr, 2003), specific estrogen receptor (ER) knockouts (Barros et al., 2009), and aromatase knockout mice (Jones et al., 2000). To summarize their findings, estradiol (E2), the physiologically active form of estrogen, has been shown to play a role in glucose utilization, lipid metabolism, and insulin sensitivity (Shi et al, 2013). Obesity and insulin resistant models were reversed by the administration of exogenous E2 (Shi and Clegg, 2009). More focus was shifted to the specific interactions of E2 and its receptors, starting with the mapping of the nuclear receptors ERα and ERβ (Dahlman-Wright et al., 2006). These receptors are present both centrally as well as in the peripheral tissues. In the brain, ERs have been shown to localize in the hypothalamus, particularly in the ventromedial hypothalamus (VMH), arcuate nucleus (ARC), as well as paraventricular nucleus of hypothalamus (PVN), where they have shown to control food intake (Butera, 2009). Peripheral ERs have been shown to be present in the liver, adipose tissue, muscle, and pancreas (Bryzgalova et al., 2008). The different functions of the two ER isoforms are beyond the scope of this thesis.

In this study, we investigated mice as whole, rather than specific functions of E2 or any other estrogen. There has always been a need for a proper treatment regimen for obesity and diabetes type II, and specific treatment paradigms for males and females have been long considered. In order to do so, there arises a need for understanding the
progression of the disease in females as well. There are already differences, i.e sexual dimorphisms in the metabolic characteristics, between males and females. For instance it has been observed that female mice are less susceptible to insulin resistance induced by an increase in FFA in the peripheral tissues (Frias et al., 2001). Many such differences have been observed in glucose tolerance, lipid oxidation and lipogenesis, β-cell function and insulin resistance (Shi and S.P.D Senthil Kumar, 2012). Additionally, there are not many studies that have elaborated the effects of MUFA and PUFA on female mice. The major directive has been on male mice, and with the above mentioned reasons it is imperative that these experiments carried out with male mice with the different diets should be reproduced using female mice.

Therefore in this study I replicated the same experiments as in Chapter II using female mice. The same short term feeding paradigm and different diets (SFA, MUFA and PUFA) were used. From existing literature, we hypothesized that female mice were much more resistant to the detrimental effects of a HFD than male mice. This study would provide important information on how high-fat diets with different fatty acids affect metabolism in males and females.

**Methods**

*Animals:*

10-12 week old adult female C57Bl/6 mice were maintained on standard 12-h light-dark cycle (lights out at 1700 h). The animals were fed with either a standard low-fat diet (Chow) or one of three high-fat diets (HFD) with matched macro- and micro-nutrient contents for 4 days (short-term). Respective diets and water provided *ad libitum* unless otherwise mentioned. All procedures were approved by the Institutional Animal Care and Use Committee of Miami University Ohio and were in strict accordance with the Guide for the Care and Use of Laboratory Animals.

*Experimental Design:*

36 female C57Bl6 mice were used with 9 animals allotted to each diet group of Chow, SFA, MUFA or PUFA and were fed with specific diet for 4 days. Fresh food was given each day to the animals. Their daily food intake and body weights were measured.
Unlike male mice, we have to take into account the female estrous cycle. Prior to classification into the different diets, the mice were analyzed for their estrous stage through vaginal cytology samples. Those animals that displayed regular estrous cycles were then selected and separated into different diet groups as well as matched for their body weights. The diet regime was started on the day of the estrous stage and after 4 days of respective diet administration, the animals were sacrificed on the day of the estrous phase with an isofluorane overdose. Blood from the hepatic portal vein was collected, centrifuged to separate plasma which was stored at -20°C for lipid profiling and leptin measurement. The liver, soleus muscle, and gonadal parametrial/ovarian white adipose tissue (PWAT), an analogous fat depot for male gonadal epididymal fat, were collected and immediately frozen in isobutene cooled by dry ice. The frozen tissues were stored at -80°C for protein extraction western blotting for pAkt and total Akt, liver TG extraction, and total RNA isolation for qPCR.

**Diets:**

The energy content of the LFD was 3.00 kcal/g and 14% calories from fat, whereas that of all three HFD enriched in different sources of dietary fat, lard oil (SFA), olive oil (MUFA), and fish oil (menhaden oil PUFA) was 4.73 kcal/g and 45% calories from fat. All HFD are formulated by Research Diets, Inc. (New Brunswick, NJ). The fatty acid in the olive oil diet was mainly the MUFA oleic acid (18:1, N-9), in the menhaden oil diet was mainly the N-3 PUFA (EPA, 20:5; docosahexaenoic acid, 22:6). For all three HFD, the protein content was 20%, and was composed of casein and L-cystine (in the ratio of 1: 0.015, by weight) and the carbohydrate content was 35%, and was composed of corn starch, maltodextrin 10, sucrose, and cellulose (in the ratio of 1: 1.4: 2.4: 0.7, by weight). HFD also contained 5.2% minerals and 1.4% vitamins, and 2.9% soybean oil to ease compounding.

**Determining Stages of the Ovarian Cycle:**

Prior to start of the diet regime, the animals were tracked by their estrous cycle. This was carried out by taking vaginal cytological samples on a daily basis. We used a 3-step staining process using Dip Quick Stain (Jorvet J-322®, Jorgensen Laboratories, Inc.) and observed the cells under a microscope. The proestrous phase is characterized by round
epithelial cells with well-defined nuclei, whereas in the estrous phase these round cells metamorphose to cornified cells. The next stage, metestrous stage can be identified by a mixture of round and cornified epithelial cells with a slight infiltration of leucocytes. Finally in the diestrous stage there is an abundance of leucocytes.

**Analytical Methods:**

Animals were overdosed with isofluorane and blood samples were collected from hepatic portal vein in heparin coated tubes. Blood samples were centrifuged and stored at -20°C until further assays was performed. Consequently liver, muscle and PWAT were collected in 2 ml tubes separately, frozen using isobutane and dry ice, and stored -80 °C.

Plasma concentrations of FFA, TG, HDL and LDL were measured using specific kits (Wako diagnostics, Richmond VA). Leptin levels were measure by ultrasensitive ELISA kits provided by CrystalChem Inc. (Downers Grove, IL)

**Liver TG Measurement:**

Liver TG extraction was adapted from the methods described by Storlien et al. (1991) and Maycock et al. (1980). In short, about 50mg of liver tissue (weight of tissue recorded) was lysed in 1 ml of 2:1 (v/v) mixture of chloroform and methanol. After 2 hours of gentle shaking, 0.3 ml of water is added to the lysed mixture for phase separation. The samples are then centrifuged at room temperature for 35 min at 2000 rpm. The top two layers (cell debris) were discarded and the bottom organic layer (containing dissolved lipids) was transferred to a new tube. The new tubes were left open overnight to allow evaporation of the organic layer.

Next day, the solute present in tubes after evaporation were redissolved in 225 µl of chloroform and mixed thoroughly to ensure complete mixing. Triplicates were prepared using the chloroform mixture in any anti-chloroform corrosive plates (we used standard PCR plates) along with the standards (Multi Calibrator lipids: TG = 107 mg/dl, Wako Chemicals, Richmond VA). The contents of the plate were evaporated in an oven at 65 °C. 25 µl of 0.9% NaCl was added, followed by 200 µl of TG or cholesterol enzyme reagent to each well. The contents of well were mixed well and transferred to an ELISA plate and the absorbance was read at 600 nm.
Liver gene expression (quantitative RT-PCR):

Liver samples were homogenized in TRI reagent (MRC, Cincinnati, OH) with consequent addition of BAN (MRC, Cincinnati, OH) and centrifuged at 12,000 g for 15 mins. RNA was extracted from the clear supernatant layer using RNeasy mini kit (Qiagen). cDNA was subsequently synthesized using the iScript kit (Bio-Rad, Hercules, CA). Quantitative PCR was performed for the following genes whose primers have been specified in Table 1 from Chapter 2.

The Genes of interest are those implicated in hepatic lipogenesis - Sterol-regulatory binding protein (SREBP)-1c, a pivotal regulator of expression of lipogenic enzyme fatty acid synthase (FAS), ACC, in hepatic mitochondrial β-oxidation of fatty acids - Peroxisome proliferator-activated receptor gamma coactivator 1-α (PGC1α) which is involved in the activity of mitochondrial β-oxidation of fatty acids, in hepatic gluconeogenesis: Insulin suppresses hepatic glucose production and gluconeogenesis by inhibiting gluconeogenic enzyme phosphoenol pyruvate carboxykinase (PEPCK).

GAPDH was used as the reference gene (F: 5'-TGCGACTTCAACAGCAACTC- 3', R: 5'–GCCTCTCTTGCTCAGTGTCC- 3'). Quantitative RT-PCR was run with a Bio-Rad iCycler (Bio-Rad, Hercules, CA) with iQ SYBR Green Supermix (Bio-Rad). The set program was 2 step cycle - amplification (95 °C for 10s) and annealing (58 °C for 30 s) for a total of 40 cycles. The amplified products were confirmed through gel electrophoresis and melt curve analysis. The samples were run in triplicates and the PCR efficiencies were calculated from the slope of the threshold cycle (C_T). The relative fold changes between the target and reference gene were calculated using the Delta-Delta CT method. In this approximation method the average difference of the C_T between GAPDH and the target gene (ΔC_T) of the control is subtracted from each ΔC_T of each sample (ΔΔC_T) from the experimental group to obtain the relative fold change.

Western Blotting:

Liver, soleus muscle, and female gonadal fat PWAT located around oviduct between uterus and ovaries were collected 15 min after saline or insulin (1 U/kg) injection. Total protein was extracted by homogenizing tissues using RIPA lysis buffer (Santa Cruz
Biotechnology; kit includes PMSF, Sodium orthovanadate, Protease inhibitor) and phosphatase inhibitor cocktail (Sigma). The homogenate was centrifuged at 10,000g with repeated transfer of supernatant to purify the supernatant of cell debris. The final supernatant was collected and stored at -80°C until use. Protein concentration will be measured using the standard BCA assay (Quick Start™ Bradford protein assay).

Western blots were run on precast 10 or 15 well gels (Mini-PROTEAN® TGX™, Bio-Rad). The samples were prepared to be loaded using the Laemmlı method. The following buffers were used: running buffer (TGS) (25 mM Tris, 192 mM glycine and 0.1% SDS (w/v)), transfer(blotting) buffer (20% v/v methanol in 1X TGS), wash buffer (TGST) (10 mM Tris, 100 mM NaCl, 0.1% Tween 20), 10% milk blocking buffer (in 1X TGST). Primary antibodies are rabbit monoclonal antibodies (pAKt, pMAPK, tAKt and tMAPK) and secondary antibodies are goat anti-rabbit IgG HRP-linked (Cell Signalling Technologies®). Antibodies were used as 5% milk buffer preparations.

The samples were run at 120 V for 5 min and at 180 V for 1 h. The gels were then separated from the cassettes to be transferred onto a nitrocellulose membrane, Trans-Blot® transfer membrane (Bio-Rad) at 80 V for 1.5 h. The transferred membrane is blocked with 10% milk for 1 h. Then the membrane was probed with the primary antibody with overnight incubation at 4°C. The primary antibody was then removed and membrane washed with wash buffer for about 4-5 times with 10 min each time. The membrane was then incubated with the secondary antibody at room temperature for 2 h followed by 4-5 washes. Finally the membrane was incubated with Amersham™ ECL™ Prime (GE Healthcare) for substrate detection. The membrane was placed in an autoradiography cassette along with a X-ray film (GeneMate, Bioexpress) and the film was developed in a dark room to visualize the bands. The densities of the bands in the films were quantified using the ImageQuant TL software (Amersham Biosciences) for 1D electrophoresis gel analysis.

Statistical analysis:
Data analysis was performed using Prism Statistical Software 5 (La Jolla, CA). Data were expressed as mean ± SEM. A one-way ANOVA followed by Tukey’s multiple comparison tests was used to analyze body weight, caloric intake, plasma and liver lipid
profile, plasma glucose level, and hepatic gene expression among four dietary groups. A two-way ANOVA followed by Bonferroni posttest was used to analyze insulin signaling by comparing dietary factor and treatment factor (insulin or saline injections). A test with P value less than 0.05 (i.e. \( P < 0.05 \)) was considered statistically significant. Post hoc multiple comparison tests were carried out with computing the 95% confidence interval (CI) of the difference between two group means and the test was considered statistically significant if this CI did not include zero.

**Results**

**Body weight, Food intake and Adiposity:**

Mice were divided into four groups receiving Chow (standard chow), SFA, MUFA, or PUFA diet, with their diets starting on their estrous phase of the ovarian cycles. Body weights and food intake were measured every day for four days. The average daily food intake of mice receiving HFDs was significantly lower than the SFA group \( [F_{(3,32)} = 37.59, P < 0.0001] \) (Figure 8A); however when the average calorific intake of all the groups were compared, only the SFA group consumed more calories than chow and PUFA groups \( [F_{(3,32)} = 4.00, P = 0.016] \) (Figure 8B). Although there were differences in the food and energy intake, the body weights at the end of four days were similar amongst all the groups \( [F_{(3,32)} = 0.18, P = 0.91] \) (Figure 8C). Following the above observations, PWAT weights at the end of four days were similar \( [F_{(3,32)} = 2.53, P = 0.07] \) (Figure 8D) and hence so were their plasma leptin levels \( [F_{(3,32)} = 2.17, P = 0.11] \) (Figure 8E).

**Hepatic and Plasma Lipid Analysis:**

Prior to sacrificing the animals, blood was collected from the hepatic portal vein, and plasma was saved for measuring plasma HDL, LDL, FFA and TG. Plasma HDL levels were not significantly different among the groups \( [F_{(3,32)} = 2.16, P = 0.11] \) (Figure 9A), however the LDL levels of SFA and MUFA groups were significantly higher than PUFA and chow groups \( [F_{(3,32)} = 9.39, P = 0.0001] \) (Figure 9B). Interestingly, when calculated for the HDL/LDL values, only MUFA mice showed significant lowered HDL/LDL values compared to chow and PUFA groups \( [F_{(3,32)} = 3.69, P = 0.02] \) (Figure 9C). Even though there were differences in the cholesterol levels, the FFA levels in plasma for the HFD groups were the
same as the chow-fed mice \[F_{(3,32)} = 0.48, P = 0.70\] (Figure 9D). Correspondingly, with the levels of FFA fed to the liver being similar in all groups, so were the liver TG levels, which showed no significant difference amongst all the groups \[F_{(3,32)} = 1.78, P = 0.17\] (Figure 9E). As seen with male mice, the plasma TG levels of HFD groups were lower than chow females, with PUFA group being significantly lower \[F_{(3,32)} = 3.76, P = 0.02\] (Figure 9F).

Expression of Genes Involved in Hepatic Lipid Metabolism:

As performed for male mice, liver expression levels of SREBP-1c, ACC, FAS and PGC1-α were measured through qPCR analysis. GAPDH was used as the reference gene. The results were plotted taking the chow group as 100% and the HFD groups were plotted in relation to the chow group.

There were no significant differences in the FAS levels between the HFD groups and the chow animals except for the PUFA animals, whose expression levels were significantly lower compared to chow as well as the other HFD fed animals \[F_{(3,32)} = 3.01, P = 0.04;\] chow vs. PUFA: \(q = 3.98, P = 0.05\] (Figure 10A). MUFA fed animals displayed higher expression levels of SREBP-1c compared to the chow group \[F_{(3,32)} = 3.47, P = 0.03;\] chow vs. MUFA: \(q = 4.37, P < 0.05\] (Figure 10B), as well as higher expression ACC levels compared to the PUFA group \[F_{(3,32)} = 3.85, P = 0.019;\] MUFA vs. PUFA: \(q = 4.77, P < 0.05\] (Figure 10C). When analyzed for PGC1-α, a gene involved in lipid oxidation, there were no significant differences between the groups \[F_{(3,32)} = 0.58, P = 0.64\] (Figure 10D).

Hepatic Gluconeogenesis and Insulin Resistance:

Food was removed from animals for 2 hours on the day of sacrifice and their circulating glucose concentration was measured using the tail clip method. Gene expression levels for PEPCK were measured from the frozen liver samples using qPCR. The glucose levels of PUFA mice were significantly higher than chow mice, whereas SFA and MUFA mice had comparable glucose levels as chow mice \[F_{(3,32)} = 5.08, P = 0.0055;\] chow vs. PUFA: \(q = 5.05, P < 0.05\] (Figure 11A). Hepatic PEPCK mRNA levels of HFD groups were comparable to the chow animals \[F_{(3,32)} = 0.74, P = 0.53\] (Figure 11B).
**Insulin Signaling in Metabolic Tissues:**

Each diet group were subdivided into two groups with matched body weights, one received insulin (i.p) and the other saline. Mice were sacrificed 15 minutes after injections. pAKT and tAKT levels were measured through western blotting of proteins extracted from PWAT, muscle, and liver. The ratio pAKT/tAKT provides information of the insulin signaling in these respective tissues.

In the PWAT, saline-treated SFA mice had elevated pAKT/tAKT basal level compared to saline-treated chow [t = 3.61, P < 0.01] and MUFA [t = 2.84, P < 0.05] mice. Thus, the PWAT of SFA female mice seemed to be already activated by endogenous insulin compared to other groups (Figure 12A). Under insulin stimulated condition, insulin-treated SFA mice had greatest pAKT/tAKT levels compared all other groups [chow vs. SFA: t = 2.82, P < 0.05; MUFA vs. SFA: t = 4.32, P < 0.001; PUFA vs. SFA: t = 4.96, P < 0.001]. Importantly, the pAKT/tAKT levels of insulin-treated mice of all four groups were significantly higher compared to those of saline-treated mice on the same diet [Chow: t = 6.26, P < 0.001; SFA: t = 5.47, P < 0.001; MUFA: t = 3.99, P < 0.01; PUFA: t = 2.37; P < 0.05], indicating that the PWAT tissues of all groups were insulin sensitive following four-day feeding. Both diet [F(3, 24) = 11.65, P < 0.0001] and insulin/saline treatment [F(1, 24) = 81.71, P < 0.0001] significantly affected pAKT/tAKT levels. The interaction between diet and insulin treatment just missed statistical significance [F(3, 24) = 2.95, P = 0.05] (Figure 12A).

In the muscle tissues, although the pAKT/tAKT baseline levels of saline-injected mice were comparable among all four groups, the insulin-injected MUFA mice had significantly greater levels of pAKT/tAKT than insulin-treated mice that were fed with SFA [t = 3.55, P <0.01] or PUFA [t = 3.11, P <0.01]. Insulin injection significantly increased pAKT/tAKT levels of mice fed with Chow [t = 2.73, P <0.05], MUFA [t = 3.20, P <0.05], or PUFA [t = 3.51, P <0.05], but not in the SFA mice [t = 1.50, P >0.05], compared to pAKT/tAKT baseline levels of saline-injected mice fed with the same diet (Figure 12B), suggesting that the insulin signaling of muscle tissue of SFA mice were not activated by insulin, whereas muscle tissues of mice fed with other diets were insulin sensitive. Both diet [F(3, 24) = 5.89, P = 0.004] and saline/insulin treatment [F(1, 24) = 20.88, P = 0.0001] had significant effect. However, there was no interaction between diet and insulin treatment [F(3, 24) = 0.66, P = 0.58].
In the liver tissue, saline/insulin injection had significant effects \( F_{(1, 24)} = 43.74, P < 0.0001 \), but diet did not affect pAKT/tAKT levels \( F_{(3, 24)} = 0.88, P = 0.47 \). There was no interaction between diet and insulin treatment \( F_{(3, 24)} = 0.50, P = 0.69 \). All groups from saline-injected mice had similar baseline levels of pAKT/tAKT. Insulin-injected mice from all four groups, Chow \([t = 3.23, P < 0.05] \), SFA \([t = 2.69, P < 0.05] \), MUFA \([t = 3.90, P < 0.01] \), and PUFA \([t = 3.76, P < 0.01] \), had significantly greater pAKT/tAKT than saline-injected mice with the same diet, indicating that liver of all groups were insulin sensitive (Figure 12C).

To summarize, except muscle tissue from SFA female mice, all tissues maintained insulin sensitivity, displaying higher pAKT/tAKT levels when injected with insulin. However, muscle tissues of SFA female mice did not have activated insulin signaling after insulin stimulation, indicating development of insulin resistance.

**Discussion**

Sexual differences in the susceptibility and development of various diseases have been observed, and although no conclusive cause has been identified, many of these have been attributed to the extra-reproductive roles of estrogen (Pettersson et al., 2012). With respect to metabolic diseases such as obesity, insulin resistance, type 2 diabetes and lipid disease, estrogens have been suggested to play a protective role with a huge amount of supporting data in rodents and humans. Prevalence of these diseases have been shown to be more incident in post-menopausal women compared to ovulating women (Shi et al., 2009) and in men age matched with pre-menopausal women (Krotkiewski et al., 1983). In a relatively recent review, the sexual bias in studies (basic and translational) with a comprehensive database search, where males are preferred was brought to light (Lisa, 2011). With mounting evidence that physiological and morphological responses are sexually dimorphic, it is imperative that research designs should include data from both sexes. Therefore we have extended the research protocol for the male mice to female mice.

Estrogens act through its receptors, particularly ERα, which has been attributed to increase energy expenditure and reduce food intake (Musatov et al., 2007). We observed similar findings in our short term study as well. As observed in our male mice, high-fat diet-fed males increased adiposity and circulating leptin levels with no change in their body
weights between the groups; the female mice, on the other hand, restricted their food intake to match their calorific needs. As a result, fat pad mass and circulating leptin levels were not different among four female groups (Figure 8). Estrogens have also been found to control body composition, particularly adiposity. Visceral fat deposition has been deemed a high risk factor for developing obesity and cardiovascular diseases (Lee et al., 2010; Kannel et al., 1991), whereas subcutaneous fat has been associated with elevated HDL and lower LDL (Snijder et al, 2005) and lower propensity for macrophage infiltration and inflammation (Cancell et al., 2006). Hence animals with more subcutaneous fat have been observed to be better protected against cardiovascular disease and diabetes. Estrogens favor accumulation of subcutaneous fat rather than visceral fat (Brown and Clegg, 2010), which is consistent with what we observed in the female mice. There was no significant change in the PWAT weights between the chow and HFD groups. Another mechanism by which estrogen has been postulated to decrease body weight is through interaction of anorexigenic neuropeptides such as insulin, leptin, serotonin or cholecystokinin (Brown and Clegg, 2010). In particular, leptin is perceived differently by males and females. E2 administration restores leptin sensitivity to OVX females and in male rats with redistribution of fat to subcutaneous depots (Clegg et al., 2006). In our short term study, females on HFD did not show any significant differences in the leptin levels compared to the chow group. Thus those females on SFA or MUFA diets seemed to be more leptin sensitive maintaining a balance between adiposity as well as caloric intake, compared to males.

Blood lipid and the hepatic lipid analysis, including those from our gene expression studies, did yield some interesting results. Estrogens being lipid soluble form esters within lipoproteins to form lipophilic conjugates (Meng et al, 1999). Although physiological roles for these estrogenic lipoproteins have not been established, some speculations provided were (i) these esterified estrogens (EE) could offer oxidative protection to the lipoproteins (Shwaery GT et al., 1997); (ii) allows estrogens to be metabolized slowly, thereby setting up hormonal reserves, particularly in lipoproteins and fat, improving activity as well as bioavailability (Tikkanen et al., 2001). In our previous chapter we discussed how the nature of the lipoproteins depend on the type of fatty acid ingested, and hence we believe
that since we have fed mice different diets rich in a particular type of fatty acid it should not be a surprise if we observed different results for each diet.

As expected, females were more resistant to the deleterious effects of HFD, particularly the SFA diet. Compared to the males using the same study design, the females on SFA diet showed comparable levels of plasma FFA measured from the hepatic portal vein, following this, there were no signs of lipid accumulation in the liver (Figure 9). This was the case in the animals fed with MUFA and PUFA too. This is clearly not the case in males fed with SFA and MUFA (Figure 4). Estrogens have been observed to affect meal size and duration, to maintain energy homeostasis, through interactions with orexigenic and anorexigenic peptides (Santollo et al., 2008; Messina et al., 2006). In the 4 days of our study we did see decreases in the amount of food eaten in the HFD groups compared to the Chow group in both the sexes. However in males this decrease in food amount was not enough to reduce amount of calories and levels of circulating FFA and Liver TG levels, although the female mice were able to do so. Gene expression of de novo lipogenic genes ACC, SREBP-1 and FAS of SFA females were not significantly different compared to Chow groups, and interestingly genes involved in β-oxidation, PGC-1α were also not expressed more. Therefore the extra lipids were not being metabolized more efficiently as one would have hypothesized. But again, we looked at gene expression only in the liver, since we did not observe any lipid accumulation in the liver this could be the reason why we did not see any significant changes in the gene expression studies. The lipoprotein profiles of female mice were not that different compared to males. Even though the LDL levels of SFA- and MUFA-fed females were higher than Chow-fed females, SFA-fed females matched their levels of HDL to maintain a comparable HDL/LDL ratio compared to Chow mice. In contrast, MUFA females had a significantly lower HDL/LDL ratio compared to Chow mice (Figure 9) which could be a cardiovascular risk factor in females.

Next, when we tested insulin sensitivity of the tissues, PWAT of all HFD fed females maintained insulin sensitive. In contrast, the muscle tissues of SFA female mice were not insulin sensitive, whereas those fed with MUFA or PUFA were. This was evident from our western blotting studies where animals that treated with insulin were able to increase their pAKT/tAKT levels 15 min after insulin treatments (Figure 12). We observed that PWAT tissues of male and female mice on SFA diets had elevated baseline levels of
pAKT/tAKT compared to chow-fed same sex counterparts, whereas this elevated baseline level of pAKT/tAKT was not evident in either muscle or liver tissues (Figure 7 and 12).

An important point to note is that we used pAKT/tAKT as a measure of insulin insensitivity, however recently discovered membrane bound estrogen receptors that are responsible for the “rapid” actions of estrogen have been found to act through the same kinases as insulin such as Akt, MAPK, etc. (Prossnitz et al., 2008, 2009). The increase in the levels of pAkt could also be due to the endogenous E2 in females. As previously mentioned, esterified estrogens have been studied to form slow releasing depots in fat tissues (Tikkanen et al., 2001), which could be the reason why we see the higher pAkt/tAkt levels and particularly in animals with the SFA diet. Nevertheless, the improved sensitivity to insulin is definitely beneficial. From our previous discussion on Randle’s cycle, fuel selection between glucose and fatty acid plays an important role in maintaining energy as well as nutritional homeostasis (Hue and Taegtmeyer, 2009). It is evident that white fat tissue being more sensitive allows for more glucose uptake and utilization rather than FFAs. This could be the reason why females fail to store fat in the visceral fat pads and could be the reason why there was no significant difference in the PWAT weights between Chow and HFD groups, in contrast to their male counterparts. This could be confirmed by analyzing mass of subcutaneous fat pads from a long-term study. Also the plasma glucose levels of the animals from each HFD group were comparable to mice that were fed Chow, except female PUFA group, with PEPCK expression, a gluconeogenic enzyme, showing no significant differences among the groups, which reiterate well-maintained insulin sensitivity in females with HFD.

Some of the similarities between males and females that we observed were with respect to the PUFA and MUFA group. PUFA was capable of suppressing expression of de-novo lipogenic gene FAS in both males and females, whereas MUFA was capable of inducing expression of de-novo lipogenic SREBP-1 only in females (Figure 10). Although these data of up-regulation of de-novo lipogenic gene during high-fat diet feeding seemed counter-intuitive, the MUFA females had similar liver TG as Chow females (Figure 9), thus the availability of lipid was not actually increased. However these observations were at the mRNA expression level, actual protein levels would paint a better picture. In contrast to females, SFA and MUFA male mice had higher liver TG (Figure 4), thus high lipid
availability in the liver, and these de novo lipogenic genes were not up-regulated (Figure 5). Some previous reports using male mice reported increased de novo lipogenic gene expressions by high-fat diet feeding (Biddinger et al., 2005; Kajikawa et al., 2009), however to our knowledge, such observations have not yet been reported in female mice. We also noticed the same decrease in plasma TG as in the males and we have discussed the possible reasons. Again, there was no such previous finding in female mice in literature search.

**Conclusions**

Estrogens are actively studied for their extra reproductive actions, particularly in the metabolic forefront. Advancements have been made in understanding the nature of its mechanism of action and its signal transduction pathways. Work is still underway to comprehend its interactions with other hormones and peptides involved in food intake and energy expenditure, as well as lipid metabolism and insulin sensitivity. Therefore it is vital that we recognize the sexual differences, if any, which I have done in this particular chapter.

As we hypothesized, females were well protected against HFD-induced metabolic diseases. We observed that female mice on SFA diets, were able to restrict their calorific intake as well as maintain lipid levels as same as the animals on a low-fat standard diet. Interestingly, the fat pads that we analyzed (gonadal PWAT) were not heavier as seen in male mice, there was no increase in plasma FFA flux into the liver, and following this there was no TG accumulation in the liver. One possible reason to account for this could be the lipid lavage to the subcutaneous depots. Estrogens have been attributed to direct lipids (including “spill over” lipids) to be stored in the subcutaneous area. As discussed in the previous section having more subcutaneous fat compared to visceral is very advantageous. Another reason could be efficient use of lipids through increased energy expenditure. Again estrogen has been credited to increase energy expenditure and decrease adiposity. The only way for us to test this hypothesis is to measure metabolic rate. We were not able to do so because of the difficulties we previously addressed. However, these laid out hypotheses need to be further tested.

We did not observe any signs of fatty liver and as a result these tissues were insulin sensitive. Drawing from our previous chapter, the “two hit hypothesis”, the female mice
were not susceptible to the first hit, at least in four days as the male mice were. The target organ “liver” was safe from fatty influx and hence suffered no “hits”. This may be the reason why we saw a higher expression of de novo lipogenic genes in the MUFA diet, but still does not explain why we did not see them in the SFA diets.

The PUFA diets being very advantageous in males, was also in females. They mirrored the Chow groups in their metabolic profiles as well as providing the mice with high calorie diet. In fact, the mice fed with PUFA had an increase in pAKT signaling strength in all the metabolic tissues. This could be because of, as previously discussed, the involvement of esterified estrogens which is one area that certainly needs to be probed further. I would also like to analyze subcutaneous fat depots to compare between subcutaneous and the visceral fat tissues, because as far as we know there have been no previous reports for a short term feeding regimen.
FIGURE 1
**Figure 1:** Body mass, body composition, and epididymal white adipose tissue (EWAT) weight measurements of male CL57B6 mice fed with the different diets for 15 weeks. Body mass (g) at week 15 (A), weekly body mass (B), lean body mass (C) and their EWAT masses (D) at the end of week 15 have been represented as means ± SEM. * P<0.05 comparing to Chow; ^P<0.05 comparing to MUFA; # P<0.05 comparing to PUFA.
FIGURE 2
Figure 2: Lipid profiles of mice under the long term study (15 weeks). Plasma HDL/LDL ratio (A), Circulating levels of plasma TG (B) and liver TG levels (C). Given are the means ± SEM. * P<0.05 comparing to Chow; # P<0.05 comparing to PUFA.
FIGURE 3
**Figure 3:** Average food intake (A), caloric intake (B), final day body weight (C), EWAT weights (D), and plasma leptin levels (E) measured in male C57Bl6 mice fed with the different diets (shown in the legend). Given are the means ± SEM (n=8). *P<0.05 when comparing to Chow group; #P<0.05 comparing to PUFA.
FIGURE 4
**Figure 4:** Lipid analysis of blood plasma from the hepatic portal vein. HDL (A), LDL (B), FFA (E) and TG (F) were measured through ELISA assays. Figure C represents the HDL/LDL ratio compared between the different groups. Liver lipid content (TG) was quantified (F). Given are the means ± SEM (n=8). *P< 0.05 comparing to Chow; #P<0.05 comparing to PUFA; ^P<0.05 comparing to MUFA.
FIGURE 5
Figure 5: Relative fold changes (PCR efficiencies) of FAS (A), SREBP-1 (B), ACC (C) and PGC1-α (D) compared to Chow group (100%). Given are the means ± SEM. *P<0.05 comparing to Chow; ^P<0.05 comparing to MUFA; #P <0.05 comparing to PUFA
FIGURE 6

A

B

Blood Glucose (mg/dL)

PEPCK mRNA Level (Relative to chow group)
**Figure 6:** Fasting blood glucose levels (mg/dl) following 2 h fasting (A). Relative fold changes (PCR efficiencies) of PEPCK (B) compared to Chow group (100%). Given are the means ± SEM.
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**FIGURE 7**
**Figure 7:** Analysis of insulin sensitivity through activation of pAKT. The western images for both pAKT and tAKT have been shown in (D) in the following order: EWAT, Muscle and Liver. The quantification for each image was done and the ratio (pAKT/tAKT) was graphed to assess insulin signaling in EWAT (A), muscle (B) and the liver (C). Given are the means ± SEM (n=4). † P<0.05 comparing to same diet saline-injected groups. *P<0.05 comparing to chow, ‡ P<0.05 comparing to SFA, ^P<0.05 comparing to MUFA.
FIGURE 8

A. Food Intake (g/day)

B. Energy Intake (kCal/day)

C. Body Mass (g)

D. PWAT Mass (g)

E. Leptin (ng/ml)
**Figure 8:** Average food intake (A), caloric intake (B), final day body weight (C), EWAT weights (D) and plasma leptin levels (E) measured in female C57Bl6 mice fed with the different diets (shown in the legend). Given are the means ± SEM (n=9). *P<0.05 when comparing to chow group; #P<0.05 comparing to PUFA.
**FIGURE 9**

A. Plasma HDL (mg/dL)

B. Plasma LDL (mg/dL)

C. HDL/LDL (AU)

D. Plasma FFA (mmol/L)

E. Liver TG (µg TG/mg)

F. Plasma TG (mg/dL)
**Figure 9:** Lipid analysis of blood plasma from the hepatic portal vein. HDL (A), LDL (B), FFA (D) and TG (F) were measured through ELISA assays. Figure C represents the HDL/LDL ratio compared between the different groups. Liver lipid content (TG) was quantified (E). Given are the means ± SEM (n=9). *P< 0.05 comparing to chow; #P<0.05 comparing to PUFA.
FIGURE 10
Figure 10: Relative fold changes (PCR efficiencies) of FAS (A), SREBP-1 (B), ACC (C) and PGC1-α (D) compared to Chow group (100%). Given are the means ± SEM. *P<0.05 comparing to Chow; #P <0.05 comparing to PUFA
**FIGURE 11**

**A**

![Bar graph showing Blood Glucose (mg/dL) for Chow, SFA, MUFA, and PUFA groups. The graph indicates that PUFA has the highest blood glucose levels compared to the other groups.](image)

**B**

![Bar graph showing PEPCK mRNA Level (Relative to chow group) for Chow, SFA, MUFA, and PUFA groups. The graph shows that PUFA has a lower PEPCK mRNA level compared to the other groups.](image)
Figure 11: Fasting blood glucose levels (mg/dl) following 2 h fasting (A). Relative fold changes of PEPCK expression (B) compared to chow group (100%). Given are the means ± SEM. *P<0.05 comparing to chow.
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**FIGURE 12**
Figure 12: Analysis of insulin sensitivity through activation of pAKT. The western images for both pAKT and tAKT have been shown in (D) in the following order: PWAT, muscle, and liver. The quantification for each image was done and the ratio (pAKT/tAKT) was graphed to assess insulin signaling in PWAT (A), muscle (B) and the liver (C). Given are the means ± SEM (n=4). † P<0.05 comparing between saline- and insulin-treated groups within each diet. *P<0.05 comparing to Chow, ‡ P<0.05 comparing to SFA, ^P<0.05 comparing to MUFA. # P<0.05 comparing to PUFA.
Chapter IV

GENERAL DISCUSSION AND CONCLUSIONS

My goal of this thesis was to learn about the development of diet induced obesity. I wanted to learn how the metabolic syndrome sets in, which of the metabolic organs are affected in the initial stages. We also wanted to know of any sexual differences or similarities that we could observe in our short term study. We have also questioned the credibility of a Mediterranean diet i.e. MUFA rich foods in this study. Therefore we carried out a study in male and female mice fed with three different HFDs for a period of 4 days.

In our study, we observed that males fed with SFA diet did show signs of developing obesity and related metabolic complications such as liver TG accumulation, and increased circulating FFA. These were mirrored in our previous long term studies (unpublished observation). From the results that were presented, I believe that initially it is the defects in the metabolic feedback that allows for fat as the selective fuel in liver and muscle (due to increased availability) which leads to increase in glucose levels in the plasma, loss of sensitivity to insulin followed by hyperinsulinemia. This hyperinsulinemia, could allow more fat storage in fat tissue (again due to high availability of FFA), leading to obesity and hyperleptinaemia. With this particular metabolic set up in the initial stages, ectopic fat accumulation would follow leading to changes in the tissues on a molecular level, such as the lipid makeup of membranes, affecting membrane bound receptors that could lead to the phosphorylation defects seen in insulin resistant individuals on long term HFD feeding.

As compared to their male counterparts fed with SFA, female mice seem to be well protected to the deleterious effects of SFA, or at least at fat and liver tissues for the span of 4 days. Female mice had their liver and fat tissues well protected against accumulation of lipids, indicated by PWAT mass (Figure 9) and similar liver TG (Figure 10). Additionally, females’ plasma lipid levels (Figure 10) show much healthier profiles than males. This particular work adds to the credibility of the role of estrogen in its protective effects against insulin resistance and metabolic diseases.

Another important aspect of this project was to ascertain the reliability of MUFA as a good alternative to SFA, but recently there has been some competition to it. We have
definitive evidence that points to MUFA being as unhealthy as SFA diets are if consumed by males. Even though we noticed some compensatory mechanisms in place, we did see some similarities between the animals fed with MUFA and SFA that could lead to the same fate as those mice fed with SFA. And as expected in females, we did not see any effects or characteristics of DIO. PUFA diets, on the other hand, as mentioned in previous reports and studies definitely confer protection from DIO for both males and females. Its effects were prominent in males where, animals on PUFA diet had higher HDL levels, lower LDL, lower plasma FFA compared to those fed with SFA or MUFA. There have been many mechanisms put forth for the protective effects of PUFA however this particular study was not intended to probe into them.

In the future, in addition to those experiments already proposed in the previous sections, it would be interesting to study reversal of DIO by PUFA diets. It would also be interesting to learn about the short term metabolic control from the “central” point of view i.e. the role of hypothalamus, its leptin signaling and as such, especially in the female mice as there are compelling studies showing estrogen's central metabolic control. In this study we have not taken into consideration the inflammatory component. That would be something to look at, because as far as we know there is no study looking at sex differences in inflammatory infiltration at the short term stage. A major study would be to compare our short term study with long term studies for both males and females, to further elucidate the issue. With respect to females in particular, elucidating estrogens role in metabolism would definitely be a promising step, particularly, how it works in concordance with other metabolic hormones such as insulin and leptin.

To summarize, from our study we have presented data on the effects of different HFDs in male and female mice. We have observed that SFA diets have stood up to their reputation of a bad diet choice and MUFA diets follow very closely in contrast to the popular belief of being a healthy alternative. PUFA diets show a tremendous promise for an alternative, however knowledge of its mechanism could definitely yield new therapeutic targets. Female mice fed with SFA were fairly protected against DIO, which definitely compels us to probe into the role estrogen in maintaining energy homeostasis in further studies.
REFERENCES


